Review Article

Hypothesized and found mechanisms for potentiation of bradykinin actions

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Potentiation of hormone actions can occur by different mechanisms, including inhibition of degrading enzymes, interaction with the hormone receptor leading to stabilization of bioactive conformation or leading to receptor homo- and hetero-oligomerization, receptor phosphorylation and dephosphorylation or can occur by directly influencing the signal transduction and ion channels.
In this review the potentiation of bradykinin actions in different systems by certain compounds will be reviewed. Despite many long years of experimental research and investigation the mechanisms of potentiating action remain not fully understood. One of the most contradictory findings are the distinct differences between the inhibition of the angiotensin I-converting enzyme and the potentiation of the bradykinin induced smooth muscle reaction.
Contradictory findings and hypothesized mechanisms in the literature are discussed in this review and in some cases compared to own results. Investigation of potentiating actions was extended from hypotension, smooth muscle reaction and cellular actions to activation of immunocompetent cells. In our opinion the potentiation of bradykinin action can occur by different mechanisms, depending on the system and the applied potentiating factor used.

Keywords: Potentiation / Bradykinin B_1 and B_2 receptors / Angiotensin I-converting enzyme / Crosstalk / Polymorphonuclear leukocytes

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Potentiation of hormone action

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 [1–3]. For therapeutically used hormones this knowledge about potentiating compounds and their action mechan-

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Abbreviations: **A-II**, Angiotensin-II; **ACE**, Angiotensin I-converting enzyme (E.C.3.4.15.1); **ACE2**, Angiotensin converting enzyme-2; **APN**, Aminopeptidase N (E.C.3.4.11.2); **APP**, Aminopeptidase P (E.C. 3.4.11.9); **ASA**, 4-azidosalicylic acid; **AT_1 receptor**, Angiotensin-II receptor AT_1; **BK**, Bradykinin; **$BKR\text{-}B_1$**, Bradykinin B_1 receptor; **$BKR\text{-}B_2$**, Bradykinin B_2 receptor; **BPP**, Bradykinin potentiating peptide; **$BPP_{9\alpha}$**, Bradykinin potentiating nonapeptide 9α [Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro]; **BPP_{11B}**, Bradykinin potentiating undecapeptide 11B [Glu-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro]; **CHO**, Chinese hamster ovary; **CPM**, Carboxypeptidase M (E.C.3.4.17.12); **CPN**, Carboxypeptidase N (E.C.3.4.17.3); **DPPIV**, Dipeptidyl peptidase IV (E.C.3.4.14.5); **EP 24.15**, Metalloendopeptidase (E.C.3.4.24.15); **EP 24.16**, Metalloendopeptidase (E.C.3.4.24.16); **GAP1**, GTPase activating-protein 1; **GLUT-4**, Glucose-4 transporter; **GPI**, Guinea pig ileum; **IC_{50}**; 50% Inhibition; **INSR**, Insulin receptor; **IP_n**, Inositol phosphates; **IP_3**, Inositol-3,4,5-trisphosphate; **IP3-K**, Inositol-1,4,5-trisphosphate kinase; **IP_4**, Inositol-1,3,4,5-tetrakisphosphate; **IRS-1**, Insulin receptor substrate-1; **NEP 24.11**, Neutral endopeptidase (E.C.3.4.24.11); **NO**, Nitric oxide; **PI3-K**, Phosphatidylinositol-3 kinase; **PIP2**, Phosphatidylinositol-4,5-bisphosphate; **PLC**, phospholipase C; **PLC_β**, phospholipase C_γ; **PLC_γ**, phospholipase C_γ; **PMN**, Polymorphonuclear leukocytes; **QSAR**, Quantitative structure activity relationship; **RUT**, Rat uterus; **SHP-2**, SH-2 domain containing tyrosine phosphatase 2.

isms is very important. With this type of information therapies can be improved; for example, by a better determination of the proper dosage, increased awareness of possible negative side effects and interactions with other drugs. Therapeutic use of potentiating compounds also requires however, knowledge of the interactions on the molecular level.

Potentiation of a hormone action also helps in the uncovering of basic processes in signal transduction. At the receptor level potentiating peptides can enhance the affinity to the ligand, enhance the receptor density, trigger homodimerization or heterodimerization with other functional membrane proteins such as enzymes and other receptors, as well as trigger phosphorylation and dephosphorylation of the cytosolic receptor loops. Bradykinin potentiating peptides may directly activate G-proteins, increase receptor density, or attenuate a signal pathway which is evoked by another hormone or biologically active compound.

It is already known from numerous studies of the potentiation of bradykinin action that there exist various potentiating peptides with distinct sequences. Since the potentiating action was estimated with different *in vivo*, *ex vivo*, cell based assays and biochemical tests, additionally using different potentiating compounds a lot of conflicting results were obtained leading to an intense difference of opinion.

