Met-Lys-bradykinin-Ser-Ser, a peptide produced by the neutrophil from kininogen, is metabolically activated by angiotensin converting enzyme in vascular tissue

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Abstract

Bradykinin (BK) is a vasoactive nonapeptide cleaved from circulating kininogens and that is degraded by angiotensin converting enzyme (ACE). It has been reported that the PR3 protease from human neutrophil releases an alternate peptide of 13 amino acids, Met-Lys-BK-Ser-Ser, from high molecular weight kininogen. We have studied vascular actions of this kinin. Its affinity for recombinant B_1 and B_2 receptors is very low, as assessed by the binding competition of [^{3}H]Lys-des-Arg^9-BK and [^{3}H]BK, respectively, but Met-Lys-BK-Ser-Ser effectively displaced a fraction of [^{3}H]enalaprilat binding to recombinant ACE. Mutant recombinant ACE constructions revealed that affinity gap between BK and Met-Lys-BK-Ser-Ser is larger for the N-terminal catalytic site than for the C-terminal one, based on competition for the substrate Abz-Phe-Arg-Lys(Dnp)-Pro-OH in an enzymatic assay. Met-Lys-BK-Ser-Ser is a low potency stimulant of the rabbit aorta (bioassay for B_1 receptors), but the human isolated umbilical vein, a contractile bioassay for the B_2 receptors, responded to Met-Lys-BK-Ser-Ser more than expected from the radioligand binding assay, this agonist being ~30-fold less potent than BK in the vein. Venous tissue treatment with the ACE inhibitor enalaprilat reduced the apparent potency of Met-Lys-BK-Ser-Ser by 15-fold, while not affecting that of BK. In the rabbit isolated jugular vein, Met-Lys-BK-Ser-Ser is nearly as potent as BK as a contractile stimulant of endogenous B_2 receptors (EC_{50} values of 16.3 and 10.5 nM, respectively), but enalaprilat reduced the potency of Met-Lys-BK-Ser-Ser 13-fold while increasing that of BK 5.3-fold. In vascular tissue, ACE assumes a paradoxical activating role for Met-Lys-BK-Ser-Ser.

Keywords: angiotensin converting enzyme; bradykinin; vascular smooth muscle; receptors for bradykinin

1. Introduction

Bradykinin (BK)-related peptides (the kinins) stimulate 2 types of G protein coupled receptors, the B_1 and B_2 receptors after being generated by kallikreins from kininogens [1]. The nonapeptide BK itself, generated by plasma kallikrein from high molecular weight kininogen, has high affinity for B_2 receptors. Lys-BK, formed by tissue kallikrein's action on kininogens, is also a B_2 receptor agonist, but with enhanced affinity at the human and rabbit B_1 receptor. The latter receptor type is optimally stimulated by kinin fragments devoid of the C-terminal arginine and secondarily produced by arginine carboxypeptidases(s), des-Arg^9-BK and Lys-des-Arg^9-BK [1].

A new pathway of interest has been recently elucidated for kinin generation: the action of the neutrophil PR3 protease on high molecular weight kininogen releases a 13-mer, Met-Lys-BK-Ser-Ser also termed PR3-kinin [2]. Preliminary pharmacologic evaluation showed the absence of an affinity for recombinant B_2 receptors, a fair affinity for human B_1 receptors in a radioligand competition assay but a discrepant low potency to activate them (phospholipase C assay), and hypotensive effects *in vivo* mediated by both receptor subtypes, given that B_1 receptors were expressed in the animals [2]. The activation of the B_2 receptor at least must be accounted for by the generation of BK, Lys-BK and/or Met-Lys-BK, which are all known B_2 receptor agonists of nanomolar potencies at recombinant human or murine B_2 receptors [1], and it was found indeed that blood plasma generates BK, Met-Lys-BK and BK fragments from Met-Lys-BK-Ser-Ser [2].

The general hypothesis of the present study was that, if Met-Lys-BK-Ser-Ser has any physiological effects on vascular tissue, relevant activating peptidase(s) must be locally present to cleave the peptide into a high affinity receptor agonist. Thus, we have characterized the vascular

actions of Met-Lys-BK-Ser-Ser with respect to BK receptor subtype affinity and possible metabolic activation by known peptidases. Affinities for 3 potential recombinant molecular targets, the BK B_1 and B_2 receptors and angiotensin converting enzyme (ACE), were estimated using separate radioligand displacement assays and an enzymatic assay. Well characterized vascular smooth muscle contractility assays, the rabbit aorta for the B_1 receptors [3], the human umbilical vein and rabbit jugular vein for the B_2 receptor [4, 5], have been exploited. These and other isolated tissues are suitable to identify activating and inactivating pathways in the sense that local metabolism distorts the concentration-effect relationship of agonist peptides. Indeed, the concentration of these peptides at the vicinity of receptors in the smooth muscle is not necessarily in equilibrium with that present in the bathing fluid of the tissue, the rate of metabolic change caused by peptidases within the tissue being occasionally faster than the rate of agonist diffusion from the bathing fluid to the extracellular space of the tissue (this paradigm is illustrated with several examples relevant for peptide receptor ligands elsewhere [6]). We provide evidence for the paradoxical activation of Met-Lys-BK-Ser-Ser by ACE in vascular tissue.

2. Materials and methods

2.1. Radioligand binding competition assays

The binding of 3 nM [^{3}H]bradykinin (Perkin Elmer Life Sciences; 90 Ci/mmol) to adherent intact HEK 293 cells stably expressing B_2R-GFP was evaluated as described [7]. The binding assay of 1 nM [^{3}H]Lys-des-Arg^9-BK (Perkin Elmer Life Sciences; 88 Ci/mmol) to HEK 293a cells stably expressing B_1R-YFP [8] was performed as described [9]. These 2 assays, based on pharmacologically functional rabbit receptors that are fluorescent (continuous stable expression judged from visual inspection) and similar to their human counterparts, were applied to construct binding competition curves for a series of unlabeled peptides. Peptidase inhibitors, including the ACE inhibitor captopril, were present in the assay buffers in the 2 binding assays for receptors. HEK 293a cells were also exploited to transiently express human recombinant ACE and perform a [^{3}H]enalaprilat binding assay assay precisely as described (2 nM radioligand concentration) [11] (the peACE vector was a gift from Prof. P. Corvol, Paris, France). A 2 nM concentration of [^{3}H]enalaprilat was used to generate binding competition curves for kinins that are high affinity substrates of this peptidase and behave as bona fide competitors this radioligand in this binding assay [12]. The fixed concentration of radioligand used in each binding assay is superior to its K_D value determined for each molecular target, in such a manner that the vast majority of binding sites are available for the competition by unlabeled peptides.

