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Research Article

Bradykinin receptors on cultured human fibroblasts were characterized using [2,3-prolyl-3,4-3H(N)]bradykinin as radioligand. During incubation with intact fibroblasts, intact [3H]bradykinin was lost much more rapidly at 37 degrees than at 4 degrees C as determined by bioassay, high-performance liquid chromatography, and ion-exchange chromatography, and is likely to be degraded. At 4 degrees, but not at 37 degrees C, bradykinin remained intact in the presence of 2 mM bacitracin, but not in the presence of soybean trypsin inhibitor or SQ-20881, an inhibitor of kininase II. Specific binding at 4 degrees C was saturable with a maximum number of binding sites of 230 +/- 18 fmol/mg protein (mean +/- SE, n = 4) and a dissociation constant of 4.6 +/- 0.5 nM (mean +/- SE, n = 4). Linear Scatchard plots, Hill coefficients close to unity (0.95-1.06), and the failure of excess bradykinin to influence dissociation kinetics are consistent with a single component binding system with no significant cooperativity. Na+ at physiological concentrations and Ca++ or Mg++ at 3-10 mM reduced binding by 25%. The relative potencies of bradykinin analogues and unrelated peptides in competing for [3H]bradykinin binding indicated a specificity of the binding sites consistent with that of a B2 type receptor. Potencies of the peptides in displacing [3H]bradykinin correlated with their abilities to release prostacyclin, determined as its metabolite 6-keto-PGF1 alpha. […]

Receptors for Bradykinin in Intact Cultured Human Fibroblasts

IDENTIFICATION AND CHARACTERIZATION BY DIRECT BINDING STUDY

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ABSTRACT Bradykinin receptors on cultured human fibroblasts were characterized using [2,3-*prolyl*-3,4-^{3}H(*N*)]bradykinin as radioligand. During incubation with intact fibroblasts, intact [^{3}H]bradykinin was lost much more rapidly at 37° than at 4°C as determined by bioassay, high-performance liquid chromatography, and ion-exchange chromatography, and is likely to be degraded. At 4°, but not at 37°C, bradykinin remained intact in the presence of 2 mM bacitracin, but not in the presence of soybean trypsin inhibitor or SQ-20881, an inhibitor of kininase II. Specific binding at 4°C was saturable with a maximum number of binding sites of 230±18 fmol/mg protein (mean±SE, $n = 4$) and a dissociation constant of 4.6±0.5 nM (mean±SE, $n = 4$). Linear Scatchard plots, Hill coefficients close to unity (0.95–1.06), and the failure of excess bradykinin to influence dissociation kinetics are consistent with a single component binding system with no significant cooperativity. Na^+ at physiological concentrations and Ca^{++} or Mg^{++} at 3–10 mM reduced binding by 25%. The relative potencies of bradykinin analogues and unrelated peptides in competing for [^{3}H]bradykinin binding indicated a specificity of the binding sites consistent with that of a B_2 type receptor. Potencies of the peptides in displacing [^{3}H]bradykinin correlated with their abilities to release prostacyclin, determined as its metabolite 6-keto-$PGF_{1\alpha}$. This system, the first in which bradykinin receptors on human cells have been characterized, should prove useful for investigation of the regulation of bradykinin-influenced biological processes.

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INTRODUCTION

The nonapeptide bradykinin is involved in many important biological processes including inflammation (1) and the regulation of blood pressure (2, 3), electrolyte fluxes, and fluid balance (4, 5). Recent evidence suggests that bradykinin might also have a role as a central neurotransmitter (6). From indirect experimental approaches (structure-activity relationships, kinetic data), it has been concluded that bradykinin exerts its characteristic effects by interacting with one or more types of specific receptors on the cell surface (7–9). Recently, using ^{125}I-[Tyr^1]kallidin (10) or [^{3}H]bradykinin (5, 11) as radioligands, bradykinin-binding sites with properties of physiologic bradykinin receptors have been identified in crude membrane preparations from several mammalian tissues. In intact tissues, bradykinin receptor stimulation appears to initiate a series of intracellular events, including activation of phospholipases A_2 and C (12, 13), the release of prostaglandins (PG)[1] (14–16), and accumulation of cyclic (c)AMP and cyclic guanosine monophosphate (16, 17). A variety of experimental manipulations, such as treatment of intact cells and tissues with steroids (12), PGE_2 (18), serotonin (18), trypsin (19), neuraminidase (20), and thiol compounds (21), have been shown to alter their responsiveness to bradykinin. Proper interpretation of the events that regulate bradykinin responsiveness, however, requires an understanding of the interaction between bradykinin and its receptor, receptor-effector coupling systems, and biological effects of bradykinin. This goal can best be achieved in an intact cell system that retains receptor-

[1] *Abbreviations used in this paper:* HPLC, high-pressure liquid chromatography; PG, prostaglandin(s); PGI_2, prostacyclin.

mediated biological responses. Cultured human skin fibroblasts have frequently been used to investigate hormone effects and genetic defects. Human fibroblasts also have been proven to be a bradykinin-responsive target tissue (12, 16, 22). Therefore, in this study, we undertook to assess bradykinin receptor binding in human foreskin fibroblasts utilizing [^{3}H]bradykinin as a physiological receptor ligand.

