Potentiation of bradykinin actions by analogues of the bradykinin potentiating nonapeptide BPP$_{9\alpha}$

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Abstract

Synthetic analogues of the bradykinin potentiating nonapeptide BPP$_{9\alpha}$ indicate significantly different structural requirements for potentiation of the bradykinin (BK)-induced smooth muscle contraction (GPI) and the inhibition of isolated somatic angiotensin I-converting enzyme (ACE). The results disprove the ACE inhibition as the only single mechanism and also the direct interaction of potentiating peptides with the bradykinin receptors in transfected COS-7 cells as molecular mechanism of potentiation. Our results indicate a stimulation of inositol phosphates (IP$_n$) formation independently from the B$_2$ receptor. Furthermore, the results with La^{3+} support the role of extracellular Ca^{2+} and its influx through corresponding channels. The missing effect of calyculin on the GPI disproves the role of phosphatases in the potentiating action. These experimental studies should not only contribute to a better understanding of the potentiating mechanisms but also incorporate a shift in the research towards the immune system, in particular towards the immunocompetent polymorphonuclear leukocytes. The chemotaxis of these cells can be potentiated most likely by exclusive inhibition of the enzymatic degradation of bradykinin. Thus the obtained results give evidence that the potentiation of the bradykinin action can occur by different mechanisms, depending on the system and on the applied potentiating factor.
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Abbreviations: AA, arachidonic acid; ABA, 4-azidobenzoic acid; ACE, angiotensin I-converting enzyme; Aloc, allyl oxycarbonyl; ASA, 4-azidosalicylic acid; BK, bradykinin; BKR, bradykinin receptor; BKR-B$_1$, bradykinin B1 receptor; BKR-B$_2$, bradykinin B2 receptor; Boc, *tert*-butyloxycarbonyl; BPA, *p*-benzoylphenylalanine; BPP, bradykinin potentiating peptide; BPP$_{9\alpha}$, bradykinin potentiating peptide 9α (Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro); BOP, benzotriazole-1-yl-oxy-tris (dimethylamino) phosphonium hexafluorophosphate; DCM, dichloromethane; Dde, *N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; Ddz, α,α-dimethyl-3,5-dimethoxy-benzyloxycarbonyl; DEAE, diethylaminoethyl; DIEA, diisopropylethylamine; DIC, di-isopropylcarbodiimide; DMEM, Dulbecco's modified Eagle's medium; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; DTE, dithioerithritol; ED, effective dose; Fmoc, 9-fluorenylmethyl oxycarbonyl; εAbu(ßPhe), erythro-α-amino-ß-phenyl-butyric acid; FR190997, 8-[2,6-dichloro-3-[*N*-(*E*)-4-(*N*-methylcarbamoyl)cinnamidoacetyl]-*N*-methylamino]benzyloxy]-2-methyl-4-(2-pyridyl-methoxy)quinoline; GPI, guinea pig ileum; HOAt, 1-hydroxy-7-azabenzotriazole; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylguanidinium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HOCr, hydroxycrotonic acid; HYCRAM, hydroxycrotonyl amidomethyl linker; IP$_3$, inositol 1,4,5-trisphosphate; IP$_n$, inositol phosphates; J526, Pyr-Trp-Pro-Lys(ASA)-Pro-Gln-Ile-Pro-Pro; J527, Pro-Trp-Pro-Lys-Pro-Gln-Ile-Pro-Pro; J725, DArg-Arg-Pro-Hyp-Gly-Thi-Ser-Pro-εAbu(ßPh)-Arg; MEM, Eagle's minimal essential medium; Mtr, methoxytrimethylbenzene sulphonyl; Pd^0, palladium *tetrakis* triphenylphosphine; PMN, polymorphonuclear leukocytes (neutrophils); Ram, ramiprilat; TBTU, 2-(1H-benzotriazol-1-yl)1,1,3,3-tetramethylguanidinium tetrafluoroborate; TFA, trifluoroacetic acid; Trt, triphenylmethyl

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1. Introduction

Hormone actions can be potentiated by different factors interacting with the receptor, by enzymatic degradation or by signal pathways. However the entire overall process has been studied in detail for only very few hormones [30,69,45].

At least 40 years ago a potentiating action was observed for the nonapeptide hormone bradykinin (BK). Indeed the history of BK isolation and characterization has long been closely related to the use of potentiating factors. Werle and coworkers [74] used snake venoms to trigger the formation of BK from plasma and to describe this tissue hormone functionally. Immediately after the isolation, chemical characterization, synthesis and functional characterization, certain snake venoms were described as bradykinin potentiating compounds. Kato and Suzuki [39,40], Ferreira et al. [26,27], and Ondetti et al. [13,59] isolated different oligopeptides with bradykinin potentiating activity from the venoms of the two snakes *Agkistrodon halys blomhoffii* and *Bothrops jararaca*, including the bradykinin potentiating nonapeptide $BPP_{9\alpha}$ (trade name TEPROTIDE).

Bradykinin potentiating peptides have also been isolated from other snakes [8,9,23–25,35] or other toxins [3,21,22,44,52,73] as well. Very recently new potentiating peptides have been isolated from the venom of *B. jararaca* [36]. Surprisingly peptides with potentiating activity have also been formed by the partial hydrolysis of proteins taken from serum [80], hemoglobin [38,66,81], milk [34,46], or wheat germ [51]. Also degradation fragments of angiotensin such as the heptapeptide 1–7 were found to potentiate the BK action [64]. In addition linear BK analogues, partial sequences [7], certain active and inactive side chain and backbone cyclic agonists are able to potentiate the BK action on GPI [68].

The angiotensin I-converting enzyme (ACE) cleaves dipeptides from the C-terminus of angiotensin I and bradykinin resulting on the one hand in the formation of the highly hypertensive hormone angiotensin II and on the other hand in the inactivation of the hypotensive BK. This enzyme has been extensively studied because of these important functions in the blood pressure regulation. ACE contains a N-terminal as well as a C-terminal catalytic domain, described in most publications as having only slight differences in their structural requirements [14,79]. More recently Cotton et al. demonstrated in an excellent investigation using domain-specific substrates and inhibitors, affinity differences between N- and C-terminal catalytic domains of about three orders of magnitude [11].

The membrane bound form of this enzyme seems to play an important role in the potentiation of the BK action. Inhibitors of this enzyme are used as drugs for treatment of different forms of hypertension and heart failure. For therapeutically used hormones this knowledge about potentiating compounds and their action mechanisms is very important. This knowledge can help to improve the therapeutic effect, to prevent not only an excessive dose, but also interaction with other drugs and side effects. On the other hand the therapeutic use of potentiating compounds requires the knowledge about the interaction with the hormone action on the molecular basis as well. As shown by Li et al. ACE may also act as a receptor for SARS coronavirus [47].

With extensive therapeutical application of bradykinin potentiating compounds such as captopril [12], enalaprilat [64], ramiprilat [75], quinaprilat [41] and lisinopril [6], studies of the molecular action mechanism have become more and more important. Many other proteases are also able to inactivate BK including the neutral endopeptidase (NEP, Neprolysin; E.C. 3.4.24.11) [42,71], metalloendopeptidase (E.C. 3.4.25.15/16) [55,58], aminopeptidase P (E.C. 3.4.11.9) [67], aminopeptidase N (E.C. 3.4.11.2) [63] and carboxypeptidase M (E.C. 3.4.17.12) [72].

