Vasopeptidase-activated latent ligands of the histamine receptor-1

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Abstract

Whether peptidases present in vascular cells can activate prodrugs active on vascular cells has been tested with 2 potential latent ligands of the histamine H_1 receptor (H_1R). First, a peptide consisting of the antihistamine cetirizine (CTZ) condensed at the N-terminus of ε-aminocaproyl-bradykinin (εACA-BK) was evaluated for an antihistamine activity that could be revealed by degradation of the peptide part of the molecule. CTZ-εACA-BK had a submicromolar affinity for the BK B_2 receptor (B_2R; IC_{50} of 590 nM, [^{3}H]BK binding competition), but a non-negligible affinity for the human H_1 receptor (H_1R; IC_{50} of 11 µM for [^{3}H]pyrilamine binding). In the human isolated umbilical vein, a system where both endogenous B_2R and H_1R mediate strong contractions, CTZ-εACA-BK exerted mild antagonist effects on histamine-induced contraction that were not modified by omapatrilat or by a B_2R antagonist that prevents endocytosis of the BK conjugate. Cells expressing recombinant ACE or B_2R incubated with CTZ-εACA-BK did not release a competitor of [^{3}H]pyrilamine binding to H_1Rs. Thus, there is no evidence that CTZ-εACA-BK can release free cetirizine in biological environments. The second prodrug was a blocked agonist, L-alanyl-histamine, potentially activated by aminopeptidase N (APN). This compound did not compete for [^{3}H]pyrilamine binding to H_1Rs. The human umbilical vein contractility assay responded to L-alanyl-histamine (EC_{50} 54.7 µM), but the APN inhibitor amastatin massively (17-fold) reduced its apparent potency. Amastatin did not influence the potency of histamine as a contractile agent. One of the 2 tested latent H_1R ligands, L-alanyl-histamine, supported the feasibility of pro-drug activation by vascular ectopeptidases.

Keywords: angiotensin converting enzyme; aminopeptidase N; bradykinin B_2 receptor; histamine H_1 receptor; human umbilical vein; prodrug.

1. Introduction

Peptidases expressed in vascular tissue are important modulators of the pharmacology of vasoactive peptides. Angiotensin converting enzyme (ACE), expressed in endothelial cells, hydrolyses both angiotensin I and bradykinin (BK). In the first case, this is a physiological activation, as the product angiotensin II is the optimal agonist of the AT_1 receptors, while the second reaction leads to BK inactivation [1]. However, we reported lately that the peptide Met-Lys-bradykinin-Ser-Ser is paradoxically activated by its reaction with ACE, that frees the BK C-terminal sequence needed to activate the cognate B_2 receptor (B_2R) [2]. The modulatory role of vascular aminopeptidase N (APN) has also been illustrated, as the blockade of this ectopeptidase, expressed in vascular smooth muscle, potentiates such peptides as Lys-des-Arg^9-BK (the optimal agonist of human and rabbit BK B_1 receptor), some peptide antagonists of B_1 receptors and angiotensin III [3, 4].

BK is a fragile peptide inactivated rapidly both in the extracellular compartment and in endosomes. The nonapeptide BK, via its preformed, phosphorylable and G protein coupled B_2R, is an excellent example of an agonist submitted to endocytosis and degradation [5]. Endosomal BK degradation obligatorily precedes the dissociation of β-arrestins from the B_2R and receptor recycling to the cell surface, as shown by several inactivation-resistant B_2R agonists that promote the persistence of this intracellular complex for at least 12 h and prolonged signaling [6, 7]. Fluorophore conjugated analogs (carboxyfluorescein- or AlexaFluor-350-ε-aminocaproyl-BK) model the intracellular inactivation of the kinin, notably because the inhibitor of the proton pump V-ATPase, bafilomycin A1, prevents the time-dependent disappearance of the fluorescent peptides in endosomes of B_2R-expressing cells [8, 9]. Free carboxyfluorescein is also released into the cytosol as a function of time in these cells, suggesting a particular strategy to release a drug cargo from BK conjugates.

A possible manner to generate diversity in drug candidates is to design ligands for more than one pharmacological target; for instance omapatrilat has been designed to block both BK-destroying peptidases ACE and neutral endopeptidase (NEP) with nanololar affinities [10]. A further step could concern ligands that successively bind to one target, and then to another upon metabolic alteration. Among several carboxylic acids that have been condensed with ε-aminocaproyl-BK, we recently reported the antihistamine drug cetirizine (Fig. 1) [9]. As other N-terminally extended analogs of BK, cetirizine-ε-aminocaproyl-bradykinin (CTZ- εACA-BK) is a full agonist the B_2R, but with a low affinity [9]. A similarly designed analog, carboxyfluorescein-εACA-BK, has an affinity for ACE that is identical to that of BK [8]. Testing the concept of a pro-drug activable by vascular tissue metabolism may be based on CTZ- εACA-BK because cetirizine has a high persistence of its binding to histamine H_1 receptor (H_1R) [11]. Conjugated cetirizine, with its carboxylic acid function engaged in an amide bond, is predicted to have a low or no affinity for the H_1R. An alternative pro-drug potentially activated by a vasopeptidase was based on the agonist histamine condensed with alanine: L-alanyl-histamine (Fig. 1) was tested as a latent H_1R agonist activable by aminopeptidase N (APN, CD13, EC 3.4.11.2), on the model of the standard chromogenic substrate of APN, L-Ala-p-nitroanilide [12]. In addition to molecular studies based on recombinant B_2R and H_1R, we exploited the human umbilical vein contractility assay naturally expressing these two receptor types that mediate important contractile responses [13,14]. This vein also expresses ACE [2, 15]. The ectopeptidase APN is present in the vascular smooth muscle cells of the umbilical artery [3, 4] and at least in the endothelium of the umbilical vein [16, 17].

2. Materials and methods

2.1. Synthesis of L-alanyl-histamine

The synthesis of L-alanyl-histamine (4-[β-(α-alanylamido)-ethyl]-1-*H*-imidazole (2-amino-N-[2-(1*H*-imidazol-4-yl)ethyl]-(2S)-propanamide) has been previously reported [18] based on Boc-L-Ala and tritylsulfenyl-histamine using the diisopropyl-carbodiimide coupling reagent. This method was slightly modified using histamine dihydrochloride and the BOP coupling reagent. Diisopropylethylamine (4 mmol, 697 µl) was added to the stirred suspension of Boc-Ala (1 mmol, 189.2 mg) (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 1 mmol, 442.3 mg), and histamine dihydrocloride (1 mmol, 184.1 mg) in 25 ml acetonitrile. The mixture was stirred overnight at room temperature. The solvent was removed in vacuum and the oily residue was dissolved in 75 mL ethyl acetate. The organic phase was extracted with 5 % $KHSO_4$ (3 × 10 ml), brine (10 ml), 5 % $NaHCO_3$ (3 × 10 ml), brine (10 ml). The organic phase was dried over anhydrous Na_2SO_4. The solvent was evaporated affording a white powder. The crude product can be used for the Boc-deprotection without further purification or could be purified by preparative HPLC on a C18 column (131 mg, 46.4 %), (2-[[(1,1-dimethylethoxy)carbonyl]amino]-N-[2-(1*H*-imidazol-4-yl)ethyl]-(2S)-propanamide, N^a-Boc-L-alanyl-histamine, $C_{13}H_{22}N_4O_3$: 282.34).

The N^a-Boc-group was cleaved according to the classical deprotection procedure. The Boc-compound (141.2 mg, 0.5 mmol) was dissolved in 25 % TFA in dichloromethane (DCM, 25 ml). After 30 min, the solution was concentrated under reduced pressure at room temperature and the residue was lyophilized from 15 ml of H_2O to give the crude product as a trifluoroacetic acid (TFA) salt of 2-amino-N-[2-(1*H*-imidazol-4-yl)ethyl]-(2S)-propanamide (L-alanyl-histamine). The crude product was purified by preparative HPLC on a C18 column. The lyophilized L-alanyl-histamine.TFA salt was dissolved in 0.25 N cold HCl (25 ml) and was lyophilized to obtain the L-alanyl-histamine.2HCl salt (53.5 mg, 41.9 %, $C_8H_{14}N_4O.2HCl$: 255.15). LC-MC m/z calculated for $[M+H]^+$ 183.23, found 183.51.

