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Agonist stimulation of B_1 and B_2 kinin receptors causes activation of the MAP kinase signaling pathway, resulting in the translocation of AP-1 in HEK 293 cells

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Abstract In response to bradykinin, phosphorylated MAP kinases (ERK-1 and ERK-2) were abundantly increased in HEK 293 cells, which overexpress the rat B_2 kinin receptor. In a similar way des-Arg^9-bradykinin stimulation of B_1 kinin receptor-overexpressing HEK 293 cells caused activation of the same species of MAP kinase. Furthermore, nuclear translocation of transcription factor AP-1 was also found in the cells after stimulation with either agonist. PD98059, a MAP kinase kinase (MEK-1) inhibitor, blocked the agonist-induced AP-1 translocation as well as the phosphorylation of the MAP kinases. This communication provides the first evidence for both B_1 and B_2 kinin receptors mediating the MAP kinase signaling pathway to activate AP-1.

Key words: Bradykinin; B_1 kinin receptor; Activator protein-1; Mitogen-activated protein kinase

1. Introduction

Kinins, which are peptides released from high-molecular-weight precursor kininogens by limited proteolysis, are known to exert a variety of physiological and pathological effects, including smooth muscle contraction, vasodilation, vascular permeability increase, and pain production [1–3]. The existence of two subtypes of kinin receptors, B_1 and B_2, has been proposed based on pharmacological grounds [4,5], and this has been substantially supported by cDNA cloning and expression [6,7]. The B_2 kinin receptor is constitutively expressed in a variety of tissues and has a high binding affinity for bradykinin (BK) and for Lys-BK (kallidin), but not for their respective carboxypeptidase degradation products, des-Arg^9-BK and des-Arg^{10}-kallidin [8]. The B_1 kinin receptor, in contrast, is expressed de novo under certain pathological conditions, and exhibits no affinity for BK or kallidin but has a high affinity for des-Arg^9-BK and des-Arg^{10}-kallidin [9]. The two receptors are known as members of the family of G-protein coupled, transmembrane seven-span proteins [10], and the signaling leads to inositol phosphate activation to increase intracellular Ca^{2+} [11]. However, precise characterization of the signaling pathways after agonist stimulation is lacking, especially in B_1 receptor-mediated signaling. There are some papers showing activation of the mitogen-activated protein (MAP) kinase pathway by BK through the B_2 kinin receptor to cause phosphorylation of cytosolic phospholipase A_2 [12] and transactivation of the epidermal growth factor receptor [13]; however, there is no information on the B_1 receptor. Transcription factor activator protein-1 (AP-1) is known to be activated not only by c-Jun N-terminal kinase (JNK) but also by the MAP kinase pathway [14]. Involvement of the MAP kinase pathway in AP-1 activation has been described in the case of angiotensin II receptors, but there is no information on kinin receptors in this regard [15].

We recently reported that des-Arg^9-BK induced hypotension via the B_1 kinin receptor, which was induced by lipopolysaccharide treatment in rats [16]; and induction of contraction of isolated rat ileum by des-Arg^9-BK allowed partial characterization of the receptor [17,18]. Recently we isolated and cloned rat cDNA of B_1 and B_2 receptors and overexpressed them in HEK 293 cells. In the study communicated here we characterized and compared B_1 and B_2 receptors in these cells, especially focussing on the cellular signalling pathway after agonist stimulation.

2. Materials and methods

2.1. Materials

Bradykinin, des-Arg^9-bradykinin, D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]BK and Hoe 140 were purchased from the Peptide Institute (Osaka). Des-Arg^{10}-Hoe 140 was from Research Biochemicals (Natick, MA). PD98059 was from Biomol (Plymouth Meeting, PA). Anti-phosphorylated MAP kinase and JNK antibodies were purchased from Promega (Madison, WI), rabbit polyclonal antibodies against Fos/Jun (anti-c-Fos, anti-c-Jun, anti-JunB, anti-JunD), from Santa Cruz Biotechnology (Santa Cruz, CA), and [^{3}H]des-Arg^{10}-[Leu^9]-kallidin and [^{3}H]BK from DuPont-New England Nuclear (Boston, MA). FR190997 [19] was generously donated by Fujisawa Pharmaceutical Co., Tsukuba Research Labs (Ibaraki). Des-Arg^9-NPC17731 was a gift from Dr. D.J. Kyle (Scios Nova, Sunnyvale, CA).

