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Pharmacological profile of the novel mammalian tachykinin, hemokinin 1

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1 The effects of the novel mammalian tachykinin, hemokinin 1 (HEK-1), have been investigated by radioligand binding and functional *in vitro* and *in vivo* experiments.

2 Similar to SP ($K_i = 0.13$ nM), HEK-1 inhibited in a concentration-dependent manner and with high affinity [^{3}H]-substance P (SP) binding to human NK_1 receptor ($K_i = 0.175$ nM) while its affinity for [^{125}I]-neurokinin A (NKA) binding at human NK_2 receptor was markedly lower ($K_i = 560$ nM).

3 In isolated bioassays HEK-1 was a full agonist at tachykinin NK_1, NK_2 and NK_3 receptors. In the rat urinary bladder (RUB) HEK-1 was about 3 fold less potent than SP. In the rabbit pulmonary artery (RPA) HEK-1 and in the guinea-pig ileum (GPI), HEK-1 was about 500 fold less potent than NKA and NKB, respectively.

4 The responses to HEK-1 were antagonized by GR 82334 in RUB ($pK_B = 5.6 \pm 0.07$), by nepadutant in RPA ($pK_B = 8.6 \pm 0.04$) and by SR 142801 in GPI ($pK_B = 9.0 \pm 0.2$) with apparent affinities comparable to that measured against tachykinin NK_1, NK_2 and NK_3 receptor-selective agonists, respectively.

5 Intravenous HEK-1 produced dose-related decrease of blood pressure in anaesthetized guinea-pigs ($ED_{50} = 0.1$ nmol kg^{-1}) and salivary secretion in anaesthetized rats ($ED_{50} = 6$ nmol kg^{-1}) with potencies similar to that of SP. All these effects were blocked by the selective tachykinin NK_1 receptor antagonist, SR 140333.

6 We conclude that HEK-1 is a full agonist at tachykinin NK_1, NK_2 and NK_3 receptors, possesses a remarkable selectivity for NK_1 as compared to NK_2 or NK_3 receptors and acts *in vivo* experiments with potency similar to that of SP.

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Abbreviations: DBP, diastolic blood pressure; GPI, guinea-pig ileum; HEK-1, hemokinin 1; HR, heart rate; RPA, rabbit pulmonary artery; RUB, rat urinary bladder; SBP, systolic blood pressure

Introduction

Mammalian tachykinins (substance P, SP, neurokinin A, NKA, neurokinin B, NKB, and two N-terminal elongated forms of NKA, neuropeptide K (NPK) and neuropeptide γ (NPγ) are coded by two different genes, termed preprotachykinin-I and preprotachykinin-II (PPT-I and PPT-II also known as PPTa and PPTb). The PPT-I gene is widely expressed in the mammalian central and peripheral nervous system, whereas the expression of the PPT-II gene is almost restricted to the central nervous system. All the mammalian tachykinins act as full agonist at three distinct receptors, termed NK_1, NK_2 and NK_3 which mediate the biological actions of tachykinins (for reviews see Maggi, 1993; 1995).

Recently, a third tachykinin gene preprotachykinin-c (PPTc) has been discovered in lymphoid B hematopoietic cells of mouse bone marrow (Zhang *et al*., 2000). The PPTc gene codes for a novel 11 amino acid tachykinin that has been termed hemokinin 1 (HEK-1): HEK-1 shares the common C-terminal sequence typical of the tachykinin peptide family and displays a high sequence homology with SP (Table 1).

Zhang *et al*. (2000) reported that HEK-1 promotes survival and maturation of murine B cells progenitors and suggested that it may act as an autocrine factor in the hematopoietic system. Despite the observation that the effects of HEK-1 on proliferation/survival of B cells progenitors are modified by a tachykinin NK_1 antagonist, the effects of HEK-1 on B cells were not reproduced by equimolar concentration of SP (Zhang *et al*., 2000). It was also reported that HEK-1 induces certain typical effects of SP, such as plasma protein extravasation and mast cell degranulation.

Zhang *et al*. (2000) hypothesized that a novel tachykinin receptor may mediate the effects of HEK-1 on B cells, but it appears also likely that this novel endogenous tachykinin could stimulate the tachykinin NK_1 receptor. This is of particular interest since an extensive cross-talk exists among the natural tachykinins and their three different receptors. For example NKA is considered to be a NK_1 receptor physiological agonist, especially in the central nervous system where NKA is abundantly present whereas its 'preferred'

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Table 1 Aminoacids sequence of hemokinin 1 compared to that of the other natural mammalian tachykinins

Hemokinin 1	Arg-Ser-Arg-Thr-Arg-Gln-Phe-Tyr-Gly-Leu-Met-NH_2
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2

Underlined are the aminoacids homologous with substance P.

receptor (NK_2) is poorly expressed (Maggi & Schwartz, 1997).

The present study was designed to investigate whether HEK-1 possesses binding affinity and agonist efficacy at mammalian tachykinin receptors and determine its profile of activity in this respect. In addition we have also compared the activity of HEK-1 in producing certain typical tachykinin-like effects *in vivo* i.e. hypotension in guinea-pigs and salivary secretion in rats.

Methods

In vitro *studies*

Receptor expression in CHO cells and membrane preparation CHO-K1 cells, stably expressing the human NK_1 and NK_2 receptor, were cultured in Ham's F12 Medium (F12) and in Minimum Essential Medium Eagle Alpha Modification (αMEM), respectively, supplemented with 2 mM L-glutamine and foetal bovine serum (10%). The cells were subcultured by using 0.25% trypsin and 1 mM ethylenediaminetetraacetate (EDTA) to detach them, and then cultured in 175 cm^2 flasks and maintained in a humidified atmosphere at 37°C with 5% CO_2.

Cells at confluence were washed out of the medium by PBS without calcium and magnesium, harvested, and washed in tris[hydroxymethyl] aminomethane buffer (TRIS, 50 mM, pH 7.4, at 4°C) containing thiorphan 10 μM, leupeptin 5 $\mu g\ ml^{-1}$, bacitracin 100 $\mu g\ ml^{-1}$, and chymostatin 10 $\mu g\ ml^{-1}$, and homogenized with a Polytron (PT 3000, Kinematica), set at 15,000 r.p.m. for 30 s. The homogenate was centrifuged at $45{,}000 \times g$ for 45 min (4°C). The pellet was resuspended to obtain 5 $mg\ ml^{-1}$ membrane protein concentration and frozen immediately in 1 ml aliquots by immersion in liquid nitrogen and then stored at −80°C until use.