METHODS

[2,3-*Prolyl*-3,4-^{3}H(*N*)]bradykinin (52 Ci/mmol) and 6-keto[^{3}H]PGF$_{1\alpha}$ (120 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [^{3}H]bradykinin, stored in 0.05 N acetic acid, had to be purified on CM-Sephadex before use, whereas solutions in ethanol proved to be more stable. Unlabeled bradykinin was purchased from Beckman Instruments, Inc. (Palo Alto, CA); bradykinin analogues and peptides from Peninsula Laboratories, Inc. (Belmont, CA); culture medium, enzyme solutions, and additives for the culture media from Gibco Laboratories (Grand Island, NY); ϵ-aminocaproic acid, 1-10 phenanthroline and *N*-methyl-D-glucamine from Sigma Chemical Co. (St. Louis, MO); bacitracin from Calbiochem-Behring Corp., American Hoechst Corp. (La Jolla, CA); soybean trypsin inhibitor from P. L. Biochemicals, Inc. (Milwaukee, WI); bovine albumin from Armour Pharmaceutical Co. (Phoenix, AZ); 6-keto-PGF$_{1\alpha}$ antiserum and standard from Seragen Inc. (Boston, MA); dextran T-70 and CM-Sephadex C-25 from Pharmacia Fine Chemicals (Piscataway, NJ). SQ-20881 and captopril were kindly provided by Squibb Pharmaceutical Inc. (Princeton, NJ). All other reagents were of analytical grade and obtained from commercial sources.

Cell culture. A single line of human fibroblasts (HF-15) from foreskin of a healthy newborn male, established by routine techniques, was used for all studies; cells were used between the 8th and 15th passages. Stock cultures were grown in Eagle's basal medium supplemented with Earle's salts, 10% fetal calf serum, and 2 mM glutamine as previously described (23). For experiments, subcultures were initiated with 1×10^6 cells in 60-mm plastic dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA). Medium was changed on day 7, and experiments were performed on day 8. For assay of intact [^{3}H]bradykinin remaining in medium after exposure to cells, fibroblasts were grown in 6-well multi-dish trays (Falcon Labware).

[^{3}H]Bradykinin-binding studies. Unless otherwise noted, all binding measurements were performed at 4°C with cell monolayers in culture dishes supported on a porous stainless steel platform covered with a film of ice water in an ice bath. Growth medium was removed and cells were washed twice with 4 ml of ice-cold Dulbecco's phosphate-buffered saline. Cells were then equilibrated for 15 min on ice with 4 ml of chilled modified Hanks' balanced salt (HBSS) solution (120 mM NaCl replaced with *N*-methyl-D-glucamine (24) and concentrations of $CaCl_2$, $MgCl_2$, and $MgSO_4$ reduced by half) supplemented with 0.05% bovine albumin, minimal essential medium amino acid mixture, 2 mM bacitracin, and 10 mM Hepes, pH 7.3. Binding was initiated by replacing medium with 2 ml of fresh medium containing the appropriate concentrations of [^{3}H]bradykinin with or without 3 μM unlabeled bradykinin. This concentration of bradykinin was determined to be optimal for differentiating specific from nonspecific binding (Fig. 6 *B*). At the indicated time thereafter, the medium was removed and cells were rapidly rinsed four times with a total of 20 ml of ice-cold modified HBSS containing 0.2% bovine albumin (pH 7.3). Cells were then rinsed twice with Dulbecco's phosphate-buffered saline, incubated with 2 ml of 0.1% trypsin (10 min, 37°C), and quantitatively transferred to vials for radioassay after addition of 15 ml of Aquasol (New England Nuclear).

Specific binding of [^{3}H]bradykinin, defined as the difference between total binding and binding in the presence of 3 μM bradykinin, usually represented >95% of the total binding (Fig. 5 *A*). Although the protein content of different subcultures varied (320–450 μg protein/dish), in a single experiment, the variation in protein per culture dish was <5%. Protein was measured according to Lowry et al. (25).

Bioassay of intact [^{3}H]bradykinin by binding to fibroblasts. The integrity of [^{3}H]bradykinin in the incubation medium after exposure to fibroblasts (used medium) was tested by its ability to bind specifically to fresh fibroblasts. Used media were transferred to chilled tubes and frozen immediately. For each experimental condition, medium containing the same amount of [^{3}H]bradykinin was prepared and treated identically but was not incubated with cells (control medium). Fibroblasts grown in multi-dish trays were used as a bioassay system. Media (control and used) were thawed (at 0°–4°C) and kept on ice; samples containing the same amounts of radioactivity were incubated with fibroblasts for 30 min at 4°C for determination of specific [^{3}H]bradykinin binding; the difference between specific binding from control and used media was taken as the amount of [^{3}H]bradykinin degraded or altered in such a way that precluded binding to fibroblasts.

Ion-exchange chromatography. To separate intact bradykinin from degraded/altered products, a modification of a previously described procedure was used (26). Samples of medium containing [^{3}H]bradykinin were supplemented with an excess of unlabeled bradykinin and diluted to a final salt concentration of <0.05 M. Samples were adjusted to pH 5.0 and applied to columns (0.2 × 0.6 cm) of CM-Sephadex C-25 equilibrated with 0.05 M ammonium acetate. Altered bradykinin products were eluted in stepwise fashion with 3 ml of 0.1 M and 8 ml of 0.2 M ammonium acetate, pH 5.0; [^{3}H]bradykinin was then eluted with 0.5 M ammonium acetate, pH 7.2.

High-performance liquid chromatography (HPLC). Fractions eluted from CM-Sephadex were lyophilized and reconstituted in 200 μl acetonitrile. Aliquots (50 μl) of the radioactive material were coinjected with nonradioactive standards (~2 μg) of Lys-bradykinin and des-Arg^9-bradykinin and chromatographed on a micro-Bondapack C_{18} column using a Varian model 5000 liquid chromatograph (Varian Associates, Inc., Instrument Group, Palo Alto, CA) (flow rate, 1 ml/min; isocratic elution for 50 min with a mixture of 80% solvent A [0.05% trifluoroacetic acid, 0.25% triethanolamine] and 20% solvent B [0.05% trifluoroacetic acid, 0.25% triethanolamine, 80% acetonitrile]). The UV absorption of the column effluent was monitored continuously with a variable wavelength spectrophotometer at 210 nm. Portions of each 1-ml fraction were assessed for radioactivity by liquid scintillation counting.