2.2. Enzymatic assay applied to recombinant ACE mutants

If Met-Lys-BK-Ser-Ser was an ACE substrate, it would be predicted that it would compete for the hydrolysis of another ACE substrate. We applied a described enzymatic assay based on the

internally quenched fluorogenic substrate Abz-Phe-Arg-Lys(Dnp)-Pro-OH, obtained from Bachem (Torrance, CA, USA), knowing that it has an approximately equal low micromolar affinity for the two separate catalytic sites of ACE [12]. The sources of enzyme were membrane preparations from HEK 293a cells transiently expressing one of the recombinant ACE mutants inactivated for either catalytic site (N1-active; C1-active; with Lys substituting for two His residues in each catalytic site [13]; also gifts of Prof. P. Corvol). The mutant ACE vectors were sequenced to confirm their identity. Membranes from transfected or untransfected (control) cells were prepared as described, with some variations [7]. Briefly, cells were homogenized (2 75-cm^2 flasks) in 0.25 ml sucrose buffer (250 mM sucrose, 20 mM tricine buffer, 1 mM PMSF, 10 mg/ml leupeptin, 2 mg/ml pepstatin, and 10 mg/ml soybean trypsin inhibitor, pH 7.5). In the sequential centrifugation steps applied, the first (600 g, 5 minutes) and second pellets (15 000 g, 5 minutes) were discarded; the third (150 000 g, 3 hours) pellets was resuspended in the same buffer as a source of membrane enzyme (normalized on the basis of protein concentration, BCA Protein Assay, Pierce). The enzymatic assay was performed as described [12]. Briefly, to 1 ml cuvettes containing Abz-Phe-Arg-Lys(Dnp)-Pro-OH (10 µM, dissolved in 0.1 M Tris HCl buffer containing 0.05 M NaCl and 10 µM $ZnCl_2$, pH 7.0), were added 5 µg of membrane proteins and, optionally, a competitor (either BK or Met-Lys-BK-Ser-Ser in variable concentrations, or the ACE inhibitor enalaprilat). The heated (37°C) cuvette, submitted to magnetic agitation, was excited at 320 nm and the emitted fluorescence read continuously at 420 nm for 600 sec using an Aminco Bowman Series 2 luminescence spectrometer. The positive and linear slope of the time trace is proportional to the reaction velocity [12] and it has been calculated for the time interval 200-600 sec for each reaction.

2.3. Vascular smooth muscle contractility assays

A local ethics committee approved the animal experimentation. Rabbit aortic rings with intact endothelium (New Zealand White, 1.5–2 kg; Charles River Canada, Montreal, QC, Canada) were suspended under a tension of 2 g in 5-ml tissue baths containing oxygenated (95% O_2/5% CO_2) and warmed (37°C) Krebs' solution as previously described [14]. Contractility studies were performed after 3 or 5.5 h of *in vitro* incubation because the response mediated by B_1 receptors is acquired in a time- and protein synthesis-dependent manner in this preparation [1]. The external jugular vein, an established bioassay for the rabbit bradykinin B_2 receptor [7], has also been isolated from the animals and mounted in organ baths as described [15]. The institutional research ethics board approved the anonymous use of human umbilical cord segments obtained after elective cesarean section deliveries. Human umbilical cords stored at 4°C were obtained within 2 hr from cesareans. Segments of umbilical veins were dissected carefully from the cords and a metal rod was inserted into the lumen. Excess connective tissue was excised and rings (2-3 mm wide) were cut. Rings of umbilical veins were suspended under 2 g of baseline tension in 5-ml organ chambers containing oxygenated (95% O_2-5% CO_2) and warmed (37°C) Krebs' solution, as described above. Umbilical vein rings, a contractile bioassay for B_2 receptor ligands [4], were randomly assigned to agonist peptides and equilibrated for 3 h before the construction of cumulative-concentration curves.

The 3 vascular preparations were used to assess the effect of peptidase inhibitors, continuously present in the bathing fluid, or of receptor antagonists (introduced 30 min before the agonist) on the apparent potency of Met-Lys-BK-Ser-Ser and of a reference agonist (the B_1 receptor agonist Lys-des-Arg^9-BK for the aorta, the B_2 receptor stimulant BK for the umbilical and jugular veins). The full cumulative concentration-effect curves was recorded for each peptide; when the effect of

Met-Lys-BK-Ser-Ser was suspected to be submaximal at the highest tested concentration, a large concentration of the reference agonist was added to record the maximal contractile effect mediated by the concerned receptor subtype. Ancillary experiments were conducted in the rabbit isolated aorta to control the effect of peptidase inhibitors on the concentration-effect relationship for angiotensin I or Lys-BK (recorded 2 or 4 h post-mounting), as explained in Results.

2.4. Drugs

Met-Lys-BK-Ser-Ser was synthesized via standard solid-phase methodology on Merrifield resin using Fmoc-chemistry, purified and analyzed (general methods as in [16]). Both BK and Lys-des-Arg^9-BK were purchased from Bachem. LF 16-0687 (1-[[2,4-dichloro-3-[(2,4-dimethylquinolin-8-yl)oxy]methyl]phenyl]sulfonyl]-N-[3-[[4-(aminoiminomethyl]-phenyl]carbonylamino]propyl]-2(S)-pyrrolidinecarboxamide, mesylate salt), a previously described nonpeptide B_2 receptor antagonist [17], was a gift from Laboratoires Fournier (Daix, France). Compound 11 (2-{(2R)-1-[(3,4-dichlorophenyl)sulfonyl]-3-oxo-1,2,3,4-tetrahydroquinoxalin-2-yl}-N-{2-[4-(4,5-dihydro-1H-imidazol-2-yl)phenyl]ethyl}acetamide) is a specific nonpeptide antagonist of the B_1 receptors documented for the rabbit form of the receptor [18] and a gift from Merck Research Laboratories (West Point, PA). Enalaprilat dehydrate was from Kemprotec Ltd. (Maltby, Middlesbrough, United Kingdom), and Plummer's inhibitor (mercaptomethyl-3-guanidinoethylthiopropanoic acid) from Calbiochem (La Jolla, CA). The latter compound is a high affinity inhibitor of arginine carboxypeptidases [19]. Other drugs were from Sigma-Aldrich (St. Louis, MO).