The protein concentration was determined by the method of Bradford (1976) with a Bio-Rad kit, using bovine serum albumin as reference standard. Immediately prior to use, frozen membrane aliquots were thawed in binding buffer (see below) and mixed to give a homogeneous membrane suspension.

Radioligand binding The buffer used for binding experiments was TRIS (50 mM, pH 7.4) containing the described enzyme inhibitors, $MnCl_2$ 5 mM, and bovine serum albumin (1 $g\ l^{-1}$). Binding assays were performed in a final volume of 0.5 ml. An incubation time of 60 min at 25°C was used.

All incubations were terminated by rapid filtration through UniFilter-96 plates (Packard) that had been pre-soaked for at least 2 h in 0.3% polyethylenimine (PEI), using a MicroMate 96 Cell Harvester (Packard Instrument Company). The tubes and filters were then washed five times with 0.5 ml aliquots of TRIS buffer (50 mM, pH 7.4, 4°C). Filters were dried and soaked in Microscint 40 (Packard Instrument Company) and bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument Company).

Preliminary experiments were performed to determine membrane proteins concentration for each receptor assay (100 $\mu g\ ml^{-1}$). [^{125}]-NKA and [^{3}H]-SP were used at 0.1 nM concentration and in both cases the bound radioligand resulted less than 10%. Non-specific binding was defined as the amount of bound radioligand in the presence of 1 μM of appropriate unlabelled ligand, and resulted less than 10% of the total bound.

All the compounds competition curves were tested in a wide range of concentrations (0.01 pM – 10 μM). Each experiment was performed in duplicate.

Functional studies in isolated organs Male albino New Zealand rabbits (2.5 – 3.0 kg), male albino Dunkin-Hartley guinea-pigs (250 – 300 g) and male albino Wistar rats (300 – 350 g), all from Charles River (Calco, Italy), were stunned and bled. Guinea-pig ileum longitudinal muscle myenteric plexus strips (GPI), rabbit pulmonary artery circular muscle strips deprived of the endothelium (RPA) and rat urinary bladder longitudinal detrusor muscle strips (RUB) were prepared as described previously (Patacchini & Maggi, 1998). All preparations were placed in 5 ml organ baths filled with oxygenated (96% O_2 and 4% CO_2) Krebs-Henseleit solution, having the following composition in mM: NaCl, 119; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1.5; $CaCl_2$, 2.5; KCl, 4.7 and glucose 11. The motor activity of GPI (load 5 mN) and RUB (load 5 mN) was recorded isotonically, while that of RPA (load 10 mN) isometrically. All experiments performed in the RUB were in the presence of indomethacin 10 μM in order to reduce the basal spontaneous activity (Patacchini & Maggi, 1998). The activity of HEK-1 at tachykinin NK_1 receptors was assayed in the RUB (Meini *et al*., 1994), in the presence of the tachykinin NK_2 receptor-selective antagonist SR 48968 (1 μM) (Emonds-Alt *et al*., 1992) to avoid involvement of NK_2 receptors in the responses studied. The activity of HEK-1 at tachykinin NK_2 receptors was assayed in the RPA (Maggi *et al*., 1990), a 'monoreceptorial' bioassay functionally devoid of NK_1 or NK_3 tachykinin receptor types mediating smooth muscle contraction. The activity of HEK-1 at tachykinin NK_3 receptors was assayed in the GPI (Patacchini *et al*., 1995), in the presence of the tachykinin NK_1 receptor-selective antagonist SR 140333 (1 μM) (Emonds-Alt *et al*., 1993) to avoid involvement of NK_1 receptors. In all preparations examined, cumulative concentration-response curves to HEK-1 were obtained, each concentration being added when the effect of the preceding one had reached a steady state. In each preparation the activity of HEK-1 was compared to that of the respective selective agonist for the tachykinin NK_1, NK_2 and NK_3 receptors. HEK-1-induced motor responses were challenged with the following tachykinin receptor-selective antagonists: GR 82334 (in the RUB; Hagan *et al*., 1991), nepadutant (in the RPA; Catalioto *et al*., 1998) and SR 142801 (in the GPI, Emonds-Alt *et al*., 1995; Patacchini *et al*., 1995). All the experiments were performed in the presence of a mixture of peptidase inhibitors: thiorphan, captopril and bestatin (1 μM each; 15 min before), to prevent peptide degradation.

In vivo *studies*

Cardiovascular effects in anaesthetized guinea-pigs Male albino Dunkin-Hartley guinea-pigs (Charles River, Calco, Italy) weighing 400–450 g were anaesthetized with urethane (1.5 g kg^{-1}, s.c.). Blood pressure was measured through an heparinized polyethylene catheter (PE50) inserted into and secured to the right common carotid artery on the one end, and in a pressure transducer connected to a MacLab8/s, on the other. Following tracheotomy, guinea-pigs were intubated (PE 260) and connected to a respirator for mechanical ventilation. The respiration rate was kept at 50 strokes min^{-1} and the respiration volume at 0.8–0.9 ml kg^{-1}. SP or HEK-1 were administered through a catheter (PE50) inserted into and secured to the left jugular vein. The body temperature was maintained constant (36.5±0.5°C) by a thermoregulated heating lamp.

About 1 h elapsed between the animal set-up and the start of the experiments, thereafter each animal received the vehicle (saline) and increasing doses (0.5 log units) of SP or HEK-1 (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 nmol kg^{-1}); the first five doses were administered at 20 min intervals, whereas 30 min elapsed between each one of the last four doses. A series of experiments was carried out in atropine-pretreated animals (1.4 μmol kg^{-1} i.v. as bolus followed by infusion of 1.4 μmol ml^{-1} in a volume of 300 μl h^{-1}), in order to evaluate whether atropine could differentially affect SP- and HEK-1-induced responses.

