Title: Potentiation of des-Arg⁹-kallidin induced vasoconstrictor responses by metallopeptidase inhibition in isolated human umbilical artery.

Authors: Pelorosso, Facundo Germán
         Brodsky, Paula Tamara
         Zold, Camila Lidia
         Rothlin, Rodolfo Pedro

Primary laboratory of origin: Departamento de Farmacología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 9, 1121, Buenos Aires, Argentina. (PFG, BPT, ZCL, RPR)
Running title page

Running title: DAKD biological inactivation by NEP, APM and ACE inhibition

Corresponding author: Rodolfo Pedro Rothlin

Address: Paraguay 2155, 9th floor, Ciudad Autónoma de Buenos Aires (1121), Argentina

Phone/Fax: +54-011-4962-0300

e-mail: farmaco3@fmed.uba.ar

Number of text pages: 25
Number of tables: 2
Number of figures: 5
Number of references: 40
Number of words in Abstract: 231
Number of words in Introduction: 304
Number of words in Discussion: 1486

Nonstandard abbreviations: HUA, human umbilical artery; ACE, angiotensin converting enzyme; NEP, neutral endopeptidase.

Section: Inflammation & Immunopharmacology
Abstract

Several metallopeptidases have been reported to be involved in bradykinin (BK) B₁ receptor agonists metabolism. Our goal was to evaluate in vitro roles of metallopeptidases (e.g., neutral endopeptidase (NEP), aminopeptidase M (APM) and angiotensin converting enzyme (ACE)) as functional inactivators of the selective BKB₁ receptor agonist, Lys-des-Arg⁹-BK (DAKD), in isolated human umbilical artery (HUA) rings. Concentration-response curves (CRCs) to DAKD were performed after a 5 h incubation period. Treatment with 10 µM phosphoramidon (NEP inhibitor) or 10 µM amastatin (APM inhibitor) potentiated DAKD elicited responses while 1 µM captopril (ACE inhibitor) had no significant effects. However, when the three enzymes were simultaneously inhibited, a significant potentiation over responses obtained under concurrent NEP and aminopeptidase M inhibition was observed. In contrast, responses induced by the peptidase resistant BKB₁ receptor agonist, Sar-D-Phe⁸-des-Arg⁹-BK, were not modified by triple peptidase inhibition. In addition, endothelial denudation failed to alter DAKD induced responses in HUA. Finally, in the presence of NEP, ACE and APM inhibition, Lys-des-Arg⁹-Leu⁸-BK, the potent BKB₁ receptor antagonist, produced a parallel, concentration dependent, rightward shift of DAKD CRCs. The obtained pKₘ (8.57) and the Schild slope not different from unity are in agreement with an interaction at a single homogeneous BKB₁ receptors population. In summary, this work constitutes the first pharmacological evidence that metallopeptidases NEP, APM and ACE represent a relevant inactivation mechanism of the endogenous BKB₁ receptor agonist, DAKD, in isolated HUA.
Introduction

Functional studies have originally indicated the existence of two bradykinin (BK) receptors in mammalian tissues (Regoli et al., 1977). Such studies were later confirmed by molecular and genomic approaches (McEachern et al., 1991 and Menke et al., 1994, for BKB₂ and BKB₁ receptor subtypes, respectively). BK and the decapeptide Lys-BK (kallidin) are endogenous agonists at BKB₂ receptors. The cleavage of these peptides by the serine peptidases, carboxypeptidase N and carboxypeptidase M, yields the naturally occurring BKB₁ receptor agonists, des-Arg⁹-BK and Lys-des-Arg⁹-BK (DAKD). Several metallopeptidases, including neutral endopeptidase (EC 3.4.24.11, NEP), aminopeptidase M (EC 3.4.11.2, APM), and angiotensin converting enzyme or kininase II (EC 3.4.15.1, ACE), have been reported to be involved in BKB₁ receptor agonists inactivation (Marceau et al., 1998).