Bradykinin potentiating peptides

At least forty years ago a potentiating action was observed for the nonapeptide bradykinin (BK). Indeed the history of BK isolation and characterization has long been closely related to the use of potentiating factors. Rocha e Silva and coworkers [4] used snake venoms to trigger the formation of bradykinin 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 *et al.* [5, 6], Ferreira *et al.* [7, 8], and Ondetti *et al.* [9, 10] 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_{9a} (trade name TEPROTIDE). These peptides were first tested on the potentiation of BK-induced contraction of the isolated guinea pig ileum (GPI).

Bradykinin potentiating peptides have also been isolated from other snakes [11–16] or venoms from scorpions [17–19] as well as venoms from spiders [20–22] (Table 1). Surprisingly peptides with potentiating activity have also been formed by the partial hydrolysis of proteins from serum [23–27], hemoglobin [28–31], milk [32, 33], or wheat germ [34]. Also degradation fragments of angiotensin such as the heptapeptide 1–7 were found to potentiate the BK action [35]. In addition linear BK analogues, partial sequences [36], as well as certain active and inactive side chain and back bone cyclic BK agonists, are able to potentiate the BK action on GPI [37]. The contractile effects of BK on GPI and rat uterus (RUT) could be shown to be potentiated by thiol reagents [38–40], including cysteine, 2,3-dimercaptopropanol and also α-thioglycerol. Cysteine and 2,3-dimercaptopropanol were up to ten times more potent at BK induced relaxation than at the contraction, whereas other potentiating factors showed nearly the same activity in both test systems. These findings indicate that different mechanisms are involved.

A very special source of BPPs is the C-type natriuretic peptide precursor from the snake venom gland and from the snake brain. These BPPs taken from the brain of *Bothrops jararaca* inhibit the angiotensin I-converting enzyme (ACE; E.C. 3.4.15.1) in a nanomolar range and potentiate the BK effects *in ex vivo* and *in vivo* experiments. The presence of BPPs within the neuroendocrine regulator C-type natriuretic peptide precursor, and their expression in brain regions, suggest that these peptides belong to endogenous vasoactive compounds. Hayashi *et al.* assumed that both the C-type natriuretic peptide and brain BPPs could be physiologically interrelated, most likely in the fluid homeostasis process [41, 42].

Methods for measuring the potentiating action

The inhibition of bradykinin degradation was the first explanation of the potentiating effect, measured as the potentiation of BK-induced contraction of isolated smooth muscle organs. Potentiation of BK action has also been investigated in *in vivo* models, by pharmacological tests on isolated organs, and on the cellular level by biochemical methods. The potentiation of BK action has been measured *in vivo* on the hypotensive effect in freely moving Wistar rats [35]. Isolated organs such as the guinea pig ileum [47], rat heart [32], rabbit jugular vein [48, 49], cerebral microvasculature (permeability) [50] and porcine coronary arteries [51] have been used for *in vitro* tests.

Initial experimentation on the potentiation of the bradykinin action was primarily performed on isolated smooth muscle organs. In the last decade, the potentiat-

Table 1. Natural sources of bradykinin potentiating peptides. Potentiating peptides from different sources differ in their chain length and amino acid sequence. Potentiating activity was tested *in vivo*, in cell cultures and with biochemical methods.

Venoms	
Snake venoms	*Bothrops jararaca* [7–9, 10, 43], *B. insularis* [11], *B. jararacussu* [12], *B. neuwiedi* [13] *Agkistrodon halys blomhoffii* [5, 6], *A. halys Pallas* [14], *A. piscivorus* [15] *Echis multisquamatus* [16]
Scorpion venoms	*Tityus serrulatus* [17], *Buthus occitanus* [18], *Buthus martensii Karsch* [19]
Spider venoms	*Latrodectus tredecimguttatus*[20, 21], *Scaptocosa raptoria* [22]
Enzymatically fragmented proteins	
Rat plasma proteins	fragmented by Cathepsin Y [23, 24]
Human serum proteins	fragmented by Trypsin [25–27]
Hemoglobin	[28–31]
Milk proteins	[32, 33]
Wheat germ	[34]
Hormones, analogues and fragments	
Degradation fragments of Angiotensin	[35]
BPPs from the C-type natriuretic peptide precursor from snake brain	[41, 42]
Insulin	[44–46]

ing activity was mainly investigated on the affinity and density of the receptor [52], the intracellular mobilization of Ca^{2+} [52, 53], release of arachidonic acid [52, 53], of inositol phosphates [52], and of nitric oxide (NO) [54]. Later studies on the molecular level of BK-evoked actions have centered increasingly on cell cultures rather than smooth muscles. These studies primarily used Chinese hamster ovary cells (CHO cells) cotransfected with the B_2 receptor and ACE, ACE mutants or neutral endopeptidase (NEP) [55]. Endothelial cells are used because they constitutively express the B_2 receptor and ACE [53].