A series of experiments was aimed to verify the activity of selective tachykinin NK_1 receptor antagonists SR 140333 (Emonds-Alt *et al*., 1993) and MEN 11467 (Cirillo *et al*., 1998) on the cardiovascular effects induced by SP or HEK-1 administered i.v. at 1 nmol kg^{-1}. SP or HEK-1 were challenged two times at 25 min intervals. The first challenge was carried out 20 min before vehicle (dimethylsulphoxide, 100 μl kg^{-1}, iv) or antagonist administration (SR 140333 or MEN 11467 at 1 μmol kg^{-1}, i.v.), the second challenge was given 5 min after the treatment.

The effect of each dose of SP and HEK-1 on blood pressure was evaluated as difference between basal cardiovascular values (the average values of 1 min before the administration of each dose of SP and HEK-1 of systolic and diastolic blood pressure and heart rate) and post-treatment values recorded for 10 min.

Salivary secretion in anaesthetized rats Male albino Wistar Rats (Charles River, Calco, Italy) weighing 400–450 g were used throughout the study. On the day of the experiment, rats were anaesthetized with urethane (1.2 g kg^{-1}, s.c.). Following tracheotomy and placement of a tracheal cannula (PE 205), the left jugular vein was cannulated (PE50) for SP or HEK-1 administrations. The body temperature was maintained constant (36.5±0.5°C) by a thermoregulated heating lamp.

About 1 h elapsed between the animal set-up and the start of the experiments, thereafter each animal received atropine (1.4 μmol kg^{-1} i.v. as bolus followed by infusion of 1.4 μmol ml^{-1} in a volume of 300 μl h^{-1}) and 10 min later the vehicle (saline) and increasing doses (0.5 log units) of SP or HEK-1 (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 nmol kg^{-1}); the first five doses were administered at 20 min intervals, whereas 30 min elapsed between each of the last four doses. Atropine pretreatment was performed in order to minimize the influence of cholinergic secretory reflexes in response to the placement of the cotton swab in the rat's oral cavity for the measurement of salivary secretion induced by SP or HEK-1.

The effect of each dose of SP and HEK-1 on salivary secretion was evaluated as difference between the weight of the cotton swab 5 min after each agonist challenge and the dry weight of the swab determined just before each administration.

A series of experiments was aimed to verify the activity of selective tachykinin NK_1 receptor antagonist SR 140333 (1 μmol kg^{-1}, i.v.) on salivary secretion induced by SP or HEK-1 (10 nmol kg^{-1} i.v.). The antagonist or its vehicle (dimethylsulphoxide, 100 μl kg^{-1}, i.v.) were administered 5 min before SP or HEK-1.

Statistical analysis All values in the text, tables or figures are mean and 95% confidence limits, or mean±s.e.mean of the given (n) number of experiments. Statistical analysis was performed by means of Student's t-test for paired or unpaired data or by means of two-way analysis of variance (ANOVA) (time × treatment, or dose × treatment) followed by Fisher's least significance tests. A P level <0.05 was considered statistically significant.

Concentration- or dose-response curves were analysed by fitting the data with GraphPad Prism programme (San Diego, CA, U.S.A.) for Macintosh in order to determine the radioligand affinity constant (K_D), and binding inhibition constant (K_i) calculated from Cheng & Prusoff (1973) equation.

Agonist activity was expressed as EC_{50} or ED_{50} values corresponding to molar concentration of peptide producing 50% of maximal effect. The apparent affinity of the antagonists was expressed in terms of apparent pK_B (negative logarithm of the antagonist dissociation constant) and, assuming a slope of −1, it was estimated by the equation: pK_B = log [dose ratio−1]−log [antagonist concentration] (Kenakin, 1993; Jenkinson, 1991).

Drugs

[^{3}H]-SP (specific activity 41 Ci $mmol^{-1}$) and [^{125}I]-NKA (specific activity 2000 Ci $mmol^{-1}$) were provided by Amersham International (Buckinghamshire, U.K.).

Hemokinin 1 was synthesized at Menarini Laboratories (Florence, Italy) by conventional solid-phase methods. NKA, [βAla^8]NKA(4–10) and GR 82334 or [D-Pro^9, (spiro-γ-lactam)Leu^{10},Trp^{11}]physalaemin(1–11) were from Neosystem (Strasbourg, France). Thiorphan, substance P, senktide, septide and [Sar^9]-SP sulfone were purchased from Bachem (Bubendorf, Switzerland). Other drugs used were: bacitracin, chymostatin, indomethacin, chlorpheniramine maleate (Sigma, St. Louis, MO, U.S.A.); atropine sulfate (Serva, Heidelberg, Germany); leupeptin (Boehringer Mannheim, Germany). The non-peptide antagonists SR 140333 or (S)1-{2-[3-(3,4-dichlorophenyl)-1-(3-isopropoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo [2,2,2]octane chloride], SR 48968 (saredutant) or (S)-N-methyl-N[4-(4-acetylamino-4-phenylpiperidino)-2-(3,4-dichlorophenyl)butyl] benzamide and SR 142801 or (S)-(N)-{1-[3-(1-benzoyl-3-(3,4-dichloro-phenyl)piperidin-3-yl)propyl]-4-phenylpiperidin/-4-yl}-N-methylacetamide, were kindly provided by Drs

X. Emonds-Alt and G. Le Fur (Sanofi Recherche, Montpellier, France). MEN 11467 (1R,2S)-2-N[1(H)indol-3-yl-carbonyl]-1-N-{N-(p-tolylacetyl)-N-(methyl)-D-3(2-Naphthyl)alanyl}-diaminocyclohexane) was synthesized by Chemistry Department of Menarini Ricerche (Pomezia, Italy).

All salts used were purchased from Merck (Darmstadt, Germany). All tissue culture materials were from Sigma (St. Louis, MO, U.S.A.).

Results

In vitro *experiments*

HEK-1 binding affinity to human recombinant NK_1 and NK_2 receptors SP displayed higher affinity than NKA or septide at the human tachykinin NK_1 receptor stably expressed in CHO cells in both homologous and heterologous competition binding experiments: K_i values of SP were about 180 and 850 fold lower than NKA and septide when competing at the [^{3}H]-SP binding site, and about 300–400 fold lower than NKA and septide when measured at the [^{125}I]-NKA binding site (Figure 1A,B, Table 2).

The binding profile of HEK-1 at human NK_1 receptor was comparable to that described for SP: HEK-1 inhibited in a concentration-dependent manner and with high affinity both [^{3}H]-SP and [^{125}I]-NKA binding to human NK_1 receptor, its K_i values being 0.175 nM (95% c.l. 0.13–0.23 nM) and 0.002 nM (95% c.l. 0.001–0.003 nM), respectively (Table 2, Figure 1A,B).

