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[Hyp³]-bradykinin and [Hyp³]-Lys-bradykinin interact with B2-bradykinin receptors and stimulate inositol phosphate production in cultured human fibroblasts

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The recently isolated, naturally occurring peptide hormones [Hyp³]-bradykinin and [Hyp³]-Lys-bradykinin were investigated for their agonist activity on solubilized binding sites from human fibroblasts. Both ligands competed with [³H]bradykinin binding in a dose-dependent fashion with potencies similar to bradykinin (BK) and Lys-BK. Biological activity was assessed by determination of inositol phosphate accumulation and cyclic 3′,5′-adenosine monophosphate synthesis in intact cultured cells. Stimulation by the hydroxylated peptides resulted in a pronounced accumulation of both parameters with similar effectiveness as BK and Lys-BK. These results indicate that [Hyp³]-BK and [Hyp³]-Lys-BK are agonists at the bradykinin receptor system with properties comparable to their non-hydroxylated analogues. This suggests that hydroxylation of kinins does not alter receptor interaction or signal transduction in cultured human fibroblasts.

Bradykinin; Kinin hydroxylation; B_2-receptor; Cyclic adenosine monophosphate; Inositol phosphate

1. INTRODUCTION

Kinins are involved in a variety of biological processes such as the regulation of blood pressure, pain, neurotransmission, inflammation and cell proliferation. They are generated from their precursors, the kininogens, by limited proteolysis through the action of kallikrein and other trypsin-like serine proteases [1]. These effects are mediated through the interaction of the ligand with at least two types of receptors, B1 and B2 [2].

The naturally occurring kinins include bradykinin (BK), Lys-BK and Met-Lys-BK. Recently, two other kinins were discovered in man. [Hydroxyproline³]-bradykinin ([Hyp³]-BK) was found in human urine [3,4]. It was also isolated from human malignant ascitic fluid and found to be active in the rat estrous uterus assay [5]. [Hyp³]-Lys-BK was obtained from human plasma's Cohn's fraction IV-4 [6] and from human purified kininogen after incubation with kallikrein [7]. In animal tissue studies, Fredrick et al. [8] reported that synthetic [Hyp³]-BK is as active as native BK in its ability to bind to bovine uterine myometrium and in its potency on the isolated rat uterus. However, data from studies in animal models cannot necessarily be applied to the human situation. Furthermore, the exact characterization of peptide hormone-receptor interaction requires the simultaneous measurement of binding characteristics and biological response parameters in order to evaluate signal transduction efficacy [9]. In this context, the functional impact of kinin hydroxylation in the human situation remains to be established.

Cultured foreskin fibroblasts have successfully been used as a human model system for the study of kinin effects and B2 bradykinin receptors [10]. Phospholipase C-induced formation of inositol phosphates has been shown to be an early occurring, sensitive parameter of BK action in these cells [11]. In addition, kinin-induced phospholipase A_2-dependent release of prostaglandins and accumulation of cyclic 3′,5′-adenosine monophosphate (cAMP) have been demonstrated in fibroblasts [10,12].

Therefore, these cells were utilized to investigate the effect of [Hyp³]-BK and [Hyp³]-Lys-BK on receptor binding properties and signal transduction processes.

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Abbreviations: BK, bradykinin; IP, inositol monophosphate; IP_2, inositol diphosphate; IP_3, inositol triphosphate; PI, phosphatidylinositol; cAMP, cyclic 3′,5′-adenosine monophosphate

2. MATERIALS AND METHODS

2.1. *Materials*

[Hyp³]-BK and [Hyp³]-Lys-BK were purchased from Peptide Institute (Osaka, Japan). DesArg⁹[Leu⁸]-BK and D-Arg⁰-[Hyp³,Thi⁵,⁸,

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D-Phe[7]-BK were generous gifts from D. Regoli (Sherbrooke, Canada). [2,3-Prolyl-3,4-^{3}H(N)]bradykinin (spec. act. 80 Ci/mmol) and myo-[2-^{3}H]inositol (spec. act. 15 Ci/mmol) were from New England Nuclear (Boston, MA). ^{125}I-cAMP radioimmunoassay reagents were purchased from Amersham (Braunschweig, FRG). All other chemicals were obtained from commercial sources and of the highest purity available.

2.2. *Cell culture*

Stock cultures of human foreskin fibroblasts were established and grown by standard procedures as described [10].

2.3. *Binding studies in solubilized receptor preparations*

Cells were grown on 145 mm dishes and incubated with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Pipes), pH 6.8, containing 4 mM 3-(3-cholamidopropyl-dimethylammonio)-1-propanesulfonate (Chaps) for 15 min at 25°C. The soluble supernatant was passed through a 2 μm membrane filter and kept at 4°C. Solubilized proteins were incubated with [^{3}H]-BK at concentrations giving approximately half-maximal saturation of specific binding sites (K_d = 2 nM). Incubations were carried out in the presence or absence of various concentrations of BK-analogues and 2 mM bacitracin at 4°C for 2 h. Free ligand was separated from protein-bound [^{3}H]-BK by rapid filtration through polyethylenimine-treated GF/C glass fiber filters (Whatman, Maidstone, UK). The filters were washed 3 times with 14% (v/v) isopropanol in H_2O and total filter-bound radioactivity was assayed by liquid scintillation counting. Non-specific binding was determined in the presence of a thousand-fold molar excess of unlabeled BK.

