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Potentiation of bradykinin actions by analogues of the bradykinin potentiating nonapeptide BPP9α

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Abstract

Synthetic analogues of the bradykinin potentiating nonapeptide BPP9α 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 dispose 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 intracellular phosphatases in the GPI 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.

Keywords: Potentiation; Bradykinin; Bradykinin potentiating peptide; Angiotensin I-converting enzyme; Inositol phosphates; Arachidonic acid; Ca2+-influx; Protein phosphatases; Polymorphonuclear leukocytes; Chemotaxis; Smooth muscle contraction; Radioligand binding

Abbreviations: AA, arachidonic acid; ABA, 4-azidobenzoic acid; ACE, angiotensin I-converting enzyme; Aloc, allyl oxycarbonyl; ASA, 4-azidosalicylic acid; BKR, bradykinin receptor; BKR-B1, bradykinin B1 receptor; BKR-B2, bradykinin B2 receptor; Box, tert-butylxycarbonyl; BPa, p-benzoylphenylalanine; BPP, bradykinin potentiating peptide; BPPα, bradykinin potentiating peptide 9α (Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro); BOP, benzotriazole-1-yl-oxy-tris (dimethylamino) phosphonium hexafluorophosphate; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; DTE, dithioerithritol; DCM, dichloromethane; Dde, N-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; Ddz, dimethyl-3,5-dimethoxy-benzyloxycarbonyl; DEEA, diethylaminoethyl; DIEA, diisopropylethylamine; DIC, diisopropylcarbodiimide; DMEM, Dulbecco’s modified Eagle’s medium; DME, N,N-dimethylformamide; DMSO, dimethylsulfoxide; DTE, diethanolthiol; ED, effective dose; Fmoc, 9-fluorenylmethyl oxycarbonyl; Fr190997, 8-[2,6-dichloro-3-[N-(E)-4-([N]-methylcarbamoyl)cinnamidoacetyl]-N-ethylamino]benzyloxy]-2-methyl-4-(2-pyridyl-methoxy)quinoline; GPA, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HOAt, 1-hydroxy-7-azabenzotriazole; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HOBr, hydrobromic acid; HYCRAM, hydroxycrotonyl amidomethyl linker; IP3, inositol 1,4,5-trisphosphate; J526, Pyr-Trp-Pro-Lys-ASA-Pro-Glu-Ile-Pro-Pro; J725, DArg-Arg-Pro-Hyp-Thi-Ser-Pro-Abu(ßPh)-Arg; MEM, Eagle’s minimal essential medium; Mtr, methoxytrimethylbenzene sulphonyl; PMN, polymorphonuclear leukocytes (neutrophils); Ram, ramspeck; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Trt, triphenylmethyl

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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 Onedetti 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 BPP9 (trade name TEPROTIDE).

Bradykinin potentiating peptides have also been isolated from other snakes [8,23–25,35] 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 hypertensive 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, Neprilysin. 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 pressure 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 B2 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 venaconstriction) 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 B2 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 (BK R) and on signal pathways.
Direct interaction of the potentiating compounds was postulated with the B1 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 B2 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 potentiation activity was mainly investigated on the affinity and density of the receptor [54], the intracellular mobilization of Ca2+ [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 B2 receptor including the hetero-oligomerization of the B2 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.

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 inhibitors [54,55], 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 B2 receptor and ACE, ACE-mutants or neutral endopeptidase (NEP) [15]. Endothelial cells have been used because they constitutively express the B2 receptor and ACE [49].

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®-linker the hydroxycrotonic ester was coupled to the aminomethyl resin Tentagel-S-NH2. The used resins were purchased from the following companies: amino methyl polystyrene, Tentagel-S-NH2 and Wang resin from RAPP Polymer (Germany), and chlorotrityl resin from NOVABiochem (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° protected amino acid and disisopropylcarbodiimide (DIC) in DCM for 4 h; 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylenurimin tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBr): Boc- or Fmoc-protected amino acids were used in a four-fold excess, solved in DMF or DCM/DMF 1:1. TBTU and HOBr were applied in the same excess, disopropylethylamine (DIEA) in a six-fold excess. The reaction time was between 4 and 12 h.