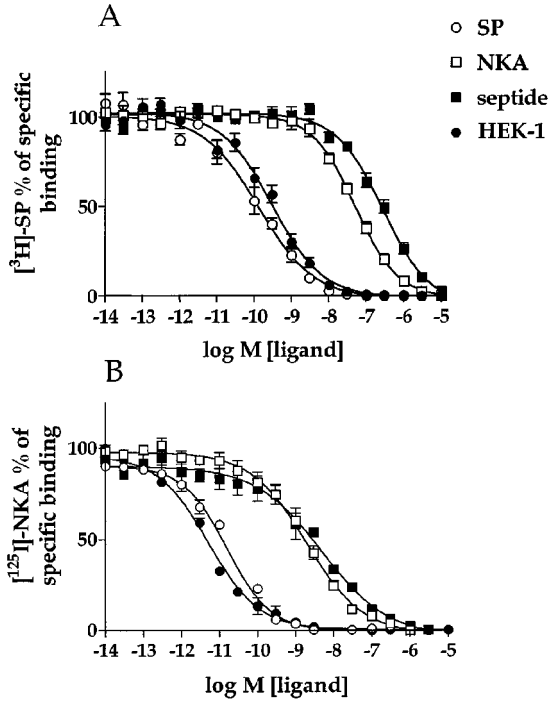

Figure 1 Competition binding curves of unlabelled SP, NKA, septide and HEK-1 at the NK_1 receptor expressed in CHO cells membranes. 0.1 nM [^{3}H]-SP (A) or [^{125}I]-NKA (B) were used as radioligand. Data shown represent the mean±s.e.mean of 3–4 experiments, each one performed in duplicate.

The estimated affinity of HEK-1 for [^{125}I]-NKA binding at the recombinant human NK_2 receptor yielded a K_i value of 560 nM (95% c.l. 470–650 nM) and it was about 250 fold less potent than NKA and about 2.5 fold more potent than SP in binding at human NK_2 receptor (Table 2, Figure 1A,B).

In vitro *functional experiments*

The agonist activity of HEK-1 was evaluated at tachykinin NK_1 receptors in the RUB in the presence of the tachykinin NK_2 receptor-selective antagonist nepadutant (0.1 μM; 30 min before HEK-1). HEK-1 (1 nM–3 μM) produced concentration-dependent contraction of RUB averaging 66±3% ($n=6$) of the maximal contraction produced by KCl (80 mM). As compared to SP or the NK_1 receptor-selective agonist [Sar^9]-SP sulfone, HEK-1 elicited full agonist responses, although showing a 3–5 fold lower potency than SP (Table 3). The involvement of tachykinin NK_1 receptors in HEK-1-induced responses in the RUB was proven by the ability of the selective NK_1 receptor antagonist, GR 82334 (10 μM; 15 min before) to produce a parallel rightward shift of the concentration-response curves to HEK-1 (Figure 2). Assuming a competitive antagonism, an apparent pK_B value of 5.6±0.07 ($n=5$) was obtained for GR 82334, similar to the corresponding value measured against [Sar^9]-SP sulphone (Table 3).

The agonist activity of HEK-1 at tachykinin NK_2 receptors was evaluated in the RPA. HEK-1 (0.1–30 μM) produced reproducible concentration-dependent contractions averaging 55±4% ($n=4$) of the maximal contraction produced by KCl (80 mM). HEK-1 yielded full agonist responses, although showing about 500 fold lower potency as compared to NKA (Table 4). The involvement of tachykinin NK_2 receptors in the response to HEK-1 was checked with the selective NK_2 receptor antagonist, nepadutant (10 nM; 15 min before) which induced a parallel rightward shift of the concentration-response curve to HEK-1. Assuming a competitive antagonism, an apparent pK_B value was calculated for nepadutant (8.6±0.04, $n=4$) (Table 4), almost superimposable to that measured against NKA.

The agonist activity of HEK-1 at tachykinin NK_3 receptors was assayed in the GPI (longitudinal muscle), in the presence of the tachykinin NK_1 receptor-selective antagonist SR 140333 (1 μM; 30 min before HEK-1). Under these conditions HEK-1 (10 nM–10 μM) induced a quickly-developing contractile responses of GPI, as did the tachykinin NK_3 receptor selective agonist senktide or neurokinin B (NKB). HEK-1 induced full agonist responses but with a 500 fold lower potency as compared to NKB (Table 5). The involvement of tachykinin NK_3 receptors in the response elicited by HEK-1 under the assay conditions was checked by using the selective NK_3 receptor antagonist SR 142801 (0.1 μM; 120 min before). SR 142801 produced a righthward shift of the concentration-response curve to HEK-1 (Figure 2) with an apparent pK_B value of 9.0±0.2 ($n=4$) which was comparable to that measured against senktide (Table 5).

In vivo *experiments*

Cardiovascular effects in anaesthetized guinea-pigs Basal cardiovascular values were comparable in animals receiving SP or HEK-1 and did not change during the course of the

Table 2 Binding affinity of tachykinins at human tachykinin NK_1 and NK_2 receptors expressed in CHO cells

	Binding affinity (nM) (95% confidence limits)		
	hNK_1/CHO receptor		*hNK_2/CHO receptor*
	[^{3}H]-SP	*[^{125}I]-NKA*	*[^{125}I]-NKA*
Hemokinin 1	0.175 (0.13–0.23)	0.002 (0.001–0.003)	560 (470–650)
Substance P	0.13 (0.07–0.21)	0.006 (0.004–0.007)	1405 (1150–1670)
Neurokinin A	24.1 (19.9–29.2)	1.6 (1.3–2.0)	2.2 (1.8–2.7)
Septide	110 (89–136)	2.5 (1.8–3.6)	NT

Binding affinity values are expressed as K_D or K_i from homologous and heterologous competition curves, respectively. Data shown represent 3–4 experiments, each one performed in duplicate, as described under Methods. NT: not tested.