Hypothesized mechanisms

It is hypothesized that many different potentiating mechanisms exist, including not just nonreceptor-mediated as well as receptor-mediated reactions, but also reactions at the receptor molecule itself (Fig. 1). One of the earliest and most plausible explanations of potentiating action is the inhibition of bradykinin destroying enzymes like ACE, NEP and other proteases under certain physiological and pathophysiological conditions. Teprotide is considered to exert its hypotensive effect by inhibition of the renin angiotensin system [56]. Since BK degradation is linked to the activity and composition of proteolytic enzymes used in the test systems, different activities of the factors can result. Influence on membrane potential, and increased Ca^{2+} influx from extracellular sources are also possible reactions. Receptor-mediated actions include the intracellular mobilization of Ca^{2+}, increased formation of IP_n, NO, arachidonic acid and prostaglandins. Further hypothetical possibilities are the non BK receptor-mediated stimulation of the pathways by a direct interaction with G-proteins and crosstalk with pathways of other receptors. Also the receptor molecule itself can be phosphorylated or dephosphorylated at the cytosolic loops, or the receptor can homo- or hetero-oligomerize. The human genome has opened new hypothetical mechanisms. Thus, orphan receptors such as bradykinin receptor GPR 100 can act as a target for potentiating factors [57] enhancing the receptor affinity to physiological concentrations [58, 59].

An interaction of the potentiating peptides with the BK receptor has been suggested. For this interaction to occur, certain mechanisms might exist. Initially the potentiating factors could act similarly to 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 might be the influ-

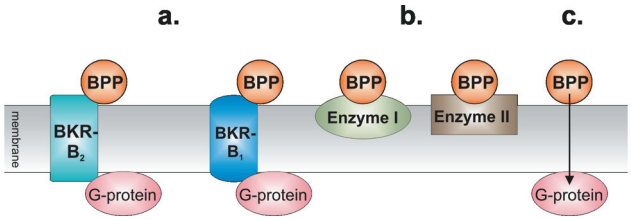


Figure 1. Possible direct interactions of bradykinin potentiating peptides with enzymes, bradykinin B_1 or B_2 receptors, and with G-proteins. The figure shows possible mechanisms for potentiation of the bradykinin action. **a)** Bradykinin potentiating peptides (BPP) can directly interact with the receptors. The stabilization of the bioactive receptor conformation leads to an amplification of bradykinin induced activation of G-proteins. **b)** Bradykinin potentiating peptides can inhibit different bradykinin degrading proteases. **c)** Bradykinin potentiating peptides can also stimulate G-proteins directly, non-receptor mediated, as shown for some other peptides [62–69].

ence of potentiating factors on receptor oligomerization, mainly homo- or heterodimerization. The influence of homodimerization by dimeric BK antagonists on the signaling was recently postulated by Chan *et al.* [60]. AbdAlla *et al.* [61] found that the active high affinity angiotensin receptor AT_1 and the BKR-B_2 are able to form heterodimeric complexes in smooth muscle cells. Most important, this heterodimerization evokes a signal enhancement, a further possible useful explanation of the potentiating effect.

Mechanisms found

Inhibition of the angiotensin I-converting enzyme

The angiotensin I-converting enzyme 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 blood pressure regulation. ACE is a membrane-bound ectoenzyme of vascular endothelial cells and is also expressed in several other cell types including male germinal cells. In somatic tissues, ACE is a glycoprotein of a single polypeptide chain of 140–170 kDa. In germinal cells, ACE is synthesized as a lower molecular mass form of 100–110 kDa, which displays catalytic properties for angiotensin I similar to the somatic ACE [70]. The somatic ACE, primarily involved in the enzymatic inactivation of BK, has two catalytic centers, slightly differing in their structural requirements for substrates and inhibitors and in their optimal catalytic conditions [70, 71]. Cotton *et al.* recently described, using domain-specific substrates and inhibitors, affinity differences between N-and C-terminal catalytic domains of about 3 orders of magnitude [72]. In 2003 Natesh *et al.* determined the crystal structure of human testicular ACE and its complex with one of the most widely used inhibitors, lisinopril, at 2.0 Å resolution [73]. Testis ACE is identical to the C-terminal half of somatic ACE, except for a unique 36-residue sequence constituting its amino terminus [74]. Thus, the structure provides an opportunity to design C-terminal catalytic domain-selective ACE inhibitors that may be exhibit new pharmacological profiles.

In addition to both isoforms of ACE, more recently ACE2 has been described as a homologue of the vasoactive peptidase ACE. Similarly to ACE, ACE2 is an integral membrane zinc metallopeptidase. ACE2 is less widely distributed than ACE in the body, being expressed in the highest concentrations in the heart, kidney and testis. ACE2 also differs from ACE in its substrate specificity, functioning exclusively as a carboxypeptidase rather than a peptidyl dipeptidase [75]. ACE2 seems to be, unlike ACE, a receptor for SARS coronavirus. ACE2 is therefore likely to be an important therapeutic target [75, 76], too.

