Autoregulation of Bradykinin Receptors and Bradykinin-induced Prostacyclin Formation in Human Fibroblasts

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Abstract. The interaction of bradykinin (BK) with its specific receptors on intact cultured human fibroblasts results in production of prostaglandins, including prostacyclin (PGI$_2$), and accumulation of cyclic AMP. Incubation of cells with 1 μM BK for 5 min at 37°C led to a marked reduction (75–90%) in BK-induced PGI$_2$ release and in total number of [^{3}H]BK-binding sites with no change in dissociation constant (6.1 and 7.6 nM for control and BK-treated cells, respectively). The decrease in receptor number did not result from BK transferred from the first incubation into the binding assay. BK-induced receptor loss was temperature dependent; exposure of cells to BK at 4°C had little or no effect on receptor number. After incubation with BK for $\cong$15 min, further incubation in the absence of BK for 30 min at 37°C almost completely restored both receptor number and BK-induced PGI$_2$ release. With more prolonged exposure to BK (>1 h), restoration of receptors was inversely related to the length of exposure and the concentration of BK. Recovery was unaffected by cycloheximide. During prolonged incubation without removal of BK, cells began to recover receptors by 5 h; >99% of the bradykinin initially present disappeared by 3 h. Bacitracin greatly retarded BK disappearance and totally prevented recovery. These observations provide direct evidence that the number of BK receptors on cultured human fibroblasts can be regulated by BK itself. In addition, it appears that BK-degrading systems, by influencing local concentrations of the peptide, may play an important role in the autoregulation of BK receptors. The presence of highly active degradation systems might serve to protect target tissues from developing chronic insensitivity to BK and, perhaps, similar peptides.

Introduction

The nonapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) bradykinin (BK)[1] has effects on a number of different tissues and cellular functions and causes, for example, vasodilation and hypotension, pain, increased intestinal motility, and chloride secretion (1–4). Moreover, recent evidence suggests a role for BK as a neurotransmitter (5). Alterations in the concentration of BK in human plasma have been reported in association with several clinical syndromes, including postgastrectomy-dumping syndrome, familial orthostatic syndromes, and carcinoid tumors (4, 6). In intact tissues, bradykinin receptor stimulation appears to initiate a series of intracellular events including activation of phospholipases A$_2$ and C (7, 8), the release of prostaglandins (9–11), and accumulation of cyclic AMP (cAMP) and cyclic guanosine monophosphate (10–14). The effects of BK on accumulation of cAMP are presumably indirect, a consequence of BK-promoted release of arachidonic acid and subsequent production of prostaglandins, which activate adenylate cyclase (11, 13, 14). We have recently identified specific [^{3}H]BK-binding sites on intact human fibroblasts that are closely linked to BK-induced PGI$_2$ formation (15). On the basis of indirect evidence, others (11, 16) suggested the possible involvement of BK receptors in refractoriness and desensitization to BK. We report here that exposure of the cultured fibroblasts to BK causes disappearance or loss of specific BK receptors and that BK-degrading enzymes may play an important part in the autoregulation of these receptors, thus influencing tissue responsiveness to BK.

Methods

[2,3-Prolyl-3,4-^{3}H(N)]BK (52 Ci/mmol) and 6-keto-[^{3}H]prostaglandin F$_{1\alpha}$ (PGF$_{1\alpha}$) (120 Ci/mmol) were purchased from New England Nuclear

1. *Abbreviations used in this paper:* BK, bradykinin; HPLC, high pressure liquid chromatography; PGE$_1$ and PGF$_{1\alpha}$, prostaglandins E$_1$ and F$_{1\alpha}$, respectively; PGI$_2$, prostacyclin.