2.5. Data analysis

Results are presented as means ± SE. When applicable, radioligand binding data were fitted by nonlinear regression to a one-site competition equation using a least-square method (Prism 4.0, GraphPad Software Inc., San Diego, CA) and IC_{50} values calculated from this procedure. The same computer program was use to draw concentration-effect curves (least square fitting of sigmoidal dose-response equation with variable slope) and to derive contractile EC_{50} values.

3. Results

3.1. Binding assays

Met-Lys-BK-Ser-Ser exhibits a very low affinity (IC_{50} > 2 µM) for recombinant B_1 and B_2 receptors, as assessed by the binding competition of [^{3}H]Lys-des-Arg^9-BK and [^{3}H]BK, respectively (Fig. 1A, B). The competing effect of the unlabeled form of each radioligand was established in these binding assays and was of nanomolar potency (table 1 reports numerical values for IC_{50} values). However, Met-Lys-BK-Ser-Ser (up to 100 µM) effectively displaced a fraction of [^{3}H]enalaprilat binding to recombinant ACE (Fig. 1C), while BK could displace virtually all radioligand from the ACE binding site. The micromolar IC_{50} values from the applied one-site competition model are reported in table 1, however the fitted curve for Met-Lys-BK-Ser-Ser is occasionally far from some experimental points. A possible explanation for these findings is a selectivity of Met-Lys-BK-Ser-Ser for one of the two catalytic sites found in tandem in ACE, for which both BK and enalaprilat have a strong affinity as a substrate (K_M 240-540 nM) and blocker (nanomolar potency), respectively [13, 20].

3.2. Enzymatic assay applied to ACE mutants

The possible selectivity of Met-Lys-BK-Ser-Ser for one of the ACE catalytic sites was tested by competition for the fluorogenic substrate Abz-Phe-Arg-Lys(Dnp)-Pro-OH used at a concentration (10 µM) superior for the reported K_m of either site (2.7 and 3.4 µM for the C- and N-domains, respectively) [12]. Membranes from untransfected HEK 293a cells did not catalyze the reaction (Fig. 2). Either the N1- or the C1-active mutant of ACE hydrolyzed the substrate (positive slope of the fluorescence intensity as a function of time) and this reaction was virtually abolished by

the addition of enalaprilat (100 nM) for either form of the recombinant enzyme. BK competed for the recombinant enzymes in a concentration-dependent manner, exhibiting nearly complete inhibition at a concentration equal to that of the substrate (10 µM). Met-Lys-BK-Ser-Ser had a lesser affinity for both forms of enzymes, the affinity gap between this peptide and BK being larger for the N1-active mutant than for the C1-active form (Fig. 2).

3.3. Contractility assays

The human isolated umbilical vein, a bioassay for the B_2 receptors, is contracted by Met-Lys-BK-Ser-Ser (Fig. 3A), this agonist being ~30-fold less potent than BK (Fig. 3B; numerical EC_{50} values in table 2). The specific nonpeptide B_2 receptor antagonist, LF 16-0687 (100 nM) displaced the concentration-effect curves of the 2 kinins to the right by 60-95-fold (Fig. 3B; table 2), suggesting that Met-Lys-BK-Ser-Ser contractile effect is ultimately mediated by B_2 receptors in this preparation. The ACE inhibitor enalaprilat (100 nM) reduces the apparent potency of Met-Lys-BK-Ser-Ser by 12-fold, while not affecting that of BK (Fig. 3; table 2).

The rabbit isolated jugular vein has been extensively used as a contractile bioassay for the BK B_2 receptors [21]. The influence of endogenously expressed ACE is stronger in this tissue, as BK is known to be potentiated by captopril treatment of the tissues [5], which has been reproduced using enalaprilat (Fig. 4B, table 2; 5.3-fold potentiation). Met-Lys-BK-Ser-Ser is nearly as potent as BK as a contractile stimulant of endogenous B_2 receptors (EC_{50} values of 16.3 and 10.5 nM, respectively), but enalaprilat reduced the potency of Met-Lys-BK-Ser-Ser 13-fold while increasing that of BK (fig. 4A), thus reproducing the large affinity gap between the 2 peptides evidenced using the radioligand binding assay.

The rabbit isolated aorta is a bioassay for the B_1 receptors and the only known nanomolar potency agonist of the human and rabbit forms of the receptor is Lys-des-Arg^9-BK [1], a finding that has been reproduced in present experiments (Fig. 5B; numerical EC_{50} values in table 2). Met-Lys-BK-Ser-Ser is a low potency stimulant of the preparation, a submaximal response having been obtained at a cumulative concentration of 613 nM (Fig. 5A). Thus, the difference of potency between the 2 stimulants is as large as in the radioligand displacement assay (Fig. 1A). Tissue treatment with the powerful and specific nonpeptide B_1 receptor antagonist, compound 11 (1 nM) [18], shifted the concentration-effect of Lys-des-Arg^9-BK to the right and abolished the effect of the submaximal concentration of Met-Lys-BK-Ser-Ser, supporting that both agents contracted the tissue via B_1 receptors. The contractile effects of Met-Lys-BK-Ser-Ser or of Lys-des-Arg^9-BK were not modified by co-incubation with the ACE inhibitor enalaprilat (100 nM) or Plummer's inhibitor (1 µM), a blocker of arginine carboxypeptidases that removes the C-terminal arginine from native kinins (Fig. 5A, B; table 2). However, both of these peptidase activities exist in the isolated rabbit aorta as shown by previously published findings reproduced here in part with tissues from the animals exploited in Fig. 5A-B. A loss of function is determined by enalaprilat treatment (100 nM) for angiotensin I (Fig. 5C; table 2) and by Plummer's inhibitor (1 µM) for Lys-BK (Fig. 5D; table 2), especially for lower concentrations of these contractile agonists, because the effect of these agonists on AT_1 and B_1 receptors of the rabbit aorta is largely attributed to their ***in situ*** conversion into angiotensin II and Lys-des-Arg^9-BK, respectively [22, 23]. The contractile potency of angiotensin II is not affected by enalaprilat (1 µM) [23] and that of Lys-des-Arg^9-BK, by Plummer's inhibitor (Fig. 5B) or other arginine analogs that share the same peptidase target [22].