Radioimmunoassay of 6-keto-PGF$_{1\alpha}$. Incubation medium was assayed directly for 6-keto-PGF$_{1\alpha}$ by a modification of the radioimmunoassay of Yaffe et al. (27). Each assay contained 100 μl of medium or standard (10–2,000 pg), 50 μl of 6-keto-PGF$_{1\alpha}$ (7,000 cpm), 50 μl of antiserum (diluted such that ~40% of the radioligand was bound) and 0.1% of gelatin in a total volume of 400 μl of 10 mM Tris/150 mM NaCl, pH 7.4. After incubation for 16 h at 4°C, antibody-bound 6-keto-PGF$_{1\alpha}$ was separated from uncomplexed tracer by the addition of 500 μl of an ice-cold 0.5%

charcoal/0.05% dextran mixture and tubes were centrifuged (1,000 *g*, 10 min, 4°C). The supernatants were decanted into scintillation vials for radioassay of antibody-bound 6-keto-[^{3}H]PGF$_{1\alpha}$ after addition of 10 ml of Aquasol.

RESULTS

Analysis of integrity of [^{3}H]bradykinin during incubation with fibroblasts

Binding assay for functional (intact) bradykinin. Incubation of [^{3}H]bradykinin for 2 h at 4°C in medium without cells reduced by only 5% the amount of radioligand capable of binding to cells (data not shown). After incubation of [^{3}H]bradykinin with cells for 30 min at 37°C or 2 h at 4°C, the amount of radioligand that remained intact in the incubation medium and could bind to fresh fibroblasts was reduced by 50%, suggesting that during the incubation [^{3}H]bradykinin was altered/degraded so as to preclude binding to fibroblasts (Fig. 1). After incubation with cells for 2 h in the presence of bacitracin, the amount of radioligand capable of binding to fresh cells was reduced by only 10% (Fig. 1). Of the agents tested, only 2 mM bacitracin almost completely prevented the loss of apparently intact (functional) bradykinin, as measured by subsequent bioassay (Table I). Neither ε-aminocaproic acid, soybean trypsin inhibitor, nor SQ-20881 (an inhibitor of kininase II) affected the disappearance of intact bradykinin (Table I). At concentrations that reduced the loss of apparently intact bradykinin, 1,10-phenanthroline (an inhibitor of metal-dependent kininases) was cytotoxic.

Separation of intact [^{3}H]bradykinin and altered products on CM-Sephadex and HPLC. Authentic [^{3}H]bradykinin could be bound to and eluted from CM-Sephadex with >90% recovery (Fig. 2). Identification of this material as bradykinin was confirmed by HPLC (Fig. 3). Of the radioactivity in medium after incubation of [^{3}H]bradykinin with cells for 2 h at 4°C with bacitracin, 90% was capable of binding to fresh fibroblasts and 86% eluted from CM-Sephadex as authentic bradykinin (Fig. 2). After incubation with cells for 1 h at 37°C, however, only 24% bound to fresh fibroblasts and 30% eluted as authentic bradykinin (Fig. 2). Most of the radioactivity was eluted with 0.2 M ammonium acetate, pH 5.0 (Fig. 2). As seen in Fig. 3, most of this material was eluted during HPLC as

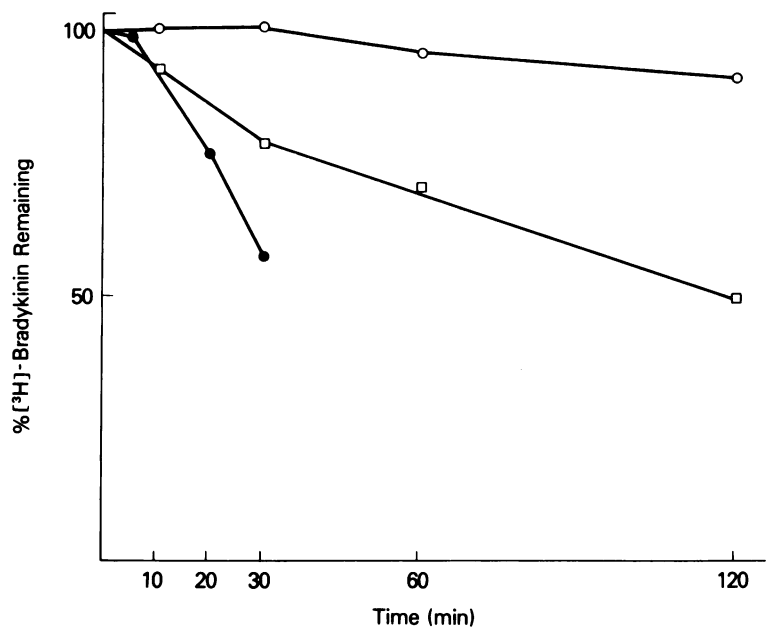

FIGURE 1 Time course of disappearance of intact [^{3}H]bradykinin during incubation with fibroblasts. Cells were incubated with 15 nM [^{3}H]bradykinin at 37° (●) and at 4°C (□) without and at 4°C with 2 mM bacitracin (○). At the indicated time, medium was removed for bioassay of [^{3}H]bradykinin by the binding to a fresh set of fibroblasts at 4°C (Methods). Data are expressed as percentage of controls as follows: % [^{3}H]bradykinin remaining = (% [^{3}H]bradykinin specifically bound from used medium/% [^{3}H]bradykinin specifically bound from control medium) × 100. These data of a representative experiment are the means of values from duplicate incubations that did not vary by more than 5%. Specific binding (100%) was 190 fmol/mg protein.