(Boston, MA). [³H]BK was analyzed by high pressure liquid chromatography (HPLC) using a micro-Bondapak C_{18} column eluted for 50 min with a mixture of 80% solution A (0.05% trifluoroacetic acid, 0.25% triethanolamine in water) and 20% solution B (0.05% trifluoroacetic acid, 0.25% triethanolamine, 80% acetonitrile). Unlabeled BK was purchased from Beckman Instruments, Inc. (Fullerton, CA); BK analogues from Peninsula Laboratories (Belmont, CA); cell culture medium and additives from Gibco Laboratories (Grand Island, NY); d,l-isoproterenol-HCl and cycloheximide from Sigma Chemical Co. (St. Louis, MO); bacitracin from Calbiochem-Behring Corp. (San Diego, CA); bovine albumin from Armour Pharmaceutical Co. (Tarrytown, NY); 6-keto-$PGF_{1\alpha}$ antiserum and standard from Seragen, Inc. (Boston, MA); dextran T-70 from Pharmacia Fine Chemicals (Piscataway, NJ). Prostaglandin E_1 (PGE_1) was generously supplied by Dr. J. E. Pike, The Upjohn Company (Kalamazoo, MI). All other reagents were of analytical grade and obtained from commercial sources.

Stock cultures of human fibroblasts (HF-15) from a healthy newborn male were maintained as described (17). Subcultures, initiated with 1 × 10⁶ cells in 60-mm dishes, were grown to confluence. Medium was replaced the day before cells were used. For experiments, after removal of growth medium, cells were washed and incubated at 37°C in Hanks' solution supplemented with 0.05% bovine albumin, minimal essential amino acid mixture, 2 mM glutamine, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.3 (hereafter referred to as medium), with or without unlabeled BK for the indicated time. To remove extracellular and surface-bound BK, cells were rinsed five times at 37°C with 4 ml of Hanks' solution containing 0.1% serum albumin, taking a total of 2.5 min, after which replicate dishes of cells were used to assess specific [³H]BK binding and BK-induced PGI_2 release.

For [³H]BK-binding studies, cell monolayers were incubated at 4°C in culture dishes supported on a porous stainless steel platform covered with a film of ice water in an ice bath. Cells were incubated for 15 min at 4°C in medium containing 2 mM bacitracin. Binding was initiated by replacing this medium with 2 ml of medium containing bacitracin and appropriate concentrations (usually 20 nM unless otherwise stated) of [³H]BK with or without 3 µM unlabeled BK. After incubation (usually 2 h) at 4°C, medium was removed and cells were rapidly rinsed four times with a total of 20 ml ice-cold modified Hanks' solution containing 0.2% bovine serum albumin (pH 7.3). Cells were then rinsed twice with ice-cold 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 [³H]BK was defined as the difference between total [³H]BK bound and that bound in the presence of 3 µM unlabeled BK (nonspecific binding) and usually represented >95% of the total [³H]BK bound.

To assess BK-induced PGI_2 release, cells were incubated for 5 min at 37°C in Hanks' solution with or without 1 µM BK. PGI_2 in the medium was determined as its metabolite 6-keto-$PGF_{1\alpha}$ by a modification of the radioimmunoassay of Jaffe et al. (18). 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, 500 µl of an ice-cold 0.5% charcoal/0.05% dextran mixture was added and tubes were centrifuged (1,000 g, 10 min, 4°C). Supernatants containing antibody-bound 6-keto-[³H]$PGF_{1\alpha}$ were decanted into vials for radioassay after addition of 10 ml of Aquasol.

BK remaining in the medium after incubation with fibroblasts was estimated by using a receptor-binding assay. BK (and closely related

analogues) but not BK degradation products compete with [³H]BK for binding by fibroblasts in proportion to their biological activity (15). Samples (1.5 ml) of used incubation medium containing BK or appropriate standards (0.5–1,000 ng/ml BK) were diluted with 0.5 ml of buffer containing 8 mM bacitracin and frozen immediately. Samples (2 ml) were thawed on ice, diluted if necessary, mixed with 100 µl of 100 nM [³H]BK and incubated with fibroblasts to determine specific [³H]BK binding. BK remaining in the used medium was calculated from a standard plot of percentage of [³H]BK specifically bound vs. BK concentration.