2.4. *Measurement of inositol phosphate production*

Fibroblasts were incubated for 48 h with 0.5 μCi/ml myo-[2-^{3}H]inositol in medium 199 (Seromed, Berlin, FRG) containing 0.1% FCS. Thereafter, supernatants were aspirated and cells were washed 3 times at 37°C with Hanks' buffered salt solution (HBSS) including 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.3. Cells were then allowed to equilibrate in the same solution containing 10 mM LiCl at 37°C for 15 min to block inositol-1-phosphatase action. Peptides were then added for 60 s in a fresh 1 ml volume of medium per dish. The reaction was terminated by replacing the medium with 600 μl/dish of 10% (w/v) ice-cold trichloroacetic acid (TCA), and freezing the dish on dry-ice. Thawed cells plus supernatant were centrifuged for 10 min at 700 × *g*. Supernatants were then extracted 6 times with 6 ml of water-saturated diethylether to remove TCA. Aliquots of the aqueous phase containing the water-soluble [^{3}H]inositol metabolites were applied on Dowex 1 × 8 anion exchange columns for separation of inositol phosphates as described by Griffin and Hawthorne [13].

2.5. *Determination of cellular cAMP*

Confluent cells were incubated with HBSS including 200 μM isobutylmethylxanthine and stimulated with or without various concentrations of peptides for 7 min at 37°C, pH 7.4. The reaction was terminated by aspirating the medium, the addition of 1 ml of 5% (w/v) ice-cold TCA and placing the dish on dry-ice. After thawing, the supernatant was extracted 5 times with 10 ml of diethylether and aliquots were assayed for cAMP content using the radioimmunoassay procedure of Amersham (Braunschweig, FRG).

3. RESULTS

Kinin receptors in cultured human fibroblasts have been tentatively characterized as a B2 subtype by the criterion of typical rank order of potency of agonists [10]. This notion is substantiated by the data shown in fig.1A. In the solubilized preparation, the prototypic B1 antagonist DesArg[9][Leu[8]]-BK at a concentration of up to 1 μM was ineffective in displacing [^{3}H]-BK, whereas the typical B2 antagonist D-Arg[0][Hyp[3], Thi[5,8],D-Phe[7]]-BK was effective in concentrations equalling those of native BK. In this B2 receptor system, [Hyp[3]]-BK and [Hyp[3]]-Lys-BK were able to displace [^{3}H]-BK from solubilized binding sites of cultured human fibroblasts in a dose-dependent manner, being at least equipotent with BK and Lys-BK (fig.1B).

After 48 h of a prior incubation of cells with 0.5 μCi/ml myo-[2-^{3}H]inositol, the incorporation of radioactivity averaged 20 ± 5%. Determination of membrane-bound [^{3}H]inositol in the cell pellet showed between 30% (BK-stimulated) and 60% (non-stimulated) of cellular radioactivity, indicating BK-induced hydrolysis of membrane-associated phospholipids. The remaining intracellular water-soluble [^{3}H]inositol fractions were further investigated. The magnitude of the accumulation and the proportions of the 3 inositol metabolites relative to each other were dependent on the concentration of the agonists applied and on the time of stimulation (data not shown). Further experiments indicated, that after 45–60 s of stimulation with kinins, IP_3 accumulation reached a

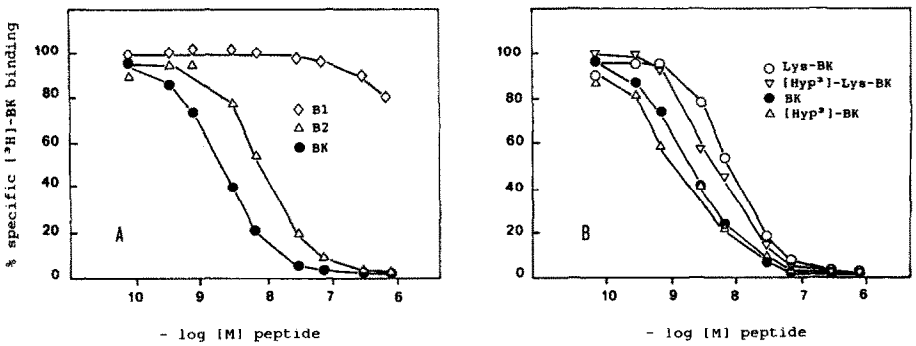

Fig.1. Displacement of specific [^{3}H]BK binding to solubilized binding sites from human fibroblasts by agonists and antagonists. (A) Effects of BK receptor antagonists. B1 = DesArg[9][Leu[8]]-BK; B2 = D-Arg[0][Hyp[3],Thi[5,8],D-Phe[7]]-BK. (B) Effects of BK, Lys-BK and [Hyp[3]]-kinins. Incubations were carried out with 2 nM [^{3}H]-BK, and the indicated concentrations of agonists and antagonists. Solubilization and binding experiments were performed as described in section 2. Data are expressed as means of triplicate cultures of a representative experiment.