BKB₂ receptors are constitutively expressed in a variety of tissues and mediate most of the in vivo effects of kinins (Bathon and Proud, 1991). On the other hand, BKB₁ receptors are not present in any significant amount in normal tissues and their expression is often inducible rather than constitutive (Regoli et al., 1978; Sardi et al., 2000). Synthesis of BKB₁ receptors can be induced during tissue isolation trauma and incubation or under certain pathophysiological conditions conveying tissue injury or inflammation (Marceau et al., 1998). Among them, a relevant role has been attributed to BKB₁ receptor function in different processes, such as endotoxic shock (Pesquero et al., 2000; deBlois and Horlick, 2001), bronchial hyperresponsiveness (Huang et al., 1999), ischemia (Tschope et al., 2001), ischemia reperfusion (Lagneaux et al., 2003) and post-ischemic neovascularization (Emmanueli et al., 2002).
Taking this into account, the aim of the present work was to evaluate, through functional studies, the possible role NEP, ECA and APM might play in biological inactivation of DAKD in isolated HUA and to validate this vessel as yet another BKB₁ receptor model preparation.
Methods

Tissue preparation. Human umbilical cords were obtained from normal full term deliveries and excised midway between the child and the placenta. Immediately, cords were placed in modified Krebs’ solution at 4ºC (of the following mM composition: NaCl 119, KCl 4.7, NaHCO3 25, KH2PO4 1.2, CaCl2 2.5, MgSO4 1.0, EDTA 0.004, D-glucose 11). Written informed consent was obtained from each parturient.

Usually within 3 h after delivery, the samples were placed onto dissecting dishes containing Krebs’ solution and arteries (internal diameter approximately 1 mm) were carefully dissected free from Wharton’s jelly using micro-dissecting instruments and cut into rings of approximately 3 mm width. Whenever necessary, endothelium was removed by gently rubbing the inner side of HUA rings with a roughened steel rod.

Functional studies. Immediately after dissection, rings were suspended in 5 ml organ baths and stretched with an optimal resting tension of 2 to 4 g (Tufan et al., 2003). Changes in tension were measured with Grass isometric force transducers (FT-03C, Grass Instruments Co., Quincy, MA, U.S.A.) and displayed on Grass polygraphs (model 7D). During the incubation period, Krebs’ solution was maintained at 37 ºC and at pH 7.4 by constant bubbling with 95 % O2: 5 % CO2. Bath solution was replaced every 15 min with fresh bubbled buffer. Rings were incubated for 5 h and then CRCs to DAKD or Sar0-D-Phe8-des-Arg9-BK were obtained by cumulative addition of agonists in 0.25 log10 increments. Since the majority of isolated smooth muscle preparations become responsive to BKB1 receptor agonists as a function of time, HUA preparations were incubated for 5 h prior to obtaining the CRCs to DAKD or
Sar⁰-D-Phe⁸-des-Arg⁹-BK. Antagonist and peptidase inhibitors were applied 30 min before and during the addition of either agonist. Neither antagonist nor peptidase inhibitors produced any significant increase in basal tone of HUA rings when applied.

At the end of each CRC, 10 μM serotonin (5-HT) was applied to determine the tissue maximal contractile response (Altura et al., 1972) which was 7.96 ± 0.24 g (n=140). All experiments were performed in parallel with rings from the same umbilical cord. Only one agonist CRC was performed in each ring.

**Drugs.** The following compounds were used for functional studies: 5-hydroxytryptamine creatine sulphate complex from RBI (Natick, MA, U.S.A.); des-Arg⁹-KD and Lys⁰-des-Arg⁹-Leu⁸-BK (des-Arg⁹-Leu⁸-KD) from Bachem Bioscience Inc. (King of Prussia, PA, USA); Sar⁰-D-Phe⁸-des-Arg⁹-BK from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA); amastatin hydrochloride ((2S,3R)-3-Amino-2-hydroxy-5-methylhexanoyl-Val-Val-Asp hydrochloride) and captopril (N-[(S)-3-Mercapto-2-methylpropionyl]-L-proline) from SIGMA Chemical Co. (St. Louis, MO, U.S.A.); phosphoramidon (N-alpha-L-rhamnopyranosyloxy(hydroxyphosphinyl)-L-Leucyl-L-Tryptophan) from Peptides International Inc. (Louisville, KY, USA).