Results

In cultured human fibroblasts, PGE_1 and isoproterenol, acting through their respective receptors, increase adenylate cyclase activity and thereby cause accumulation of cAMP (16). BK presumably increases the cAMP content of these and other cells indirectly as a consequence of BK-induced prostaglandin production (10, 11, 13, 14). Incubation of fibroblasts with 1 µM BK at 37°C markedly reduced the rise in cAMP produced by a subsequent 5-min exposure to BK without altering responses to PGE_1 or isoproterenol (Table I).

In an earlier study, Scatchard analysis of equilibrium binding of [³H]BK to human fibroblasts at 4°C indicated a dissociation constant (K_d) of 4.6 ± 0.1 nM and maximum binding (B_{max}) of 230 ± 18 fmol [³H]BK bound/mg protein (mean±SE, $n = 4$) (15). BK-induced PGI_2 release and specific binding of [³H]BK were decreased by prior incubation of fibroblasts with BK at 37°C. Both were reduced maximally, ~75%, after 5–10 min of exposure to 1 µM BK and remained at this level for 60 min (Fig. 1). (In other experiments, both were reduced by 75–90% in 10 min.) The apparent loss of receptors was temperature dependent and observed when cells were incubated with BK at

Table I. Effect of Prior Incubation with BK on cAMP Accumulation in Response to BK, PGE_1, and Isoproterenol

Additions for 5 min	cAMP content	
	No BK	1 µM BK
	pmol/mg protein	pmol/mg protein
None	24±2.6	24±2.6
Bradykinin, 1 µM	193±4.9	41±1.8
PGE_1, 1 µM	911±32	1,180±54
Isoproterenol, 1 µM	124±2.0	141±3.4

Cells were incubated with or without 1 µM BK for 15 min at 37°C, then washed and incubated for 5 min with additions as indicated. Medium was rapidly replaced with ice-cold 5% trichloroacetic acid. cAMP in the cell extracts was determined by radioimmunoassay (New England Nuclear) after removal of trichloroacetic acid with trioctylamine/Freon (19). Data are means±SEM of values from triplicate incubations.
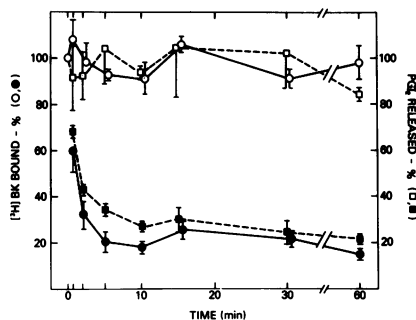
Figure 1. Effect of incubation with BK on subsequent [³H]BK binding and BK-induced PGI₂ release by fibroblasts. Cells were incubated at 37°C in medium with (●, ■) or without (○, □) 1 μM BK, then washed at 37°C and [³H]BK binding (○, ●) or BK-induced PGI₂ release (□, ■) was assayed as described in Methods. Percentage of initial [³H]BK binding (275±5 fmol/mg protein = 100) or of initial BK-induced PGI₂ release (11.5±2 ng/dish = 100) is plotted as a function of time of prior incubation with BK. Data are means±SD of values from triplicate incubations.

37° and not at 4°C. Exposure of cells to 1 μM BK for 30 min at 4°C (followed by washing at 37°C as described in Methods) reduced [³H]BK binding only by 10% (from 220±8 fmol/mg protein in control cells incubated at 37°C without BK to 204±fmol/mg protein in BK-treated cells) in contrast to the much greater decrease in binding of [³H]BK observed after incubation with BK at 37°C (to 59±11 fmol/mg protein). The reduction in [³H]BK binding was dependent on the concentration of BK present during a 15-min incubation and closely
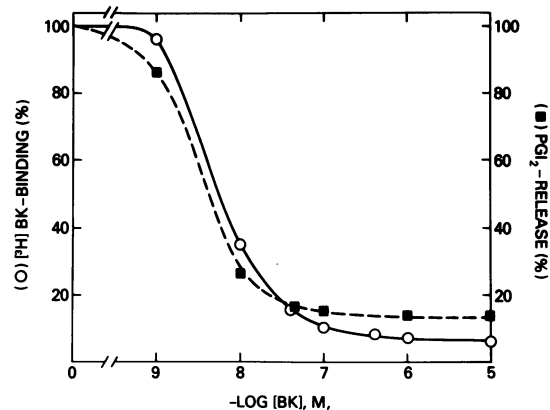