2.2. Other drugs

BK was purchased from Bachem Biosciences (King of Prussia, PA) and histamine dihydrochloride, cetirizine dihydrochloride, pyrilamine maleate and amastatin from Sigma-Aldrich (St. Louis, MO). LF 16-0687 (anatibant; 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_2R antagonist [19], was a gift from Laboratoires Fournier (Daix, France). CTZ- εACA-BK has been previously described [9]. The vasopeptidase inhibitor omapatrilat was kindly provided by Bristol-Myers Squibb (Princeton, NJ).

2.3. Radioligand binding studies

$[^3H]$BK ([2,3-prolyl-3,4-^{3}H(N)]-bradykinin, 85.7 Ci/mmol) and $[^3H]$pyrilamine ([pyridinyl-5-3H]-pyrilamine, 20 Ci/mmol) were purchased from PerkinElmer (Boston, MA) and American Radiolabeled Chemicals, Inc. (St. Louis, MO). In preliminary experiments, Chinese-hamster ovary (CHO) cells were found suitable to express good levels of both H_1R and myc-B_2R; they were grown in Dulbecco's modified Eagle's medium, supplemented with fetal bovine serum (10%) and antibiotics. Cells were transiently transfected with the myc-B_2R vector [6], which codes for a fully functional N-terminally myc-tagged rabbit BK B_2 receptor, or a vector coding for human H_1R (clone HRH0100000 inserted into pcDNA3.1, obtained from University of Missouri-Rolla cDNA Resource Center, Rolla, MO) (transfection methods as in Gera et al., [8]).

The binding of either radioligand to adherent intact CHO cells transiently expressing H_1R or myc-B_2R (plates of 24 wells) was evaluated in the following manner: cells were washed with an assay buffer common to both binding assays (ice-cold phosphate-buffered saline [PBS], pH 7.4, supplemented with 0.02% NaN_3, 0.1% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 µM captopril). 0.5 ml of this buffer was left in each cell well, to which were optionally added cold competitors and the radioligand. After 90 min incubation at 0°C, cell wells were washed 3 times with 1 ml of ice-cold PBS. The wells' supernatants were discarded and the cells were dissolved in 1 ml NaOH 0.1 N. This suspension was transferred in a scintillation vial containing 7 ml of Ecolite Plus and counted for radioactivity.

The binding assays were applied to construct saturation curves for both radioligands by varying the concentration of each radioligand. Non-specific binding was obtained in matched cell wells co-treated with unlabeled BK (1 µM) or pyrilamine (1 µM) and subtracted from total binding to evaluate specific binding. A second series of experiments involved the competition of a fixed concentration of each radioligand with a panel of unlabeled drugs used at variable concentrations to evaluate their affinity at each receptor from binding competition curves.

2.4. Radioreceptor assay

A variation of the competition protocol (radioreceptor assay) was used to evidence the possible metabolic activation of the latent antihistamine effect of CTZ-εACA-BK by cellular systems competent to show the intracellular or extracellular degradation of BK. CTZ-εACA-BK (1 µM) was added to a simplified version of the PBS-based binding buffer (without PMSF of sodium azide for both cell systems, and also without captopril for ACE-expressing cells) that was pre-warmed and replaced the culture medium of the following cells in 35 mm petri dishes: (1) HEK 293 cells stably expressing the fluorescent construction B_2R-GFP, previously used to model the intracellular endosomal inactivation of BK [7]; (2) HEK 293a cells that transiently expressed recombinant human ACE, to model the extracellular inactivation of BK [2] (the corresponding peACE expression vector was a generous gift from Prof. P. Corvol, Paris, France). After 30 min of incubation at 37°C, the cells supernatants were harvested, centrifuged to remove any debris and frozen for further use. The thawed supernatants (0.5 ml/well), to which were added 2 nM [^{3}H]pyrilamine, were later directly used without dilution in the binding assay to H_1R expressed in CHO cells (see above). Displacement of [^{3}H]pyrilamine binding was taken as evidence of the release of free cetirizine from CTZ-εACA-BK.

2.5. β-arrestin$_1$ condensation assay

CHO cells were co-transfected with a vector encoding β-arrestin$_1$-cherry (kind gift from Dr. Martin Beaulieu, Université Laval, Quebec City, Canada) and another one encoding one of the receptors under study. The cells were stimulated with drugs (37°C, 30 min) and the red epifluorescence was then observed. The goal of the experiment was to monitor the endosomal condensation of the β-arrestin (as in [8]) in response to a given compound: if the condensation occurs, the ligand is an agonist. If no condensation is noted, the agent may not bind the studied type of receptor or may be an antagonist of this receptor.

2.6. Contractility assay

The anonymous use of human umbilical cords obtained after elective caesarean section deliveries was approved by a local institutional research ethics board. The preparation of the umbilical vein rings was described elsewhere [20]. This vein is a suitable contractile bioassay for both the B_2R and

H_1R [13, 14]. After a 2.5-h equilibration period during which tissues were periodically washed with fresh Krebs buffer, tissues were randomly assigned to one of the experimental groups in several protocols further described in Results.

2.7. Immunofluorescence of peptidases in the human umbilical vein

The conversion of the latent H_1R ligands in the venous contractility assay described above is dependent on the presence of peptidases in the vascular structure, minimally ACE and APN. Five-µm-thick paraffin tissue section of the human umbilical vein in situ were rehydrated and processed for the immunofluorescence of ACE using anti-ACE polyclonal antibodies C28 (dilution 1:50) raised against the C-terminal sequence of human ACE [21] (gift from Prof. P. Corvol, INSERM U36, Paris, France) and of APN (CD13) using the mouse monoclonal anti-CD13 antibodies (BD Pharmingen, clone WM-15, dilution 1:50) (general immunofluorescence methods as in Morissette et al., [22]). Other sections were stained using anti-α-actin monoclonal antibodies (dilution 1:100, clone 1A4, Sigma-Aldrich) to identify vascular smooth muscle. Staining was revealed using the appropriate AlexaFluor-conjugated secondary antibodies and sections were observed (epifluorescence, 100 ×).

2.8. Data analysis

Numerical values are reported as means ± s.e.m. The data from competition assays involving radioligand binding were fitted by nonlinear regression to a one-site competition equation to derive IC_{50} values (Prism 5.0, GraphPad Software Inc., San Diego, CA). The contractility concentration-effect data were analyzed with Prism (sigmoidal dose-response curve with variable slope) to obtain EC_{50} values and their 95% confidence limits (C.L.). χ^2 statistics (comparison of frequencies) was used to determine qualitative drug effect on the endosomal labeling by recombinant β-arrestin$_1$, the categories being: presence or absence of robust labeling.

3. Results

3.1. Radioligand binding studies

As judged from the results of radioligand binding assays, CHO cells were found suitable to transiently express both recombinant human histamine H_1R and the tagged construction myc-B_2R (Fig. 2); the latter has an intact pharmacological profile relative to the wild type BK B_2R [6]. Thus, the specific binding of [^{3}H]pyrilamine to H_1R is apparently saturable with a calculated K_D of 3.19 nM and an extrapolated B_{max} of 123 fmol/well (Fig. 2A). Other cells that expressed myc-B_2R bound negligible amounts of the tritiated antihistamine (Fig. 2A). Conversely, the specific binding of [^{3}H]BK to myc-B_2R was important (B_{max} 42.9 fmol/well), of high affinity (K_D 1.67 nM), but non-existent in other CHO cells that expressed H_1R (Fig. 2B).

These separate binding assays were exploited to calculate the potency of a panel of unlabeled drugs at each receptor type by competition of a fixed concentration of each radioligand (2 nM of [^{3}H]pyrilamine at the H_1R, 3 nM of [^{3}H]BK at myc-B_2R; Figs. 2C, D). The unlabeled antihistamines were potent competitors at the H_1R (calculated IC_{50} of 5.5 and 532 nM for pyrilamine and cetirizine, respectively). The natural agonist histamine itself has a low absolute affinity at H_1R (IC_{50} 55.5 µM). Of note, CTZ-εACA-BK was capable of competing for [^{3}H]pyrilamine binding to H_1R (IC_{50} 11.0 µM, thus only ~20-fold less potent than cetirizine). The typical B_2R ligands BK (agonist) and LF 16-0687 (antagonist) were inactive to displace [^{3}H]pyrilamine binding from H_1R at pharmacologically relevant concentrations.

The same set of non-radioactive agents were tested as competitors of [^{3}H]BK binding to myc-B_2R (Fig. 2D). The non-peptide antagonist LF 16-0867 and the peptide agonist BK exhibited the highest affinity in the competition assay (IC_{50} of 6.1 and 11.5 nM, respectively), followed by CTZ-εACA-BK (690 nM). The typical H_1R ligands (pyrilamine, cetirizine, histamine) did not significantly compete for [^{3}H]BK binding to myc-B_2R at 10 µM (Fig. 2D).