Potentiation of BK action has also been studied in in vivo models by pharmacological tests on isolated organs and on the cellular level by biochemical methods. In the in vivo models, potentiation of BK action has been measured on the pressor response to intravenous BK in conscious rabbits [68] or on the hypotensive effect in freely moving Wistar rats [65]. Isolated organs such as the guinea pig ileum (GPI) [54], rat heart [34], rabbit jugular vein [17,33], cerebral microvasculature (estimation of permeability) [58] and porcine coronary arteries [76] have been used for in vitro tests.

Yet even at the onset of the search for the molecular mechanism of BK potentiation, certain contradictory findings have been observed. The inhibition of ACE by various peptide and nonpeptide compounds did not correlate well with the potentiating activity [5,10]. Furthermore, the maximum of the BK-induced contraction of guinea pig ileum can be enhanced by potentiating compounds [77]. Also the action evoked by enzymatically stable BK agonists can be potentiated in some test systems [78]. Repeated exposure of porcine coronary arteries to BK has led to receptor desensitization. The addition of the potentiating compounds quinaprilat or angiotensin 1–7 fully restored the relaxant effect at a point when BK alone was no longer able to induce relaxation [76]. At the molecular level the co-immunoprecipitation of ACE and the B_2 receptor with an anti receptor antibody clearly indicates an interaction of both partners on the cell membrane [50]. Despite all these contradictory and to some degree confusing findings Regoli and coworkers [29] and Dendorfer et al. [17] have demonstrated that in their test systems (rabbit isolated aorta and venoconstriction) the potentiation by therapeutically used ACE inhibitors results exclusively from the inhibition of enzymatic BK degradation. Nevertheless, the group of Regoli found under influence of ACE inhibitors, a resensitization of the rabbit jugular vein [31] and a change in the density of B_2 receptors in rat spinal cord [60].

To understand the molecular basis for these discrepancies many approaches have been undertaken to elucidate the influence of potentiating compounds on the different bradykinin destroying enzymes, on the bradykinin receptors (BKR) and on signal pathways.

Direct interaction of the potentiating compounds was postulated with the B_1 receptor [37]. Phosphorylation of the receptor by protein kinase C leads to internalization [19] (desensitization) and dephosphorylation by phosphatase SHP-2 to resensitization [18,49]. Bradykinin potentiating factors can influence both desensitization and resensitization, as well as the hetero-oligomerization of the B_2 receptor including the interaction with ACE [54] and other degrading enzymes [15]. At the level of signal pathways, a crosstalk with other pathways induced by other hormones [43] or nonreceptor-mediated intracellular reactions [28] have been observed.

As initial experimentation on the potentiation of the bradykinin action was primarily performed on isolated smooth muscle organs, in the last decade the potentiating activity was mainly investigated on the affinity and density of the receptor [54], the intracellular mobilization of Ca^{2+} [49,54], the release of arachidonic acid [49,54], of inositol phosphates [54], and of nitric oxide [37]. Later studies on the molecular basis of bradykinin-evoked actions have been performed more and more on cell cultures instead of smooth muscles. These studies have used primarily Chinese hamster ovary cells (CHO cells) cotransfected with the B_2 receptor and ACE, ACE-mutants or neutral endopeptidase (NEP) [15]. Endothelial cells have been used because they constitutively express the B_2 receptor and ACE [49].

The aim of our experiments was to contribute to a better understanding of the potentiating mechanisms, and to prove or disprove postulated action mechanisms. For these studies we used three different systems: the smooth muscle contraction, transiently transfected COS-7 cells and polymorphonuclear leucocytes. We began to find differences between ACE inhibition and potentiation of the BK action. We were interested in determining the role of Ca^{2+}-uptake from extracellular sources and its release from sarcoplasmatic reticulum for the potentiating action. We tested the direct interaction with the B_2 receptor in COS-7 cells and checked the influence of potentiating peptides on receptor dephosphorylation and selected signal transduction reactions.

Since BK plays an important role in inflammatory processes we studied the potentiation on immunocompetent polymorphonuclear leucocytes. However it was not only our aim to verify or negate the contradictory opinions regarding the mechanism of bradykinin potentiation, we would also like to show that there simultaneously exist different mechanisms, depending on the assay and on the potentiating factors used.

2. Methods

2.1. Synthesis of peptides

Boc-, Fmoc- and Ddz-amino acids were purchased from BACHEM (Switzerland) and ORPEGEN-Pharma (Germany). Boc-Pro-OCr-OH was synthesized according to the general procedure published by Gothe et al. [32]. To form the HYCRAM^{TM}-linker the hydroxycrotonic ester was coupled to the aminomethyl resin Tentagel-S-NH$_2$. The used resins were purchased from the following companies: amino methyl polystyrene, Tentagel-S-NH$_2$ and Wang resin from RAPP Polymere (Germany), and chlorotrityl resin from NOVA-Biochem (Germany). The peptides were synthesized on a PSS-80 automatic synthesizer (Applied Protein Technologies, USA) or on the semiautomatic synthesizer SP-650 (BACHEM, Switzerland). Each step was monitored by the Kaiser test. Couplings were carried out after neutralization by repeated washings (5–10 times) of the resin with 5% DIEA in DCM in a two-fold excess of N^α-protected amino acid and diisopropylcarbodiimide (DIC) in DCM for 4 h; 2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethylguanidinium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBt): Boc- or Fmoc- protected amino acids were used in a four-fold excess, solved in DMF or DCM/ DMF 1:1. TBTU and HOBt were applied in the same excess, diisopropylethylamine (DIEA) in a six-fold excess. The reaction time was between 4 and 12 h.

Deprotecting procedures: Boc-deprotection was performed with TFA/DCM 1:1 without any scavenger in two steps, 5 min treatment followed by washing with DCM and second treatment for 20 min; Ddz-deprotection was carried out with 5% TFA in DCM in two steps, 5 and 20 min; Fmoc-deprotection was achieved with 20% piperidine in DMF in two steps, 5 and 20 min.

2.2. Potentiation of the BK-induced contraction of GPI

The ileum (1.5 cm) of the guinea pigs was suspended in a 5 ml organ bath containing Tyrode solution at 37 °C. Isotonic contractions were recorded under a resting tension of 500 mg. BK was applied in a concentration of 1×10^{-8} M that corresponded to an ED_{25} to ED_{30} of BK under these experimental conditions. Generally this effect was increased twice by $BPP_{9\alpha}$ (20 nM) that was used as standard. The bradykinin potentiating peptides or the ACE inhibitors were given 5 min before application of BK. The potentiating effects of the analogues were calculated as per cent of the BK-induced contraction and compared with the effect caused by $BPP_{9\alpha}$ (=1.0 or 100%) in the same experiment.

2.3. Ca^{2+}-dependence of the potentiation of the BK-induced contraction of GPI

To investigate the influence of Ca^{2+} on the BK-potentiating action of $BPP_{9\alpha}$ (20 nM BK, 20 nM $BPP_{9\alpha}$), La^{3+} (1.8 mM) was used as an inhibitor of the Ca^{2+} influx in Ca^{2+}-free or Ca^{2+}-containing Tyrode solution. LaCl$_3$ was always given 1 min before or after the BK-induced contraction and before the application of $BPP_{9\alpha}$ (see Fig. 3).