The potentiation can not be exclusively reduced to inhibition of ACE

Yet even at the onset of the search for the molecular mechanism of BK potentiation, certain findings have been contradictory. The inhibition of ACE by various peptide and nonpeptide compounds have not correlated well with the potentiating activity [38, 39, 77]. Furthermore, the maximum of a BK-induced submaximal contraction of guinea pig ileum can be enhanced by potentiating compounds [78]. Also the action evoked by enzymatically stable BK agonists can be potentiated in some test systems [79]. In addition to these contradictory results, we also found that affinity labeled $BPP_{9\alpha}$ and some of its analogues are able to inhibit the $BPP_{9\alpha}$ induced potentiation of BK action on GPI. The BK action alone was not influenced. Because the expected inhibition of ACE by these labeled peptides should lead to a permanent potentiation of BK action other interactions are indicated [80]. 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 [51]. 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 [81].

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

BPPs have been intensively investigated. Derived from the natural sequences of bradykinin potentiating peptides numerous analogues have been synthesized.

Based on these synthetic analogues as well as structure activity relationship and conformational shape have been studied [82, 83]. Comparisons of smooth muscle contraction to ACE inhibition showed distinct differences between both activities. Conformational analyses by different spectroscopic methods i.e. circular dichroism, electronspin-resonance, and fluorescence, indicate a turned shape and allow to some degree a differentiation between conformations favorable for potentiation of GPI contraction or inhibition of ACE [84, 85].

The inhibitory and potentiating activities of some analogues selected from our very recent original publication [86] are listed in Table 2. As shown in that table, 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 a quotient was calculated from both activities. For the analogue [1-Pro]-$BPP_{9\alpha}$ this quotient was accounted to 1. We found the most distinct differences in the nonapeptide analogues and partial sequences labeled with azidosalicylic acid (ASA). Some quotients reach 10^5. The Table indicates strongly different structural requirements for both biological activities, and is inconsistent with the exclusive reduction of potentiating action to the inhibition of ACE. This conclusion might be drawn 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 [87], whereas the structural requirements for substrates and inhibitors of ACE seems to be less species independent. Thus, despite of some existing species specificities, ACE of nine different mammalian species could be tested with the same substrate and inhibited by captopril [88]. Interestingly, the potentiating activity is quite less influenced by amino acid replacements or modifications of the lead peptide BPP_{9a} than the inhibition of ACE.

Quantitative Structure Activity Relationship (QSAR) studies with activities taken from the literature became very difficult because of the use of differing biological tests applied from various authors. Only recently approaches have been undertaken to analyze and to predict biological activities [89–91].

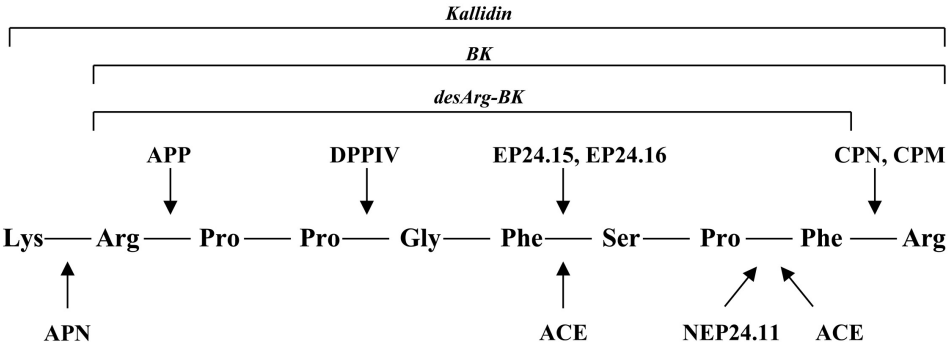
Using qualitative structure activity relationship studies, Cushman *et al*. [92] created a model of the active center of the bradykinin degrading proteolytic enzyme ACE. Based on this model, inhibitors of this enzyme were used as drugs for treatment of different forms of hypertension and heart failure. Because of the extensive therapeutic application of such bradykinin potentiating compounds such as captopril [92], enalaprilat [93], ramiprilat [94], quinaprilat [95] and lisinopril [96] studies on the molecular action mechanisms have become increasingly important.

Despite all these contradictory and to some degree confusing findings, Fortin and Regoli *et al.* [97] and Dendorfer *et al.* [48] have demonstrated that in their test systems (isolated rabbit aorta and venoconstriction) the potentiation by therapeutically used ACE inhibitors results exclu-

Table 2. Selected analogues of bradykinin potentiating peptide $BPP_{9\alpha}$ with distinct differences between potentiation of the bradykinin induced contraction of isolated guinea pig ileum (GPI) and inhibition of isolated angiotensin I-converting enzyme (ACE). The distinct differences between potentiation of bradykinin induced contraction of guinea pig ileum and inhibition of isolated guinea pig angiotensin-I converting enzyme disprove the assumption that this potentiation results exclusively from ACE inhibition. To quantify the differences between potentiation and inhibition a quotient was calculated from both activities. For the analogue with the highest ACE inhibitory activity [1-Pro]-$BPP_{9\alpha}$ the quotient was accounted to 1. Compound 8 shows about 160% potentiation compared to $BPP_{9\alpha}$, but to reach the same inhibition of ACE as the 1-Pro analogue a 10^5 higher concentration is required, resulting in a quotient of 10^5 [86].