Table 1

Effect of bradykinin (BK) and BK analogues on accumulation of water-soluble inositol phosphate metabolites

Peptides	[^{3}H]IP	[^{3}H]IP$_2$	[^{3}H]IP$_3$
None (buffer)	5415 ± 516	546 ± 119	483 ± 129
Bradykinin	16672 ± 1070	14081 ± 442	4386 ± 213
Met-Lys-BK	14547 ± 1760	12040 ± 1519	4408 ± 512
[Hyp^3]-BK	13372 ± 1923	11971 ± 1480	3973 ± 405
[Hyp^3]-Lys-BK	15263 ± 1115	12758 ± 2061	4971 ± 182
Tyr^8-BK	8973 ± 307	5029 ± 197	2830 ± 79
Tyr^5-BK	7418 ± 1121	1616 ± 163	1071 ± 170
DesArg^9-BK	6522 ± 1565	2196 ± 440	1432 ± 395
Angiotensin II	6069 ± 2012	2558 ± 1028	1150 ± 420

Prelabeled cells were stimulated for 60 s at 37°C with 0.1 μM of the peptides, except for DesArg^9-BK and angiotensin II, which were used at a concentration of 10 μM to test for non-specific effects. Inositol phosphates were separated and measured as described in section 2. Numbers represent dpm ^{3}H/dish as the mean ± SE of triplicate cultures

maximum which was not superceded after 5 min. At 60 s, the largest specific increases observed were for the IP_2 and IP_3 fractions (table 1). A variety of BK analogues were studied for their potency to elicit a PI response at 60 s stimulation time (table 1). [Hyp^3]-BK and [Hyp^3]-Lys-BK led to a pronounced accumulation of inositol phosphates, as did the non-hydroxylated kinins BK and Met-Lys BK. Tyr^8-BK and Tyr^5-BK were less effective. DesArg^9-BK and angiotensin II, serving as controls in the B2 system had only minimal effects, even at a concentration of 10^{-5} M.

In addition to phospholipase C activation, the effect of the hydroxylated kinins on phospholipase A_2-dependent signal generation was investigated. Kinin-induced cAMP accumulation was measured by radioimmunoassay. Stimulation by [Hyp^3]-BK and [Hyp^3]-Lys-BK resulted in an up to 16-fold increase of cAMP levels above control values in intact cells (table 2). At 0.5 μM of kinin concentration, no significant differences between hydroxylated and the corresponding non-hydroxylated derivatives were observed. Again, DesArg^9-BK was ineffective.

Table 2

Effect of bradykinin and BK analogues on cAMP production

Peptides	pmol cAMP/mg protein
None (buffer)	21
Bradykinin	367
Lys-BK	368
[Hyp^3]-BK	317
[Hyp^3]-Lys-BK	334
DesArg^9-BK	17

Confluent monolayers of human fibroblasts were incubated with HBSS containing 0.5 μM of the indicated peptides for 7 min at 37°C. The reaction was terminated and cAMP content measured as described in section 2. Values represent means of duplicate determinations

4. DISCUSSION

The recent identification of hydroxylated kinins from human sources [3–6] has raised several questions. Hydroxylation of the kinin acceptor sequence of Pro-Pro-Gly is postulated to occur within the kininogen molecule due to posttranslational modification by prolyl-4-hydroxylase [6,7,14]. This enzyme hydroxylates Xaa-Pro-Gly sequences within procollagen, where Xaa is typically proline or alanine [15]. In vitro studies have demonstrated that the 'free' peptides BK [16] and Lys-BK [17] serve as substrates for this enzyme, but whether this holds true for in vivo situations is presently unknown. From data on the action of [Hyp^3]-kinins on muscle contraction [7,8] one has to postulate that these compounds exert their effects through specific receptors. In this report we have investigated specific binding properties and signal transduction processes of the hydroxylated kinins in cultured human foreskin fibroblasts, a B2 receptor system.

From our data it can be concluded that the hydroxylated kinins have binding properties with the B2 receptor similar to the non-hydroxylated kinins. Possible interactions of these compounds with B1 receptors in other cell types remain to be established. Quantitative assessment of phosphoinositol responses to a variety of BK analogues including hydroxylated kinins showed correlation with their capability to displace [^{3}H]-BK binding.

These results are consistent with the hypothesis, that hydroxylated kinins are capable of activating both phospholipase C-dependent PI responses and phospholipase A_2-mediated cAMP generation with similar efficiency as native BK. Hence, other possible physiological implications of kinin hydroxylation, e.g. on tissue distribution or elimination kinetics of the modified ligands, have to be considered to elucidate the role of posttranslational kinin modification.

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