TABLE I

Effect of Peptidase Inhibitors on [^{3}H]Bradykinin Degradation/ Alteration by Fibroblasts

Peptidase inhibitor	Specifically bound [^{3}H]bradykinin: Control medium	Specifically bound [^{3}H]bradykinin: Used medium	[^{3}H]Bradykinin degraded
	cpm/dish		%
None	1,190±75	580±40	52
Bacitracin, 2 mM	1,770±40	1,640±50	7
EACA, 0.5 M	1,400±65	710±65	49
SQ-20881, 10 μM	1,460±110	740±30	50
SBTI, 0.01%	1,180±15	575±30	51

[^{3}H]Bradykinin, 15 nM, in 2 ml of binding medium with or without peptidase inhibitors was incubated for 120 min at 4°C without (control medium) or with fibroblasts (used medium). Intact [^{3}H]bradykinin in medium samples was then determined by binding to a fresh set of fibroblasts as described in Methods. Specifically bound [^{3}H]bradykinin is reported as mean±SD of values from triplicate assays. EACA, ε-aminocaproic acid; SBTI, soybean trypsin inhibitor.

unidentified radioactive material (retention time of 3 min); only a small fraction of the applied radioactivity eluted with authentic bradykinin (retention time 18 min). In these experiments (Fig. 3) or in others in which fibroblasts were incubated at 37°C with 1 μM or 3 mM [^{3}H]bradykinin (data not shown), no accumulation of des-Arg^9-bradykinin was observed as monitored by HPLC. Of the radioactivity specifically bound to cells during incubation at 4°C for 2 h with bacitracin, >90% could be released by a brief (10 min, 4°C) incubation with acetic acid (0.2 M, pH 2.5) and presumably represented surface-bound material (28, 29). Virtually all of this acid-extracted ^{3}H bound to CM-Sephadex and eluted as authentic bradykinin (data not shown). These data suggested that in the presence of 2 mM bacitracin at 4°C [^{3}H]bradykinin was not appreciably altered, either in the incubation medium or when bound to fibroblasts. Kininases I and II are not likely to be important in the formation of the products which accumulate when [^{3}H]bradykinin is incubated with fibroblasts at 37°C, since inhibitors such as SQ-20881 did not prevent the disappearance of intact (functional) bradykinin (Table I) and des-Arg^9-bradykinin did not apparently accumulate (data not shown).

Effect of pH and ionic composition on [^{3}H]bradykinin binding to intact fibroblasts

Alteration of pH of the incubation medium between 6.5 and 8.0 did not affect either total or specific binding of [^{3}H]bradykinin to intact fibroblasts. Na^+, at physiological concentrations, and Ca^{++} and Mg^{++} (3–10 mM) reduced binding by ~25% (Table II). For most studies of [^{3}H]bradykinin binding to intact fibroblasts, a modified HBSS (pH 7.3) was used with 120 mM Na^+

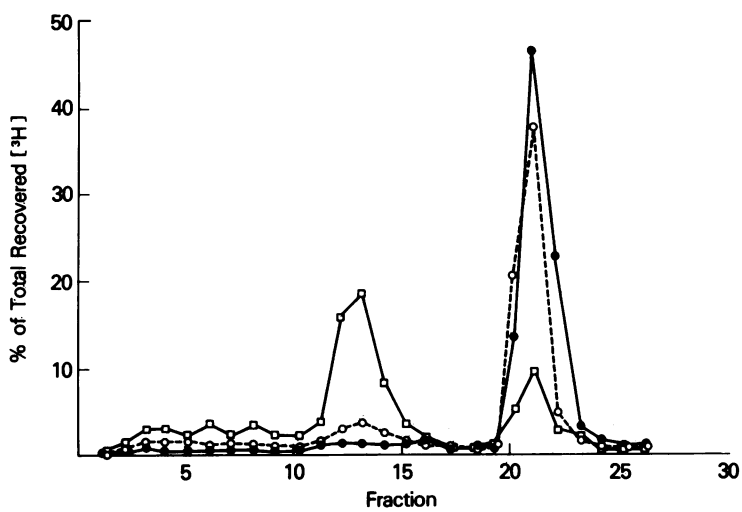

FIGURE 2 Elution from CM-Sephadex of authentic [^{3}H]bradykinin and of [^{3}H]bradykinin incubated with fibroblasts. Medium containing 15 nM [^{3}H]bradykinin was incubated with cells for 60 min at 37°C (□) or for 120 min at 4°C in presence of 2 mM bacitracin (○). Another sample of medium was not incubated with cells (●). Products of bradykinin degradation and authentic bradykinin were eluted as described in Methods. Recoveries of ^{3}H were >85%. Fraction volume = 1 ml.

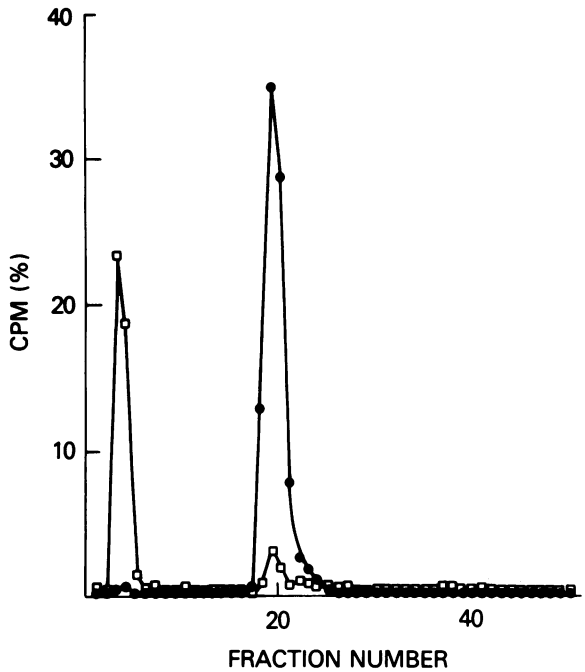

FIGURE 3 HPLC profile of fractions eluted from CM-Sephadex. The material eluted with 8 ml of 0.2 M ammonium acetate, pH 5.0 (□) and with 8 ml of 0.5 M ammonium acetate, pH 7.2 (●) was collected, lyophilized, and subjected to HPLC (Methods). Radioactivity is expressed relative to total radioactivity recovered from the HPLC run, which was 3.2×10^5 and 7.6×10^5 cpm, respectively. Typical retention times for nonradioactive standards were 13 min for Lys-bradykinin, 18 min for bradykinin, and 40 min for des-Arg^9-bradykinin.