No.	Sequence	Potentiation (%)	ACE inhibition IC_{50} (M)	Quotient
1	**Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (BPP_{9a})**	**100**	$\mathbf{3 \times 10^{-9}}$	**7.0**
2	**Pro-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro ([1-Pro]-$BPP_{9\alpha}$)**	**115**	$\mathbf{5 \times 10^{-10}}$	**1.0**
3	Pro-Trp-Pro-Leu-Pro-Gln-Ile-Pro-Pro	90	1×10^{-5}	2.5×10^4
4	Pro-Trp-Pro-Lys-Pro-Lys-Tyr-Pro-Pro	90	5×10^{-5}	1.3×10^5
5	Pro-Trp-Pro-Phe-Pro-Gln-Ile-Ala-Pro	70	3×10^{-6}	1.0×10^4
6	Leu-Pro-Gln-Ile-Pro-Pro	40	7×10^{-6}	4.0×10^4
7	Arg-Pro-Gln-Ile-Pro-Pro	55	7×10^{-6}	3.0×10^4
8	**Pyr-Trp-Pro-Lys(ASA)*-Pro-Gln-Ile-Pro-Pro**	**160**	$\mathbf{7 \times 10^{-5}}$	$\mathbf{1.0 \times 10^5}$
9	Pro-Trp-Pro-Lys-Pro-Gln-Ile-Pro-Pro	70	3×10^{-8}	0.1×10^3

* 4-azidosalicylic acid.



ACE:	Angiotensin I-converting enzyme (kininase II, E.C.3.4.15.1)
APN:	Aminopeptidase N (CD13; Ala-X aminopeptidase, E.C. 3.4.11.2)
APP:	Aminopeptidase P (X-Pro aminopeptidase, E.C. 3.4.11.9)
CPN, CPM:	Carboxypeptidase N (E.C. 3.4.17.3), Carboxypeptidase M (E.C.3.4.17.12) (kininase I)
DPPIV:	Dipeptidyl peptidase IV (CD26; E.C.3.4.14.5)
EP24.16, EP24.15:	Metalloendopeptidase (E.C.3.4.24.16/ 3.4.24.15)
NEP24.11:	Neutral endopeptidase (Neprolysin; CD10; E.C.3.4.24.11)

Figure 2. Enzymes involved in degradation of bradykinin. Bradykinin can be inactivated by different proteases. Depending on the test system used a different set of enzymes is involved in bradykinin degradation.

sively 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 [98] and a change in the density of B_2 receptors in rat spinal cord [99].

Other degrading enzymes

To understand the molecular basis for these discrepancies many approaches have been undertaken to elucidate the influence of potentiating compounds on different bradykinin destroying enzymes (Fig. 2), on the bradykinin receptors and on signal pathways. These studies include at the level of proteolytic enzymes, beside ACE, the neutral endopeptidase (NEP, Neprilysin; E.C.3.4.24.11) [100, 101], metalloendopeptidase (E.C.3.4.25.15/16) [50, 102], aminopeptidase P (E.C.3.4.11.9) [103], aminopeptidase N (E.C.3.4.11.2) [104] and carboxypeptidase M (E.C. 3.4.17.12) [105].

Therefore inhibitors of some of these enzymes are also used in studies on BK potentiation. Aminopeptidase P inactivates BK by hydrolyzing the Arg^1-Pro^2 bond. An inhibitor of this enzyme, Apstatin, can potentiate the vasodepressor response to BK and can significantly reduce cardiac ischemia damage [106]. Maggiora *et al.* predict that inhibitors of aminopeptidase P will exhibit cardioprotective effects by potentiating endogeneously released BK, possibly acting synergistically with ACE inhibitors in this regard [107].

Influence on the bradykinin receptor

Direct interaction of the potentiating compounds with the B_1 receptor has been postulated [55]. Phosphorylation of the receptor at its C-terminal part leads to internalization (desensitization) [108–110] and dephosphorylation by phosphatase SHP-2 to resensitization [111]. Bradykinin potentiating factors can influence homo- and heterooligomerization of the B_2 receptor including the interaction of that receptor with ACE [51] and other degrading enzymes [55] (Fig. 3). Very recently the spontaneous formation of a B_1 and B_2 bradykinin receptor complex has been described [112]. In cells that coexpress B_1 and B_2 receptors, these receptors directly interact to enhance signaling. This enhanced signaling capacity is similar to the potentiation of the hormone action. Thus, this finding contains a new possible mechanism for the potentiation of BK action. If BK potentiating factors can stimulate the formation of the B_1-B_2 receptor complex, they can also potentiate the action.