Table 3 Agonist activity of hemokinin 1 at tachykinin NK_1 receptors in the rat isolated urinary bladder

	pEC_{50}	*E_{max}* (% of [Sar9]-SP sulphone)	*GR 82334* (pK_B)
Hemokinin 1	7.0±0.1	96±4	5.6±0.07
Substance P	7.7±0.2	100	NT
[Sar^9] substance P sulphone	7.7±0.1	100	5.9±0.1
Septide	7.8±0.1	100	7.0±0.1

All the experiments were performed in the presence of the tachykinin NK_2 receptor-selective antagonist SR 48968 (1 μM; 30 min before) and indomethacin (10 μM). GR 82334 (10 μM) apparent affinity was estimated after 15 min incubation period. Each value is the mean±s.e.mean of 5–7 experiments. NT: not tested.

Table 4 Agonist activity of hemokinin 1 at tachykinin NK_2 receptors in the rabbit isolated pulmonary artery

	pEC_{50}	*E_{max}* (% of neurokinin A)	*Nepadutant* (pK_B)
Hemokinin 1	5.6±0.04	90±2	8.6±0.04
Neurokinin A	8.3±0.1	100	8.6±0.07

Nepadutant (10 nM) apparent affinity was estimated after 15 min incubation period. Each value is the mean±s.e.mean of four experiments.

Table 5 Agonist activity of hemokinin 1 at tachykinin NK_3 receptors in the guinea-pig isolated ileum

	pEC_{50}	*E_{max}* (% of senktide)	*SR 142801* (pK_B)
Hemokinin 1	6.7±0.2	98±3	9.0±0.2
Neurokinin B	9.4±0.3	100	NT
Senktide	9.1±0.2	100	9.3±0.3

All experiments were in the presence of the tachykinin NK_1 receptor-selective antagonist SR 140333 (1 μM; 30 min before). SR 142801 (100 nM) apparent affinity was estimated after 120 min incubation period. Each value is the mean± s.e.mean of 4–6 experiments. NT: not tested.

experiments: the pooled basal values were 57±1 mmHg, 35±1 mmHg and 262±6 beats min^{-1}, $n=20$, for systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR), respectively.

Both SP and HEK-1 (0.01–100 nmol kg^{-1} i.v., $n=10$) induced a dose-related hypotension that was maximal at the dose of 10 nmol kg^{-1} (Figure 3A,B). For SBP, the ED_{50} values (95% confidence limits in brackets) were 0.4 nmol kg^{-1} (0.3–0.5 nmol kg^{-1}) and 0.2 nmol kg^{-1} (0.1–0.4 nmol kg^{-1}) for SP and HEK-1, respectively. For DBP, the ED_{50} values were 0.2 nmol kg^{-1} (0.1–0.4 nmol kg^{-1}) and 0.1 nmol kg^{-1} (0.07–0.2 nmol kg^{-1}) for SP and HEK-1, respectively.

HEK-1 induced a slightly deeper hypotension than SP for diastolic blood pressure at 0.3 and 1 nmol kg^{-1} (Figure 3B). Both peptides induced bradycardia, however this effect was not clearly dose-related (Figure 3C).

The time-courses of cardiovascular effects induced by SP or HEK-1 (0.01–10 nmol kg^{-1}) are compared in Figure 4A,B. Up to a dose of 3 nmol kg^{-1} the hypotensive effects faded within 10 min; at higher doses the recovery was slower but complete within 20 min (data not shown). A complete recovery from SP- or HEK 1-induced bradycardia occurred within 2–3 min up to the dose of 10 nmol kg^{-1} (Figure 4C). Atropine pretreatment (1.4 μmol kg^{-1} i.v., followed by infusion of 1.4 μmol ml^{-1} in a volume of 300 μl h^{-1}) did not significantly affect the cardiovascular responses induced by SP or HEK-1 (data not shown).

In order to perform a pharmacological investigation on the cardiovascular effects induced by SP or HEK-1, a dose of 1 nmol kg^{-1} was selected on the basis of the previous dose-response studies. Therefore, the effects induced by HEK-1 were assessed in animals treated with selective NK_1 receptor antagonists SR 140333 or MEN 11467 (1 μmol kg^{-1}, i.v.) or vehicle (dimethylsulphoxide, 100 μl kg^{-1}, i.v.). As shown in Figure 6, at 5 min from SR 140333 administration, hypotension and bradycardia induced by HEK-1 were completely prevented; similar results were obtained following MEN 11467 treatment (data not shown). Likewise, the cardiovascular effects induced by SP were abolished by SR 140333 (Figure 6A–C).

Salivary secretion in anaesthetized rats In atropine-pretreated rats (1.4 μmol kg^{-1} i.v., followed by infusion of 1.4 μmol ml^{-1} in a volume of 300 μl h^{-1}), both SP and HEK-1 (0.1–100 nmol kg^{-1}, i.v.) induced a dose-related salivary secretion: SP was slightly more effective than HEK-1 and statistically significant differences were detected at doses of 3, 10, 30, and 100 nmol kg^{-1} (Figure 5). However the ED_{50} (95% c.l.) values were not significantly different being 5 (3–10) nmol kg^{-1} and 6 (3–10) nmol kg^{-1} for SP and HEK-1, respectively.

Both SP- and HEK-1-induced salivary secretion (10 nmol kg^{-1} i.v.) were antagonized by SR 140333 (1 μmol kg^{-1} i.v., 5 min before) pretreatment (Figure 6D).