4. Discussion

We found that the kinin generated by the neutrophil protease PR3, Met-Lys-BK-Ser-Ser, has only a micromolar affinity for BK B_2 receptors (Fig. 1B; table 1), as previously reported [2]. The affinity of the PR3 kinin is marginally better at B_1 receptors (Fig. 1A), but not as strong as previously reported by Kahn et al. [2]. Further, these authors reported that Met-Lys-BK-Ser-Ser activated phospholipase C in cells only at 1 µM, at variance with their own binding results, but possibly consistent with our affinity findings. Therefore, any significant vasoactive effect of this peptide must derive from its cleavage, leading to more conventional receptor agonists. Our basic hypothesis was that the peptidases present in freshly isolated blood vessels would process Met-Lys-BK-Ser-Ser into fragments that are active on kinin receptors.

The most salient findings are that Met-Lys-BK-Ser-Ser is a contractile agonist of the human umbilical or rabbit jugular veins variably less potent than BK but more potent than anticipated from the radioligand binding competition assay, and that the specific ACE inhibitor enalaprilat, further abates the effect of Met-Lys-BK-Ser-Ser (Figs. 3, 4), suggesting that the peptidase activates this peptide by removing the C-terminal dipeptide. The resulting peptide, possibly Met-Lys-BK, is antagonized by a low concentration of the specific B_2 receptor antagonist LF 16-0687 in the umbilical vein. We recently identified ACE in endothelial cells of the human umbilical vein (tissue sections, immunohistochemistry) [11]; in cultured endothelial cells derived from this vessel, the regulated expression of ACE has been described [11, 24, 25]. As previously reported [15], enalaprilat did not influence the apparent potency of BK in the contractility assay. The alternate ACE inhibitor captopril also failed to potentiate BK in this preparation [4, 26], suggesting that ACE is not abundant enough in the tissue to impair the equilibrium between BK

concentrations in the bathing fluid and those at the vicinity of B_2 receptors that mediate the contraction of venous muscle cells. However, ACE presence is functionally revealed by the metabolic activation of Met-Lys-BK-Ser-Ser, and even more so in the rabbit jugular vein, a thinner tissue already known to be more influenced by endogenous ACE on the basis that ACE inhibition potentiates BK [5]. Thus, Met-Lys-BK-Ser-Ser reaches a nanomolar potency in this bioassay, being almost equipotent to BK in untreated jugular veins. However, enalaprilat co-treatment re-established the wide potency gap between the 2 peptides in favour of BK.

The competition of [^{3}H]enalaprilat binding to ACE by ACE inhibitors or substrates has been previously reported [10, 11]. Both enalaprilat and BK have rather similar affinities for either catalytic site of somatic ACE [13, 20], leading to apparently seamless radioligand saturation or competition curves. However, the irregular competition curve of [^{3}H]enalaprilat binding to recombinant ACE generated by Met-Lys-BK-Ser-Ser (Fig. 1C) suggested to us the possibility of a preferential occupancy of one catalytic site by this peptide. ACE mutants essentially confirmed this hypothesis, the gap of potency between BK and Met-Lys-BK-Ser-Ser as an ACE competitor being larger for the N-terminal active site than for the C-terminal one (Fig. 2). The absolute micromolar kinin levels needed to inhibit ACE in either the binding or enzymatic assay should not be directly compared to affinity for receptors, because ACE is much more abundant than receptors in relevant cells and it mediates an irreversible hydrolysis reaction at a velocity that does not need to be maximal. We used an empirical pharmacological approach to identify peptidase(s) relevant for the activation of the 13-mer peptide in vascular tissue, but recent work identified peptides ending by Ser-Ser, Ser-Tyr and Ser-Pro as possessing an affinity for ACE in a fish protein hydrolysate [27]. These peptides acted as hydrolysis inhibitors of an ACE substrate, but it was not determined whether they were themselves ACE substrates.

Kahn et al. [2] stressed the activation of kinin B_1 receptors as a salient effect of Met-Lys-BK-Ser-Ser. This 13-mer has a mediocre micromolar potency as a contractile agonist of the rabbit isolated aorta (Fig. 5), the preparation on which the pharmacologic profile of the B_1 receptors was initially defined [21]. The effect of Met-Lys-BK-Ser-Ser was nevertheless mediated by B_1 receptors, as suggested by the effect of a low concentration of the nonpeptide antagonist compound 11. Met-Lys-des-Arg^9-BK and Lys-des-Arg^9-BK are both high affinity stimulants of the rabbit B_1 receptor (nanomolar EC_{50} values) [21]. However, there is no evidence for a metabolic activation of Met-Lys-BK-Ser-Ser in the rabbit aorta, the [^{3}H]Lys-des-Arg^9-BK binding competition assay showing that only a micromolar concentration of the 13-mer peptide displaced a fraction of the radioligand from B_1 receptors (Fig. 1A) and congruent with the potency in the contractile bioassay. Rabbit aortic contractions induced by various peptides are influenced by inhibitors of ACE, arginine-carboxypeptidases and others peptidases, as mentioned in Results (Fig. 5C, D) and elsewhere [3, 6]. It remains possible that the conversion of Met-Lys-BK-Ser-Ser into effective B_1 receptor agonist(s) occurs *in vivo*, if not importantly in the rabbit aortic model.

BK is degraded by ACE *in vivo* and ACE inhibitors may exert a fraction of their beneficial cardiovascular action through the potentiation of endogenous BK on B_2 receptors in humans [28-30]. Present results suggest that cell surface in the endothelium activate Met-Lys-BK-Ser-Ser, presumably by removing the C-terminal dipeptide Ser-Ser. While the BK-inactivating role of ACE is widely acknowledged, this ectopeptidase assumes a paradoxical activating role for this newly found kinin generated by the neutrophil PR3 protease.

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Figure legends

Fig. 1. Competition of the binding of 3 radioligands at their respective recombinant molecular target by Met-Lys-BK-Ser-Ser or a reference kinin. A. Competition of [^{3}H]Lys-des-Arg^9-BK (1 nM) binding to stably expressed B_1R-YFP in HEK 293a cells. B. Competition of [^{3}H]BK (3 nM) binding to stably expressed B_2R-GFP in HEK 293 cells. C. Competition of [^{3}H]enalaprilat (2 nM) binding to transiently expressed human somatic ACE. Results are expressed as the residual specific bindings (means ± SE of *n* duplicate determinations).

Fig. 2. Competition of the hydrolysis of Abz-Phe-Arg-Lys(Dnp)-Pro-OH by kinins in enzymatic assays based on ACE mutants. Values are means ± SE of the number of replicates indicated by *n*. See Results for full description.