At the level of signal pathways, a crosstalk with other pathways induced by other hormones [44–46] or nonreceptor mediated intracellular reactions [62, 113] has been observed.

To study the influence of bradykinin potentiating peptides on the bradykinin B_2 receptor, COS-7 cells were transfected with the gene of the human receptor [86].

The obtained radioligand binding curves show no differences between displacement of [^{3}H]-BK by BK alone and

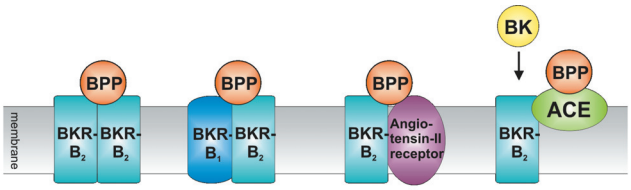


Figure 3. Influence on bradykinin B_1 or B_2 receptors by homo- and heterodimerization. Interaction of bradykinin potentiating peptides (BPP), with the receptor subtypes B_1 and B_2 or with the angiotensin-I converting enzyme can induce homo- or heterodimerization of receptors or complex formation between receptor and membrane bound enzyme. In all cases an amplification of the bradykinin action can be expected. The formation of the heterodimeric complex between BKR-B_2 and ACE or NEP triggered by BPP is described in the literature [47, 52, 53]. Heterodimerization of BKR-B_1 and BKR-B_2 [112], and of the angiotensin-II receptor AT_1 with BKR-B_2 [61] is detected without BPP. Homodimerization of the BKR-B_2 is only postulated [60, 114].

in the presence of potentiating peptides. Thus, the potentiating peptides have no effect on COS-7 cells in the binding of bradykinin, indicating that the peptides used are not able to enhance the receptor affinity. Our radioligand binding curves show no influence of the potentiating peptides on the receptor capacity.

This finding clearly demonstrates that the analogues of BPP_{9a} used in this study do not act as allosteric effectors stabilizing the active receptor conformation and do not induce homooligomerization of the receptor. Because COS-7 cells do not contain ACE, a heterodimerization of the B_2 receptor with somatic ACE as demonstrated by Marcic *et al.* [81] is not possible in our system. With this result we can not exclude a changed 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. 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 [81].

Influence of bradykinin potentiating peptides 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. 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 [86]. Results with La^{3+}, an inhibitor of the uptake of Ca^{2+} from extracellular space, agree well with the finding of Marcic *et al.* [53], who described the inhibition of resensitization of the BK receptor in CHO cells by La^{3+}. These results clearly underline the important role of extracellular Ca^{2+} for the BK-induced smooth muscle contraction and also are evidence that mobilization of intracellular Ca^{2+} is necessary for the potentiation by the peptides.

Bradykinin induces the formation of inositol phosphates in COS-7 cells transiently expressing the BK receptor. Analogues of the potentiating nonapeptide are not able to augment the BK induced intracellular concentration of inositol phosphates. The most striking result is the significant enhancement of the basal level by both potentiating peptides in the absence of bradykinin, possibly indicating a nonreceptor mediated pathway [86].

Bradykinin triggers the release of [^{3}H]-labeled arachidonic acid from labeled phospholipids, presumably through activation of the phospholipase A_2 by a G_a-protein. The level of arachidonic acid is significantly enhanced by BK in transfected COS-7 cells. Potentiating peptides slightly, but significantly, enhance the BK-mediated release of labeled arachidonic acid. Without BK neither potentiating peptide has any significant influence on the basal level [86]. Contrary to the missing effect of potentiating peptides on the BK induced enhancement of inositolphosphates the release of arachidonic acid is significantly increased. The molecular mechanism behind this acceleration remains unknown.

According to Rodrigues *et al.* [25] and Fernandes *et al.* [26] kinin potentiation by peptides, obtained from tryptic digestion of human serum proteins which does not involve ACE inhibition, is not due to an increased affinity to the receptor but may be involved in post receptor events linked to phospholipase A2.

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 (Fig. 4). Dephosphorylation of cytosolic Ser-, Thr- and Tyr-residues of the BK receptor results from activated protein phosphatases. Calyculin is known as a potent inhibitor of the protein phosphatases 1 and 2A. In our experiments calyculin did not influence the potentiation of the BK-induced contraction of guinea pig ileum. Application of calyculin, neither before nor after BK administration, did not change the potentiating effects of BPP_{9a} and ramiprilat on smooth muscle contractility [86].