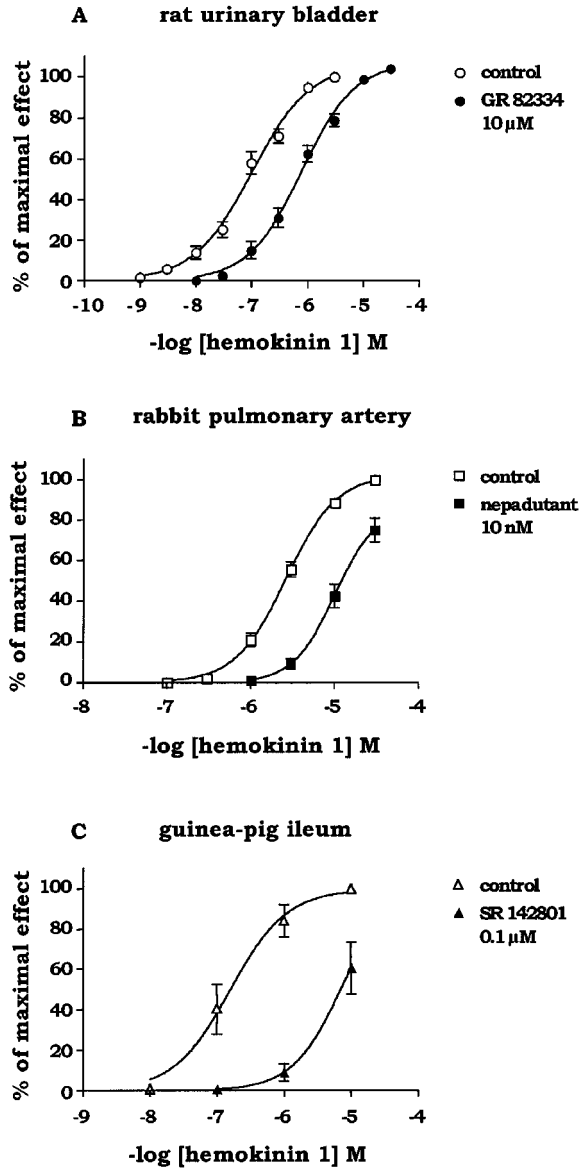

Figure 2 Concentration-response curves of the contractile effect of HEK-1 in rat urinary bladder (RUB, panel A), rabbit pulmonary artery (RPA, panel B), guinea-pig ileum (GPI, panel C) under appropriate experimental conditions for recording the tachykinin NK_1, NK_2 and NK_3 receptor-mediated responses, respectively. The experiments in the RUB were performed in the presence of SR 48968 (1 μM) and indomethacin (10 μM). The experiments in GPI were conducted in the presence of the tachykinin NK_1 receptor antagonist SR 140333 (1 μM). Concentration-response curves to HEK-1 are shown either in the absence or in the presence of GR 82334 (10 μM), nepadutant (10 nM) and SR 142801 (0.1 μM) in the RUB, RPA and GPI, respectively. Each value is the mean$\pm$s.e.mean of 4–7 experiments.

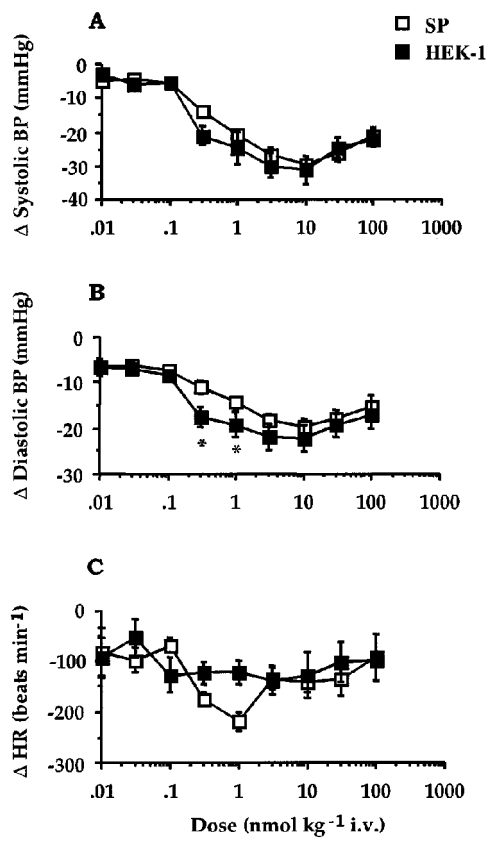

Figure 3 Dose-dependent (0.01–1000 nmol kg^{-1}) hypotensive (panels A and B) and bradycardic (panel C) effect of HEK-1 and SP in anaesthetized guinea-pigs. The maximal effect on systolic and diastolic blood pressure was reached at 10 nmol kg^{-1} of either agonist. *$P<0.05$ significantly different from the response induced by SP. Each value is the mean$\pm$s.e.mean of 10 experiments.

Discussion

Zhang *et al*. (2000) showed that a novel preprotachykinin gene (PPTc) is selectively expressed by certain hematopoietic cells of mouse bone marrow but failed to detect it in the brain or spleen. No information is available to date about possible expression of the PPTc gene in the peripheral/enteric nervous system or in primary afferent neurons. The undecapeptide HEK-1, produced through the expression of the PPTc gene, could thus act as an hematopoietic autocrine factor promoting maturation/survival of B cell progenitors (Zhang *et al*., 2000). Notably, the effects of HEK-1 in promoting maturation/survival of B cells progenitors were not shared by SP, although being blocked by a selective NK_1 receptor antagonist (Zhang *et al*., 2000). Thus a number of basic questions arise from the paper of Zhang *et al*. (2000), including the possibility that a novel, distinct type of tachykinin receptor mediates the effects of HEK-1 in hematopoietic cells.

It has to be noted that remarkable species-related differences are known to exist in tachykinin receptor pharmacology: however these differences are chiefly linked to variable, species-dependent, affinities of receptor antagonists and not to agonist activity of natural tachykinins. Therefore, although the estimate of HEK-1 agonist affinity and efficacy in this study has been obtained at tachykinin receptors from different species, we'll discuss these results as representative of HEK-1 agonist activity at tachykinin receptors in general.

Our data provide evidence that HEK-1 is a full agonist at the three known tachykinin receptors: in each one of the three assays presented here (RUB for NK_1 receptor, RPA for NK_2 receptor and GPI for NK_3 receptor) HEK-1 produced full agonist responses as compared to the natural tachykinins SP, NKA or NKB, respectively. Receptor selective antago-

nists (GR 82334, nepadutant and SR 142801) were used in each assay to verify the assumption that the responses to HEK-1 were indeed ascribable to stimulation of tachykinin receptors. This conclusion is further substantiated by the results of radioligand binding experiments at recombinant human NK_1 and NK_2 receptors. From these results it turned out that HEK-1 is almost equipotent to SP in activating NK_1 receptors whereas it is about 500 fold less potent than NKA or NKB in activating NK_2 and NK_3 receptors, respectively. This set of results indicates a 'SP-like' profile of action for HEK-1 at tachykinin receptors: in other words, HEK-1 has preferential affinity for NK_1 over NK_2/NK_3 receptors.