Preparation of all stock solutions and their subsequent dilutions were performed in glass bidistilled water. Stock solutions were stored in frozen aliquots and thawed and diluted daily.

**Expression of results and statistical analysis.** All data are expressed as mean ± SEM. The number of experiments n represents the number of rings from different cords tested. Responses are expressed as percentage of tissue maximum response elicited by 10 μM 5-HT. The estimates of EC₅₀ values (i.e.,
the agonists concentration that produces 50% of the maximal response), the maximal response ($E_{\text{max}}$) and the slope factor ($n_H$) were obtained using ALLFIT (DeLean et al., 1978). Briefly, responses obtained for each agonist concentration in each ring tested in the same group were averaged and then fitted to a four parameter logistic model expressed as

$$Y = a - E_{\text{max}} + \frac{d}{1 + \left(\frac{X}{EC_{50}}\right)^{n_H}}$$

where $Y$ is the response; $X$, the arithmetic dose and $a$, the response when $X = 0$. $EC_{50}$ were transformed into $pEC_{50}$ (-log $EC_{50}$). When using antagonists, statistical differences in $n_H$ were determined by constraining $n_H$ of treated tissues equal to $n_H$ of control values and assessing for significance by using $F$ statistics where $P$ values lower than 0.05 were taken to indicate significant differences (DeLean et al., 1978). When criteria for competitive antagonism were satisfied, that is, the antagonist produced a parallel rightward shift of the agonist curve without attenuation in maximum response, antagonist $pA_2$ values and slopes of Schild regressions were calculated by graphical extrapolation as described by Arunlakshana and Schild (1959). Where the slope of the Schild plot was not significantly different from unity, the regression was recalculated with Schild slope constrained to unity and the affinity value obtained was then referred to as $pK_B$. The measured slope of Schild plot is presented in order to illustrate whether agonist-antagonist interactions are consistent with an involvement of one or more receptor subtypes in mediating response of the agonist (Kenakin, 1992). Statistical analysis was performed by means of unpaired Student’s t-test or one-way analysis of variance (ANOVA) followed by Tukey’s post-test, when appropriate.
Results

Contractile effects of DAKD on isolated HUA.

Cumulative addition of DAKD to HUA rings, after a 5 h incubation period, produced a concentration dependent contraction. The estimated pEC$_{50}$ was 7.82 ± 0.28 (n=8) and the maximum response ($E_{\text{max}}$) was 48.7 ± 7.5 % of maximal contraction obtained with 5-HT 10 µM (Figure 1). Endothelium removal did not modify DAKD induced responses in HUA rings (pEC$_{50}$ 8.00 ± 0.15, n=8, $P>0.05$; $E_{\text{max}}$ 56.8 ± 10.0 %, $P>0.05$).

Potentiation of DAKD elicited responses in HUA by NEP and APM inhibition.

Treatment with phosphoramidon 10 µM, a selective NEP inhibitor, significantly augmented maximum DAKD elicited contractions in HUA (control: $E_{\text{max}}$ 49.3 ± 7.1 %, n=26; treated: $E_{\text{max}}$ 87.4 ± 1.1 %, n=6, $P<0.05$; Figure 2A; Table 1). In addition, a significant leftward shift of DAKD induced responses was observed by treatment of HUA rings with amastatin 10 µM, a selective APM inhibitor (control: pEC$_{50}$ 7.42 ± 0.09, n=26; treated: pEC$_{50}$ 8.24 ± 0.17, n=8, $P<0.05$; Figure 2B; Table 1). Maximum responses produced by DAKD were not significantly modified by treatment of rings with amastatin 10 µM (control: $E_{\text{max}}$ 49.3 ± 7.1 %, n=26; treated: $E_{\text{max}}$ 67.7 ± 14.0 %, n=8, $P>0.05$; Figure 2B; Table 1).