Deprotecting procedures: Boc-deprotection was performed with TFA/DMC 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 \times 10^{-8} M that corresponded to an ED25 to ED50 of BK under these experimental conditions. Generally this effect was increased twice by BPP9 (20 nM BK, 20 nM BPP9), La3+ (1.8 mM) was used as an inhibitor of the Ca2+ influx in Ca2+-free or Ca2+-containing Tyrode solution. LaCl3 was always given 1 min before or after the BK-induced contraction and before the application of BPP9 (see Fig. 3).

2.3. Ca2+ -dependence of the potentiation of the BK-induced contraction of GPI

To investigate the influence of Ca2+ on the BK-potentiating action of BPP9 (20 nM BK, 20 nM BPP9), La3+ (1.8 mM) was used as an inhibitor of the Ca2+ influx in Ca2+-free or Ca2+-containing Tyrode solution. LaCl3 was always given 1 min before or after the BK-induced contraction and before the application of BPP9 (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(BA) contraction of the guinea pig ileum. Caly- 
culin was applied before BPP(BA) 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% CO2, 95% air atmosphere. Sub-
confluent cells were transfected in 24-well plates with BKR-
B2-cDNA (1 μg/cDNA/well) by the DEAE-dextran tech-
nique, and cells were used 48–72 h after transfection. Prepa-
ration of COS-7 membranes was done as described previ-
ously [57].

2.7. Receptor binding assay

Competition binding studies were performed using 
[3H]BK (102 Ci/mmol, NEN Life Science Products, USA) and intact COS-7 cells (initial density (1–2) × 10^5 cells/well) in 24-well plates expressing the BKR-B2 [56]. Cells were in-
cluded in binding buffer containing 0.8 nM of [3H]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 concen-
trations of J527 alone for 90 min at 4 °C. Non-specific binding 
was measured in the presence of 1 μM unlabeled BK. To esti-
mate binding properties at 37 °C membranes of COS-7 cells 
were used. Membranes (0.15 mg/tube) were incubated with 
0.8 nM of [3H]BK or with 0.8 nM of [3H]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 bind-
ing). 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 scintil-
lation 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-[3H]inositol (10–25 Ci/mmol, NEN Life Science Pro-
ducts, USA) for 24 h. Cells were stimulated in DMEM with-
out 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 
IPs was measured with J526 or J527 (0.1 μM), too. Prepa-
ration of stimulation and determination of the levels of in-
ositol 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 
[3H]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 con-
taining 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 
[3H]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 sedi-
mentation to occur. The leukocyte-rich plasma was layered 
over an equal volume of Histopaque-1077, and the gradi-
ent was centrifuged for 30 min at 700 × g. Contaminating 
erythrocytes were eliminated by lysis with ammonium chlor-
ide (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 × 10^{-5}/0.5 ml, pipetted into the top of Boyden chamber 
which were separated by a 3 μm pore filter (Sartocon SM 
11302) from the lower port. 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% CO2, 95% air 
at high humidity, the filters were fixed and stained with methy-
lene 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_x) 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

BPP9 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™ resin was used. In the case of CI-trityl resin, cyclization of the C-terminal dipeptide was avoided by sterical hindrance at the resin. Using the Boc-strategy with the HYCRAM™ 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] BPP9 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 BPP9, 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 BPP9 and by the ACE inhibitor ramiprilat as well. Both potentiators enhance the isotonic measured contractions after 5 min preincubation (Fig. 1). The pD2-values for BK were shifted in the presence of 20nM BPP9 from 7.15 ± 0.11 (n = 8) to 7.84 ± 0.19 (n = 4) and by ramiprilat (20nM) 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-eAba(cPh)-Arg [16] to higher affinities. The pD2-value of this BK analogue was shifted by BPP9 (20nM) 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 B2 receptor

To study the influence of bradykinin potentiating peptides on the bradykinin B2 receptor COS-7 cells were transfected

1. Wang-resin: Fmoc-strategy

\[
\begin{align*}
    & \text{Boc} \\
    & \text{Fmoc} - \text{Pro} - \text{Trp} - \text{Pro} - \text{Arg} - \text{Pro} - \text{Glu} - \text{Ile} - \text{Ala} - \text{Pro} - \text{O} \\
    & \text{double couplings with TBTU/HOBt}
\end{align*}
\]