2.2. Stable expression of rat B_1 or B_2 kinin receptors in HEK 293 cells

cDNAs for rat B_1 and B_2 kinin receptors were obtained by means of the polymerase chain reaction using Sprague-Dawley rat genomic DNA as a template. Primers (B_2: sense 5′-GAAATGTTCAACATCACCACGC-3′, antisense 5′-GATGGCTTGTGTTCACTGCTTGTT-3′, B_1: sense 5′-ATGGCGTCCGAGGTCTTGTT-3′, antisense 5′-TTATAAAGTCCCCAGAACC-3′) were synthesized according to the nucleotide sequence of rat B_1 and B_2 kinin receptors reported by Chao et al. [20,21]. The obtained DNAs were subcloned into the pCR 3.1 expression vector (Invitrogen, San Diego, CA) and sequenced by the dideoxy method. The plasmid DNA transfection of human embryonic kidney cells (HEK 293) was performed by the use of Cell Fectin (Life Technologies, Grand Island, NY) and the cells showing the highest expression of B_1 or B_2 kinin receptor mRNAs

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Abbreviations: BK, bradykinin; AP-1, activator protein-1; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases

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were selected among G418-resistant transformants (HEK 293/B_1 or HEK 293/B_2).

2.3. Agonist stimulation of B_1 or B_2 kinin receptor-overexpressed cells

HEK 293, HEK 293/B_2 or HEK 293/B_1 cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA). After an 18 h incubation in 4 ml of serum-free RPMI 1640 medium (1×10^5 cells/60 mm dish), stimulation was started by the addition of BK or des-Arg^9-BK to the medium and terminated by removing the medium by aspiration, followed by the addition of 1.4 ml of ice-cold phosphate-buffered saline. In the case of the experiment using a MAP kinase inhibitor, the cells were incubated with the inhibitor, PD98059, at 37°C for 20 min, and then BK or des-Arg^9-BK was added.

2.4. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides containing consensus sequences were radiolabeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL) at their 3′ end with a Klenow fragment and then purified with a MicroSpin column (Pharmacia, Uppsala). The sense sequences of synthesized oligonucleotides used were as follows: AP-1, 5′-CGCTTGATGATGCAGC-CGGAA-3′; mutated AP-1, 5′-CGCTTGACGC-AATCGCCGGAA-3′; NF-κB, 5′-TTAACAGAGGGGACTTTCC-GAG-3′; and CREB, 5′-AGAGATTGCCTGACGTCAGAGAGC-TAG-3′.

Nuclear extracts of the cells were prepared as follows: the suspended cells in 200 μl of ice-cold buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin; pH 7.9) were incubated for 10 min on ice, Nonidet P-40 was added to the suspension at a final concentration of 0.4% (v/v) and the mixtures were vortexed vigorously for 10 s; and then the nuclei were pelleted by centrifugation at $15\,000\times g$ for 1 min. The pellets were suspended in 30 μl of buffer C (20 mM HEPES, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin; pH 7.9) by vigorous mixing for 5 min at 4°C. After centrifugation at $10\,000\times g$ for 5 min, the supernatants were used as nuclear extract. The nuclear extracts were incubated with 1 ng of radiolabeled AP-1 or NF-κB oligonucleotide in binding buffer (10 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 0.7 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, 10% (v/v) glycerol, 1 μg/ml poly(dI-dC)·poly(dI-dC), pH 7.9) for 30 min at 25°C. For competition assay, 100-fold excess of unlabeled oligonucleotide was added to the binding reaction prior to the addition of the radiolabeled probe. For supershift analysis, 1 μg of the indicated antibodies were added following the binding reaction for 1 h at 4°C. Then these incubation mixtures were electrophoresed in 4.5% native polyacrylamide gels with Tris borate-EDTA buffer. The gels were dried and analyzed with a BAS2000 (Fuji Photo Film, Kanagawa).