replaced by equimolar *N*-methyl-D-glucamine (24) and with the concentrations of Mg^{++} and Ca^{++} reduced by half.

Characteristics and specificity of binding of [^{3}H]bradykinin to intact fibroblasts

Binding of [^{3}H]bradykinin by fibroblasts reached apparent equilibrium within 1 h at 4°C (Fig. 4). The rate of association was faster with 20 nM [^{3}H]bradykinin (a saturating concentration) than with a 4 nM [^{3}H]bradykinin. At 37°C, binding of [^{3}H]bradykinin rapidly reached a maximum and then declined progressively (data not shown).

At 4°C, [^{3}H]bradykinin dissociated from fibroblasts with a half-time of 90 min (Fig. 5). At 37°C, dissociation was extremely rapid, displaying an upwardly concave curve. Dissociation at either temperature was unaffected by the addition of 3 μM unlabeled bradykinin. In the experiment presented in Fig. 5, >90% of the cell-associated radioactivity at zero time and after 90 min at 4°C was extracted by brief treatment with

TABLE II
Effect of Cations on [^{3}H]Bradykinin Binding by Fibroblasts

Ion concentration	Specifically bound [^{3}H]bradykinin	
	Ca	Mg
mM		
0.3	102±2.1	96±2.4
1	96±1.4	92±1.2
3	84±0.9	84±4.2
10	74±2.1	73±2.8
	Na	
17.5	97±2.2	
35	95±1.3	
70	85±1.6	
140	72±3.3	

Cells were incubated with 15 nM [^{3}H]bradykinin with and without 3 μM bradykinin for 60 min at 4°C (Methods). Medium composition was varied as indicated. Ions were added as the chloride salts and osmolarity maintained by addition of *N*-methyl-D-glucamine. Data are means±SD of values from triplicate determinations expressed relative to binding in the absence of the individual cation = 100.

acetic acid. This procedure (28, 29) is reported to discriminate between surface-bound and internalized ligands. Only 50% of the radioactivity remaining after 40 min at 37°C was extracted with acetic acid, suggesting that some of the bound radioligand had shifted to an acid-resistant compartment during incubation at 37°C (29).

Because apparent equilibrium of binding was not attained at 37°C and since [^{3}H]bradykinin was extensively altered/degraded and apparently transferred from the cell surface to another cell-associated compartment at this temperature, most binding studies were performed for 2 h at 4°C, where alteration and internalization of this radioligand were minimal. Specific binding (comprising >95% of total binding) was saturated at 20 nM [^{3}H]bradykinin (Fig. 6 *A*). Scatchard analysis (30) of these data (Fig. 6 *B*) indicated that the equilibrium dissociation constant (K_d) of the binding was 4.1 nM and the maximum binding capacity (B_{max}), 266 fmol/mg protein. In three other experiments, similar results were observed, giving a K_d of 4.6±0.5 nM and B_{max} of 230±18 fmol [^{3}H]bradykinin bound per milligram protein (mean±SE). Scatchard plots were linear up to concentrations of 40 nM [^{3}H]bradykinin (Fig. 6 *B*), and Hill plots (31) of equilibrium binding data (Fig. 6 *B*, *inset*) had slopes close to unity (0.95–1.06). Such analyses indicate a single category of binding sites with no cooperative interactions.

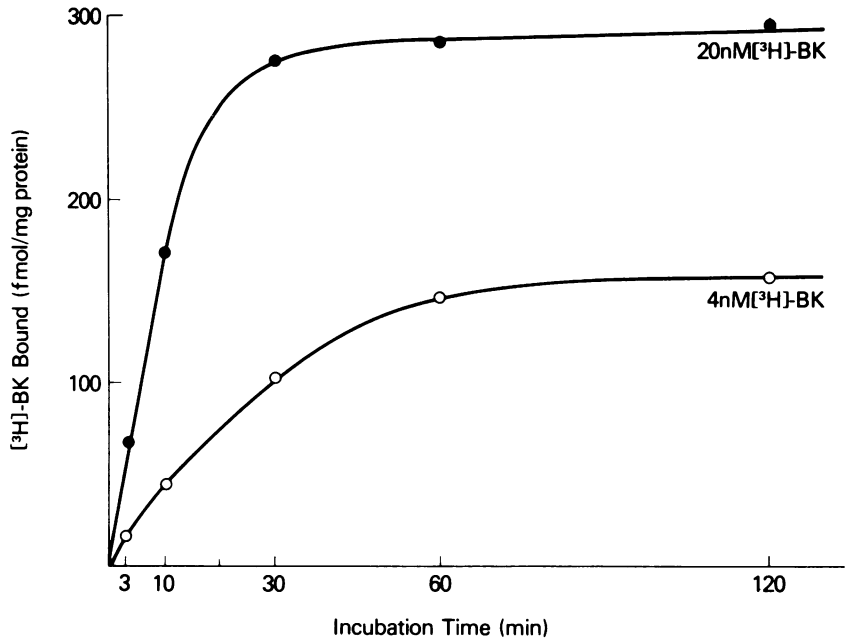

FIGURE 4 Time course of [^{3}H]bradykinin ([^{3}*H*]-*BK*) binding by fibroblasts at 4°C. Cells were incubated for the indicated times with 4 nM (O) or 20 nM (●) [^{3}H]bradykinin at 4°C for determination of specific binding as described in Methods. Data are the means of values from triplicate determinations. Standard deviations were <5%.