2. Chlorotrityl-resin: Fmoc-strategy

\[
\begin{align*}
    & \text{Boc} \\
    & \text{Boc} - \text{Pro} - \text{Trp} - \text{Pro} - \text{Lys} - \text{Pro} - \text{Lys} - \text{Tyr} - \text{Pro} - \text{Pro} - \text{O} \\
    & \text{double couplings with TBTU/HOBt}
\end{align*}
\]

3. HYCRAM-resin: Boc-, Dde-strategy

\[
\begin{align*}
    & (\text{Aloc})_2 \\
    & \text{Dde} - \text{Boc} - \text{Boc} - \text{Aloc} - \text{Pro} - \text{Trp} - \text{Pro} - \text{Arg} - \text{Pro} - \text{Glu} - \text{Ile} - \text{Pro} - \text{Pro} - \text{O} \\
    & \text{double couplings with TBTU/HOBt}
\end{align*}
\]

Scheme 1. Strategies for synthesis of BPP9 analogues using different linkers and resins (Wang resin, chlorotrityl resin, HYCRAM™ resin) and different protecting group combinations.
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 dose-response 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).

### Table 1

Analogues of the bradykinin potentiating peptide BPP\(_{18}\) (TEPOTIDE) with distinct differences between potentiating 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\(_{50}\) (nM) | Quotient |
|----------|--------------------|-----------------|------------------|---------|
| BPP\(_{18}\) | Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro | 100 | \(3 \times 10^{-4}\) | 7.0 |
| [1-Pro]-BPP\(_{18}\) | Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro | 115 | \(5 \times 10^{-4}\) | 1 |
| J527 | Pro-Trp-Pro-Lys-Pro-Gln-Ile-Pro-Pro | 70 | \(3 \times 10^{-4}\) | 0.1 \(10^{3}\) |
| ASA-Arg-Pro-Gln-Ile-Pro-Pro | 50 | \(1 \times 10^{-4}\) | 0.5 \(10^{3}\) |
| Pro-Trp-Pro-Phe-Pro-Gln-Ile-Pro-Pro | 90 | \(5 \times 10^{-4}\) | 0.1 \(10^{3}\) |
| J526 | Pro-Trp-Pro-Lys-Pro-Gln-Ile-Pro-Pro | 70 | \(3 \times 10^{-4}\) | \(1.0 \times 10^{4}\) |
| ASA-Arg-Pro-Gln-Ile-Pro-Pro | 50 | \(7 \times 10^{-5}\) | \(3.0 \times 10^{3}\) |
| ASA-Arg-Pro-Gln-Ile-Pro-Pro | 90 | \(2 \times 10^{-4}\) | 71 |
| ASA-Arg-Pro-Gln-Ile-Pro-Pro | 40 | \(7 \times 10^{-4}\) | \(4.0 \times 10^{5}\) |
| ASA-Arg-Pro-Gln-Ile-Pro-Pro | 55 | \(7 \times 10^{-4}\) | \(3.0 \times 10^{6}\) |
| ASA-Pro-Arg-Pro-Gln-Ile-Pro-Pro | 50 | \(7 \times 10^{-4}\) | \(1.0 \times 10^{5}\) |
| ASA-Pro-Arg-Pro-Gln-Ile-Pro-Pro | 110 | \(6 \times 10^{-3}\) | \(0.1 \times 10^{5}\) |
| ASA-Pro-Arg-Pro-Gln-Ile-Pro-Pro | 70 | \(8 \times 10^{-3}\) | \(2.6 \times 10^{5}\) |
| ASA-Pro-Arg-Pro-Gln-Ile-Pro-Pro | 40 | \(4 \times 10^{-3}\) | \(2.3 \times 10^{5}\) |

### Notes

1. 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\(_{18}\), the quotient was accounted to 1.
2. * 4-Azidosalicylic acid.
3. ** 4-Azidobenzoic acid.
4. *** p-Fluorophenylalanine.
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 BPPs 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 BPPs, 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 phosphatidylinositol 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 [3H]-labeled arachidonic acid from labeled phospholipids, presumably through activation of phospholipase A_{2} by a G_{alpha}-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 BPPs or ramipril.
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 BPP9/H9251 (20 nM) in Tyrode-solution with (A) and without [Ca\(^{2+}\)] (B). The arrows indicate the addition of BK, BPP9, lanthanum or calcium ions. W = wash period. Curves are examples of five separate experiments.