2.5. SDS-PAGE/immunoblotting

The cells at a density of 5×10^6 cells/ml in PBS containing 0.1% SDS were lysed, applied to SDS-polyacrylamide gels, and electrophoresed as previously reported [22]. Then the proteins were electroblotted onto nitrocellulose membranes. The membranes were blocked for 1 h in 10 mM TBS containing 0.1% Tween-20 (TBS-T) and 3% skim milk. After the membranes had been washed with TBS-T, antibody against phosphorylated MAP kinase was added at a 1:4000 dilution in TBS-T, and incubation was carried out for 40 min. After washing of the membranes with TBS-T, horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) was added at a 1:10 000 dilution in TBS-T, and the membrane was incubated for 40 min. After a final washing of the membranes with TBS-T, protein bands were visualized with an ECL Western blot analysis system (Amersham).

3. Results and discussion

3.1. Functional properties of the B_1 and B_2 kinin receptors in receptor-overexpressed cells

Nucleotide sequences of the rat B_2 kinin receptor gene obtained here from the Sprague-Dawley rat as well as from the Brown Norway rat were completely homologous with the published sequence [20], whereas B_1 receptor cDNAs obtained from both strains diverged from the GenBank-registered sequence [21] at positions 310, 311, and 314, where bases G, C, and A were found instead of C, A and G, respectively. This resulted in a change in the amino acid at position 104 from Q to A and at position 105 from C to E.

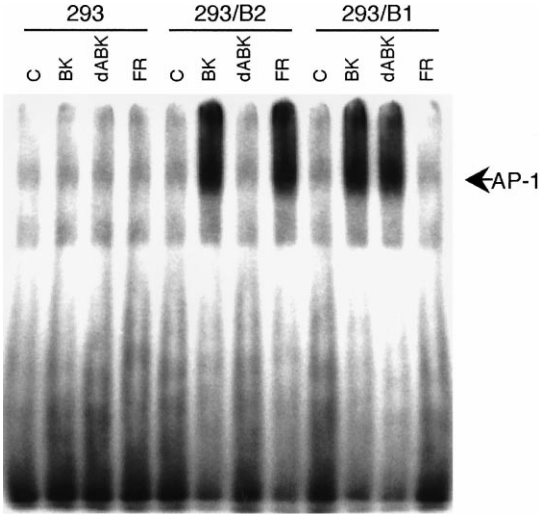

Fig. 1. Translocation of AP-1 in the B_1 or B_2 receptor-overexpressed HEK 293 cells by the activation with BK or des-Arg^9-BK. Untransfected HEK 293 cells (293), transfected HEK 293 cells with B_2 receptor (293/B2) or transfected HEK 293 cells with B_1 receptor (293/B1) were stimulated with BK (1 μM, BK), des-Arg^9-BK (1 μM, dABK), FR190997 (1 μM, FR) or vehicle (lane C) for 120 min. Nuclear extracts were prepared from the cells and subjected to EMSA as described in Section 2. Patterns representative of those found in three independent experiments are shown.

To confirm the functional properties of the B_1 and B_2 kinin receptors thus obtained, we prepared cells overexpressing these receptors (HEK 293/B_1 and HEK 293/B_2, B_1 and B_2 receptors, respectively) and examined them for their ligand binding and ligand induced calcium influx (data not shown). HEK 293/B_1 and HEK 293/B_2 cells each showed receptor-specific ligand binding ([^{3}H]des-Arg^{10}-[Leu^9]-kallidin for B_1 receptor and [^{3}H]BK for B_2 receptor) and also responded with an increase in intracellular calcium when stimulated with each receptor-specific ligand (des-Arg^9-BK for B_1 receptor and BK for B_2 receptor). These agonist-induced increases in intracellular calcium were inhibited by the corresponding receptor-specific antagonists, i.e. des-Arg^9-NPC17731 for the B_1 receptor and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]BK for the B_2 receptor. These binding properties and intracellular calcium response are mostly compatible with observations made on human B_2 or B_1 kinin receptor-overexpressed cells [23,24]. Thus HEK 293/B_1 and HEK 293/B_2 cells were proved to express the agonist-stimulated functions through each receptor type.