Lack of potentiation of DAKD elicited responses in HUA by ACE inhibition

Neither pEC$_{50}$ nor maximum responses to DAKD were modified by treatment with the selective ACE inhibitor, captopril 1 µM (Figure 2C, Table 1).
Effects of triple peptidase inhibition on DAKD induced responses in HUA.

CRCs to DAKD obtained in the presence of phosphoramidon 10 µM, amastatin 10 µM and captopril 1 µM yielded a pEC$_{50}$ value of 8.86 ± 0.09 and a maximum response value of 87.3 ± 2.0 % (n=6; Figure 3; Table 1). The potency of DAKD induced responses in the presence of triple peptidase inhibition was significantly increased over CRCs obtained in the presence of phosphoramidon 10 µM and amastatin 10 µM (pEC$_{50}$ 8.13 ± 0.11, n=8, P<0.05; Figure 3; Table 1).

Effects of triple peptidase inhibition on Sar$^0$-D-Phe$^8$-des-Arg$^9$-BK induced responses in HUA.

Sar$^0$-D-Phe$^8$-des-Arg$^9$-BK produced a concentration dependent contractile response of HUA rings (pEC$_{50}$ 6.84 ± 0.13, E$_{\text{max}}$ 70.3 ± 11.8 %, n=9; Figure 4). Exposure to captopril 1 µM, phosphoramidon 10 µM and amastatin 10 µM did not produce modifications of Sar$^0$-D-Phe$^8$-des-Arg$^9$-BK induced responses (pEC$_{50}$ 6.83 ± 0.11, E$_{\text{max}}$ 65.3 ± 11.6 %, n=9, P>0.05; Figure 4).

Antagonism of des-Arg$^9$-Leu$^8$-KD on DAKD induced responses in HUA

In rings pretreated with captopril 1 µM, phosphoramidon 10 µM and amastatin 10 µM, exposure to increasing concentrations of the selective BK B$_1$ receptor antagonist, des-Arg$^9$-Leu$^8$-KD (10, 30 and 100 nM) produced a competitive rightward shift of DAKD induced responses (Figure 5A; Table 2). Schild plot yielded a pA$_2$ value of 8.34 ± 0.14 and a slope of 1.29 ± 0.30. Since the slope was not significantly different from unity, regression was recalculated with the slope value constrained to unity and the pK$_B$ was obtained (pK$_B$ 8.57 ± 0.12; Figure 5B).
Discussion

In HUA, inhibition of NEP’s enzymatic activity caused a significant potentiation of vasoconstrictor responses induced by DAKD. NEP, a membrane bound M13 zinc metallopeptidase, was first detected in the brush border of animal kidney by Kerr and Kenny (1974). Since then, NEP presence has been described in many vascular tissues such as rat aorta smooth muscle, adventitial and endothelial cells (González et al., 1998), human endothelial cells (Llorens-Cortes et al., 1992) and cardiomyocytes (Kokkonen et al., 1999). Gafford et al. (1983) have shown that the selective BKB₂ receptor agonist, BK, is hydrolyzed by NEP. The primary site of hydrolysis in BK was reported to be at the Pro⁷-Phe⁸ bond. However, a secondary site for cleavage, Gly⁴-Phe⁵ was detected in that study. It has been proposed that the primary site for cleavage of DAKD by NEP might be at the Gly⁴-Phe⁵ bond (Marceau et al., 1998). Additionally, in functional studies, NEP’s ability to inactivate different vasoactive peptides in isolated smooth muscle preparations has been evaluated. For instance, BK-induced relaxation of vascular as well as bronchial smooth muscle has been shown to be potentiated by inhibition of NEP (Miyamoto et al., 2002; Frossard et al., 1990). However, to our knowledge, no reports have been made regarding any functional relevance of NEP on BKB₁ receptor agonists’ elicited responses.