2.4. Inhibition of phosphatase activity

Calyculin (100 nM) was used to estimate the involvement of BK-induced (20 nM BK) and potentiated (20 nM BK,

20 nM $BPP_{9\alpha}$) contraction of the guinea pig ileum. Calyculin was applied before $BPP_{9\alpha}$ was given at the maximum of the BK-induced contraction or 1 min before the BK- and BPP-induced effect was elicited (see Fig. 6).

2.5. Inhibition of isolated angiotensin I-converting enzyme

Inhibitory activities were determined with an enzyme preparation from the guinea pig lung, using Benzoyl-Gly-His-Leu as the substrate. This method is described elsewhere [70].

2.6. Cell culture and transfection of COS-7 cells

Cell culture and transfection of COS-7 cells have been described in detail [2]. COS-7 cells (ATCC) were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum and antibiotics, and were kept in a humidified 5% CO_2, 95% air atmosphere. Subconfluent cells were transfected in 24-well plates with BKR-B_2-cDNA (1 μg cDNA/well) by the DEAE-dextran technique, and cells were used 48–72 h after transfection. Preparation of COS-7 membranes was done as described previously [57].

2.7. Receptor binding assay

Competition binding studies were performed using [^{3}H]BK (102 Ci/mmol, NEN Life Science Products, USA) and intact COS-7 cells (initial density $(1–2) \times 10^5$ cells/well) in 24-well plates expressing the BKR-B_2 [56]. Cells were incubated in binding buffer containing 0.8 nM of [^{3}H]BK with different concentrations of BK in the absence or the presence of J526 (0.1 μM) or J527 (0.1 μM), or in increasing concentrations of J527 alone for 90 min at 4 °C. Non-specific binding was measured in the presence of 1 μM unlabeled BK. To estimate binding properties at 37 °C membranes of COS-7 cells were used. Membranes (0.15 mg/tube) were incubated with 0.8 nM of [^{3}H]BK or with 0.8 nM of [^{3}H]BK and 100 nM J526 for 20 min at 37 °C in binding buffer in the absence (total binding) or in the presence of 1 μM BK (non-specific binding). Thereafter, the samples were filtered through Whatman GF/B glass fiber filters pretreated with 0.1% (w/v) aqueous polyethylenimine. The filters were washed three times with 5 ml of ice-cold binding buffer, transferred into scintillation vials, dried and analyzed for bound radioactivity by scintillation counting.

2.8. Phosphatidylinositol turnover

COS-7 cells expressing the BKR were grown in 24-well plates (5×10^4 to 10^5 cells/well) and labeled with 4 μCi/ml *myo*-[^{3}H]inositol (10–25 Ci/mmol, NEN Life Science Products, USA) for 24 h. Cells were stimulated in DMEM without captopril with 3×10^{-8} M BK for 5 min at 37 °C, or

with 3×10^{-8} M BK in the presence of J526 (0.1 μM), or with 3×10^{-8} M BK in the presence of J527 (0.1 μM). Basal IP_n was measured with J526 or J527 (0.1 μM), too. Preparation of stimulation and determination of the levels of inositol phosphates by anion exchange chromatography using AG-X8 as a resin were performed as recently described in detail [2].

2.9. Arachidonic acid release

COS-7 cells were transfected and labeled with 0.4 μCi/ml [^{3}H]arachidonic acid (60–100 Ci/mmol, NEN Life Science Products, USA) for 24 h as described previously [57]. The cells were washed three times with label-free DMEM containing 0.5% (v/v) fetal calf serum and then stimulated in 0.5 ml in the same medium with 3×10^{-8} M BK for 30 min in the absence or the presence of J526 (0.1 μM) or J527 (0.1 μM) at 37 °C. Assays were terminated by removing the medium from the cells. The medium was measured for [^{3}H]arachidonic acid release by scintillation counting.

2.10. Preparation of human polymorphonuclear leukocytes (PMN) [62]

Heparinized venous blood from healthy volunteers was mixed with 1% dextran sulphate in 0.9% NaCl at a ratio of 1:1 (v/v) and left for 50 min at 37 °C for erythrocyte sedimentation to occur. The leukocyte-rich plasma was layered over an equal volume of Histopaque-1077, and the gradient was centrifuged for 30 min at $700 \times g$. Contaminating erythrocytes were eliminated by lysis with ammonium chloride (0.98%), and the pure PMN were washed twice with Eagle's minimal essential medium (MEM) and resuspended (1×10^6 PMN/ml).

2.11. Chemotaxis assay

Chemotaxis was quantified using the Boyden chamber technique as described previously [61]. PMN were suspended in Eagle's MEM containing 20 mM HEPES (pH 7.3) and $5 \times 10^5/0.5$ ml, pipetted into the top of Boyden chamber which were separated by a 3 μm pore filter (Sartorius SM 11302) from the lower part. Peptides were put into the lower stimulus compartment. Inhibitors or potentiating peptides were applied to the PMN for 15 min at 37 °C and then to the upper compartment of the chamber. After an incubation period of 3 h at 37 °C in an atmosphere of 5% CO_2, 95% air at high humidity, the filters were fixed and stained with methylene blue. Using an image analyzer (Chemotaxis Analyzer HCA-100, HA-SOTEC, Rostock, Germany), the PMN were counted at every 10 μm interval from the original monolayer to the distal surface in 10 high power fields of each duplicate filter. An average locomotion index (LI_{m+}) was quantified and expressed as the migration index in comparison to the control (control = 1.0).

2.12. Statistical analysis

Mostly the values are presented as means ± S.E.M. Statistical analysis was evaluated with the Student's t-test for independent samples or for paired samples, depending on the experimental protocol. Details are described in the legends for each figure.

3. Results

3.1. Synthesis of peptides

BPP$_{9\alpha}$ and a variety of analogues were synthesized according to the three different strategies in Scheme 1 on the solid phase. To prevent dioxopiperazine formation, chlorotrityl resin or HYCRAM^{TM} resin was used. In the case of Cl-trityl resin, cyclization of the C-terminal dipeptide was avoided by sterical hindrancy at the resin. Using the Boc-strategy with the HYCRAM^{TM} resin prevents ring formation and thereby loss of loading. Syntheses on Wang resin provided very low yields. After removal from the resins all the peptides were purified by semipreparative HPLC and chemically characterized. Some of the analogues are prepared for photoaffinity labeling of the binding protein.

3.2. Inhibition of the isolated angiotensin I-converting enzyme

The inhibitory activities of all analogues listed in Table 1 were determined on isolated ACE from guinea pig lungs. As most organs, particularly guinea pig ileum, contain different BK degrading enzymes, we did not use an organ homogenate to estimate the inhibition of BK degradation. We used the isolated ACE to test the inhibition of hydrolysis of a synthetic tripeptide substrate by the potentiating peptides. As shown in Table 1, distinct differences exist between potentiation of the BK-induced contraction of the guinea pig ileum and the inhibition of the isolated ACE.

To quantify this difference we calculated a quotient from both activities. For the analogue [1-Pro]-BPP$_{9\alpha}$ this quotient was accounted to 1. Most distinct differences were found by nonapeptide analogues and partial sequences labeled with azidosalicylic acid (ASA). Some quotients reach 10^5. Thus the compound J526 shows about 160% potentiation compared to BPP$_{9\alpha}$, but to enrich the same inhibition of ACE as the 1-Pro analogue a 10^5 higher concentration is required, resulting in a quotient of 10^5.