Figure 2. Effect of BK concentration during initial incubation on subsequent [³H]BK binding and BK-induced PGI₂ release. Cells were incubated for 15 min at 37°C with the indicated concentration of BK, then washed and specific [³H]BK binding (○) or BK-induced release of PGI₂ (■) was assayed. Control [³H]BK binding (100%) was 198 fmol/mg protein, and PGI₂ release was 14.5 ng/dish. Data, reported as in Fig. 1, are means of values from triplicate incubations. SD were in the range as shown in Fig. 1.
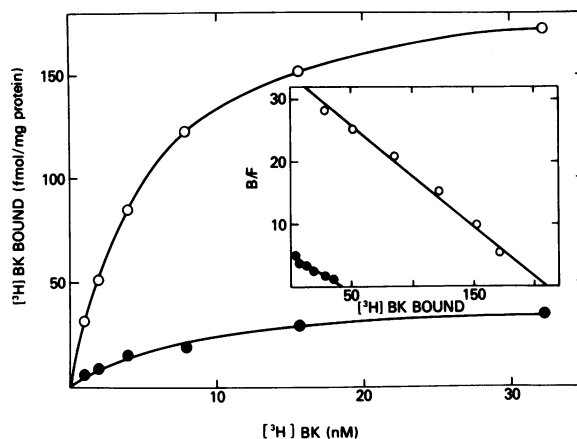

Figure 3. Binding of [³H]BK by fibroblasts before and after incubation with BK. Cells were incubated with (●) or without (○) 1 μM BK for 15 min at 37°C, washed, and then incubated with the indicated concentrations of [³H]BK with or without 3 μM unlabeled BK for 2 h at 4°C to determine specific binding. (*Inset*) Scatchard plot of the data. Lines were computed by linear regression analysis (r = 0.99 and 0.93, respectively) B/F, bound/free. In control cells, the K_d was 6.1 nM and B_{max} 211 fmol/mg protein; respective values for the BK-treated cells were 7.6 nM and 42 fmol/mg protein. Data are means of values from triplicate assays.

paralleled the reduction in BK-stimulated PGI₂ release (Fig. 2). The concentration that caused a half-maximal reduction (ED₅₀) in both was 4–6 nM. The reduction in specific [³H]BK binding resulting from incubation of cells with BK for 15 min appeared to reflect a decrease in binding capacity without any significant alteration in affinity (Fig. 3).

The apparent loss of receptors at 37°C is not simply due to carry-over of free or bound BK from the first incubation. After incubation with saturating (20 nM) [³H]BK at 4°C for as long as 2 h and washing at 4°C (which serves to dilute and remove free BK with little or no loss of cell-bound ligand [15]), maximal binding of [³H]BK was ≥200 fmol/mg protein. Incubation (10 min, 4°C) with acetic acid (0.2 N, pH 2.8) released >90% (93±3, n = 4) of the radioactivity specifically bound to the cells (20). Virtually all of this acid-extracted material bound to CM-Sephadex and eluted as authentic BK (15). This indicates that at 4°C most of the ligand is surface-bound and represents intact BK, although as discussed by Wiley and Cunningham (25) the acid extraction procedure is not entirely quantitative and could reflect extraction of a small amount of material not bound to the cell surface. Of the BK specifically bound during incubation of cells with saturating concentrations of [³H]BK (20 nM) at 4°C (236 fmol/mg protein), >90% was released (218 fmol/mg protein) by washing at 37°C as described in Methods. Further incubation with excess, unlabeled BK at either 4° or 37°C released virtually no radioactivity, suggesting that washes at 37°C were efficient in removing readily accessible, presumably