Fig. 4. Effect of BPP9 analogues J526 and J527 on basal level of inositol phosphate production in COS-7 cells transiently transfected with BKR-B2 cDNA. Cells were prelabeled with 4\(\mu\)Ci/ml myo\(^{[3H]}\)-inositol for 24 h and than stimulated with 3\(\times 10^{-8}\) M bradykinin for 5 min in absence or in presence of J526 (0.1 \(\mu\)M) or J527 (0.1 \(\mu\)M). Inositol phosphate formation was determined in quadruplicates. Shown are the mean \(\pm\) S.E.M. of four independent comparative experiments.

Fig. 5. Influence of BPP9 analogues J526 and J527 on the BK-induced increase in arachidonic acid production in COS-7 cells which have transiently expressed the bradykinin B2 receptor. Cells were prelabeled with 0.4\(\mu\)Ci/ml \([3H]\)arachidonic acid for 24 h and stimulated with 3\(\times 10^{-8}\) M BK for 30 min in absence or in presence of J526 (0.1 \(\mu\)M) or J527 (0.1 \(\mu\)M). 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 BPP9 (20 nM). The arrows indicate the addition of BK, BPP9, 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-B1 and BRK-B2, 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 \(\pm\) 0.03) of BK (10\(^{-8}\) M) is significantly \((p < 0.5; \text{t-test for paired samples}; n = 12)\) enhanced in the presence of phosphoramidon (MI = 1.19 \(\pm\) 0.03) (not shown).

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

As shown in Fig. 7 BPP9 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-...
Pro-c-Abu(Ph)-Arg [16], but is unable to enhance the migratory activity of the nonpeptide agonist FR190997 [4] and the BKR-B1 agonist desArg⁹-BK. BPP₉/H₉⁵₁ (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. [Hyp³,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-
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 as 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 heterologimerization. AbdAlla et al. [1] found that both the active high affinity angiotensin receptor AT1 and the BKR-B2 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-B2. The displacement curves show no differences between the displacement of [3H]BK by BK alone nor in the presence of potentiating peptides. This finding clearly demonstrates that the analogues of BPP9 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 B2 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 Ca2+. In a variety of publications the enhancement of the intracellular Ca2+-level is used as a qualitative or quantitative proof of the potentiating action. Our results with the inhibition of Ca2+ uptake from extracellular sources by La3+ indicates that the potentiation of BK-action requires mobilization of Ca2+ 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 La3+ agree well with the finding of Marcic et al. [49], who described the inhibition of resensitization of the BK receptor in CHO cells by La3+.

The inositol phosphates (IPn) formed in transiently with the B2 receptor transfected COS-7 cells opens the Ca2+ channels of the endoplasmatic reticulum leading to an increase of cytosolic Ca2+. But, both potentiating nonapeptides J526 and J527 are unable to enhance the BK-induced formation of IPn. Surprisingly, both potentiating peptides increase the basal level of IP3 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 IP3 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 calcylxin on the potentiation might indicate that the inhibited phosphatases are not involved into resensitization.
besides GPI and COS-7 cells the PMN represent our third system for studies about the potentiation mechanism. These cells constitutively express B₁ as well as B₂ receptors and have a cell specific set of proteases involved in BK degradation. In this system the potentiating nonapeptide BPPᵊ and ramiprilat accelerate the BK-induced migration. In contrast to Ignjatovic et al. [27], who found on other cells a concentration-dependent direct activation of the B₁ receptor in the absence of kinins, we could not detect any activity of the potentiating nonapeptide without administration of B₁ or B₂ 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 BPPᵊ 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 BPPᵊ, 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 potentiation 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 BPPᵊ 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 La³⁺ gave evidence that the potentiation of smooth muscle contraction requires extracellular Ca²⁺ 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 Ca²⁺ concentration. Application of potentiating peptides to COS-7 cells which were transiently transfected with the B₂ 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 BPPᵊ 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 leukocytes seems to be nearly exclusively evoked by the ACE inhibition. On these B₁ and B₂ receptor containing cells no direct interaction with the B₁ 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.

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