3.3. Potentiation of the BK-induced contraction of the isolated guinea pig ileum

One of the earliest pharmacological tests used for bradykinin was the contraction of the isolated guinea pig ileum (GPI). In our test the contraction of GPI can be potentiated by BPP$_{9\alpha}$ and by the ACE inhibitor ramiprilat as well. Both potentiators enhance the isotonic measured contraction after 5 min preincubation (Fig. 1). The pD$_2$-values for BK were shifted in the presence of 20 nM BPP$_{9\alpha}$ from 7.15 ± 0.11 ($n = 8$) to 7.84 ± 0.19 ($n = 4$) and by ramiprilat (20 nM) to 7.87 ± 0.22 ($n = 4$). Both potentiators are also able to shift the cumulative GPI contraction curves for the enzymatically more stable bradykinin analogue DArg-Arg-Pro-Hyp-Gly-Thi-Ser-Pro-εAbu(βPh)-Arg [16] to higher affinities. The pD$_2$-value of this BK analogue was shifted by BPP$_{9\alpha}$ (20 nM) from 6.65 ± 0.14 ($n = 6$) to 7.15 ± 0.18 ($n = 3$) and by ramiprilat to 7.16 ± 0.15 ($n = 3$).

3.4. Influence on the bradykinin B$_2$ receptor

To study the influence of bradykinin potentiating peptides on the bradykinin B$_2$ receptor COS-7 cells were transfected
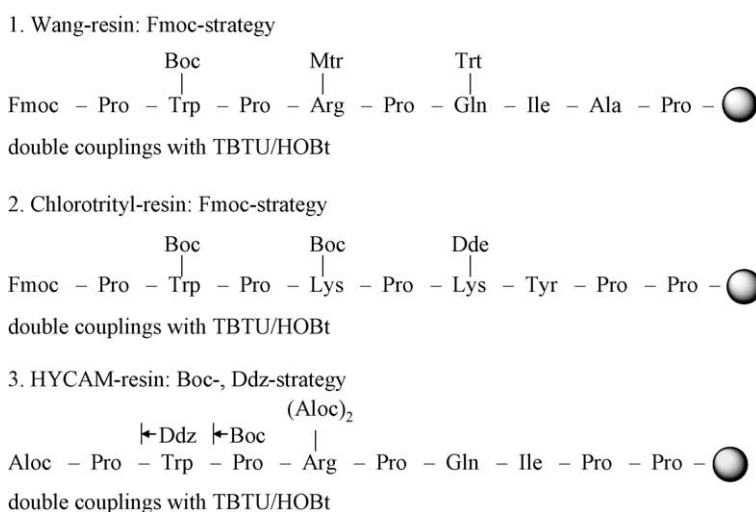
Scheme 1. Strategies for synthesis of BPP$_{9\alpha}$ analogues using different linkers and resins (Wang resin, chlorotrityl resin, HYCRAM^{TM} resin) and different protecting group combinations.

Table 1
Analogues of the bradykinin potentiating peptide BPP$_{9\alpha}$ (TEPROTIDE) with distinct differences between potentiation of the BK-induced contraction of the isolated guinea pig ileum (GPI) and inhibition of the isolated angiotensin I-converting enzyme (ACE)

Compound	Aminoacid sequence	Potentiation (%)	ACE-inhibition: IC$_{50}$ (M)	Quotient
BPP$_{9\alpha}$	Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	100	3×10^{-9}	7.0
	Pro-Trp-Pro-Phe-Pro-Gln-Tyr-Pro-Pro	100	8×10^{-9}	19
	Pro-Trp-Pro-Leu-Pro-Lys-Tyr-Pro-Pro	100	6×10^{-8}	0.1×10^3
	Pro-Trp-Pro-Leu-Pro-Gln-Ile-Pro-Pro	90	1×10^{-5}	2.5×10^4
[1-Pro]-BPP$_{9\alpha}$	Pro-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	115	5×10^{-10}	1
	Pro-Trp-Pro-Arg-Pro-Gln-Ile-Ala-Pro	70	3×10^{-7}	1×10^3
	Pro-Trp-Ala-Arg-Pro-Gln-Ile-Pro-Pro	75	3×10^{-8}	93
	Pro-Trp-Pro-Leu-Pro-Leu-Ile-Pro-Pro	50	1×10^{-7}	0.5×10^3
	Pro-Trp-Pro-Phe-Pro-Gln-Ile-Pro-Pro	90	5×10^{-8}	0.1×10^3
J527	Pro-Trp-Pro-Lys-Pro-Gln-Ile-Pro-Pro	70	3×10^{-8}	0.1×10^3
	Pro-Trp-Pro-Lys-Pro-Lys-Ile-Pro-Pro	75	2×10^{-8}	63
	Pro-Trp-Pro-Lys-Pro-Lys-Tyr-Pro-Pro	90	5×10^{-5}	1.3×10^5
	Pro-Trp-Pro-Leu-Pro-Lys-Ile-Pro-Pro	85	3×10^{-8}	82
	Pro-Trp-Pro-Phe-Pro-Gln-Ile-Ala-Pro	70	3×10^{-6}	1.0×10^4
	Pro-Trp-Pro-Phe-Pro-Gln-Tyr-Ala-Pro	58	3×10^{-8}	0.1×10^3
	Pro-Trp-Pro-Arg-Pro-Gln-Tyr-Pro-Pro	76	2×10^{-8}	61
	Pyr-Trp-Pro-Lys-Pro-Gln-Ile-Pro-Pro	65	2×10^{-8}	72
	Pro-Trp-Pro-Arg-Pro-Lys-Ile-Pro-Pro	50	7×10^{-8}	0.3×10^3
	Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	65	2×10^{-8}	71
	Leu-Pro-Gln-Ile-Pro-Pro	40	7×10^{-6}	4.0×10^4
	Arg-Pro-Gln-Ile-Pro-Pro	55	7×10^{-6}	3.0×10^4
Analogues and partialsequences with the photolabels ASA[a] and ABA[b]				
	ABA-Pro-Trp-Pro-Phe-Pro-Gln-Tyr-Pro-Pro	93	1×10^{-7}	0.3×10^3
	ASA-Pro-Trp-Pro-Leu-Pro-Gln-Ile-Pro-Pro	110	3×10^{-7}	0.7×10^3
	ASA-Pro-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	90	6×10^{-9}	16
	Pro-Trp-Pro-Lys(ASA)-Pro-Lys-Ile-Pro-Pro	99	1×10^{-8}	23
	Pro-Trp-Pro-Lys-Pro-Lys(ASA)-Ile-Pro-Pro	103	8×10^{-8}	0.2×10^3
	Pro-Trp-Pro-Lys(ABA)-Pro-Lys-Tyr-Pro-Pro	88	3×10^{-8}	79
	Pro-Trp-Pro-Leu-Pro-Lys(ASA)-Ile-Pro-Pro	86	5×10^{-7}	1.4×10^3
	ABA-Pro-Trp-Pro-Phe-Pro-Gln-Tyr-Ala-Pro	20	1×10^{-5}	1.1×10^5
J526	Pyr-Trp-Pro-Lys(ASA)-Pro-Gln-Ile-Pro-Pro	160	7×10^{-5}	1.0×10^5
	ASA-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	110	6×10^{-8}	0.1×10^3
	ASA-Leu-Pro-Gln-Ile-Pro-Pro	70	8×10^{-6}	2.6×10^4
	ASA-Arg-Pro-Gln-Ile-Pro-Pro	40	4×10^{-5}	2.3×10^5
Analogues with the photolabel Bpa[c]				
	Bpa-Pro-Trp-Pro-Lys-Pro-Lys-Tyr-Pro-Pro	70	7×10^{-5}	2.3×10^5
	Bpa-Pro-Trp-Pro-Lys-Pro-Lys-Tyr(I)-Pro-Pro	75	5×10^{-8}	0.2×10^3
	Bpa-Pro-Trp-Pro-Phe-Pro-Gln-Tyr-Ala-Pro	20	1×10^{-6}	1.1×10^4
	Bpa-Pro-Trp-Pro-Arg-Pro-Gln-Tyr-Pro-Pro	72	2×10^{-8}	65
	Pro-Bpa-Pro-Arg-Pro-Gln-Tyr-Pro-Pro	63	2×10^{-8}	74
	Pro-Trp-Pro-Bpa-Pro-Gln-Tyr-Pro-Pro	90	3×10^{-8}	77