In contrast in CHO cells coexpressing B_2 receptor and ACE and in human pulmonary endothelial cells, that

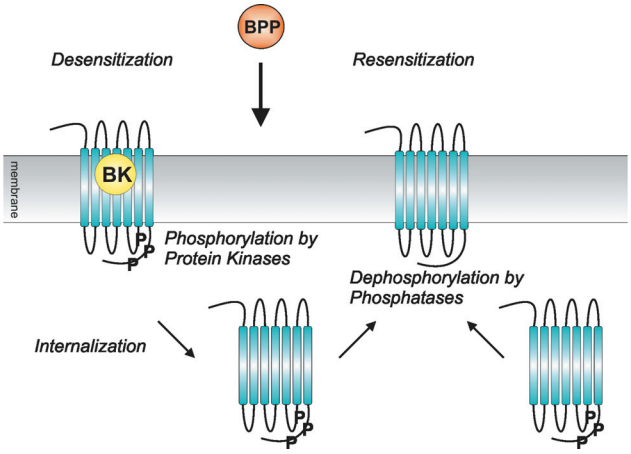


Figure 4. Influence on phosphorylation and dephosphorylation of the bradykinin receptor. As experimentally shown bradykinin potentiating peptides influences the desensitization and resensitization of the tissues or cells containing the bradykinin receptor. After bradykinin binding the receptor is phosphorylated at its cytosolic part and internalized. Dephosphorylation by phosphatases leads to receptor reintegration into the membrane and thus to resensitization. It can be assumed that bradykinin potentiating peptides can interact with kinases or phosphatases. Inhibition of internalization and accelerated resensitization lead to an enhanced receptor density, which could be considered as a further possible mechanism for potentiating action.

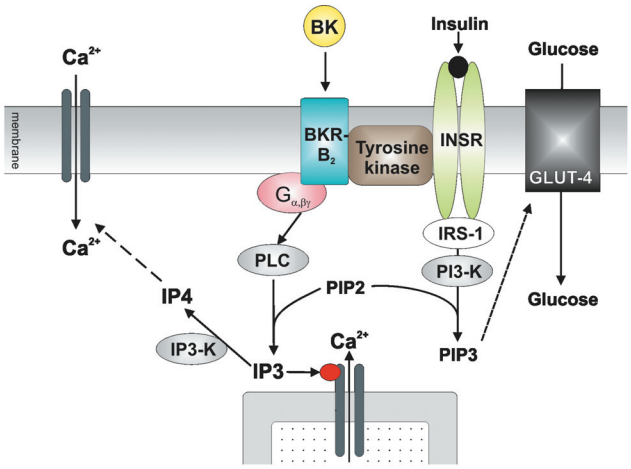


Figure 5. Potentiation of bradykinin action by insulin. Bradykinin stimulates via its B_2 receptor (BKR-B_2) and G-protein activation the phospholipase Cβ (PLC) which produces inositol-1,4,5-trisphosphate (IP_3) from phosphatidylinositol-4,5-bisphosphate (PIP2). This IP_3 can be phosphorylated by the inositol-1,4,5-trisphosphate kinase (IP3-K) to inositol-1,3,4,5-tetrakisphosphate (IP_4). IP_4 enhances the cytosolic Ca^{2+} level by opening a Ca^{2+} influx channel [106], by promoting Ca^{2+} release from sarcoplasmatic reticulum and by inhibition of IP_3 dephosphorylation [45,46], thus leading to an autocatalytically enhancement of Ca^{2+} signal [45,46]. Increased cytosolic Ca^{2+} activates the insulin receptor (INSR). The phosphatidylinositol-3 kinase (PI3-K), which is activated through the insulin receptor, and PLC_β, activated through BKR-B_2, compete for PIP2. Thus, inhibitors of PI3-K can enhance the BK induced IP_3 formation in the presence of insulin [45,46]. A direct interaction between the bradykinin B_2 receptor and the insulin receptor by a genistein and tyrphostin sensitive tyrosine kinase is postulated [46], resulting in a potentiation of bradykinin evoked IP_4 production and in enhancement of insulin-mediated glucose uptake through insertion of glucose-4 transporters (GLUT-4) into the membrane [44].

constitutively express both proteins, phosphatase inhibitors such as calyculin blocked the ability of angiotensin I-converting enzyme inhibitors to resensitize the receptor to bradykinin without altering the primary effects of bradykinin [115]. We believe that the differences result from different test systems and time scales.

Crosstalk of signal pathways evoked by bradykinin and insulin receptors

A number of studies on patients with essential hypertension or non-insulin-dependent diabetes mellitus have indicated minor improvements in glucose homeostasis and correction of dislipidaemia, indicating that BK can enhance the insulin-mediated glucose transport across cell membranes and thereby utilization of glucose [44]. On the other side insulin enhances the BK response in rat skeletal myoblasts [45] and in neonatal rat cardiomyocytes [46]. The BK-induced IP_3 formation can be enhanced in myoblasts and cardiomyocytes by insulin partially through PLC_γ. IP_3 can be metabolized by IP_3 kinase to IP_4, which promotes the Ca^{2+}-mobilizing function of IP_3 and inhibits IP_3 dephosphorylation. The IP_3 kinase is activated in a calmodulin-dependent manner via the insulin receptor. Since intracellular Ca^{2+} together with calmodulin is involved in regulation of insulin receptor conformation and consequently its activity this effect leads to an autocatalytical enhancement of the Ca^{2+} signal. IP_4 stimulates Ca^{2+} uptake from extracellular space. This IP_4-mediated Ca^{2+} influx is possibly influenced by GAP1and Ras as shown for fibroblasts [116]. Ca^{2+} uptake is stimulated by insulin. In the crosstalk between these two signal pathways a tyrosine kinase, highly sensitive to the kinase inhibitors genistein and tyrphostin, appears to play an important role. However, the signaling pathway between the bradykinin B_2-receptor and the insulin receptor remains partially unclear. Figure 5 gives to some degree an explanation for this potentiating action.