In summary, our results constitute the first functional evidence that NEP might be involved in the biological inactivation of the naturally occurring BKB₁ receptor agonist, DAKD, in HUA biophase.

APM is a transmembrane M1 zinc metallopeptidase. Proud et al. (1987) have reported that APM is able to cleave the amino terminal Lys- of DAKD and Lys-BK into des-Arg⁹-BK and BK, respectively. Palmieri et al. (1989) characterized
APM activity in cultured porcine aorta endothelium and smooth muscle cells and suggested that vascular APM may modulate vasoactive peptide levels in vivo, particularly within the microenvironment of endothelial and smooth muscle cell surface receptors. Correspondingly, it has been demonstrated that APM inhibition can potentiate in vitro responses produced by angiotensin III in different isolated vessels (Robertson et al., 1992; Li et al., 1997). In addition, in lipopolysaccharide-pretreated rabbits, inhibition of APM by previous treatment with amastatin, increased duration of the hypotension induced by DAKD (Drapeau et al., 1991). The authors suggested that APM represents an inactivation pathway for the potent natural BKB₁ receptor agonist since its metabolite, des-Arg⁹-BK, is a much less potent BKB₁ receptor agonist. The present study, in agreement with these hypotheses, demonstrates that in HUA, inhibition of APM significantly potentiates DAKD induced contractile responses suggesting that this enzyme might be another important pathway of kinins inactivation in this tissue. According to available bibliography there is no previous evidence of potentiation of in vitro contractile responses by DAKD in human isolated vascular tissue induced by APM inhibition.

ACE is a single chain transmembrane M2 zinc metallopeptidase which converts angiotensin I into angiotensin II by removing the C-terminal dipeptide from the former (Skeggs et al., 1956). This enzyme has been described in endothelial cells of many origins (Erdos, 1990), in human heart (Kokkonen et al., 1999) and in rat aorta smooth muscle (Arnal et al., 1994). ACE cleaves the C-terminal tripeptide from BKB₁ receptor agonists, yielding BK₁₋₅ but at a slower rate, and with less affinity than the removal of the C-terminal dipeptide from BK (Marceau et al., 1998). It has been shown that ACE inhibition potentiates in vitro effects of
BK in vascular tissues (Miyamoto et al., 2002). However, in our model, ACE inhibition by captopril did not modify DAKD induced contractile responses. Similarly, Babiuk et al. (1982) failed to observe any potentiation of vasoconstrictor responses elicited by des-Arg⁹-BK or DAKD in isolated rabbit aorta by ACE inhibition. Nevertheless, in the present study, when ACE, NEP and APM were simultaneously inhibited DAKD induced responses in HUA were potentiated when compared with those obtained in conditions of concurrent APM and NEP inhibition.

Taken together, the above-mentioned results support our hypothesis that the potentiation of DAKD induced responses in HUA was due to impairment of its biotransformation in biophase. In addition, the absence of potentiation of responses elicited by Sar[D-Phe⁸]des-Arg⁹-BK, a peptidase resistant BKB₁ receptor agonist (Drapeau et al., 1993), by triple enzymatic inhibition substantiates our hypothesis. On the other hand, a direct agonist effect of ACE inhibitors has been proposed by Ignjatovic et al. (2002) who observed in several cell culture models that enalaprilat produced an agonist-like action which they considered belonged to stimulation of BKB₁ receptors. However, Fortin et al. (2003) have failed to observe such effect when evaluating responses to captopril, enalaprilat and zofenoprilat on isolated rabbit aorta and mouse stomach, two BKB₁ receptor model preparations. In addition, in the same study, enalaprilat produced negligible vasomotor effects on HUV, yet another extensively characterized BKB₁ receptor model. The absence of vasoconstrictor responses observed with captopril in HUA suggests a lack of activity of ACE inhibitors on BKB₁ receptors in this tissue.