3.2. β-arrestin$_1$ condensation in CHO cells

An assay based on β-arrestin condensation at the level of endosomes, where many phosphorylated receptors are translocated (including the B_2R [8]), was performed to confirm the agonist or antagonist status of some ligands in co-transfected CHO cells that expressed either recombinant H_1R or myc-B_2R along with β-arrestin$_1$-cherry (Fig. 3). BK and CTZ-εACA-BK, used at concentrations that displaced [^{3}H]BK binding from myc-B_2R (Fig. 2D) effectively condensed the arrestin at the level of multiple granular structures located in the cytosol (Fig. 3). Histamine was inactive in this respect in cells that expressed myc-B_2R but was active in those expressing H_1R (the frequency of the response being significant relative to control, but smaller than that achieved with BK stimulation of myc-B_2R). The histamine H_1R did not mediate arrestin condensation in response to either BK sequences (Fig. 3), either because BK has no affinity at this receptor (as proven by Fig. 2B) or because the CTZ conjugate is an antagonist ligand of the H_1R.

3.3. Metabolic activation of CTZ-εACA-BK in the human umbilical vein

HEK 293 cells that stably express the fluorescent receptor B_2R-GFP exhibit the endocytosis of this construction in response to CTZ-εACA-BK (5 µM) and this translocation was prevented by treatment with LF 16-0687 [9]. Whether the intracellular metabolism of the conjugated peptide in endosomes releases free cetirizine has been tested using the competition assay (binding of 2 nM [^{3}H]pyrilamine to H_1R expressed in CHO cells, Fig. 4A). HEK 293 cells expressing high levels of B_2R-GFP were stimulated at 37°C with CTZ-εACA-BK (1 µM, 30 min) and the cell supernatant was then harvested, frozen, and later applied undiluted to the [^{3}H]pyrilamine competition assay. The conjugated peptide, which competes very little for this binding at 1 µM (Fig. 2C), was not rendered more active by preincubation with cells that expressed B_2R-GFP (Fig. 3A). Alternatively, the latent antihistamine effect of CTZ-εACA-BK could be activated by extracellular enzymatic systems that initiate BK degradation, primarily ACE. CTZ-εACA-BK (1 µM) was incubated with HEK 293a cells that transiently expressed human recombinant ACE; the supernatants were again tested as competitors in the [^{3}H]pyrilamine binding assay (Fig. 4B). Again, no gain of potency was observed at H_1Rs.

The human isolated umbilical vein is a tissue that expresses peptidases such as ACE and APN (Supplementary Fig. 1) and endogenous B_2 and H_1 receptors. In this preparation, CTZ-εACA-BK exerted mild antagonist effects on histamine-induced contraction that were not modified by omapatrilat, an inhibitor of ACE and neutral endopeptidase, or by a B_2R antagonist that prevents endocytosis of the BK conjugate. These conclusions are supported by the online data supplement (Supplementary Results, Supplementary Figs. 2-4 and Supplementary Tables 1 and 2). Thus, based on the lack of gain of function, there is no evidence that CTZ-εACA-BK can release free cetirizine in biological environments.

3.4. Pharmacology and metabolic activation of L-alanyl- histamine

When used at concentrations up to 300 µM, L-alanyl-histamine did not compete intensely enough with for [^{3}H]pyrilamine binding to H_1Rs expressed by CHO cells to calculate an IC_{50} value (Fig. 5A), whereas authentic histamine displaced the radioligand (IC_{50} 75 µM in this set of experiments). In the human umbilical vein contractility assay, L-alanyl-histamine, is a contractile agent less potent than histamine (Fig. 5B, numerical parameters in Table 1). Pretreatment of tissue with the APN inhibitor amastatin had contrasting effects on the apparent potency of the 2 agonists: it did not affect that of histamine, but reduced 17-fold that of L-alanyl-histamine, supporting that the latter derivative is a pro-drug metabolically releasing histamine *in situ*.

4. Discussion

It was hypothesized that peptidases expressed in blood vessels, and specifically in the robust contractility assay based on the human umbilical vein, would release H_1R ligands from peptide-like pro-drugs. In the present study, the previously reported conjugate CTZ-εACA-BK [9] was further characterized as a dual ligand of both B_2R and H_1R based on radioligand competition assays (Fig. 2C, D), and categorized as an agonist and probable antagonist of these respective receptors, based on the arrestin condensation assay (Fig. 3). As the peptide BK is metabolized via several degradative pathways in endosomes following B_2R-mediated internalization [23], the endosomal release of cetirizine from a prodrug that is a B_2R agonist was an alternate possibility. The direct affinity for the H_1R in a binding assay conducted at ice temperature was unexpected and constituted a first limitation of the approach, because the metabolic release of free cetirizine would have increased its potency by about 20-fold only at the H_1R (compare the relative potencies in Fig. 2C). Thus, despite the facts that the fluorescent conjugate CF-εACA-BK binds to ACE with an affinity equal to BK and that this peptide apparently releases free carboxyfluorescein after endocytosis [8], metabolic release of free cetirizine from the analog peptide CTZ-εACA-BK was not pharmacologically detected under the forms of a potent antagonism of histamine or of a competitor for the binding of [^{3}H]pyrilamine to recombinant H_1Rs. The drug-peptide conjugate behaved as a weak H_1R antagonist in the umbilical vein under all circumstances, with or without a wide spectrum peptidase inhibitor or B_2R blockade.

The proof-of-concept of the metabolic activation of a H_1R latent ligand is provided by the very simple compound L-alanyl-histamine. Oddly, this compound had been previously synthesized as a carbonic anhydrase activator [18]; we rather re-discovered it as an analogue of some of the highly efficient APN substrates, such as L-Ala-p-nitroanilide (see Introduction). As predicted, L-alanyl-histamine has practically no affinity for the H_1R (Fig. 5A), but naturally expressed APN released histamine from it (detected as a contractile agent in the umbilical vein, Fig. 5B). The APN inhibitor amastatin abated the conversion of L-alanyl-histamine into a H_1R stimulant, consistent with the metabolic activation model. Thus, the potency of L-alanyl-histamine as a contractile agent in the isolated vein (Fig. 5B) is determined by the enzymatic velocity of the APN, the diffusion rates of the pro-drug into, and that of the produced histamine out of the tissue and, possibly, by histamine degradation within the tissue (the general problem of non-equilibrium pharmacological effects on isolated tissues is discussed elsewhere [14]).

The interest of activation of pro-drugs by ectopeptidases will essentially depend on the distribution of these enzymes in tissues. We have previously shown the activation of a B_2R agonist from the peptide Met-Lys-BK-Ser-Ser by ACE [2]. ACE is essentially endothelial (Supplementary Fig. 1), renal and plasmatic and there may be valid reasons for locally stimulating the endothelial B_2Rs with selectivity, e.g., elicit vasodilation without stimulation of afferent nerve terminals where B_2Rs are also present. APN is rather widely expressed [24], but is overexpressed during tumoral angiogenesis and in certain tumors [16, 17, 25, 26], raising for instance the possibility to use L-alanyl-histamine as a vasodilator adjuvant to deliver chemotherapeutic agents.

One of the two tested latent H_1R ligands, L-alanyl-histamine, supported the feasibility of pro-drug activation by vascular ectopeptidases.

Authorship

L.G. designed and synthesized novel pharmacologically active compounds, participated to the general design of the project. C.R. and X.C.-M. executed most experiments. F.M. designed the experiments and drafted the manuscript.

Conflict of interest

The authors have no competing interests for this article.

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Table 1. Parameters of concentration-effect curves generated by contractility experiments in rings of human umbilical veins (experiments reported in Fig. 5B).

pre-treatment (2.5 h)	agonist (3 h)	parameters	
		EC_{50} (μM)	EC_{50} 95% C.L.
saline vehicle	histamine	0.13	0.09-0.20
amastatin 3 μM	histamine	0.19	0.10-0.36
saline vehicle	L-alanyl-histamine	54.7	40.2-74.5
amastatin 3 μM	L-alanyl-histamine	916	739-1136

Fig. legends

Fig. 1. Structure of the tested prodrugs and potential enzymatic reactions that may release H_1R ligands from them. A. Cetirizine-ε-aminocaproyl-BK (CTZ-εACA-BK), a known BK B_2R agonist. B. L-Alanyl-histamine. Some of the arrows indicate putative hydrolysis sites by enzymes (ACE: angiotensin converting enzyme; NEP: neutral endopeptidase; APN: aminopeptidase N).