It is interesting to consider these results with a comparison of HEK-1 primary sequence vs SP, NKA and NKB (Table 1). HEK-1 shares the common C-terminal sequence Phe-X-Gly-Leu-MetNH_2 typical of the tachykinin peptide family. It is noteworthy that the C-terminal pentapeptide fragment possesses low but sizeable binding affinity and agonist efficacy at the three tachykinin receptors (Ingi *et al*., 1991; Cascieri *et al*., 1992). The residue in position X of SP and HEK-1 sequence is aromatic (Phe and Tyr, respectively) whereas a Val residue is present in the corresponding sequence of NKA/NKB. Notably an aromatic residue (Tyr, as in the case of HEK-1) is also present in physalaemin, a non-mammalian tachykinin with SP-like pharmacological profile (preferential NK_1 receptor agonist). On the other hand the N-terminal sequence of mammalian tachykinins is highly divergent (Table 1): HEK-1 and SP share a positively-charged Arg residue in position 11 and a Gln residue

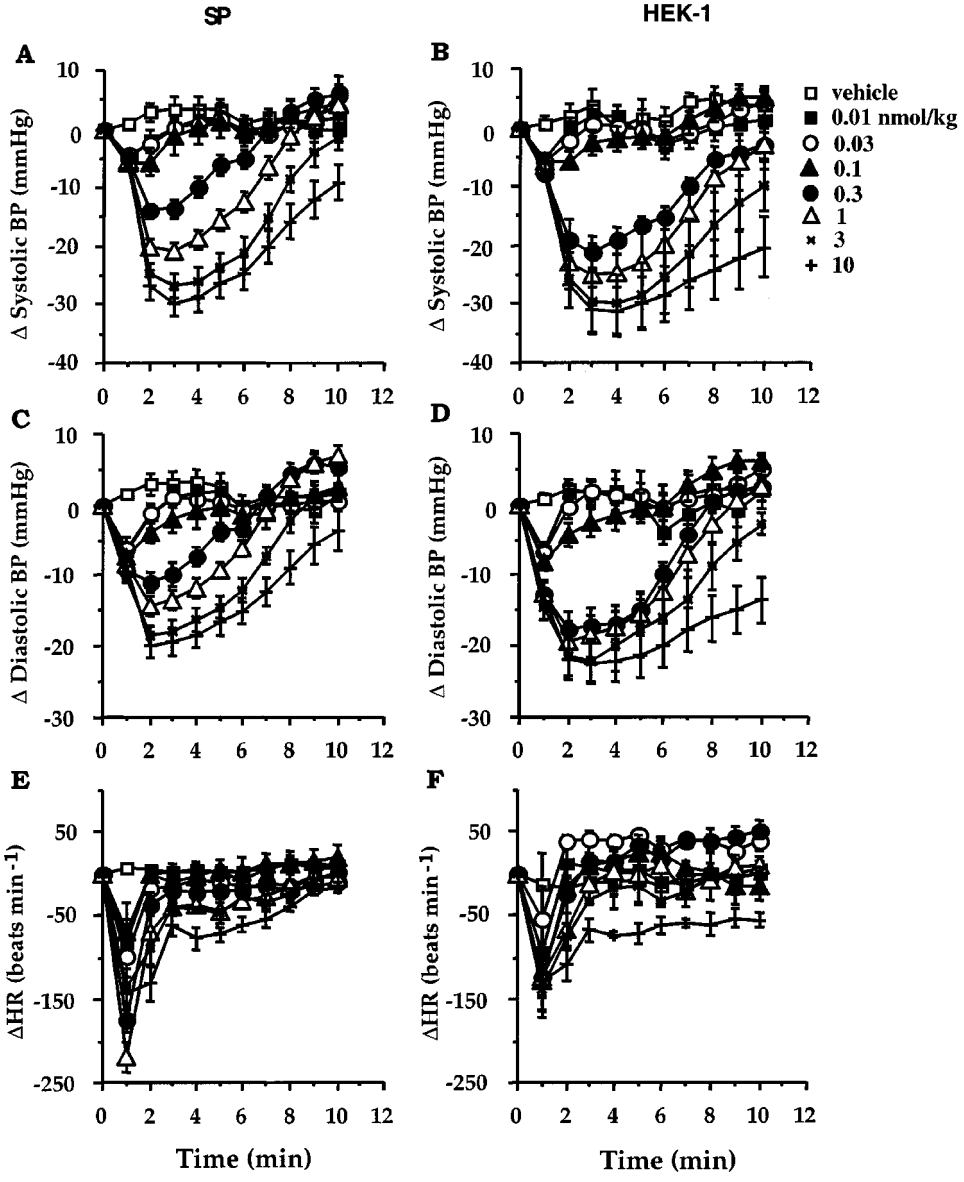

Figure 4 Time-courses of the cardiovascular effects of intravenous administered SP and HEK-1 (0.01 – 10 nmol kg^{-1}) on systolic (A,B), diastolic (C,D) and heart rate (E,F) in anaesthetized guinea-pigs. The maximal response was reached within 1 – 3 min from administration of each peptide. Each value is the mean ± s.e.mean of 10 experiments and represents the difference between the peak effect and the basal value at each dose.

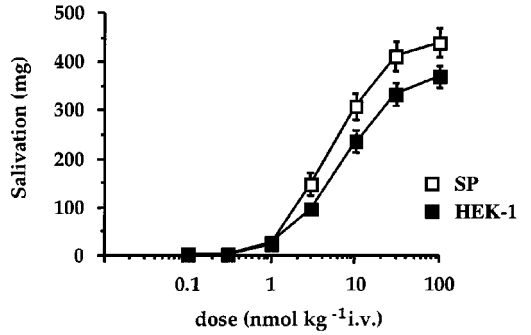

Figure 5 Salivary secretion produced by SP and HEK-1 (0.1–100 nmol kg^{-1} i.v.) in atropine-pretreated (1.4 μmol kg^{-1} i.v., followed by infusion of 1.4 μmol ml^{-1} in a volume of 300 $\mu l\ h^{-1}$) anaesthetized rats. The saliva was collected for a period of 5 min after administration of the peptide. Each value is the mean ± s.e.mean of 10 experiments.