Fig. 3. Contractility studies in the human isolated umbilical vein, a bioassay for the B_2 receptors. A. Cumulative concentration-effect curve for Met-Lys-BK-Ser-Ser as modified by the ACE inhibitor enalaprilat (100 nM) or the specific B_2 receptor antagonist LF 16-0687 (100 nM). B. Cumulative concentration-effect curve for BK as modified by the same two drugs. The control curves were constructed in the presence of the DMSO vehicle of enalaprilat. Separate tissues from the same individuals were used as controls. Values are means ± SE of the number of replicates indicated by *n*.

Fig. 4. Contractility studies in the rabbit isolated jugular vein, a bioassay for the B_2 receptors. A. Cumulative concentration-effect curve for Met-Lys-BK-Ser-Ser as modified by the ACE inhibitor enalaprilat (100 nM). B. Cumulative concentration-effect curve for BK as modified by

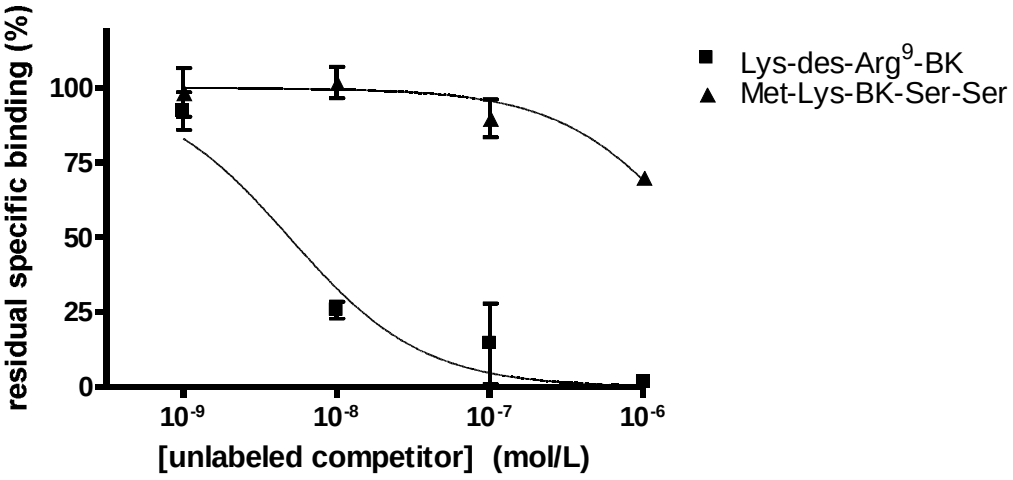
the same drug. The control curves were constructed in the presence of the DMSO vehicle of enalaprilat. Values are means ± SE of the number of 4 replicates.

Fig. 5. Contractility studies in the rabbit isolated aorta, a bioassay for the B_1 receptors. A. Cumulative concentration-effect curve for Met-Lys-BK-Ser-Ser as modified by the specific B_1 receptor antagonist compound 11 (1 nM), the ACE inhibitor enalaprilat (100 nM) or the arginine carboxypeptidase inhibitor Plummer's inhibitor (1 μM). The effect of the maximal tested agonist concentration differed between groups (ANOVA $P<0.001$); only the effect of Met-Lys-BK-Ser-Ser in the presence of compound 11 was different from that of control tissue (Dumnnet's test, $P<0.01$). B. Cumulative concentration-effect curve for Lys-des-Arg^9-BK as modified by the same three drugs. In A and B, the maximal effect mediated by B_1 receptors (100% reference) was ascertained by adding des-Arg^9-BK (10 μg/ml) at the end of the cumulative concentration-effect construction. The control curves were constructed in the presence of the DMSO vehicle of enalaprilat. C, D. Loss of apparent potency for angiotensin I- or Lys-BK-induced contraction of the rabbit aorta in the presence of enalaprilat or Plummer's inhibitor, respectively. All values are means ± SE of 4 replicates. Des-Arg^9-BK (11.1 μM) was applied to tissue at the end of the construction of the concentration-effect curves in A, B and D to ascertain the maximal effect mediated by B_1 receptors.

Figure 1

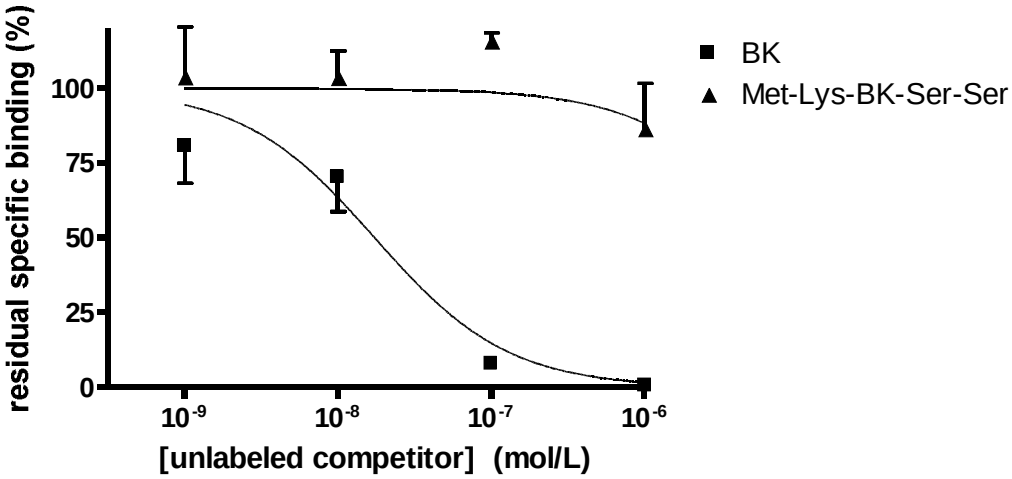
A. binding of 1 nM [^{3}H]Lys-des-Arg^9-BK to B_1R-YFP