Fig. 2. Radioligand binding assays to CHO cells transiently expressing recombinant receptors (human histamine H_1R or bradykinin myc-B_2R, as indicated). A. Saturation of [^{3}H]pyrilamine binding. B. Saturation of [^{3}H]BK binding. C. Competition of [^{3}H]pyrilamine (2 nM) binding to H_1R by a panel of unlabeled agents. D. Competition of [^{3}H]bradykinin (3 nM) binding to myc-B_2R by a panel of unlabeled agents. Specific binding values (either fmol/well or percent of control) are the means ± S.E.M. of the number of duplicate determinations indicated by *n*. Derived numerical affinity estimates are reported in Results.

Fig. 3. Endosomal condensation of β-arrestin$_1$-cherry in CHO cells that expressed either H_1R or myc-B_2R. Cells were stimulated for 30 min as indicated (red epifluorescence, 1000 ×). Histograms at the right represent the proportion of cells with robust endosomal labeling for cells expressing either H_1R (white bars) or myc-B_2R (grey ones). Numbers at the right of histograms represent the numbers of cells evaluated during several days of experiment. χ^2 statistics: only proportions significantly different from control ones were indicated with a P value.

Fig. 4. Competition of [^{3}H]pyrilamine (2 nM) binding to H_1R by a panel of conditioned media prepared to reveal the metabolic activation of the latent antihistamine activity of CTZ-εACA-BK. A. Media transferred from untreated or CTZ-εACA-BK-treated HEK 293 cells that stably expressed B_2R-GFP. B. Media transferred from untreated or drug-treated HEK 293a cells that transiently expressed ACE. Presentation as in Fig. 2.

Fig. 5. Pharmacology and metabolic activation of L-alanyl-histamine. A. Competition of [^{3}H]pyrilamine (2 nM) binding to H_1R expressed in CHO cells by histamine and its derivative. Presentation as in Fig. 2C. B. Cumulative concentration-effect curves constructed for histamine or L-alanyl-histamine (separate tissues pretreated or not with amastatin 3 µM). A maximal concentration of histamine (1 mM) was added at the end of the curves constructed with L-alanyl-histamine to evaluate the full effect mediated by endogenous H_1Rs. Values are expressed as the % of the maximal histamine-induced maximal contractile response in each tissue and are means ± s.e.m. of 4 determinations in separate tissues. The curve parameters computed from the data (EC_{50}, C.L.) are reported in Table 1.