surface-bound BK and that residual radiolabel (~200 cpm representing ~18 fmol/mg protein) was not surface-bound but presumably internalized. In other experiments, cells were incubated with 20 nM [³H]BK for 15 min at 37°C, then washed at 37°C as described in Methods or in the same manner at 4°C. Cells were then incubated (10 min, 4°C) with acetic acid (0.2 N, pH 2.8). After washing at 37°C and incubating with acetic acid, released radioactivity represented only ~6 fmol/mg protein, <10% of that released from cells washed at 4°C. Since maximal binding was ≥200 fmol/mg protein, this amount of residual BK, even if surface-bound, could not account for the observed 90% reduction in [³H]BK binding resulting from incubation with 20 nM BK (Fig. 2). After acid treatment, the remaining ³H (presumably internalized peptides) was completely (>95%) extracted with ethanol. Radioactivity in the ethanol extract, analyzed by HPLC as described in Methods, was associated entirely with altered/degraded products of BK (data not shown). This indicates that after incubation and washing at 37°C only a negligible amount of radiolabel remains susceptible to extraction with acetic acid (and presumably is bound to the cell surface); residual radiolabel represents both internalized and degraded/altered products of BK.

With cells that had been exposed to 1 μM BK for 15 min, incubation at 37°C for 30 min after removal of BK restored [³H]BK binding and BK-induced PGI₂ release almost completely (Fig. 4). Exposure of cells for 15 min to 0.03, 0.3, or 3 μM BK caused ~80% loss of receptors, and restoration of receptor number was virtually complete after a subsequent 60-min incubation (Fig. 5). After incubation with 0.3 or 3 μM BK for 60 min or more, however, a further 60-min incubation resulted in only partial restoration of [³H]BK binding (Fig. 5). In other experiments, BK was not removed and cells began to recover [³H]BK-binding sites by 3 h; binding was restored to ~50% of maximal by the eighth hour (Fig. 6 B). In this experiment, >99% of the BK initially present disappeared in 3 h and by 5 h no BK was detectable (Fig. 6 A). When bacitracin was present, the BK concentration was still >100 nM after 8 h and there was no recovery of [³H]BK-binding sites. The nature of the presumed fibroblast BK-degrading systems has not been determined. Degradation, however, does not occur in the medium as a result of peptidase secretion or leakage (unpublished observations). Inhibitor experiments and HPLC analysis of the degradation products of BK indicate that neither kininase I nor kininase II is likely to be involved (15). During incubation at 37°C with human fibroblasts, immunoreactive substance P also disappeared, suggesting that the presumed degradative system is not specific for bradykinin (data not shown).

Discussion

As reported here, incubation of human fibroblasts with BK at 37°C rapidly caused a decrease in specific [³H]BK binding; the
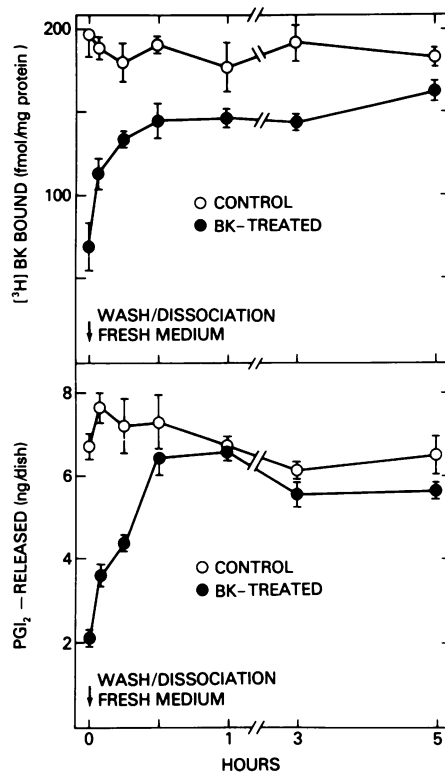

Figure 4. Recovery of specific [³H]BK binding and BK-induced PGI₂ release after brief exposure to BK. Cells were incubated with (●) or without (○) 1 μM BK for 15 min at 37°C, washed, and further incubated at 37°C in 2 ml of fresh medium. At the indicated time, specific [³H]BK binding (upper) and the BK-induced PGI₂ release (lower) were measured. Data are means±SD of values from triplicate incubations.

number of binding sites was reduced but their affinity for BK was apparently unaltered. The decrease in binding sites was closely paralleled by a decrease in BK-induced PGI₂ production, indicating that these high affinity specific binding sites have the functional characteristics of receptors. In addition, it would appear that in the fibroblasts virtually all BK receptors are linked to the cellular effector system, i.e., there is no significant fraction of "reserve" or "spare" receptors.