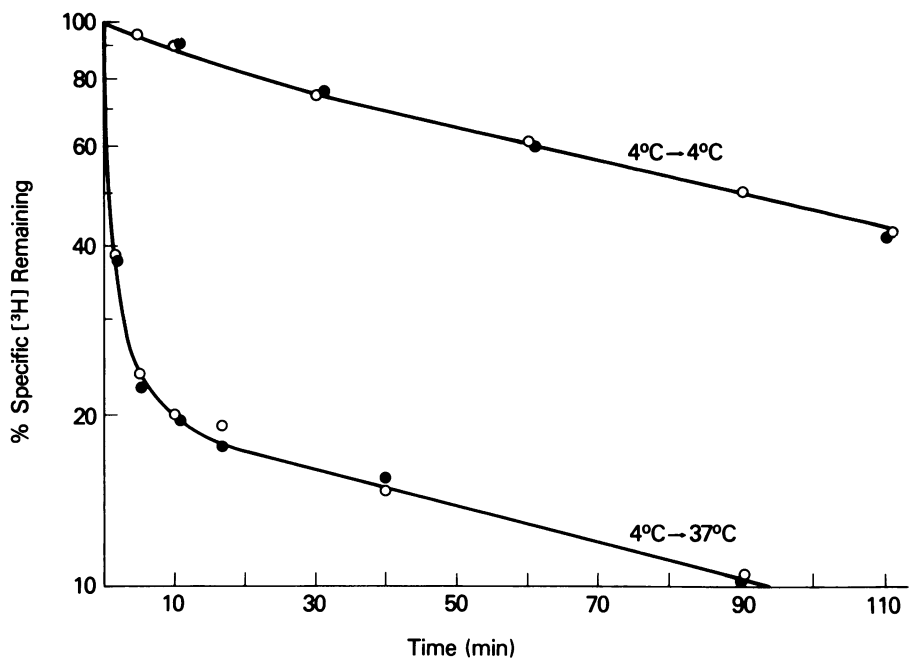

FIGURE 5 Dissociation of [^{3}H]bradykinin from fibroblasts at 4° and 37°C. Cells were incubated with 1.5 nM [^{3}H]bradykinin for 120 min at 4°C. Medium was removed and cells were rinsed at 4°C as for binding assays. Then (zero time), 6 ml of either ice-cold or prewarmed (37°C) incubation medium without (O) or with (●) 3 μM unlabeled bradykinin was added. At indicated times, cell-associated radioactivity was measured as described in Methods. Specifically bound [^{3}H]bradykinin is reported as a percentage of that at zero time. Points represent means of duplicate determinations.

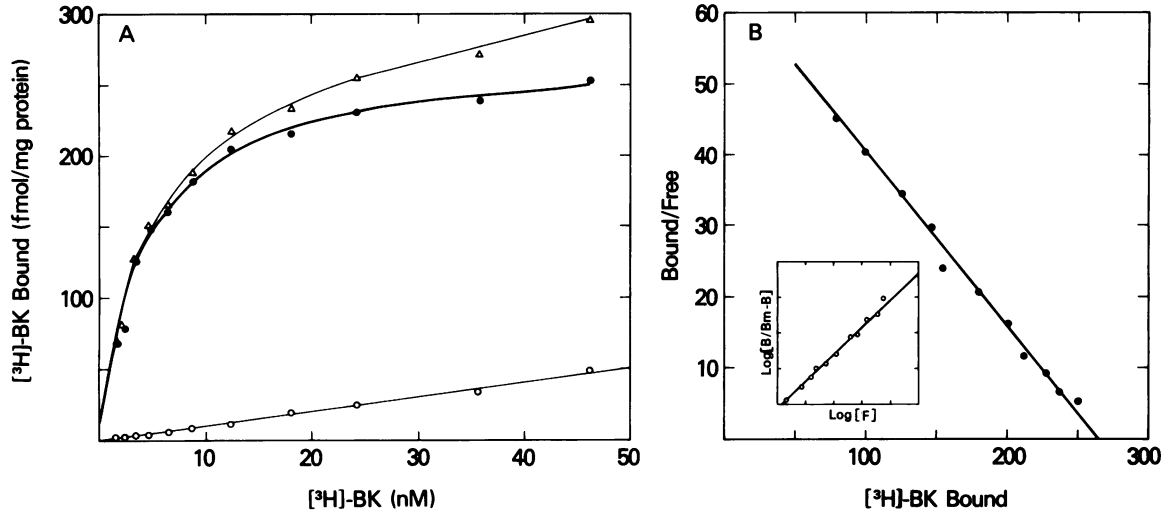

FIGURE 6 Equilibrium binding of [^{3}H]bradykinin ([^3H]-*BK*) to fibroblasts as a function of [^{3}H]bradykinin concentration. (*A*) Cells were incubated for 2 h at 4°C with the indicated concentration of [^{3}H]bradykinin (1.5 to 50 nM) without (△) or with (○) 3 μM unlabeled bradykinin. The difference between total (△) and nonspecific (○) binding is considered to be specifically bound [^{3}H]bradykinin (●). Each point represents the mean of triplicate determinations. Standard deviations are within the size of the symbols. (*B*) Scatchard plot of data from Fig. 5 *A*. Free (F) refers to the difference between added and bound (B) radioligand. The line was computed by linear regression analysis (r = 0.99). The equilibrium dissociation constant estimated from the slope ($-1/K_D$) was 4.1 nM. *Inset:* Hill analysis of the same data. B_m represents specific binding at saturation as determined from Scatchard analysis. The Hill coefficient is 0.95.