Figure 1

Fig. 1

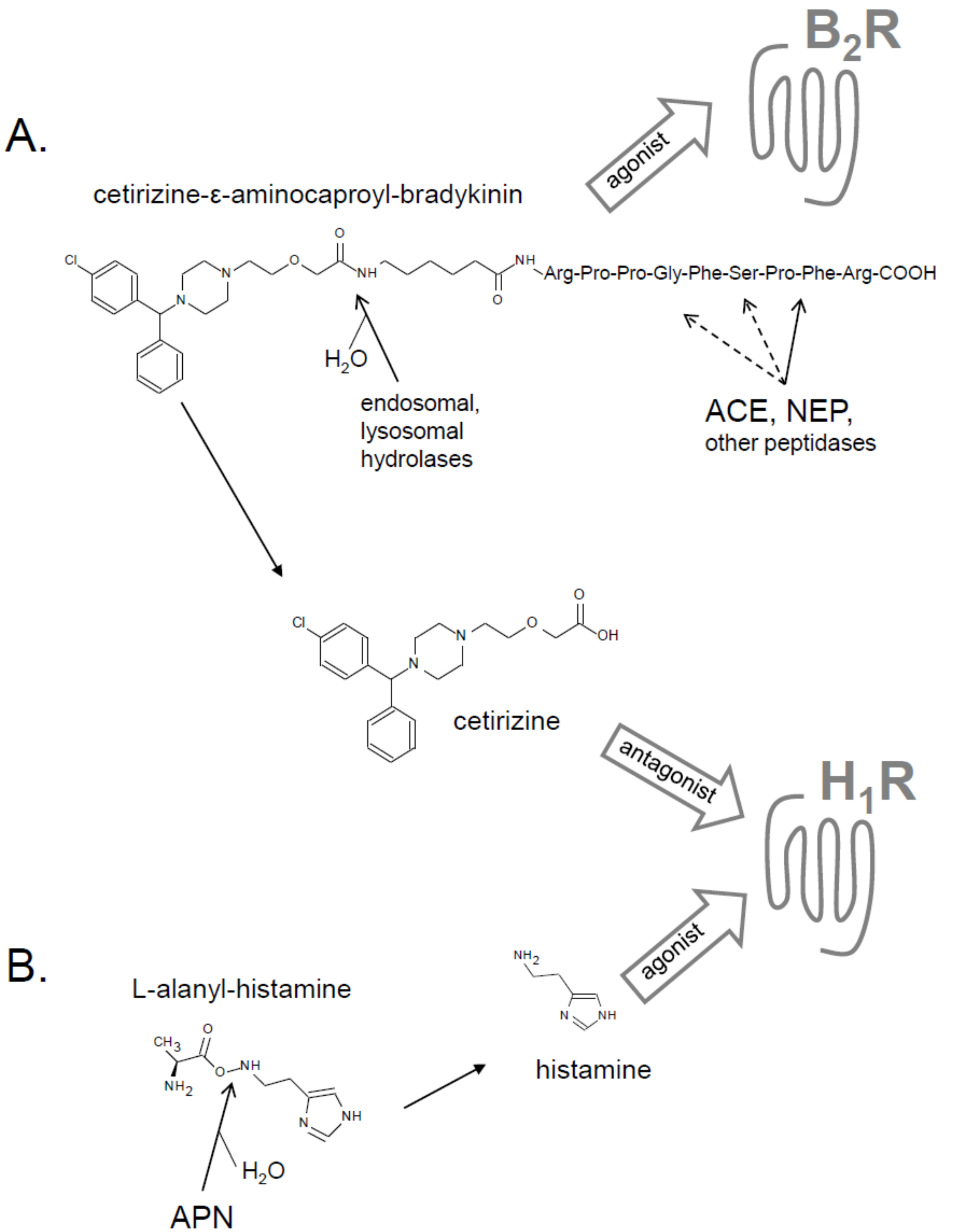


Figure 2

Fig. 2

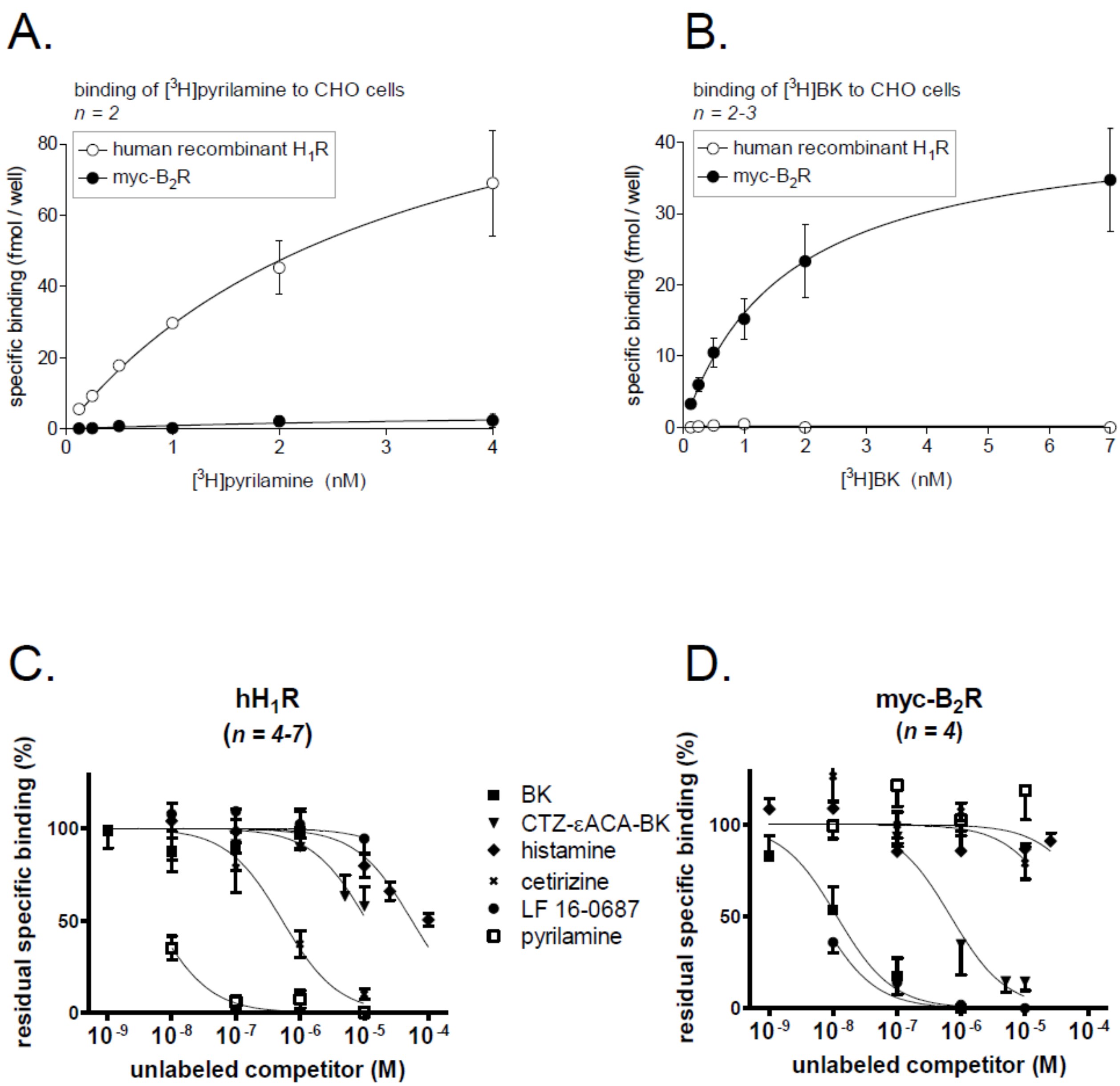


Figure 3

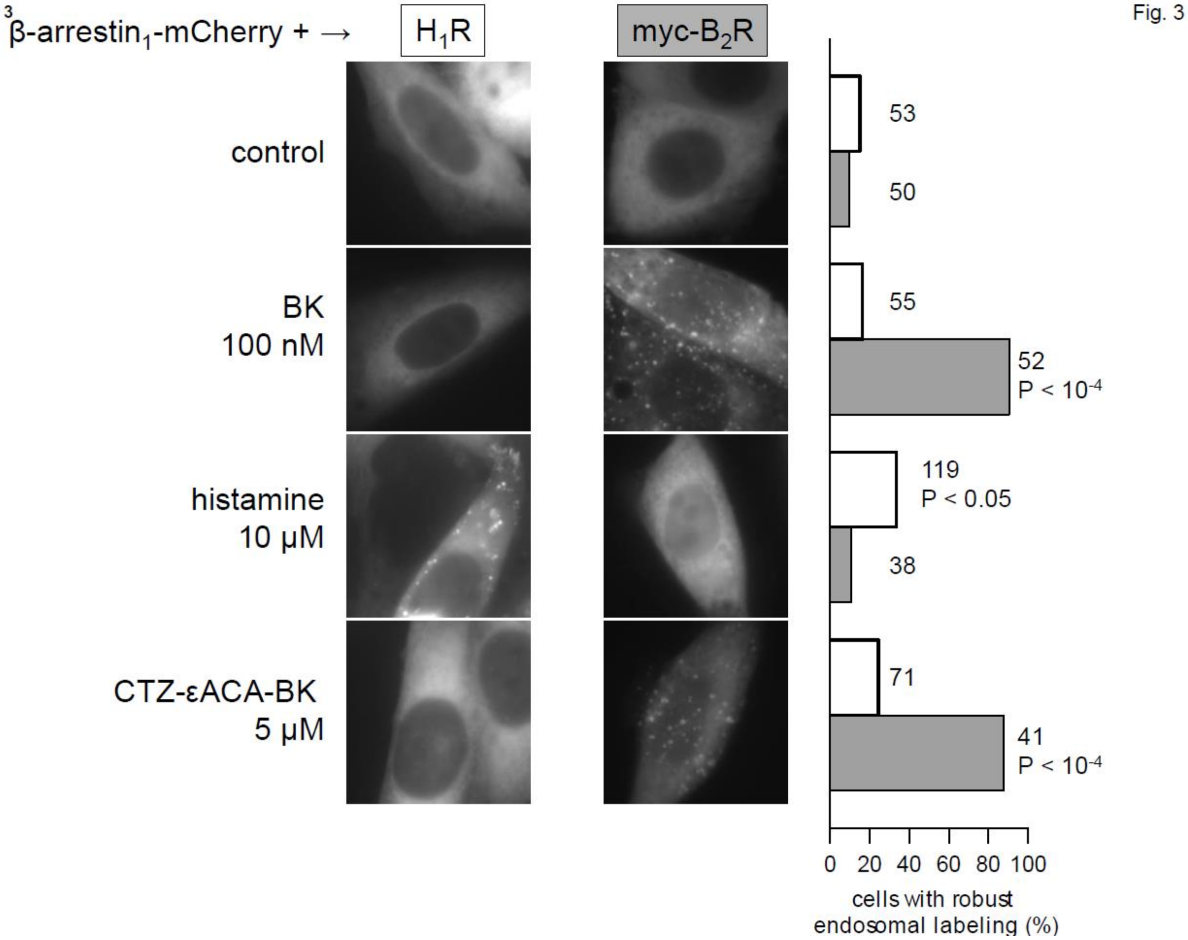


Fig. 3
β-arrestin1-mCherry + →
H1R
myc-B2R
control
BK
100 nM
histamine
10 µM
CTZ-εACA-BK
5 µM
53
50
55
52
P < 10-4
119
P < 0.05
38
71
41
P < 10-4
0 20 40 60 80 100
cells with robust
endosomal labeling (%)