n = 4



B. binding of 3 nmol/L [^{3}H]BK to B_2R-GFP

n = 3-4



C. binding of 2 nM [^{3}H]enalaprilat to ACE

n = 2-7

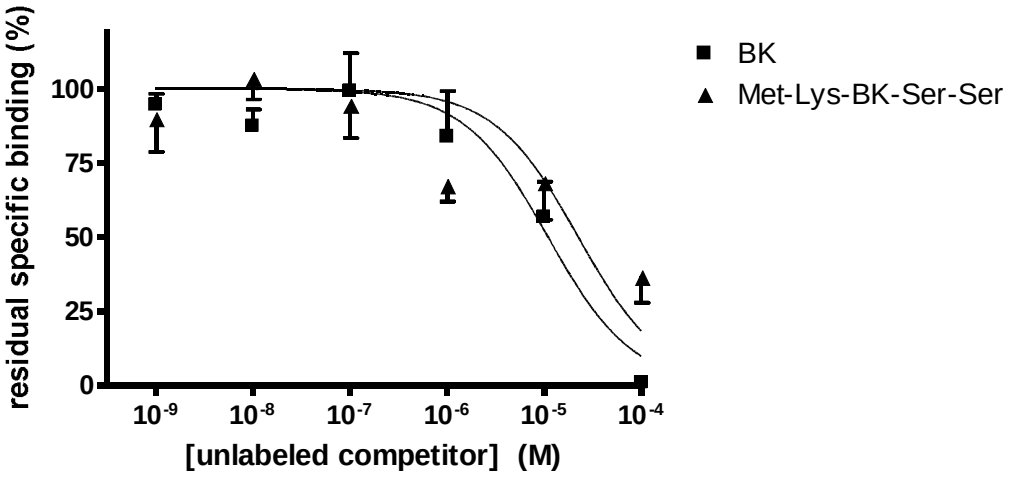


Figure 2

Fig. 2

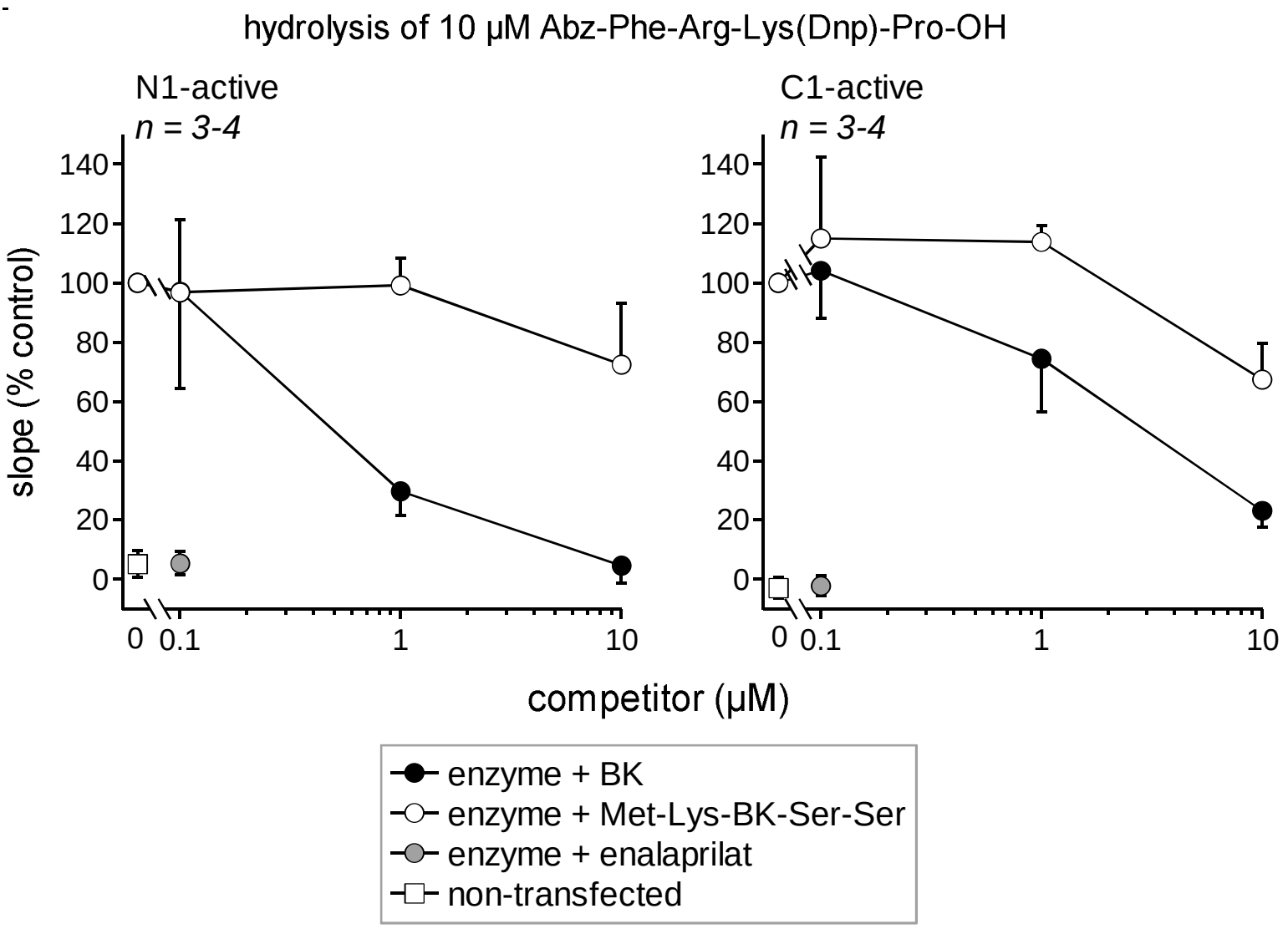


Figure 3

Fig. 3

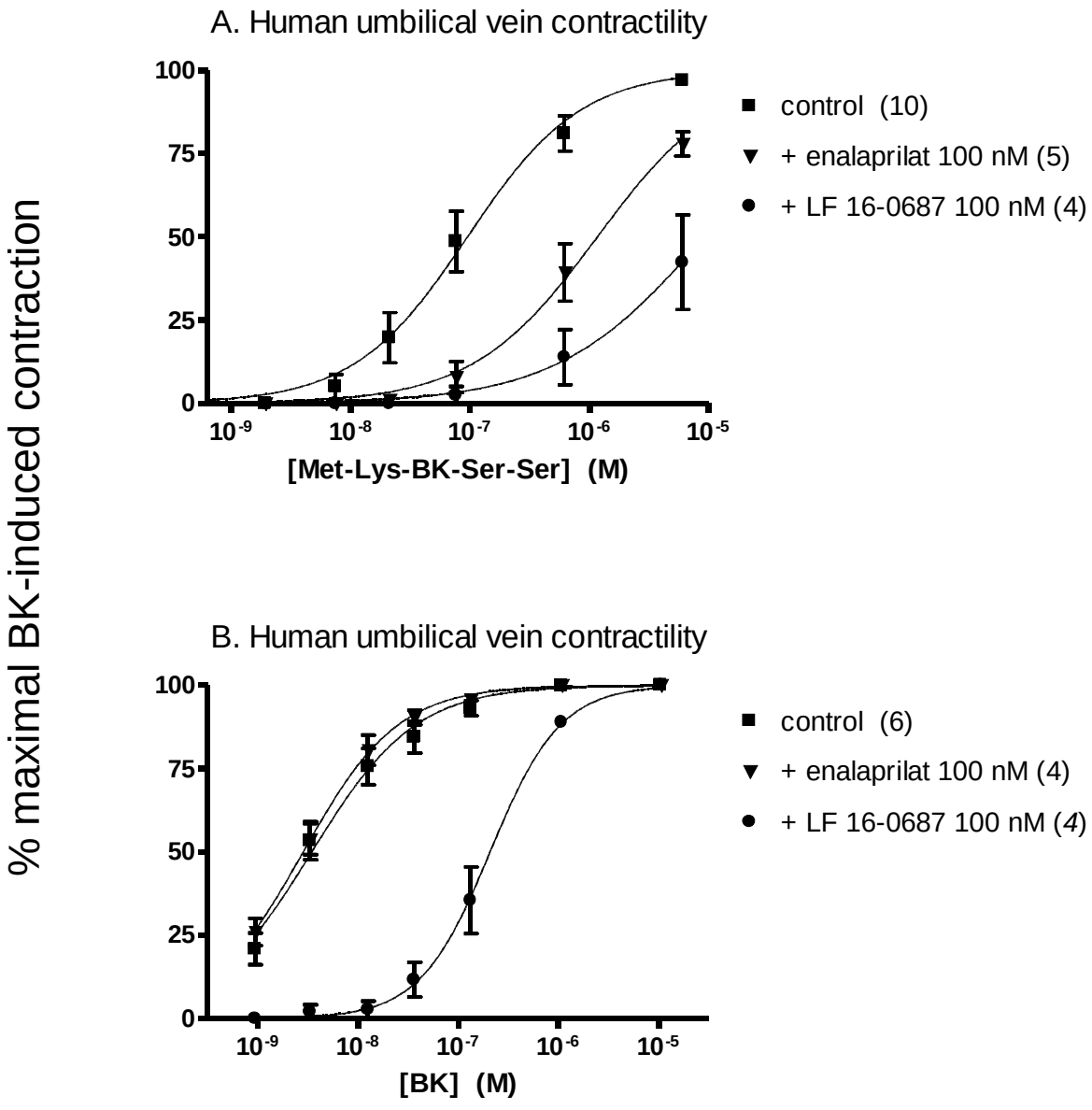


Figure 4

Fig. 4

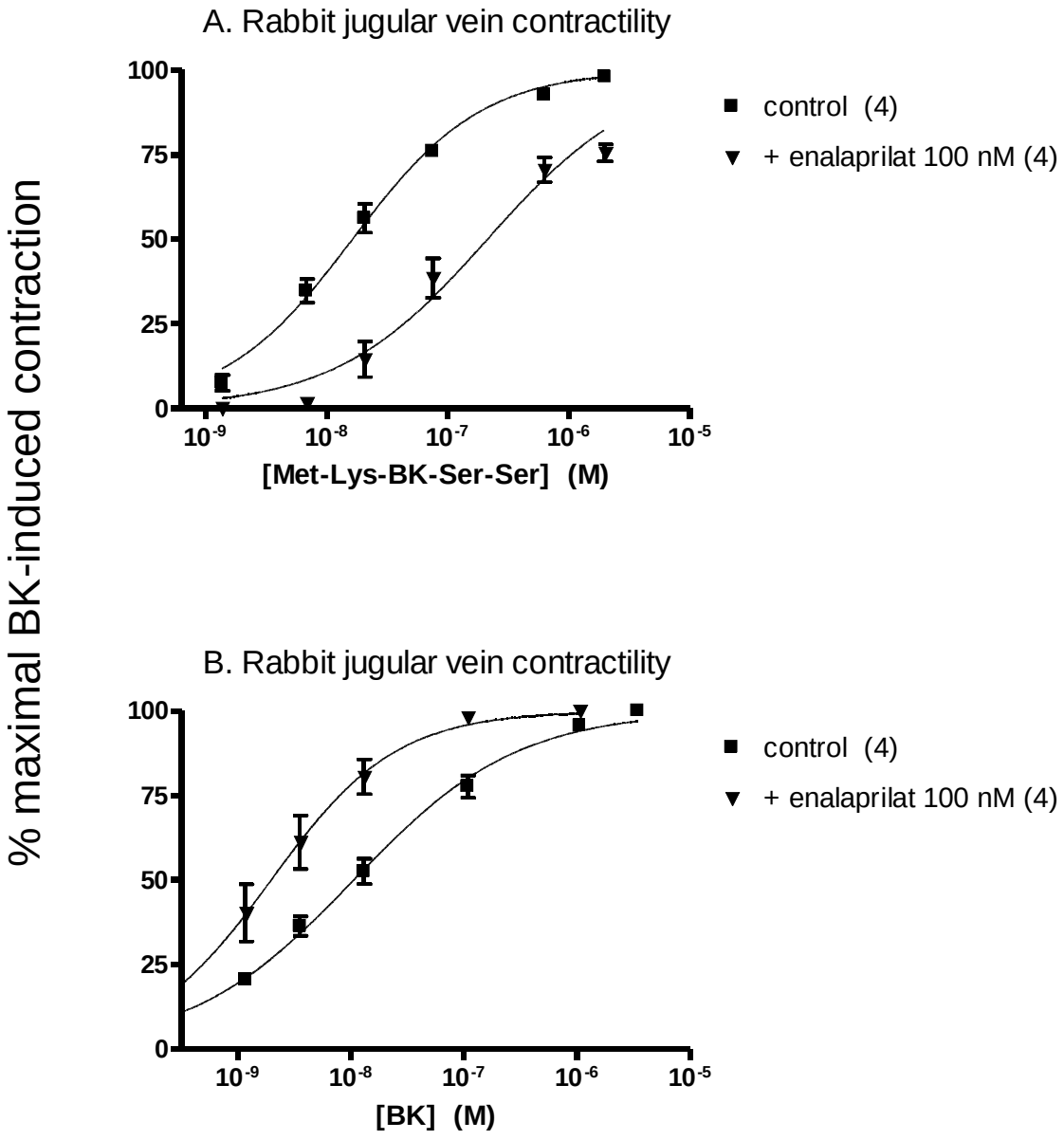


Figure 5

Fig. 5

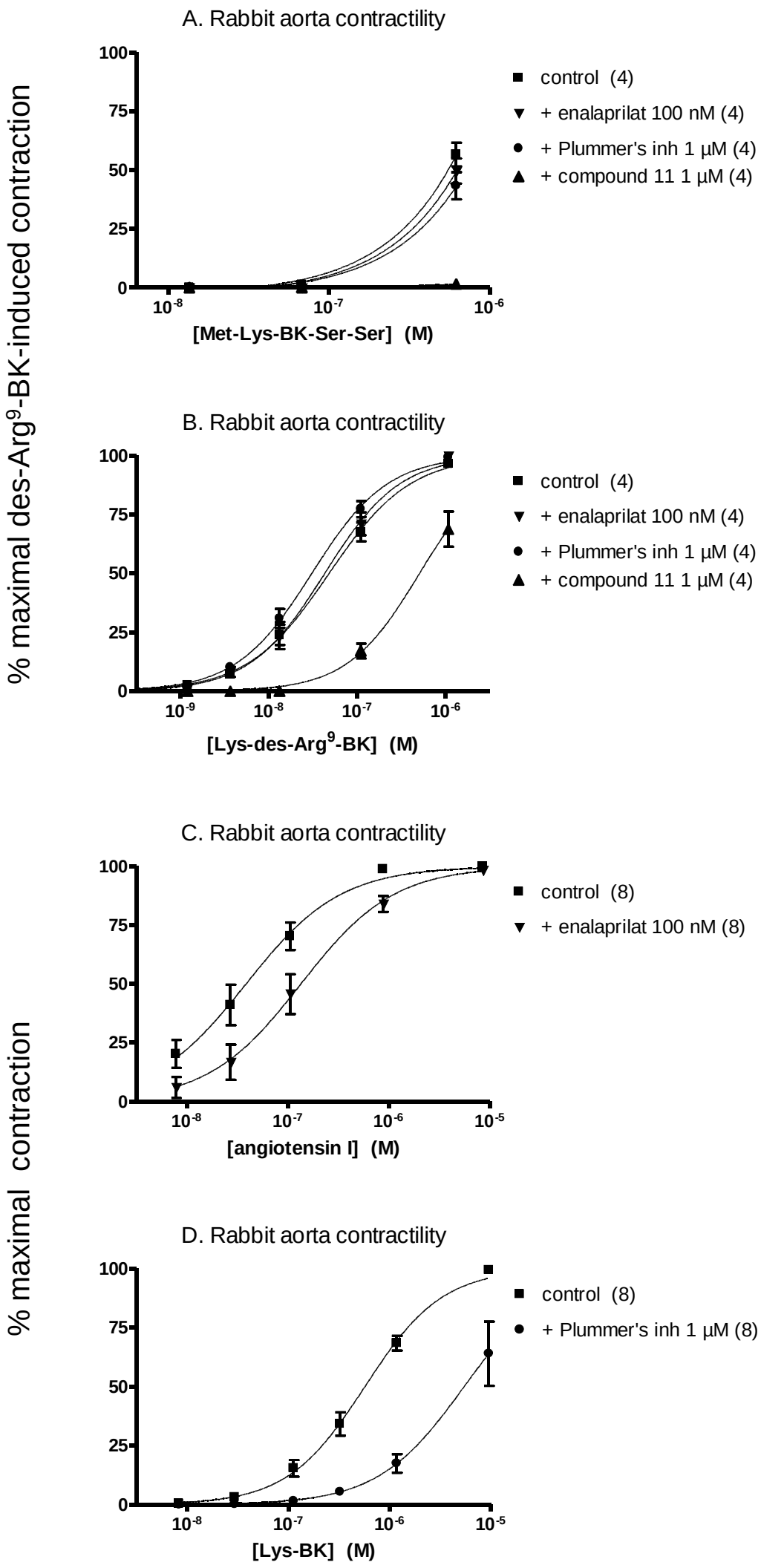


Table 1

Table 1. Parameters derived from radioligand binding competition assays.

	Met-Lys-BK-Ser-Ser		reference peptide		
Molecular target	**IC_{50}**	**log (IC_{50}) ± SE**	**identity**	**IC_{50}**	**log (IC_{50}) ± SE**
B_2R-GFP	7.6 µM[a]	-5.12 ± 0.51	BK	17.5 nM	-7.76 ± 0.14