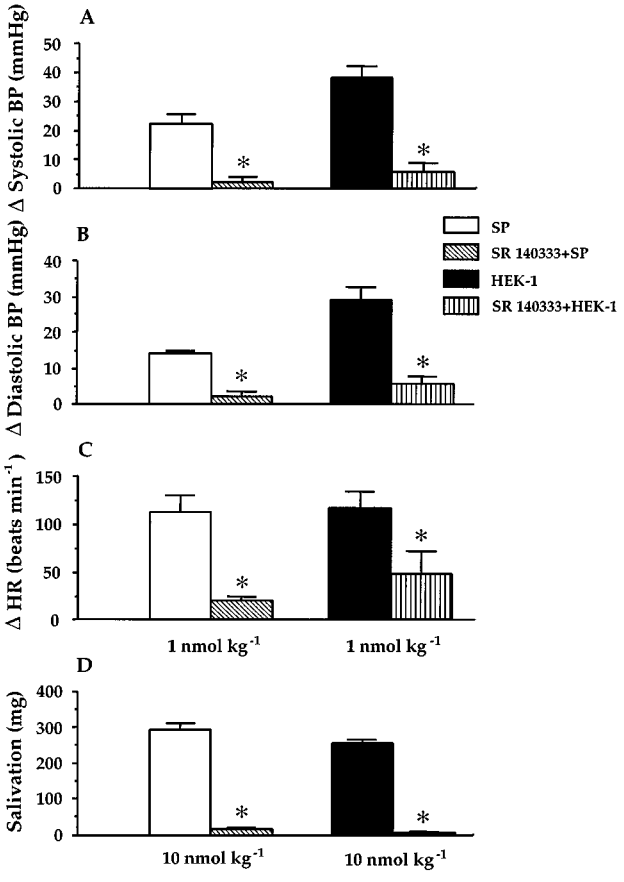

Figure 6 Effect of the tachykinin NK_1 receptor antagonist SR 140333 (1 μmol kg^{-1} i.v., 5 min before agonist) on SP and HEK-1 (1 nmol kg^{-1} i.v.)-induced inhibition of systolic (A), diastolic (B) blood pressure and heart rate (C) in anaesthetized guinea-pigs and on SP and HEK-1 (10 nmol kg^{-1} i.v.)-induced salivary secretion (D) in anaesthetized rats. Empty columns for SP and full columns for HEK-1 represent the control responses in vehicle-treated animals. Each value is the mean ± s.e.mean of four experiments. $^*P<0.05$ significantly different from the control response.

in position 6, but diverging residues at all other positions of the N-terminal sequence. No other match exists in the N terminal region of HEK-1 and natural tachykinins with the only exception of Thr in position 8 which is shared by NKA (SP bears a Pro residue in this position). Two main theories exist to account for the relative contribution of N- and C-terminal regions of tachykinin peptide sequence in binding and activating tachykinin receptors: according to the address-message model (Ingi *et al*., 1991; Yokota *et al*., 1992; Fong *et al*., 1992; Gether *et al*., 1993) residues in the N-terminal region determines selectivity for a given receptor, whereas residues in the C-terminal region would determine receptor activation (Cascieri *et al*., 1992). However several results from mutational studies do no fit into this theory (Werge, 1994; Huang *et al*., 1995) and suggest that the N-terminal region of natural tachykinins would rather act by influencing the conformation of the C-terminal region thus indirectly determining receptor selectivity. The present results indicate that HEK-1, while having relatively little sequence homology with SP and other natural tachykinins in the N-terminal region, still exhibits a remarkable SP-like profile of receptor selectivity (NK_1 receptor preferring): at first sight, this would speak against the general validity of the address-message model, an hypothesis which can be further checked by mutational studies assessing whether residues of NK_1 receptor which are critical for binding/agonist efficacy of SP are also crucial for binding/efficacy of HEK-1.

With regard to NK_1 receptor agonist pharmacology, the evidence gathered in the past few years indicate that SP, physalaemin and other full length NK_1 receptor selective agonists, such as [Sar^9]-SP sulphone, on the one hand, vs NKA, NKB and certain synthetic receptor selective agonists tailored on the C-terminal sequence of SP, such as septide, on the other, act in differential manner on the NK_1 receptor (Petitet *et al*., 1992; Maggi *et al*., 1993; Meini *et al*., 1994). In particular it has been proposed that two main families of agonist-bound conformers of the NK_1 receptor may exist: the first 'SP-preferring conformer' binds SP with very high affinity and septide-like ligands with much lower affinities; the second, called 'general-tachykinin conformer' binds the majority of tachykinin peptides with equal, relatively high affinities (Hastrup & Schwartz, 1996; Maggi & Schwartz, 1997 for review). Recent results by Holst *et al*. (2001) indicate that coupling with different G proteins is a major determinant of the agonist-dependent pharmacology of the NK_1 receptor. Our results indicate that HEK-1 acts in a SP-like manner also in this respect since: (a) as for SP, the binding affinity of HEK-1 was consistently higher in displacing both [^{125}I]-NKA and [^{3}H]-SP from human NK_1 receptors (Hastrup & Schwartz, 1996) and (b) the selective NK_1 receptor antagonist GR 82334 antagonized HEK-1 induced RUB contractions with apparent affinity similar to that measured against [Sar^9]-SP sulphone and about 1 log unit less than that measured against septide (cf. Meini *et al*., 1994).

Bioassays such as salivary secretion or fall in blood pressure have been especially useful for the characterization of SP as relatively selective NK_1 receptor agonist *in vivo* (Maggi *et al*., 1987). Although some differences were detected between SP and HEK-1 in producing these effects, the behaviour of the two peptides in producing hypotension in guinea-pigs and salivary secretion in rats was almost superimposable suggesting that HEK-1 is a potent NK_1 receptor agonist *in vivo*. Indeed, the cardiovascular effects induced by HEK-1 in guinea-pigs were abolished by structurally unrelated NK_1 receptor antagonists such as SR 140333 and MEN 11467, indicating that, as for the cardiovascular effects induced by SP in this species (Hirayama *et al*., 1993), HEK-1-induced hypotension and bradycardia are

entirely mediated by NK_1 receptors. Likewise SR 140333 antagonized in a similar manner both SP and HEK-1-induced salivary secretion, confirming that, as in the case of other tachykinins (Bremer *et al*., 2001), also the secretory effect of HEK-1 is due to NK_1 receptor stimulation.

In conclusion our data indicate that the novel mammalian tachykinin, HEK-1 is a NK_1 receptor preferring agonist that interacts with NK_1 receptor in SP-like rather than septide-like manner and that it is a powerful SP-like receptor agonist *in vivo*.

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