Figure 4

Fig. 4

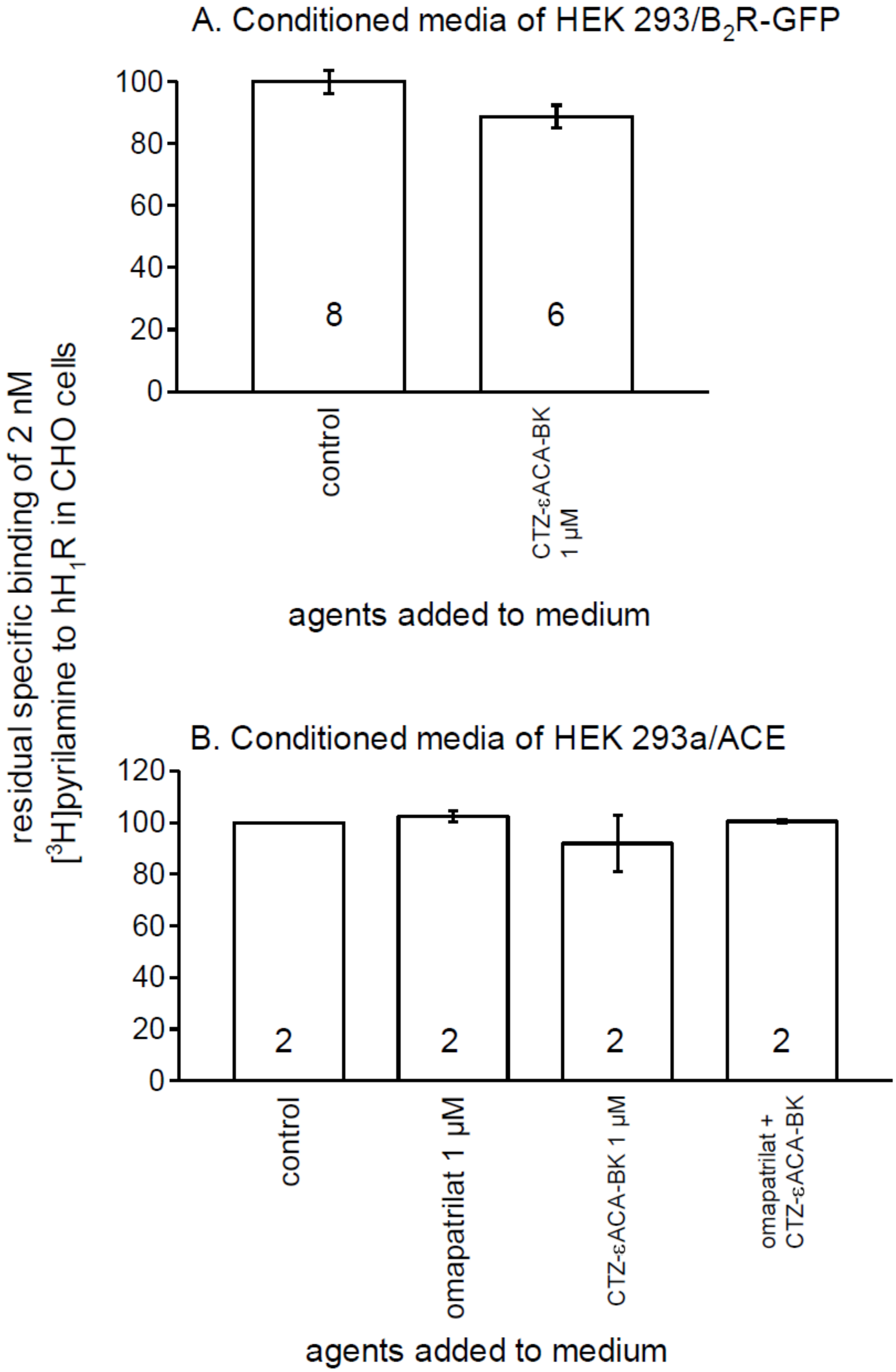
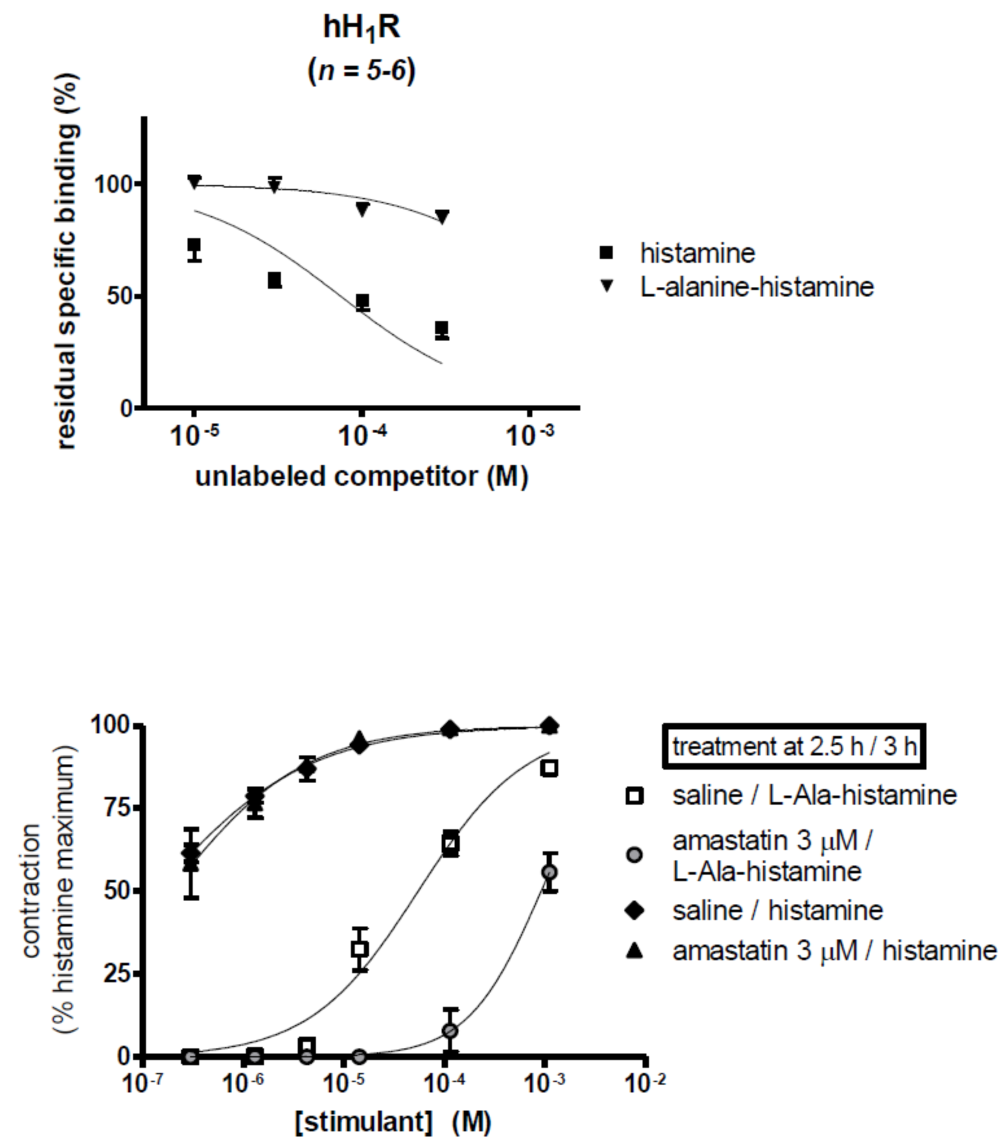


Figure 5

Fig. 5

A.

B.



Supplementary material to Gera et al., Vasopeptidase-activated latent ligands of the histamine receptor-1

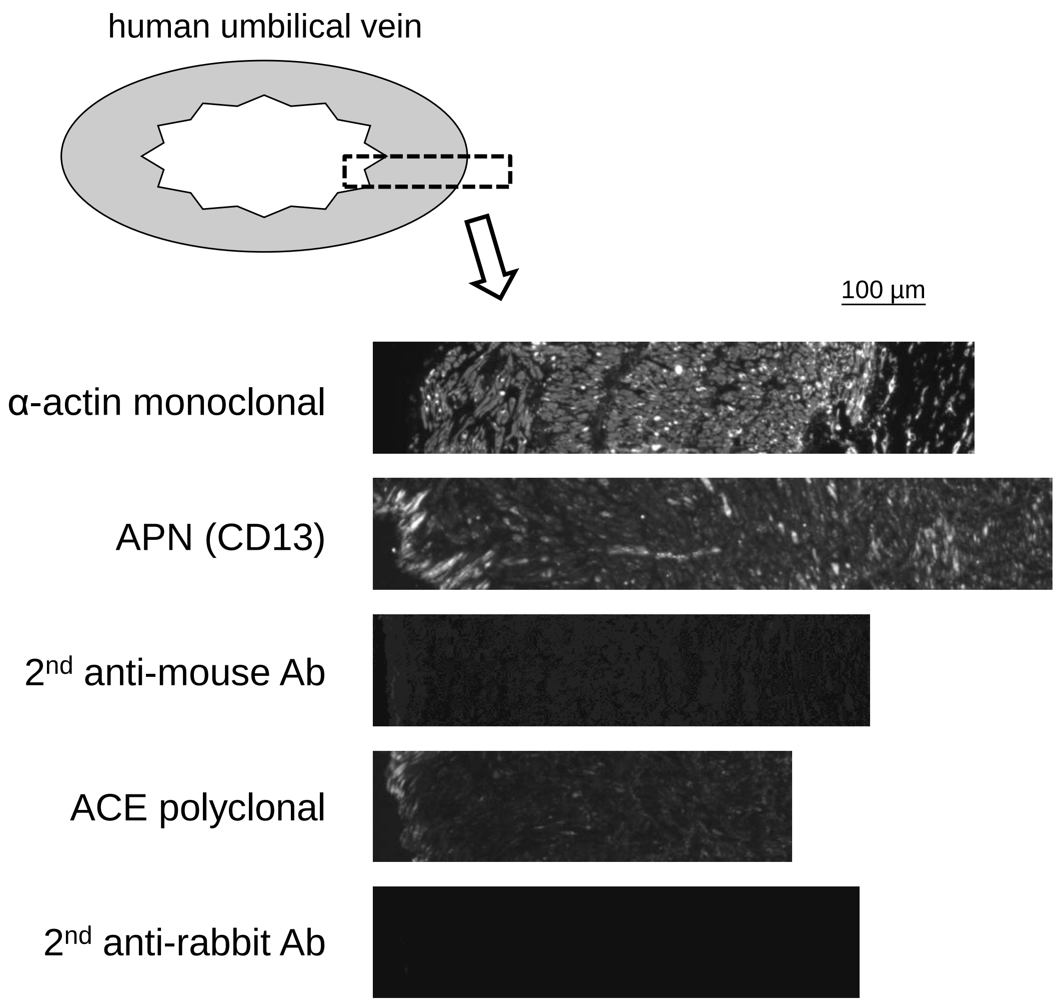
Supplementary Results: Lack of evidence for the metabolic activation of CTZ-εACA-BK in the isolated human umbilical vein

The contractility assay exploited in the present studies is based on the human umbilical vein. ACE was identified essentially at the luminal surface of venous tissue section (immunofluorescence, Supplementary Fig. 1) consistent with the endothelial expression of this ectopeptidase. Aminopeptidase N seemed expressed by both the endothelium, as previously reported for cultured human umbilical vein endothelial cells [16], and the smooth muscle cells, although generally less intensely in the latter case (the muscle cell layer can be identified by comparison with cells positive for α-actin, Supplementary Fig. 1).