B_1R-GFP	2.25 µM[a]	-5.65 ± 0.11	Lys-des-Arg^9-BK	4.9 nM	-8.31 ± 0.11
ACE	22.4 µM[b]	-4.649 ± 0.22	BK	11.0 µM	-4.96 ± 0.16

[a] extrapolated value

[b] calculated using the one-site model; however, the competition curve has an irregular shape.

Table 2

Table 2. Parameters derived from contractility assays.

Assay tissue	**agonist**	**co-treatment**	**EC_{50}**	**log(EC_{50}) ± SE**
Human umbilical vein	Met-Lys-BK-Ser-Ser	control	95.9 nM	-7.01 ± 0.07
		enalaprilat	1.17 µM	-5.93 ± 0.07
		LF 16-0687	9.18 µM	-5.04 ± 0.20
	BK	control	3.46 nM	-8.46 ± 0.05
		enalaprilat	2.84 nM	-8.55 ± 0.04
		LF 16-0687	208 nM	-6.68 ± 0.06
Rabbit jugular vein	Met-Lys-BK-Ser-Ser	control	16.3 nM	-7.79 ± 0.04
		enalaprilat	211.9 nM	-6.67 ± 0.07
	BK	control	10.5 nM	-7.98 ± 0.03
		enalaprilat	1.97 nM	-8.71 ± 0.07
Rabbit aorta	Met-Lys-BK-Ser-Ser	control	471 nM	---[a]
		enalaprilat	620 nM	---[a]
		Plummer's inh	860 nM	---[a]
		compound 11	>3 µM[b]	---[a]
	Lys-des-Arg^9-BK	control	47.8 nM	-7.32 ± 0.04
		enalaprilat	42.6 nM	-7.37 ± 0.05
		Plummer's inh	30.4 nM	-7.52 ± 0.03
		compound 11	504 nM	-6.30 ± 0.05
	Ang I	control	38.3 nM	-7.41 ± 0.07
		enalaprilat	133.8 nM	-6.87 ± 0.08
	Lys-BK	control	581 nM	-6.24 ± 0.05
		Plummer's inh	1.83 µM	-5.74 ± 0.03

[a] insufficient data to calculate

[b] extrapolated value

Graphical Abstract