To quantify the difference between potentiation and inhibition we calculated a quotient from both activities. For the analogue with the highest ACE-inhibitory activity [1-Pro]-BPP$_{9\alpha}$ the quotient was accounted to 1.

[a] 4-Azidosalicylic acid.
[b] 4-Azidobenzoic acid.
[c] *p*-Benzoylphenylalanine.

with the gene of the human receptor. Expression, chemical and functional characterization of the expressed receptor is described in a preceding publication [56]. Radioligand binding studies with tritium labeled bradykinin [^{3}H]BK were performed on intact cells. Fig. 2 shows the displacement curves of [^{3}H]BK by BK alone and in the presence of the both potentiating nonapeptide analogues J526 and J527. Both peptides were used in such a concentration (0.1 μM) as necessary for the maximum potentiating effect on the isolated smooth muscle organ GPI. The potentiating peptides have no effect on the binding of bradykinin, indicating that the peptides used are neither able to enhance the affinity nor the number of active receptors. An unexpected finding is that analogue J527 is able to displace slightly [^{3}H]BK in concentrations of 0.1 μM (20% displacement) to 1 μM (40% displacement) (not shown).
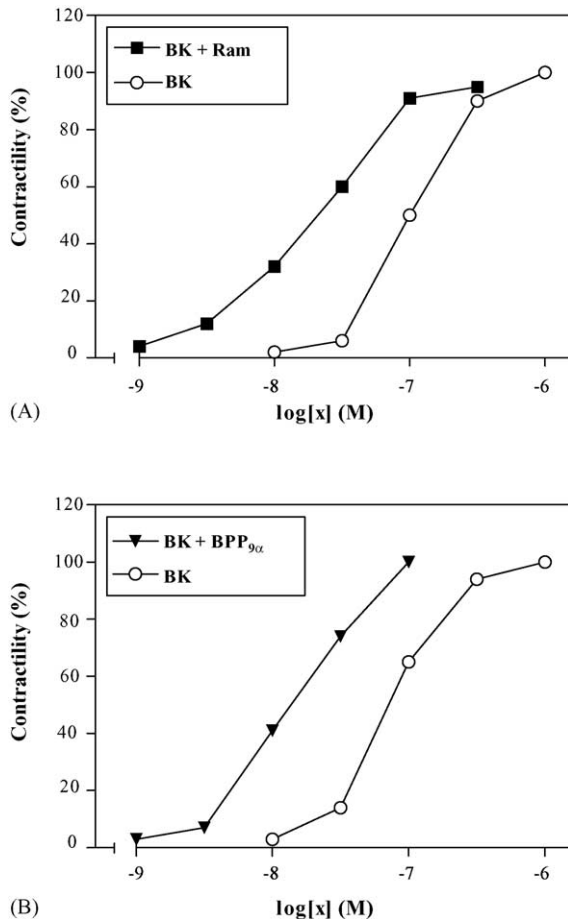
3.5. Influence of bradykinin potentiating peptides on signal pathways

3.5.1. Influence on Calcium uptake

Lanthanium ions (La^{3+}) act as inhibitor of the uptake of Ca^{2+} from extracellular space. We used La^{3+} to estimate the influence of $BPP_{9\alpha}$ on Ca^{2+} uptake. BK induces the contraction of the GPI only in the presence of extracellular calcium ions. The lowest extracellular concentration of Ca^{2+} necessary for a detectable contraction is 1.8 mM. In a Ca^{2+} containing Tyrode buffer, BK induces the contraction of the isolated GPI, which can also be potentiated in the presence of 1.8 mM La^{3+} (Fig. 3). Bradykinin alone is unable to contract the GPI in the presence of La^{3+}. The ileum contracts only after addition of $BPP_{9\alpha}$, as shown in part A of Fig. 3. Part B of this figure, reflecting the results in the Ca^{2+} free Tyrode buffer, clearly underlines the important role of extracellular Ca^{2+} for the BK-induced smooth muscle contraction. Part A also shows evidence that mobilization of intracellular Ca^{2+} is necessary for the potentiation by the peptides.

3.5.2. Influence on the phosphatidyinositol turnover

Bradykinin induces the formation of inositol phosphates in COS-7 cells transiently expressing the BK receptor. Both analogues of the potentiating nonapeptide are not able to augment the BK-induced intracellular concentration of inositol phosphates. Rather it seems that J527 reduces the BK-induced IP_n formation. The most striking result from Fig. 4 is the significant enhancement of the basal level by both potentiating peptides in the absence of bradykinin, possibly indicating a nonreceptor-mediated pathway.

3.5.3. Influence on release of arachidonic acid

Bradykinin triggers the release of [^{3}H]-labeled arachidonic acid from labeled phospholipids, presumably through activation of phospholipase A_2 by a G_α-protein. The level of arachidonic acid is significantly enhanced by BK as shown in Fig. 5. Both potentiating peptides J526 and J527 slightly, but significantly, enhance the BK-mediated release of labeled arachidonic acid. Without BK both peptides have no significant influence on the basal level.

3.5.4. Influence on protein phosphatases

Desensitization and resensitization are processes at the receptor level elicited by cytosolic receptor phosphorylation, followed by internalization or dephosphorylation, which is followed by reintegration into the cell membrane. Dephosphorylation of cytosolic Ser- and Thr-residues of the BK receptor results from activated protein phosphatases. Calyculin is known as a potent inhibitor of the protein phosphatases 1 and 2A. We used this inhibitor to check the involvement of these phosphatases in the potentiation of bradykinin action. As Fig. 6 illustrates calyculin has no influence on the potentiation of the BK-induced contraction. Application of calyculin, neither before nor after BK administration, changes the potentiating effects of $BPP_{9\alpha}$ or ramiprilat.