Unspecific potentiation

It should be noted that agonists other than BK can also be enhanced in their activity by BPPs. Thus, we could show that compared to BK, BPP_{11B} is able to enhance the contraction of GPI by 30% to 50%, induced by angiotensin II, eledoisin, histamine and also potassium chloride [62]. This result clearly indicates an unspecific, nonreceptor-

mediated element of the potentiating mechanism, possibly by direct G-protein activation. This kind of interaction was shown for mast cells [63–65, 69], human or rat leukemia cell lines [65], rat cerebral cortical membranes [66], and for isolated G-proteins [67, 68] using different peptides such as mastoparan, mellitin, substance P, adreno- corticotropic hormone (1–24), bradykinin [69], and was reviewed in reference [113].

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

Because BK plays an important role in inflammatory processes in which PMN are involved, the elucidation of the action mechanism of potentiating factors on different immunocompetent cells remains a challenge to biologists and immunopharmacologists.

Bradykinin stimulates the migration of PMN corresponding to its concentration gradient (Fig. 6. This effect can be characterized as a 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 [117]. 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. The migratory capacity of BK for PMN can also be potentiated by the ACE inhibitor ramiprilat. $BPP_{9\alpha}$ can enhance the migration of PMN induced as well as by BK and by the enzymatic stable BK agonist D-Arg^0-[Hyp^3,Thi^5,ε-Abu(βPhe)^8]-BK [118], but is unable to enhance the migratory activity of the nonpeptide agonist FR190997 [119] and the BKR-B_1-agonist desArg^9-BK.

In contrast to Ignjatovic *et al*. [54], who found in other cells a concentration-dependent direct activation of the B_1 receptor in the absence of kinins, no activity of the potentiating nonapeptide could be detected without administration of B_1 or B_2 agonists to the PMN cells. Though the migratory activity of the proteolytic stabilized BK-analogue is potentiated, the action of the nonpeptide agonist FR190997 could not be accelerated by BPP_{9a} or 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_{9a}, 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 a reduced intrinsic activity of the nonpeptide agonist FR190997 compared to BK itself, although its dose-response curve is shifted to the left [117].

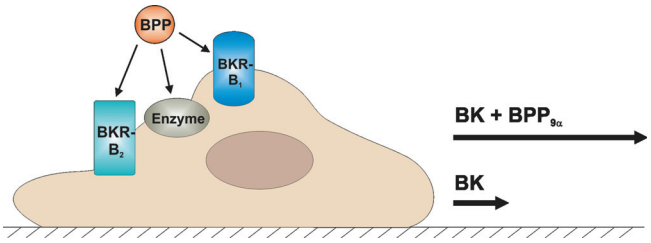


Figure 6. Influence of bradykinin potentiating peptides (BPPs) on migration of polymorphonuclear leukocytes (PMN). Polymorphonuclear leukocytes (PMN) contain both bradykinin receptor subtypes B_1 and B_2 and the neutral endopeptidase (NEP). Bradykinin induces a true chemotactic migration of these cells. The migration can be accelerated by the bradykinin potentiating nonapeptide $BPP_{9\alpha}$, but only in the presence of enzymatically labile bradykinin agonists, thus disproving a direct stimulation of B_1 or B_2 receptor by $BPP_{9\alpha}$ and indicating a potentiating action by inhibition of enzymatic bradykinin degradation.

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 beside the oligopeptides isolated from snake venoms have been used in these studies.

The broadening of the research field provides a clear indication that the potentiation of the bradykinin action can occur by different mechanisms, depending on the system used and on the applied potentiating factor. 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 enzymatic not fully stable bradykinin agonists, the species dependency of the bradykinin receptor, the very different protease compositions of the tissues and cell lines used in the different studies, and from the different structural requirements for both catalytic centers of ACE. Furthermore, we have to keep in mind the different densities of BK receptors, their localization in microdo-

mains 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 receptor independent signal transduction, on G-protein trafficking pattern in the cells and on activators or regulators of G-protein signaling. Additionally the potentiating effect can be differentiated into specific and unspecific. Consequently, the search for potentiation of hormone actions is strongly related to the very recent research on multifunctional signal proteins.

In our opinion the contradictory explanations regarding the mechanism of potentiation primarily result from different factors including the very high complexity of the systems involved (the kallikrein-kinin and renin-angiotensin systems), as well as the varying *in vivo* and *in vitro* tests used. Furthermore, we suggest that there exist different potentiating compounds with different mechanisms of action.

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