Correlation of [^3H]bradykinin binding with prostacyclin (PGI_2) formation

Several bradykinin analogues and other peptides were tested for their abilities to compete with [^{3}H]bradykinin binding (Fig. 7 *B*). Lys-bradykinin, Met-Lys-bradykinin, Tyr-bradykinin, and [Tyr^8]-bradykinin were able to displace specifically bound hormone to the same extent as bradykinin, provided their concentrations were high enough. Des-Arg^9-bradykinin, the product of kininase I action, and the unrelated peptides antiotensin II, neurotensin, and histamine failed to inhibit [^{3}H]bradykinin binding significantly.

The ability of the peptides to stimulate release of PGI_2 from fibroblasts was also assessed (Fig. 7 *A*, Table III). The rank order of potency was the same for binding and PGI_2 release (bradykinin > Lys-bradykinin > Met-Lys-bradykinin ≅ Tyr-bradykinin ≫ Tyr^8-bradykinin ≫ Tyr^5-bradykinin ⋙ angiotensin II ≅ des-Arg^9-bradykinin) (Table III).

As seen in these and other (unpublished) experiments, the EC_{50} values for bradykinin and analogues in stimulating production of PGI_2 (i.e., the concentration of bradykinin and analogues that produces half-maximal stimulation of prostaglandin formation) were greater, by at least an order of magnitude, than the K_d for bradykinin binding or concentrations of the more potent analogues to displace [^{3}H]bradykinin. Although the reasons for this discrepancy are not completely understood, it should be emphasized that PGI_2 production was measured at 37°C in the absence of bacitracin (in our hands, radioimmunoassay of 6-keto-$PGF_{2\alpha}$ was unreliable in the presence of bacitracin).

DISCUSSION

Bradykinin causes accumulation of cAMP in cultured human fibroblasts apparently as a result of enhancing PG production (16). Several experimental manipulations (12, 18, 19, 21) can influence the magnitude of these biological responses to bradykinin. To determine whether alterations in specific bradykinin receptors are involved in the changes in responsiveness, it was necessary to establish conditions for assay of bradykinin receptors on the intact cells that retain receptor-mediated biological responses. Before a quantitative analysis of bradykinin binding, it was necessary to evaluate the integrity of bradykinin during incubation with the fibroblasts. During incubation with fibroblasts, especially at 37°C, intact (functional) bradykinin was rapidly lost as assessed by several criteria. At 37°C, bradykinin was rapidly altered so as to preclude binding to fresh fibroblasts and to be readily separated from authentic bradykinin during HPLC and ion-exchange chromatography. Loss of intact (functional) bradyki-

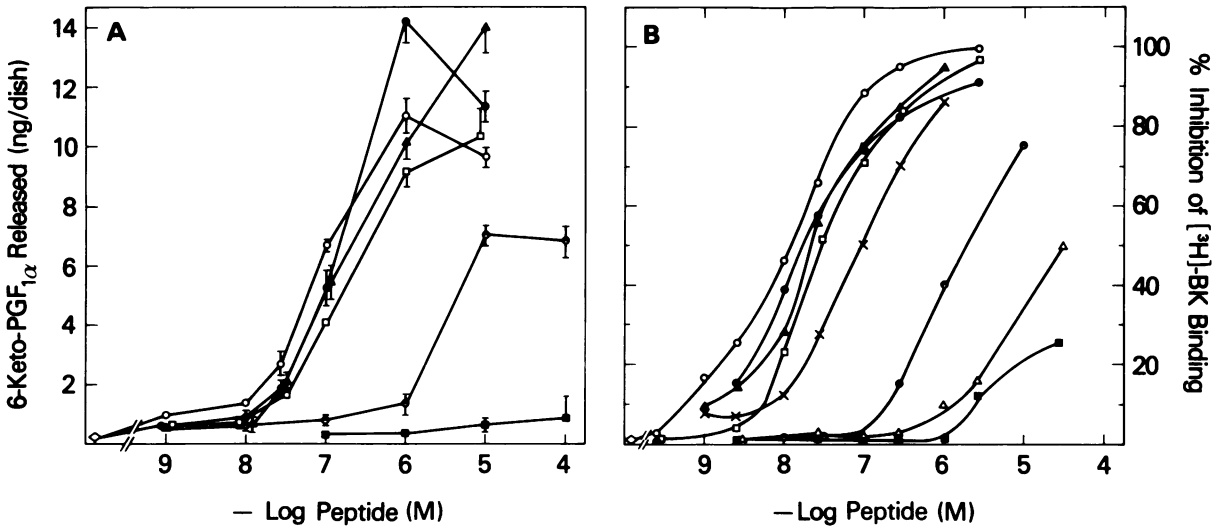

FIGURE 7 Effects of bradykinin analogues and other peptides on PGI_2 release and [^{3}H]bradykinin ([^{3}*H*]-*BK*) binding. (*A*) Cells were incubated for 5 min at 37°C in HBSS with the indicated addition. Medium was saved and assayed as described in Methods for 6-keto-$PGF_{1\alpha}$, the metabolite of PGI_2, which accumulated in the incubation medium. One experiment with six different compounds is shown. Relative potencies of other analogues are listed in Table III. Values are the mean±SD of triplicate determinations. (*B*) Cells were incubated for 120 min at 4°C with 15 nM [^{3}H]bradykinin and the indicated addition. 100% inhibition refers to complete inhibition of specific binding by 3 μM bradykinin. ○, bradykinin; ●, Lys-bradykinin; ▲, Met-Lys-bradykinin; □, Tyr-bradykinin; ×, [Tyr^8]bradykinin; ⊖, [Tyr^5]bradykinin; △, angiotensin II; ■, des-Arg^9-bradykinin. Values are the means of duplicate determinations. The combined results of three different experiments are depicted. Standard deviations are within the symbols.