Fig. 1. Concentration–response curves to bradykinin on the guinea pig ileum without and after preincubation (5 min) of 20 nM ramiprilat (A) or 20 nM $BPP_{9\alpha}$ (B). Contractions are expressed as a percentage of the maximum BK-induced response (10^{-8} M BK) before addition of ramiprilat or bradykinin potentiating peptides. The values are accounted from one representative experiment, which was repeated three to eight times.
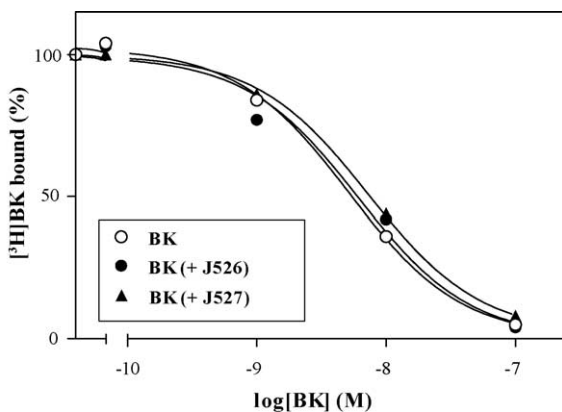

Fig. 2. Competition curves of bradykinin (BK), BK with J526 (0.1 μM), or BK with J527 (0.1 μM) for specific binding of [^{3}H]BK to COS-7 cells expressing the bradykinin B_2 receptor. Shown are representative experiments performed in quadruplicate determinations and repeated once with the same result.
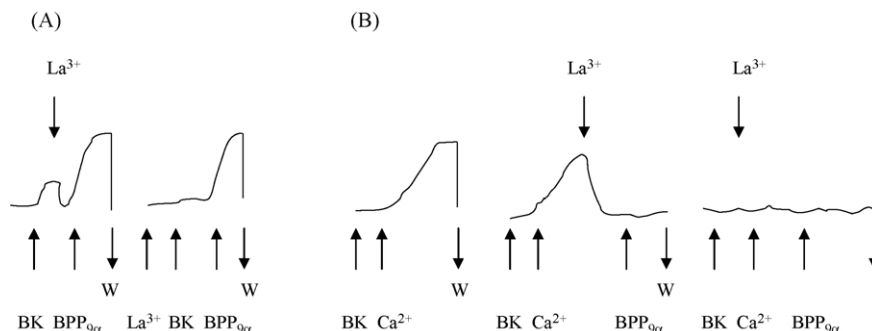
Fig. 3. Effect of La^{3+} (1.8 mM) on the potentiation of a BK-induced (20 nM) contraction of the guinea pig ileum by $BPP_{9\alpha}$ (20 nM) in Tyrode-solution with (A) and without $[Ca^{2+}]$ (B). The arrows indicate the addition of BK, $BPP_{9\alpha}$, lanthanium or calcium ions. W = wash period. Curves are examples of five separate experiments.
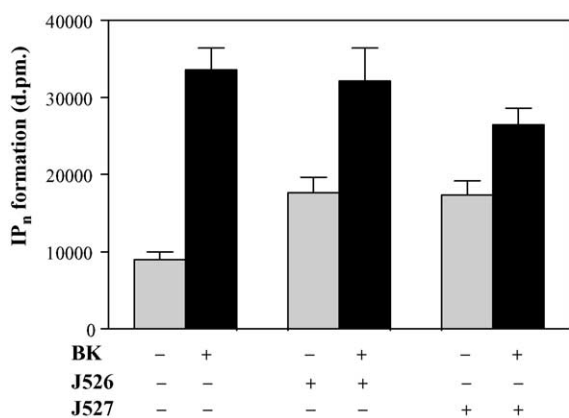

Fig. 4. Effect of $BPP_{9\alpha}$ analogues J526 and J527 on basal level of inositol phosphate production in COS-7 cells transiently transfected with BKR-B_2 cDNA. Cells were prelabeled with 4 μCi/ml myo[^{3}H]-inositol for 24 h and than stimulated with 3×10^{-8} M bradykinin for 5 min in absence or in presence of J526 (0.1 μM) or J527 (0.1 μM). Inositol phosphate formation was determined in quadruplicates. Shown are the mean $\pm$ S.E.M. of four independent comparative experiments.
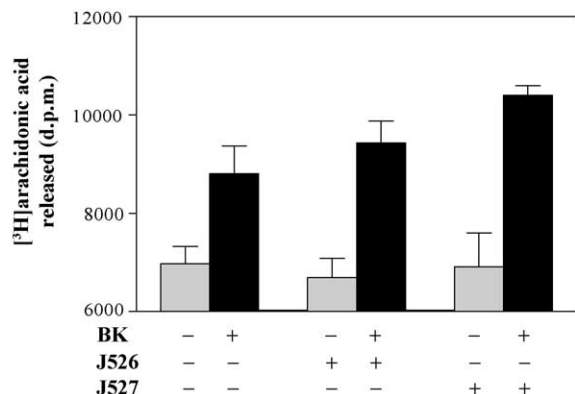

Fig. 5. Influence of $BPP_{9\alpha}$ analogues J526 and J527 on the BK-induced increase in arachidonic acid production in COS-7 cells which have transiently expressed the bradykinin B_2 receptor. Cells were prelabeled with 0.4 μCi/ml [^{3}H]arachidonic acid for 24 h and stimulated with 3×10^{-8} M BK for 30 min in absence or in presence of J526 (0.1 μM) or J527 (0.1 μM). Arachidonic acid release was six-fold determined. Results are expressed as the mean $\pm$ S.E.M.
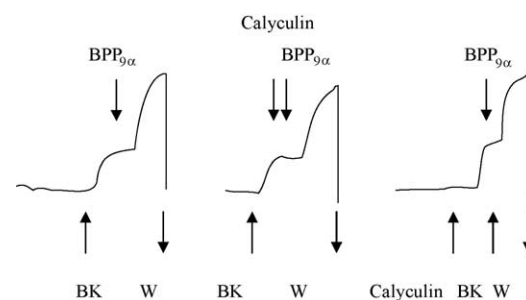

Fig. 6. Influence of calyculin (100 nM) on the potentiation of a BK-induced (20 nM) contraction of the guinea pig ileum by $BPP_{9\alpha}$ (20 nM). The arrows indicate the addition of BK, $BPP_{9\alpha}$ and calyculin. W = wash period. Curves are examples of four to six separated experiments.

3.6. Influence of potentiating factor on the migration of polymorphonuclear leukocytes (PMN)

Bradykinin stimulates the migration of PMN corresponding to its concentration gradient. This effect could be characterized as true chemotaxis. These cells contain both types of BK receptors, BKR-B_1 and BRK-B_2, as demonstrated using BK agonists and antagonists in the migration assay [62]. They also contain the complete system for synthesis and release of bioactive kinins. Degradation of BK proceeds in PMN mainly by the neutral endopeptidase NEP (E.C. 3.4.24.11). The BK-induced accelerated migration of PMN can be potentiated after preincubation (5 min) of the cells with the NEP inhibitor phosphoramidon (10^{-7} M). The migration index (MI = 1.12 ± 0.03) of BK (10^{-8} M) is significantly ($p < 0.5$; t-test for paired samples; $n = 12$) enhanced in the presence of phosphoramidon (MI = 1.19 ± 0.03) (not shown).

The migratory capacity of BK for PMN can also be potentiated by the ACE inhibitor ramiprilat. The migation index of BK (10^{-7} M) without ramiprilat is MI = 1.30 ± 0.06, in the presence of ramiprilat (10 nM) MI = 1.69 ± 0.26, measured in six experiments (not shown).