The fresh rings of umbilical veins were allowed 2.5 h of equilibration post-mounting in organ tissues baths. Protocol I (schematic representation in Supplementary Fig. 2A) was designed to verify that authentic cetirizine (25 nM) antagonized histamine-induced contraction (concentration-effect curve constructed at time 4 h) without affecting that induced by BK (curve constructed at 3 h). This prediction was fully verified, the apparent potency of histamine being reduced 50-fold without a significant loss of the extrapolated maximal effect (Supplementary Fig. 2B, C; EC_{50} and E_{max} statistics reported in Supplementary Table 1).

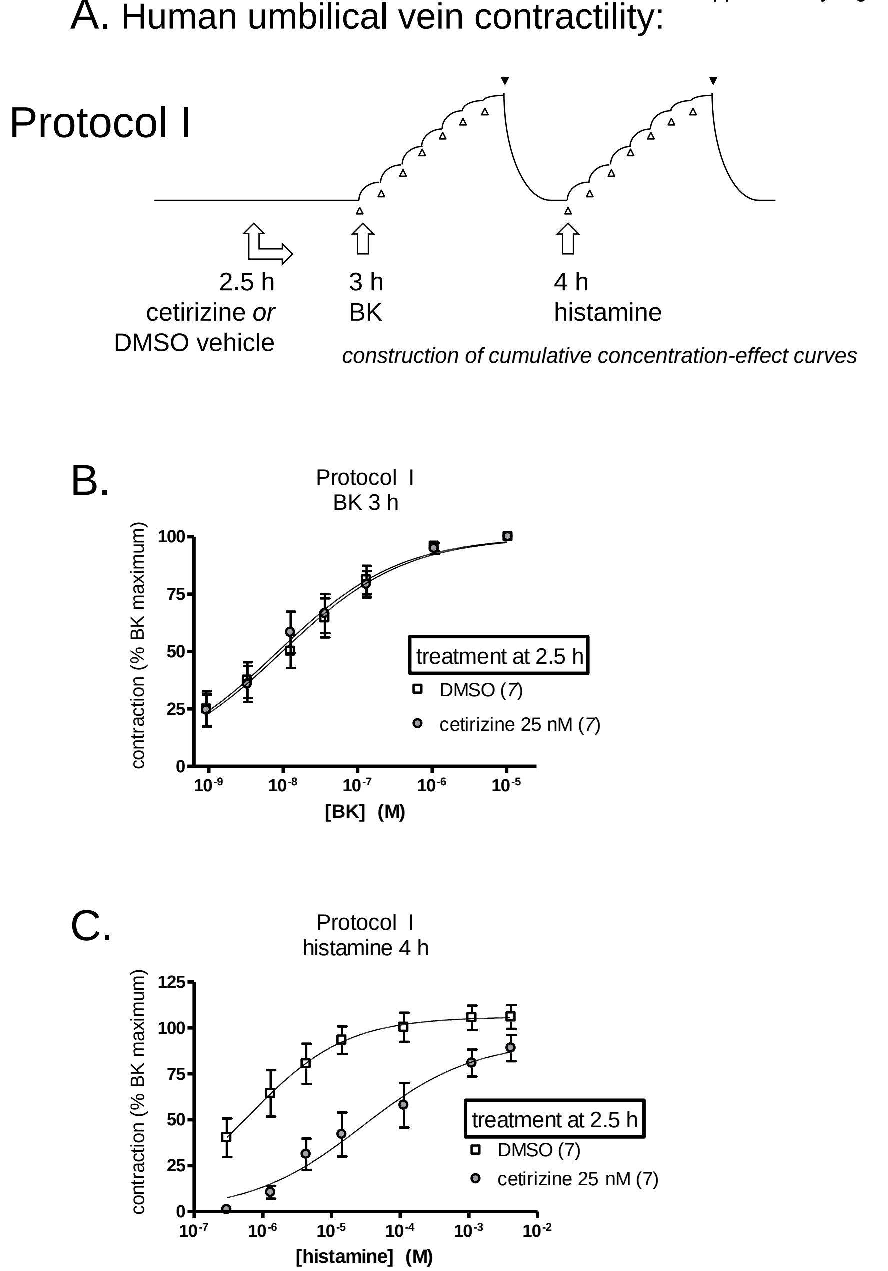
Whether CTZ-εACA-BK could antagonize histamine in the vein contractility was then tested (Protocol II, schematically represented in Supplementary Fig. 3A; results in Supplementary Fig. 3B and Supplementary Table 2). LF16-0687, a potent nonpeptide B_2R antagonist, was present in all tissues to isolate effects on H_1Rs from B_2R-dependent effects, such as contraction or endocytosis of the ligand (LF16-0687 inhibits the endocytosis of a comparable agonist, carboxyfluorescein-εACA-BK; [8]). At the relatively low concentration of 0.59 µM, CTZ-εACA-BK shifted the concentration-effect curve of histamine to the right by 6.6-fold (compare experimental conditions A and B in Supplementary Fig. 3B). This significant B_2R-independent antagonism is compatible with either an extracellular release of cetirizine from CTZ-εACA-BK in the venous tissues or with a direct effect of the conjugated peptide at H_1Rs. Additional experimental conditions in Protocol II were included to address the possible activation of the latent anti-histamine properties of CTZ-εACA-BK by kininases naturally expressed in vascular tissues. Omapatrilat has been designed to block both BK-destroying peptidases angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) with nanololar affinities [9]. This inhibitor did not abate the antagonism of histamine exerted by CTZ-εACA-BK. Thus, results do not support the metabolic activation of the antihistamine properties of the peptide.

Stimulation of venous tissue with CTZ-εACA-BK should lead to the endocytosis of the peptide conjugate via endogenous B_2Rs with possible hydrolysis and release of free cetirizine in the endosomes and diffusion to cell surface H_1Rs. This was tested using contractility protocol III (Supplementary Fig. 4A) where the B_2R antagonist LF16-0687 was optionally used to inhibit B_2R-mediated endocytosis of the conjugated peptide. These experiments exploited a larger concentration of the conjugated peptide (5.9 µM) or a BK concentration (3.3 nM) that are both submaximal in the venous contraction assay [8]. Thus, precontraction of tissues with CTZ-εACA-BK at time 3 h followed by ample washout was associated with the antagonism of histamine at time 4 h (Supplementary Fig. 4B, Supplementary Table 2). Pretreatment of tissues with LF16-0687 virtually abolished CTZ-εACA-BK-induced contraction (Supplementary Fig. 4C), but did not influence the persistent antagonism of histamine (Supplementary Fig. 4B, C, Supplementary Table 2). Precontraction of tissues with BK was abolished by LF16-0687 (Supplementary Fig. 4C), but BK, associated or not with LF16-0687, did not influence the apparent potency of histamine (compare experimental conditions C and D in Supplementary Fig. 4B with the effect of histamine in the control group of Supplementary Fig. 3B). Thus, tissues once exposed to CTZ-εACA-BK, whether the B_2R was blocked or not, exhibited persistent antagonism of histamine. These observations did not support the metabolic release of free cetirizine via B_2R-mediated endocytosis of the conjugated peptide.



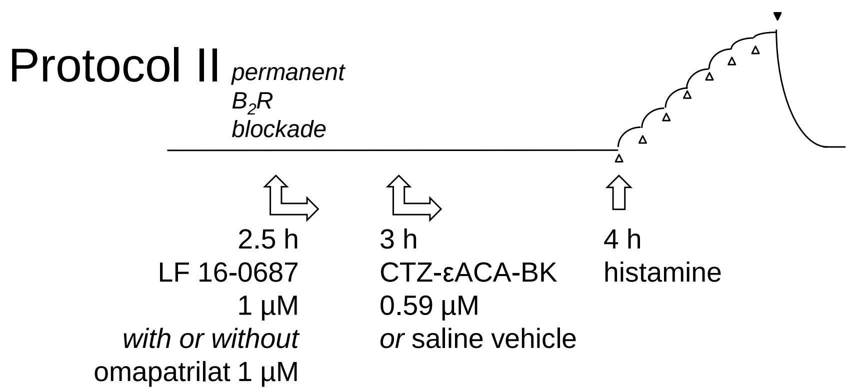
Supplementary Fig. 1. Immunofluorescence of ACE and aminopeptidase N (APN) in sections of the human umbilical vein. The intimal direction is at the left of images. Controls for identifying contractile smooth muscle cells using anti-α actin antibodies and for non-specific staining by secondary antibodies alone were included.