3.2. Agonist-induced translocation of AP-1 in the B_1 or B_2 receptor-overexpressed cells

Using these receptor-overexpressed cells, we examined the signal transduction pathway after B_1 and B_2 receptor stimulation by a specific agonist. First of all we determined whether transcription factors AP-1 and NF-κB were activated. Untransfected HEK 293 cells showed no increased AP-1 binding activity, but B_2 receptor stimulation with BK but not with des-Arg^9-BK induced translocation of AP-1 into the nuclear

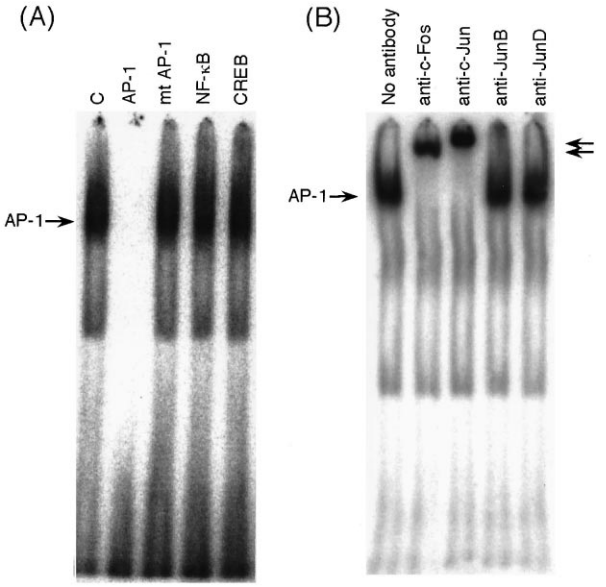

Fig. 2. Effect of cold competitors and antibodies on the pattern of AP-1 complex in EMSA. Nuclear extracts were prepared from HEK 293/B_1 cells stimulated by des-Arg^9-BK (1 μM, 120 min), incubated for 30 min with or without 100-fold excess of unlabeled AP-1, mutated AP-1 (mt AP-1), NF-κB, or CREB probes (A). Antibodies (1 μg) were added following the binding reaction for 1 h at 4°C (B), and then assayed for AP-1 binding activity as described in Section 2. Arrows to the right indicate the specific supershifted complexes. Similar results were obtained in two other independent experiments.

fraction in HEK 293/B_2 cells; and also stimulation of HEK 293/B_1 cells with des-Arg^9-BK showed this translocation (Fig. 1). However, stimulation with not only des-Arg^9-BK but also BK showed increased AP-1 binding activity in HEK 293/B_1 cells that expressed only B_1 receptor, and this activation by BK was inhibited by a B_1 antagonist. By a preliminary experiment, HEK 293/B_1 cells did not express B_2 agonist binding, i.e. [^{3}H]BK binding or displacement of [^{3}H]des-Arg^{10}-[Leu^9]-kallidin with BK (data not shown). Therefore, the action of BK on the B_1 receptor may be explained by the degradation of BK to des-Arg^9-BK during the 120 min incubation, with the latter acting on the B_1 receptor. That is, the increased AP-1 binding may not be a consequence of the direct action of BK itself. BK is well known to be labile in biological fluids, being cleaved in a few minutes by peptidases on the cell membrane [8]. Then we examined the effect of the novel B_2 agonist FR190997, which was reported to be a non-peptide stable agonist [19]. This compound induced AP-1 binding activity only in HEK 293/B_2 cells but not HEK 293/B_1 cells (Fig. 1). We could not find any NF-κB activation in these cells (data not shown).