As shown in Fig. 7 $BPP_{9\alpha}$ can enhance the migration of PMN induced as well by BK and by the enzymatically stable BK agonist D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-
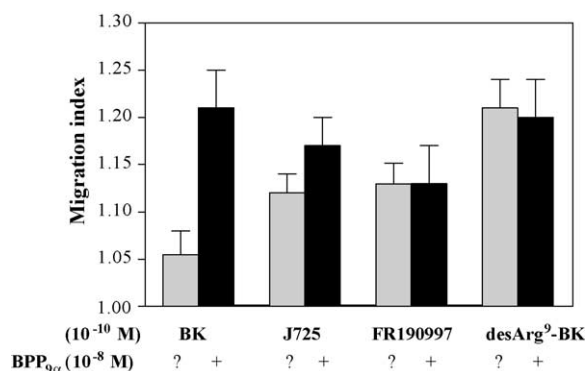
Fig. 7. Influence of $BPP_{9\alpha}$ on $BKR-B_2$ and $BKR-B_1$ agonists-induced migration of polymorphonuclear leukocytes (PMN). Cells in the upper compartment of the chamber were preincubated (15 min) with $BPP_{9\alpha}$ (10^{-8} M). Bradykinin, the nonpeptide agonist FR190997 or desArg^9-BK (10^{-10} M) were given into the lower compartment. Results are expressed as migration index (control = 1.0) and represent mean ± S.E.M. of five to seven experiments.

Pro-εAbu(βPh)-Arg [16], but is unable to enhance the migratory activity of the nonpeptide agonist FR190997 [4] and the $BKR-B_1$ agonist desArg^9-BK. $BPP_{9\alpha}$ (10 nM) is able to potentiate the dose–response curve of the BK-induced migration of the PMN (Fig. 8A). It potentiates the BK-induced effect in a concentration-dependent way (Fig. 8B).

4. Discussion

The potentiating action, despite many long years of experimental research and investigation, remains a phenomenon not fully understood. Beginning with studies on the potentiation of smooth muscle contraction more than 30 years ago, the search has been extended to other organs such as the vascular system, to different bradykinin degrading enzymes, to immunocompetent cells, and in the last decade to molecular mechanisms at the level of the bradykinin receptors and signal pathways, primarily studied on cell cultures. Other potentiating peptides and peptidomimetics besides the oligopeptides isolated from snake venoms have been used in these studies.

Generally, some of the contradictory findings and therefore explanations in the literature seem to result not only from the complexity of the system, but also from: the use of enzymatically not fully stable bradykinin agonists e.g. [Hyp^3,Tyr(Me)^8]-BK [31,53], the species dependency of the bradykinin receptor, the very different protease compositions of the tissues and cell lines used in the different studies, and the different structural requirements for both catalytic centers of ACE. Furthermore, we have to keep in mind the different density of the BK receptors, their localization in microdomains in the plasma membrane, the presence of certain other hormone receptors, the different signal pathways in the used tissues and cells, a possible influence on G-protein independent signal transduction, on G-protein trafficking pattern in the cells and on activators or regulators of G-protein signal-
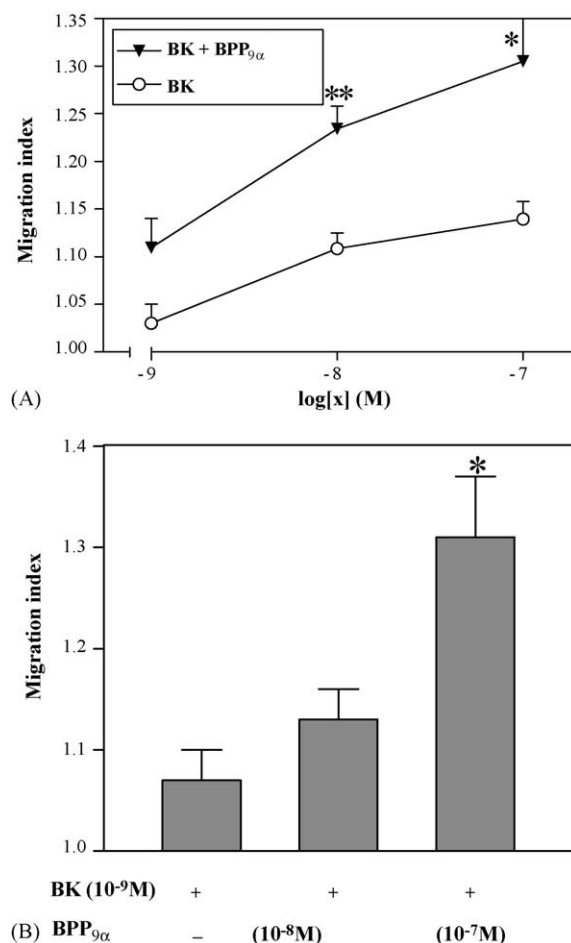

Fig. 8. Effect of $BPP_{9\alpha}$ on the BK-induced migration of human polymorphonuclear leukocytes (PMN) in Boyden chambers. (A) Concentration–response curves of BK without and in presence of 10 nM $BPP_{9\alpha}$. Cells in the upper compartment of the chamber were preincubated (15 min) with $BPP_{9\alpha}$ and BK was given into the lower compartment. (B): The BK-induced (10^{-9} M) migration is dose-dependently potentiated by $BPP_{9\alpha}$ in concentrations of 10^{-8} and 10^{-7} M. Results are expressed as migration index (control = 1.0) and represent mean ± S.E.M. of 3–11 experiments. Values are significantly different from control values without $BPP_{9\alpha}$: $^{**}p < 0.01$, $^{*}p < 0.05$ (Student's t-test).

ing [48]. Additionally the potentiating effect can be differentiated into an unspecific and specific part. Consequently, the search for potentiation of hormone actions is strongly related to the very recent research on multifunctional signal proteins.

4.1. Distinct differences between potentiation of the BK-induced contraction of the isolated guinea pig ileum and the inhibition of the isolated angiotensin I-converting enzyme

The isolation, chemical and functional characterization of oligopeptides taken from the venoms of different snakes provided lead structures for a number of potentiating peptides. Long ago these oligopeptides gave some evidence of different structural requirements for potentiation of smooth mus-

cle contraction and inhibition of ACE. Our synthesized analogues and fragments of the highly active nonapeptide BPP$_{9\alpha}$ show in some cases differences between potentiation and enzyme inhibition of more than five orders of magnitude. This result indicates strongly different structural requirements for both biological activities, and is inconsistent with the exclusive reduction of potentiating action to the inhibition of ACE, as discussed by Regoli and his coworkers [31] and Dendorfer et al. [17]. We might draw this conclusion despite the different sources for the isolated organs and the ACE. Since the BK receptor is species dependent, the human BKR-B$_2$ shares about 80% sequence homology with that of the guinea pig [20], whereas the ACE seems to be nearly species independent. Interestingly, the potentiating activity is much less influenced by amino acid replacements or modifications of the lead peptide BPP$_{9\alpha}$ than the inhibition of ACE.

From the analogues listed in Table 1, it is clearly evident that the potentiation of GPI contraction is less sensitive to amino acid replacements and modifications in the peptide sequence than the inhibition of ACE. Thus potentiating activities vary between 160% and 20%, whereas the IC$_{50}$ values for ACE inhibition vary between 5×10^{-10} and 7×10^{-5} M. Both activities show no correlation. The estimated values indicate that the potentiating activity allows beside amino acid replacements, a shortening of the sequence as well as substitutions of the N-terminus or side chains by aromatic moieties. The inhibitory activity seems to depend on the whole sequence. The replacement of the basic amino acid Arg at position 4 by neutral aliphatic or aromatic amino acids influences the ACE inhibition only slightly, on the other hand the acylation of the Lys side chain at the same position by ASA reduces the IC$_{50}$ value by four orders of magnitude. According to Cotton et al. [11] position 4 seems to be crucial for inhibitory activity. But, our results did not provide clear structure activity relationships.