NEP, APM and ACE have been characterized in endothelial cells of several
species including man (Walsh et al., 1993; Llorens-Cortes et al., 1992; Erdos, 1990). However, when we evaluated DAKD-elicited contractile responses in HUA, no differences were observed between responses obtained in intact and deendothelized rings. The lack of potentiation of responses induced by DAKD in HUA rings without endothelium suggests that, in this tissue, the enzymatic activity of NEP, APM and ACE responsible of biological inactivation of this agonist is not endothelial.

The only work to date that proposed the presence of BKB\textsubscript{1} receptors mediating contraction in HUA was carried out in presence of indomethacin, with low oxygen tension in Krebs media, and with unusually low obtained maximum responses (Abbas et al., 1998). In our study, CRCs to DAKD obtained in the presence of triple enzymatic inhibition, yielded a maximum effect approximately 2-fold and a potency approximately 30-fold higher when compared to those obtained in control HUA rings. Taking into account this marked potentiation, we considered that the enzymatic inhibition of BKB\textsubscript{1} receptor agonist metabolism was the best condition for a more reliable pharmacological characterization of the receptor population involved in DAKD elicited responses in HUA. In this condition, a highly selective BKB\textsubscript{1} receptor antagonist, Lys-des-Arg\textsuperscript{9}-[Leu\textsuperscript{8}]-BK, competitively antagonized DAKD induced responses yielding a pK\textsubscrpt{B} value of 8.57. This finding is in agreement with previously reported affinities of Lys-des-Arg\textsuperscript{9}-[Leu\textsuperscript{8}]-BK for the human BKB\textsubscript{1} receptor (\(K\textsubscript{i} 1.3 \text{ nM}, \) Menke et al., 1994). In addition, the obtained Schild slope was not different from unity, consistent with involvement of a single homogeneous receptor population in DAKD-elicited responses (Kenakin et al., 1992).

Although evidence from different models suggests that the BKB\textsubscript{2} receptor
subtype mediates most of kinin actions under physiological conditions, the rapid and long lasting up-regulation of BKB$_1$ receptors after inflammatory insult implies that this receptor may become the dominant subtype in mediating chronic inflammation (Dray and Perkins, 1993). The relevance of the BKB$_1$ receptor subtype in different pathophysiological conditions has been reported. BKB$_1$ gene deletion prevents endotoxic shock by lipopolysacharide (LPS) in mice (Pesquero et al., 2000) and, in accordance with this report, deBlois and Horlick (2001) have suggested that haemodynamic and inflammatory phenomena observed after LPS treatment in green monkeys, a non-human primate model, are mainly mediated by BKB$_1$ receptor activation. Additionally, associated with other inflammatory stimuli, it has been described that BKB$_1$ receptor mRNA expression is increased in an allergen induced bronchial hyperresponsiviness model in rats (Huang et al., 1999). Moreover, allergic lung inflammation in ovalbumin-sensitized mice is diminished by systemic treatment with a BKB$_1$ receptor antagonist. The role of BKB$_1$ receptors in ischemic processes has also been studied. Lagneaux et al. (2003) have shown that des-Arg$^9$-BK infusion in an isolated rat heart low-flow ischemia-reperfusion model significantly reduced infarct size. Additionally, Tschope et al. (2001) have reported that the cardio protective effects of AT$_1$ receptor blockade after experimental myocardial infarction, by coronary artery ligation, are partly mediated by secondary activation of the BKB$_1$ receptor pathway. Moreover, Emmanueli et al. (2002) have established a functional role for the BKB$_1$ receptor in post ischemic neovascularization by means of a murine model of limb ischemia. According to the authors, B$_1$ signaling plays an essential role in reparative angiogenesis by modulating endothelial cell proliferation and survival.
In addition, they suggest that potentiation of this mechanism may exert a therapeutic effect by accelerating spontaneous tissue healing. Taken together, all these data underline the potential relevance of BKB1 receptor mediated actions in different pathological conditions. Taking into account the present results, the possible potentiation of BKB1 receptor agonists induced responses by NEP, APM and/or ACE inhibition in the above mentioned pathophysiological models could be tested. Data obtained from these future studies and from the present one could potentially contribute to the development of new therapeutic strategies for these pathologies.