Reduction in number of BK receptors and in BK-induced PGI₂ release exhibited the same dependence on BK concentration. Half-maximal decreases in both were produced with ~6 nM BK, a concentration approximating the K_d for the BK receptor in these cells. With several BK analogues, the rank order of potency in causing receptor loss (21) was similar to the order of potency in competing for specific [³H]BK binding or stimulating PGI₂ production (15). Thus, there is close correlation between agonist occupancy of the BK receptor and receptor loss. In addition to receptor occupancy, however, a further tem-
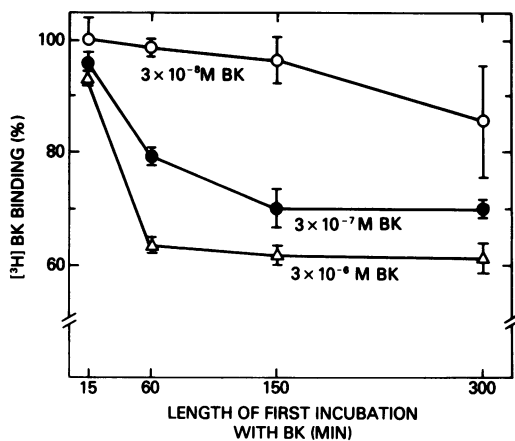
Figure 5. Effect of BK concentration and of length of incubation with BK on the recovery of BK receptors. Cells were incubated at 37°C without or with 0.03 (o), 0.3 (●), or 3 (△) μM BK. At the indicated time, cells were washed and then incubated for 60 min at 37°C with 2 ml of fresh medium before measurement of specific [³H]-BK binding. Specific [³H]BK binding by cells incubated with BK for the indicated time is reported as percentage of that by cells incubated for the same period without BK and subsequently treated in the same way. These values ranged from 253 to 314 fmol/mg protein. Data are means±SD of values from triplicate incubations.

Figure 6. BK degradation and specific [³H]BK binding during long term incubation with BK with and without bacitracin. Cells were incubated at 37°C with (o, ●) or without (□, ■) 1 μM BK without (o, □) or with (●, ■) 2 mM bacitracin. Medium was collected at the indicated time for assay of intact BK remaining as described in Methods (*A*). Points at 3, 5, and 8 h represent concentrations of BK below the detection limit of the assay (i.e., 0.5 ng/ml). After removal of medium, cells were washed and specific [³H]BK binding was determined (*B*). Data are presented as means±SD of values from triplicate incubations.

perature-dependent step(s) must be involved, since cells incubated with BK at 4°C rather than 37°C, although they bound BK, did not exhibit a significant reduction in receptor number or in BK-induced PGI₂ release. As incubation of cells with BK did not alter their responsiveness to PGE₁ or isoproterenol, the loss or disappearance of BK-binding sites appears to represent specific autoregulation of the BK receptor, i.e., homologous desensitization (22).