nin at 4°C was almost completely inhibited by bacitracin, which was much less effective at 37°C. Although we ascribe the loss in biological activity (ability to bind to cells) and altered chromatographic properties to destruction of bradykinin, the definitive structure of the presumed degradation products accumulated during incubation has not been identified. Since des-Arg^9-bradykinin did not accumulate during incubation of fibroblasts with bradykinin, and since neither SQ-20881, an inhibitor of kininase II, nor soybean trypsin inhibitor prevented the loss of intact bradykinin, these cells may lack both kinase I and kininase II (angiotensin I-converting enzyme). The latter enzyme has been found in cultured endothelial cells but not human fibroblasts or smooth muscle cells (33). Since immunoreactive substance P also disappeared during incubation with these fibroblasts,[2] the presumed degradation process(es) are most likely not specific for bradykinin. Of the [^{3}H]bradykinin bound to fibroblasts at 4°C, virtually all remained intact and could be released by brief exposure of cells to acetic acid, a procedure that has been reported to extract only surface-bound, not internalized, radioligands (28, 29). During incubation at 37°C, however, bradykinin was not only extensively altered/degraded but was also transferred into a compartment resistant to extraction with acetic acid, and binding of bradykinin to fibroblasts did not reach equilibrium. Therefore, bradykinin binding to intact fibroblasts was assessed at 4°C at apparent equilibrium under conditions where disappearance of intact free and surface-bound bradykinin and internalization of the radioligand were minimized.

The binding of [^{3}H]bradykinin exhibited the specificity, saturability, and kinetics typical of other peptide-reactor interactions. The binding sites most likely represent a single class without significant cooperative interactions, since Scatchard plots (at least up to 40 nM [^{3}H]bradykinin) were linear, Hill coefficients were near unity, and the presence of excess bradykinin did not affect the rate of dissociation of [^{3}H]bradykinin from the cells. The presence of a second class of sites with higher affinity for bradykinin cannot, however, be ruled out by these data. The bradykinin receptor on the cultured human fibroblasts can be classified as of the B_2 subtype as defined by Regoli and Barabe (7, 8), since des-Arg^9-bradykinin was ineffective both in displacing [^{3}H]bradykinin from binding sites and in stimulating production of PGI_2.

Despite the differences in assay systems and tissues, comparison of our binding data in intact cells with

[2] Saria, A. Unpublished observations.

TABLE III

Relative Potencies of Bradykinin Analogues in Stimulating PGI_2 Release and Inhibiting [^3H]Bradykinin Binding by Fibroblasts

Peptide	PGI_2 release	[^{3}H]Bradykinin binding
Bradykinin	100	100
Lys-bradykinin	65	60
Met-Lys-bradykinin	44	43
Tyr-bradykinin	37	41
[Tyr^8]bradykinin	18	12
[Tyr^5]bradykinin	1.9	0.6
Des-Arg^9-bradykinin	<0.3	<0.03
Angiotensin II	<0.3	<0.03

Potencies in competing with [^{3}H]bradykinin binding were calculated by the equation outlined by Cheng and Prusoff (35). The effects of the analogues on PGI_2 release were compared by estimating the ratio of R_{max}/K_a for each analog relative to R_{max}/K_a for native bradykinin (=100). R_{max} represents PGI_2 released in response to a maximally effective dose of analogue and K_a, the concentration of analogue required to elicit a half-maximal response. To determine potencies, 5–8 concentrations of each peptide were assayed in triplicate. Experimental conditions were as described in the legend to Fig. 6.

those derived from studies of [^{3}H]bradykinin binding by mammalian cell membranes (5, 11) revealed similarities in dissociation constants, rates of dissociation, ion dependence, and relative potency of bradykinin analogues in inhibiting binding. The reduction in specific binding produced by Na^+, Mg^{++}, and Ca^{++} was less in human fibroblasts than in membrane preparations (11). These effects, especially of Na^+, may be of importance, since solute concentrations have been reported to alter effects of bradykinin on PGE_2 production in kidney (36). In the intact fibroblasts, the binding capacity (per milligram protein) was higher than that of most membrane preparations and the optimal pH for binding was in the physiological range. The number of sites for ^{125}I-[Tyr^1]kallidin in bovine myometrium (10) was similar to that in the fibroblasts, although affinity of the more avid sites was higher in the myometrial tissue.

As pointed out by Odya and Goodfriend (9), in view of the lack of a specific antagonist for the B_2 receptor, it is important in characterizing receptors for bradykinin to distinguish between binding of bradykinin to physiological receptors and degradation systems. The use of intact cells has allowed us to correlate binding with enhancement of PGI_2 release, a biological effect of bradykinin that results from the presumed interaction of bradykinin with its specific receptor. The good correlation between the relative potency of several bradykinin analogues in competing for [^{3}H]bradykinin binding and in increasing PGI_2 release (Table III) is consistent with the proposal that the [^{3}H]bradykinin-binding sites are identical to the bradykinin receptors that mediate the release of PG. Our studies demonstrate for the first time the feasibility of using intact human cells to quantify and characterize bradykinin receptors by direct radioligand-binding studies. The intact cell system offers the possibility of establishing directly relationships between bradykinin receptor binding and biological phenomena such as desensitization. In addition, with cultured fibroblasts the interaction of bradykinin with a human bradykinin receptor can be studied. Thus, this model may also be suitable to investigate a possible role for bradykinin receptors in genetically determined disease processes, such as essential hypertension.

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