In summary, this work constitutes the first pharmacological evidence that metallopeptidases NEP, APM and ACE represent a relevant inactivation mechanism of the endogenous BKB1 receptor agonist, DAKD, in isolated HUA.
Acknowledgments

We wish to thank the Instituto Médico de Obstetricia (Buenos Aires) for their efforts in providing umbilical tissues.
References


McEachern AE, Shelton ER, Bhakta S, Obernolte R, Bach C, Zuppan P, 

Menke JG, Borkowski JA, Bierilo KK, MacNeil T, Derrick AW, Schneck 

Miyamoto A, Murata S and Nishio A (2002) Role of ACE and NEP in 
bradykinin-induced relaxation and contraction response of isolated porcine 

Palmieri FE, Bausback HH and Ward PE (1989) Metabolism of 
vasoactive peptides by vascular endothelium and smooth muscle 

Pesquero JB, Araujo RC, Heppenstall PA, Stucky CL, Silva JA Jr, 
Walther T, Oliveira SM, Pesquero JL, Paiva AC, Calixto JB, Lewin GR and 
Bader M (2000) Hypoalgesia and altered inflammatory responses in mice 

metabolism in human nasal secretions during experimentally induced allergic 

Regoli D, Barabe J and Park WK (1977) Receptors for bradykinin in 

Regoli D, Marceau F and Barabe J (1978) De novo formation of vascular 


Footnotes

a) This research was supported by grants from the University of Buenos Aires (U.B.A; Grant M-049). Facundo Germán Pelorosso is a research fellow of the U.B.A.

b) Reprint requests: Rodolfo Pedro Rothlin

Address: Paraguay 2155, 9th floor, Ciudad Autónoma de Buenos Aires (1121), Argentina

Phone/Fax: +54-011-4962-0300

email: farmaco3@fmed.uba.ar
Legends for figures

Figure 1. Lack of effect of endothelium removal (□) on control (■) DAKD induced responses. The points represent the mean of 8 determinations made after the 5 h equilibration period; vertical lines show S.E.M. Abscissa scale: -log$_{10}$ of molar concentration.

Figure 2. A. Potentiation of control (■; n=26) DAKD induced responses by treatment with phosphoramidon 10 µM (□; n=6). * expresses significant differences between maximal responses. B. Potentiation of control (■; n=26) DAKD induced responses by treatment with amastatin 10 µM (□; n=8). * expresses significant differences between pEC$_{50}$. C. Lack of effect on control (■; n=26) DAKD induced responses in HUA rings by exposure to captopril 1 µM (□; n=9). Vertical lines show S.E.M. Abscissa scale: -log$_{10}$ of molar concentration.

Figure 3. Further potentiation of DAKD induced responses in presence of phosphoramidon 10 µM and amastatin 10 µM (■; n=8) by addition of captopril 1 µM (□; n=6) on HUA rings. Vertical lines show S.E.M. Abscissa scale: -log$_{10}$ of molar concentration. * expresses significant differences between pEC$_{50}$.

Figure 4. Lack of potentiation of Sar$^0$-D-Phe$^9$-des-Arg$^9$-BK induced responses in control (■; n=9) HUA rings when exposed to phosphoramidon 10 µM, amastatin 10 µM and captopril 1 µM (□; n=9). Vertical lines show S.E.M. Abscissa scale: -log$_{10}$ of molar concentration.
Figure 5. A. Concentration response curves for DAKD on control (■; n=10) HUA rings and on tissues exposed to 10 nM (□; n=7), 30 nM (●; n=7) or 100 nM (○; n=5) des-Arg⁹-Leu⁸-KD for 30 min. All rings were exposed to phosphoramidon 10 µM, amastatin 10 µM and captopril 1 µM for 30 min. Vertical lines show S.E.M. Abscissa scale: -log₁₀ of molar concentration. * expresses significant differences between pEC₅₀. B. Schild plot of concentration-response curves for DAKD on tissues exposed to des-Arg⁹-Leu⁸-KD 10 nM, 30 nM or 100 nM.
TABLE 1