Because of the extremely rapid dissociation of BK from its receptors at 37°C (15), it was possible to remove essentially all of the BK present in the medium and bound to the cell surface during the initial "desensitizing" incubation simply with five washes at 37°C before assay of [³H]BK binding at 4°C. The efficacy of this procedure was documented by several types of evidence. First, although the fibroblasts bind BK at 4°C, this does not lead to a reduction in receptor number. Any ligand not removed by washing at 37°C should have decreased the subsequent binding of [³H]BK. In addition, retained ligand would be expected to increase the apparent affinity for BK (23), whereas the reduction in binding capacity induced by incubation of the cells with BK at 37°C was associated with little or no change in K_d. Direct measurement of surface-bound (acid-released) radioligand showed that the amount remaining after cells were washed at 37°C could account for only a small fraction (~3%) of the reduction in binding sites. This experiment also revealed that after incubation at 37°C part of the cell-associated [³H] is not released with acid and is presumably intracellular.
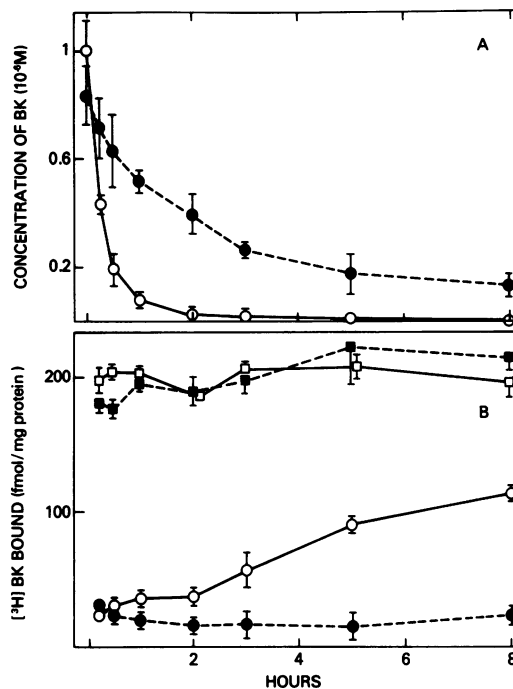
This material consisted exclusively of altered/degraded products of BK.

BK-induced receptor loss and restoration appear to involve several processes. After brief (15–30 min) exposure to BK, restoration of receptors was essentially complete within 30 min after removal of BK. The reappearance of BK receptors was accompanied by a parallel recovery in BK-induced PGI₂ release, indicating a functional coupling of the restored receptors with prostaglandin synthesis. In contrast to the relatively rapid and complete restoration of receptors after brief incubation (15 min) with BK, only partial recovery of binding occurred when cells had been exposed to BK for >1 h. Recovery was inversely related to the concentration of BK as well as the duration of exposure. During prolonged incubation of fibroblasts without removal of BK, partial recovery of BK receptors (~40–60%) was observed within 6 h. Under these conditions, however, substantial degradation of BK occurred so that within 3–5 h the concentration of BK was <0.5 nM. Similar recovery of responsiveness during incubation with angiotensin II and substance

P as well as BK has been reported (16). Bacitracin, which had no effect on the binding of BK to cells or on the time course of BK-induced down-regulation, markedly retarded BK degradation and completely prevented recovery of BK receptors during an 8-h incubation. Similarly, putative BK receptors in rat uterus were reportedly decreased after infusion of the kininase II inhibitor Captopril (24). Processes other than peptide degradation appear to influence the rate and extent of receptor reappearance on the cell surface, since the full complement of receptors was not restored even several hours after removal or complete destruction of BK in the incubation medium. It is of interest that recovery of receptors, after either brief or prolonged exposure to BK, was not inhibited by cycloheximide (21).

The observations reported here might be described by a model in which BK receptors cycle between active (accessible to the peptide and coupled to PGI_2 production) and inactive states and that prolonged receptor occupancy leads to incomplete reactivation of inactive receptors. Although the molecular mechanisms underlying these phenomena remain to be elucidated, the temperature-dependent accumulation of BK degradation products in an apparently intracellular compartment suggests that internalization of a ligand-receptor complex may be involved as is the case with several other hormones (25–27). In the sense that BK degradation minimizes both the concentration to which cells are exposed and the duration of exposure, it could be of major importance in regulating the number of functional receptors and thereby the responsiveness of cells to the peptide. The presence of highly active degradation systems might serve to protect target tissues from developing a chronic insensitivity to BK and perhaps similar peptides. In addition to providing direct evidence that BK induces the homologous desensitization of its specific receptor as do many other peptide hormones (22, 26), our experiments are consistent with the view that autoregulation of the BK receptor and its biological function is controlled by peptide-degrading systems. These findings could prove useful in understanding the impact of therapeutic interventions aimed at altering plasma or tissue content of BK (28) and might also serve as a model for the regulation of receptors for other hormones (e.g., opioid peptides, histamine) that are rapidly degraded in their target tissues.

Acknowledgments

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