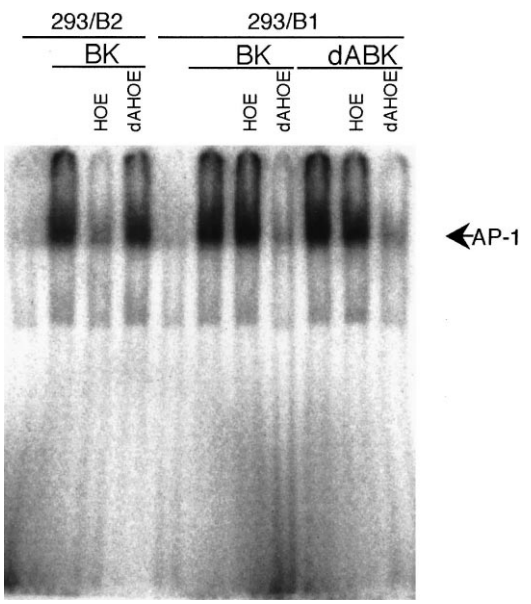

Fig. 3. Effect of B_1 and B_2 receptor antagonists on the translocation of AP-1 in HEK 293 cells following the activation with agonist. HEK 293/B_2 or HEK 293/B_1 cells were pretreated with Hoe 140 (3 μM, HOE), a B_2 antagonist, or with des-Arg^{10}-Hoe 140 (3 μM, dAHOE), a B_1 antagonist, and then stimulated with BK (1 μM) or des-Arg^9-BK (1 μM, dABK) for 120 min. Nuclear extracts were prepared from the cells and subjected to EMSA as described in Section 2. Results representative of those found in three independent experiments are shown.

3.3. *Detection of nuclear factors bound to the AP-1 consensus oligonucleotide by supershift analysis*

Competitive and supershift analyses were performed with nuclear extracts prepared from des-Arg^9-BK-stimulated HEK 293/B_1 cells. The AP-1 complex was specifically competed by the 100-fold addition of unlabeled AP-1 probe, but not by the 100-fold addition of NF-κB, CREB, or mutated AP-1 probes (Fig. 2A). To identify nuclear proteins that interacted with the AP-1 consensus oligonucleotide, supershift analyses were performed by means of specific antibodies against Jun/Fos families. The c-Jun and c-Fos antibodies effectively supershifted the protein-DNA complex (indicated by arrows to the right of the EMSA in Fig. 2B), although antibodies to either JunB or JunD did not result in a discernible supershift. Similar result was observed in BK-stimulated HEK

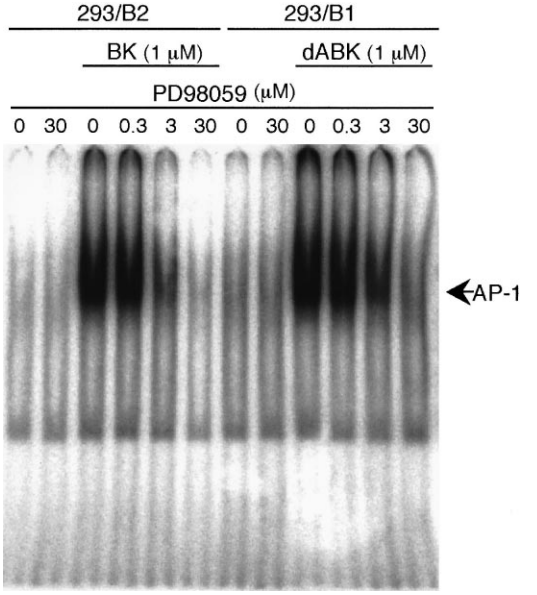

Fig. 4. Effect of MEK inhibitor PD98059 on the translocation of AP-1 in HEK 293 cells following activation with agonist. HEK 293/B_2 or HEK 293/B_1 cells were pretreated with PD98059 (0.3, 3, 30 μM) and then stimulated with BK (1 μM) or des-Arg^9-BK (1 μM, dABK) for 120 min. Nuclear extracts were prepared from the cells and subjected to EMSA as described in Section 2. Similar results were found in two other independent experiments.

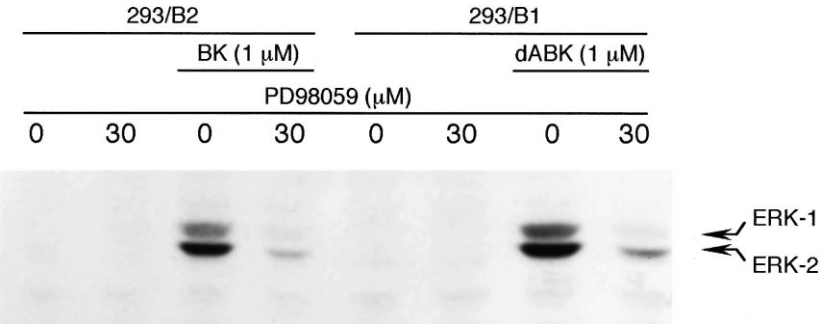

Fig. 5. Immunoblot analysis of phosphorylated ERK in HEK 293 cells following activation with agonist. HEK 293/B_2 or HEK 293/B_1 cells were stimulated with BK (1 μM) or des-Arg^9-BK (1 μM, dABK) for 5 min in the absence or presence of PD98059 (30 μM), and lysates were then processed for immunobloting as described in Section 2. Another independent experiment gave similar results.