Effects of peptidase inhibition on DAKD induced responses in HUA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$pEC_{50}$</th>
<th>$E_{max}$</th>
<th>$n_H$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>7.42 ± 0.09</td>
<td>49.3 ± 7.1</td>
<td>0.79 ± 0.12</td>
<td>26</td>
</tr>
<tr>
<td>P</td>
<td>7.27 ± 0.09</td>
<td>87.4 ± 1.1</td>
<td>0.89 ± 0.16</td>
<td>6</td>
</tr>
<tr>
<td>A</td>
<td>8.24 ± 0.17</td>
<td>67.7 ± 14.0</td>
<td>0.72 ± 0.17</td>
<td>8</td>
</tr>
<tr>
<td>C</td>
<td>7.54 ± 0.15</td>
<td>66.1 ± 12.3</td>
<td>0.67 ± 0.13</td>
<td>9</td>
</tr>
<tr>
<td>P + A</td>
<td>8.13 ± 0.11</td>
<td>92.3 ± 3.1</td>
<td>0.70 ± 0.11</td>
<td>8</td>
</tr>
<tr>
<td>P + C</td>
<td>7.32 ± 0.10</td>
<td>85.7 ± 1.8</td>
<td>0.73 ± 0.12</td>
<td>9</td>
</tr>
<tr>
<td>A + C</td>
<td>8.37 ± 0.05</td>
<td>81.4 ± 4.5</td>
<td>1.32 ± 0.17</td>
<td>15</td>
</tr>
<tr>
<td>P + A + C</td>
<td>8.86 ± 0.09</td>
<td>87.3 ± 2.0</td>
<td>0.94 ± 0.16</td>
<td>6</td>
</tr>
</tbody>
</table>

* Significant differences vs. control ($P<0.05$)
† Significant differences vs. single inhibition ($P<0.05$)
‡‡ Significant differences vs. dual inhibition ($P<0.05$)

$E_{max}$ is expressed as percentage of maximum responses obtained with 5-HT 10 µM. Values are expressed as mean ± SEM. Abbreviations: P, phosphoramidon 10 µM; A, amastatin 10 µM; C, captopril 1 µM.
TABLE 2

Effects of des-Arg⁹-Leu⁸-KD on DAKD induced responses in HUA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nM</th>
<th>pEC₅₀</th>
<th>Eₘₐₓ</th>
<th>nₜ</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>8.54 ± 0.08</td>
<td>87.2 ± 3.3</td>
<td>1.02 ± 0.16</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>des-Arg⁹-Leu⁸-KD</td>
<td>7.62 ± 0.12*</td>
<td>86.9 ± 6.4</td>
<td>0.99 ± 0.20</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>7.42 ± 0.12*</td>
<td>80.9 ± 5.5</td>
<td>1.29 ± 0.28</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>6.69 ± 0.06*</td>
<td>85.4 ± 7.4</td>
<td>1.56 ± 0.61</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

* Significant differences vs. control (P<0.05)

Eₘₐₓ is expressed as percentage of maximum responses obtained with 5-HT 10 µM. Values are expressed as mean ± SEM.
(Figure 1)
(Figure 2A)

A.

Isometric contraction
(% 5-HT 10^{-5} M)

- log [DAKD] (M)
(Figure 3)

Isometric contraction
(% 5-HT $10^{-5}$M)

- log [DAKD] (M)
Isometric contraction (% 5-HT 10^{-5}M)

- log [Sar-D-Phe^{8}-des-Arg^{9}-BK] (M)
(Figure 5A)

A.

![Graph showing isometric contraction (% 5-HT 10^-5M) vs. -log [DAKD] (M)](image-url)