Discussing the potentiation of the BK-induced contraction of GPI we have to keep in mind that this tissue also contains other proteases than ACE involved in BK degradation. But this finding is not able to explain the strong differences between both activities as expressed in the ratio in Table 1.

4.2. Direct interaction with the bradykinin receptors

It is reasonable to assume an interaction of the potentiating peptides with the BK receptor. For this interaction certain possible mechanisms exist. At first, the potentiating factors could act like allosteric effectors stabilizing the active receptor conformation. Secondly, these factors could influence the phosphorylation and dephosphorylation of cytosolic parts of the receptor, resulting in desensitization and resensitization of the receptor. A third possible mechanism consists in the influence of potentiating factors on receptor heterooligomerization. AbdAlla et al. [1] found that both the active high affinity angiotensin receptor AT$_1$ and the BKR-B$_2$ are able to form heteromeric complexes in smooth muscle cells. Most importantly, this heterodimerization evokes a signal enhancement, a further possible useful explanation of the potentiating effect.

For checking these possibilities we used COS-7 cells transiently transfected with the human BKR-B$_2$. The displacement curves show no differences between the displacement of [^{3}H]-BK by BK alone nor in the presence of potentiating peptides. This finding clearly demonstrates that the analogues of BPP$_{9\alpha}$ used in this study do not act as allosteric effectors stabilizing the active receptor conformation. Because COS-7 cells do not contain ACE, a heterodimerization of the B$_2$ receptor with somatic ACE as demonstrated by Erdos and Marcic [19] is impossible in our system. Our radioligand binding curves show no influence of the potentiating peptides on the receptor capacity, indicating that under the test conditions the number of intramembranal receptors was not changed. This result excludes an internalization or reintegration of the receptor under the influence of potentiating peptides. But, we would not exclude these processes after heterodimerization with ACE or with the angiotensin receptor.

4.3. Influence on signal pathways

The contraction of smooth muscles requires calcium ions. Consequently the potentiation of BK-induced contraction of GPI depends on extracellular and intracellular Ca^{2+}. In a variety of publications the enhancement of the intracellular Ca^{2+}-level is used as a qualitative or quantitative proof of the potentiating action. Our results with the inhibition of Ca^{2+}-uptake from extracellular sources by La^{3+} indicates that the potentiation of BK-action requires mobilization of Ca^{2+} from intracellular stores. On the other hand, in the beginning phase of the BK-evoked GPI contraction extracellular calcium is needed. Thus, our results with La^{3+} agree well with the finding of Marcic et al. [49], who described the inhibition of resensitization of the BK receptor in CHO cells by La^{3+}.

The inositol phosphates (IP$_n$) formed in transiently with the B$_2$ receptor transfected COS-7 cells opens the Ca^{2+} channels of the endoplasmatic reticulum leading to an increase of cytosolic Ca^{2+}. But, both potentiating nonapeptides J526 and J527 are unable to enhance the BK-induced formation of IP$_n$. Surprisingly, both potentiating peptides increase the basal level of IP$_n$ in the absence of BK. Possibly this may be due to an unspecific, nonreceptor-mediated part of the potentiating action.

Contrary to the absent effect of both potentiating peptides on the BK-induced enhancement of inositol phosphates, the BK-induced release of arachidonic acid is significantly increased. Furthermore, contrary to the IP$_n$ formation, no influence of either potentiating peptides on the basal level could be found. We continue to be unable to explain the potentiation of arachidonic acid release because no effect on the receptor has been detected. The missing effect of calyculin on the potentiation might indicate that the inhibited phosphatases are not involved into resensitization.

4.4. Influence on migration of polymorphonuclear leukocytes

Besides GPI and COS-7 cells the PMN represent our third system for studies about the potentiating mechanism. These cells constitutively express B_1 as well as B_2 receptors and have a cell specific set of proteases involved in BK degradation. In this system the potentiating nonapeptide $BPP_{9\alpha}$ and ramiprilat accelerate the BK-induced migration. In contrast to Ignjatovic et al. [37], who found on other cells a concentration-dependent direct activation of the B_1 receptor in the absence of kinins, we could not detect any activity of the potentiating nonapeptide without administration of B_1 or B_2 agonists. Though the migratory activity of the proteolytic stabilized BK analogue J725 [16] is potentiated, the action of the nonpeptide agonist FR190997 [4] could not be accelerated by $BPP_{9\alpha}$ and ramiprilat. It is difficult to explain this finding. In agreement with the weaker acceleration of the stabilized BK analogue, the effect of the NEP inhibitor phosphoramidon and the concentration-dependent potentiation induced by $BPP_{9\alpha}$, we might suppose that in this test system the potentiation occurs possibly exclusively by inhibition of the enzymatic BK degradation. But, we also have to consider a different mechanism for the peptide and nonpeptide agonist. This assumption is supported in that test by the strongly reduced intrinsic activity of the nonpeptide agonist FR190997 compared to BK itself.

5. Conclusions

During the last three decades many different and controversial explanations on the potentiating mechanism have been derived from experiments performed in vivo, in vitro and at the cellular level.

The distinct and in some cases very significant differences between the inhibitory and potentiating activity of the synthesized $BPP_{9\alpha}$ analogues clearly indicate, in our opinion, that on guinea pig ileum this mechanism cannot be exclusively reduced to enzyme inhibition. The observed differences even exceed the different structural requirements for N- and C-terminal catalytic centers of ACE. The experiments with La^{3+} gave evidence that the potentiation of smooth muscle contraction requires extracellular Ca^{2+} which is taken up through opened channels. The increase of the intracellular level of inositol phosphates, triggered by potentiating peptides, leads to an additional enhancement of the intracellular Ca^{2+} concentration. Application of potentiating peptides to COS-7 cells which were transiently transfected with the B_2 receptor and did not contain endogenous ACE, shows no increase in receptor affinity or density. These results disprove the described suggestion of direct interaction of BPP with the BK receptor.

The described receptor resensitization by potentiating peptides could result from dephosphorylation of the activated receptor. Our results obtained with the phosphatase inhibitor calyculin disprove the influence of $BPP_{9\alpha}$ on the calyculin sensitive phosphatase. No difference between potentiation neither in the absence nor in the presence of the inhibitor could be observed.

The release of arachidonic acid is one of the prerequisites for inflammatory processes. The potentiating peptides have no effect on the basal level but they significantly increase the BK-induced release in COS-7 cells. This result indicates the possible involvement of potentiating peptides in inflammatory processes without affecting ACE.

Contrary to that mechanism the potentiation of the migratory activity of polymorphonuclear leucocytes seems to be nearly exclusively evoked by the ACE inhibition. On these B_1 and B_2 receptor containing cells no direct interaction with the B_1 receptor could be observed.

Our results together with those from the literature may lead to the general conclusion that different potentiating factors can act on different organs, tissues and cells by different mechanisms. Furthermore, BPP can evoke both receptor-mediated and nonreceptor-mediated intracellular reactions. In our opinion the contradictory explanations describing the potentiation mechanism result on the one hand from the very high complexity of the kallikrein–kinin and renin–angiotensin systems and on the other hand from the different in vivo and in vitro tests used.

Acknowledgements

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