293/B_2 cells (data not shown). These results demonstrate that c-Jun/c-Fos heterodimers are major components of the AP-1 consensus oligonucleotide binding complex activated by agonist-stimulation of B_1 and B_2 kinin receptors.

3.4. *Effect of B_1 and B_2 antagonists on the agonist-induced AP-1 activation*

Pretreatment with B_2 antagonist Hoe 140 but not with B_1 antagonist des-Arg^{10}-Hoe 140 inhibited BK-induced AP-1 activation in HEK 293/B_2 cells. On the other hand, des-Arg^9-BK and BK-induced AP-1 activation in HEK 293/B_1 cells was inhibited by the pretreatment with des-Arg^{10}-Hoe 140, but not with HOE 140 (Fig. 3). These results indicate that BK-induced AP-1 activation in HEK 293/B_2 cells is mediated by the B_2 receptor and that des-Arg^9-BK and BK-induced AP-1 activation in HEK 293/B_1 cells occurs via the B_1 receptor, with BK acting as des-Arg^9-BK on the B_1 receptor for the above-stated reason. Then, we studied further the signal transduction of these two receptors.

3.5. *Involvement of MAP kinase pathway in agonist-induced AP-1 activation*

We examined the possible involvement of MAP kinases in the AP-1 activation by B_1 and B_2 receptors. As shown in Fig. 4, pretreatment with PD98059, a MAP kinase kinase MEK-1 inhibitor [25,26], dose-dependently suppressed the AP-1 activation in both HEK 293/B_1 and HEK 293/B_2 cells when the cells were stimulated with des-Arg^9-BK and BK, respectively.

Further, we explored factors in the MAP kinase pathways and found activation of MAP kinases ERK-1 and ERK-2, as shown in Fig. 5. Phosphorylated ERK-1 and ERK-2 were detected in the HEK 293/B_1 and HEK 293/B_2 cells by immunoblotting with antibody against phosphorylated ERKs following a 5 min stimulation with each agonist. Pretreatment with PD98059 also suppressed ERK activation in both types of cells, suggesting that agonist stimulation of B_1 and B_2 receptors causes activation of ERK-1 and ERK-2 in the MAP kinase pathway via MEK-1. However, phosphorylated JNK-1 and JNK-2 were not detected, either before or after the cells were stimulated with agonist (data not shown). These results suggest that the MAP kinase ERK but not JNK, could be involved in B_1 and B_2 receptor-mediated AP-1 activation. Huang et al. reported that microinjection of an ERK-specific substrate peptide into mouse epidermal cells inhibited the induction of AP-1 activity and that the overexpression of ERK-1 augmented AP-1 activation in their evaluation of the role of ERKs in tumor promotion [27]. Therefore, based on current information a possible pathway could be the following: phosphorylated ERKs may activate AP-1 through phosphorylation of TCF/EIK-1 and thereby induce c-Fos synthesis or act indirectly through unknown downstream mediators; but further precise study is necessary to clarify the mechanism of the activation of AP-1.

In this study we demonstrated that both B_1 and B_2 receptor activation by agonists caused AP-1 activation in cells overexpressing either receptor, but could not find any trace of NF-κB activation during the time period examined. However, there is a report showing bradykinin-induced NF-κB activation in human fibroblasts that led to IL-1 gene expression [28]. This discrepancy may be attributable to different cell types or animal species of receptors. With respect to AP-1 activation, stimulation of various autacoid receptors, such as those for endothelin and angiotensin II, has been reported to induce AP-1 activation through the MEK/ERK signaling pathway. Although BK was already known to activate MAP kinase pathway through the B_2 kinin receptor [12,13], there had been no information on the B_1 receptor in this regard until now. We have demonstrated herein that both B_1 and B_2 receptors activate AP-1 through the MAP kinase pathway, suggesting that agonist stimulation of these receptors could contribute to the expression of AP-1-dependent genes and thus play a crucial role in physiological and pathological effects. The fact that this pathway was activated similarly through both B_1 and B_2 receptors may suggest a similar functional activity of these two receptors, although these receptors are expressed under biologically different circumstances [4,29].

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