Supplementary Fig. 2

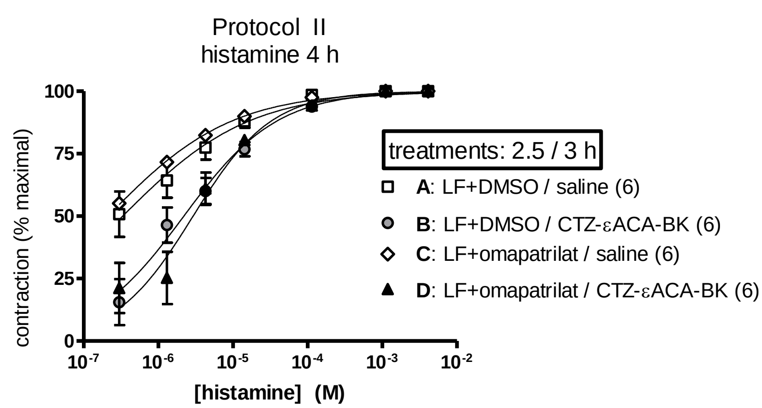


Supplementary Fig. 2. A. Schematic representation of the protocol I used to analyze the contractile response to BK and histamine in rings of human umbilical veins. Imaginary tracings illustrate contraction (vertical axis) as a function of time (horizontal axis). Indicated time points are relative to tissue mounting in organ baths. Open arrowheads indicate the injection of contractile agonists; closed arrowheads, the first of a series of stimulant washouts. B, C. Cumulative concentration-effect curves constructed for BK (B) and histamine (C) in prococol I. Values are expressed as the % of the maximal BK-induced maximal contractile response in each tissue and are means ± s.e.m. of the number of determinations indicated between parentheses.

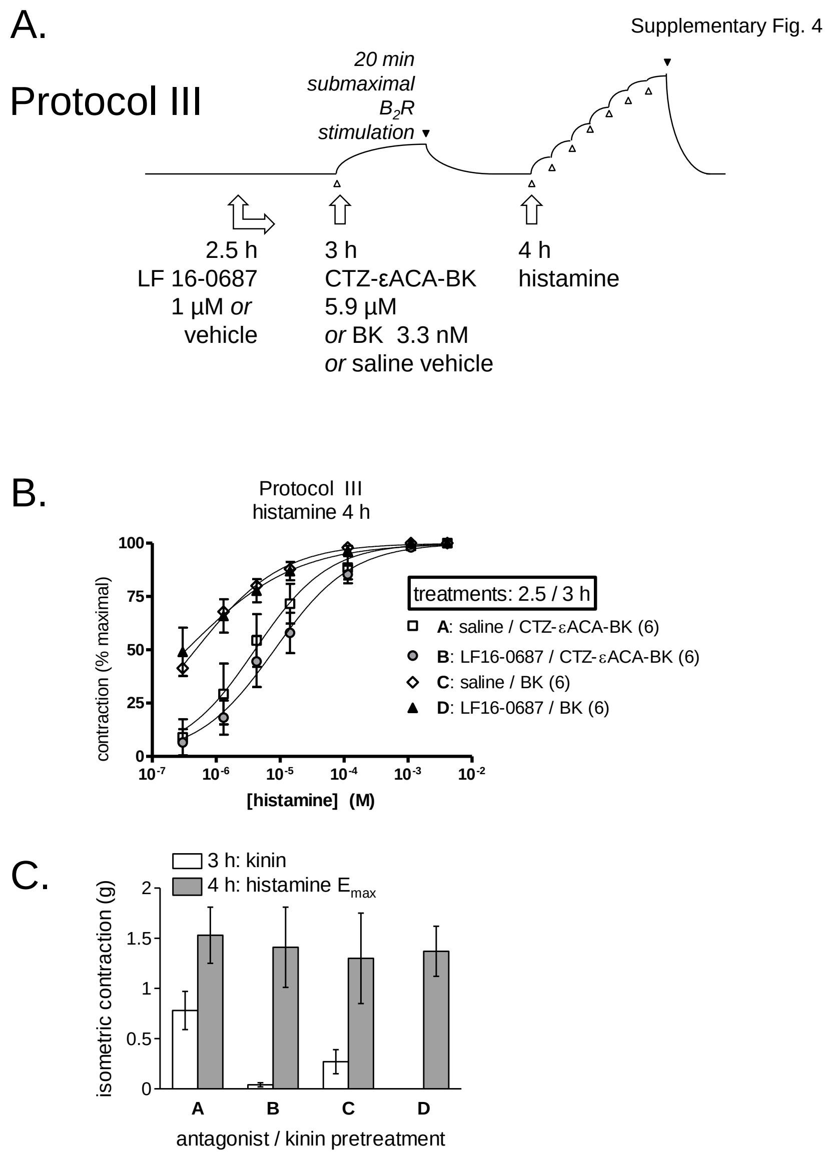
A.



B.



Supplementary Fig. 3. A. Schematic representation of the protocol II used to attempt the generation of an anti-histamine activity from ectopeptidase-mediated degradation of CTZ-εACA-BK in rings of human umbilical veins. Presentation as in Supplementary Fig. 1A. B. Cumulative concentration-effect curves constructed for histamine in prococol II (values are expressed as the % of the maximal histamine-induced maximal contractile response in each tissue and are means ± s.e.m. of the number of determinations indicated between parentheses). The groups of tissues corresponding to the combination of successive treatments are indicated either by a letter (A-D) as in panel C. The curve parameters computed from the data (EC_{50}, E_{max}) are reported in Supplementary Table 1.



Supplementary Fig. 4. A. Schematic representation of the protocol III used to attempt the generation of an anti-histamine activity from B_2R-mediated internalization and endosomal degradation of CTZ-εACA-BK in rings of human umbilical veins. Presentation as in Supplementary Fig. 1A. B. Cumulative concentration-effect of histamine constructed at time 4 h as a function of successive pretreatments (presentation as in Supplementary Fig. 2B). C. In experiments reported in panel B, the results of the maximal histamine-induced contraction (E_{max}, in g) did not differ between groups, but that of a kinin measured at 3.5 h was virtually abolished by pretreatment with LF16-0687 (column A vs. B: $P<0.01$; column C. vs. D: $P<0.05$; Mann-Whitney test). The groups of tissues corresponding to the combination of successive treatments are indicated by a boldface letter (A-D) as in panel B.

Supplementary Table 1. Parameters of concentration-effect curves generated by contractility experiments in rings of human umbilical veins.

Treatment 2.5 h	BK (3 h)		histamine (4 h)			
Protocol I	EC_{50} (nM)	EC_{50} 95% C.L.	EC_{50} (µM)	EC_{50} 95% C.L.	E_{max} (% BK max)	E_{max} 95% C.L.
DMSO	9.7	5.9-15.9	0.56	0.28-1.52	106.1	93.4-118.7
cetirizine 25 nM	8.5	5.1-14.1	28.1	5.00-157	62.9	67.1-118.7

Supplementary Table 2. Parameters of the concentration-effect curves generated for histamine by protocols II and III in rings of human umbilical veins.

	Treatment 2.5 h	Treatment 3 h	histamine (4 h)	
			EC_{50} (µM)	EC_{50} 95% C.L.
Protocol II	A: LF16-0687 1 µM + DMSO	saline	0.33	0.18-0.62
	B: LF16-0687 1 µM + DMSO	CTZ-εACA-BK 0.59 µM	2.20	1.57-3.08
	C: LF16-0687 1 µM + omapatrilat 1 µM	saline	0.21	0.12-0.37
	C: LF16-0687 1 µM + omapatrilat 1 µM	CTZ-εACA-BK 0.59 µM	2.93	2.01-4.25
Protocol III	A: saline	CTZ-εACA-BK 5.9 µM	4.05	2.34-7.03
	B: LF16-0687 1 µM	CTZ-εACA-BK 5.9 µM	8.16	5.13-12.97
	C: saline	BK 3.3 nM	0.48	0.35-0.65
	D: LF16-0687 1 µM	BK 3.3 nM	0.34	0.16-0.72