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RESEARCH PAPER

Effects of the somatostatin receptor subtype 4 selective agonist J-2156 on sensory neuropeptide release and inflammatory reactions in rodents

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Background and purpose: Substance P (SP) and calcitonin gene-related peptide (CGRP) released from capsaicin-sensitive sensory nerves induce local neurogenic inflammation; somatostatin exerts systemic anti-inflammatory actions presumably via sst_4/sst_1 receptors. This study investigates the effects of a high affinity, sst_4-selective, synthetic agonist, J-2156, on sensory neuropeptide release *in vitro* and inflammatory processes *in vivo*.
Experimental approach: Electrically-induced SP, CGRP and somatostatin release from isolated rat tracheae was measured with radioimmunoassay. Mustard oil-induced neurogenic inflammation in rat hindpaw skin was determined by Evans blue leakage and in the mouse ear with micrometry. Dextran-, carrageenan- or bradykinin-induced non-neurogenic inflammation was examined with plethysmometry or Evans blue, respectively. Adjuvant-induced chronic arthritis was assessed by plethysmometry and histological scoring. Granulocyte accumulation was determined with myeloperoxidase assay and IL-1β with ELISA.
Key results: J-2156 (10–2000 nM) diminished electrically-evoked neuropeptide release in a concentration-dependent manner. EC_{50} for the inhibition of substance P, CGRP and somatostatin release were 11.6 nM, 14.3 nM and 110.7 nM, respectively. J-2156 (1–100 $\mu g\,kg^{-1}$ i.p.) significantly, but not dose-dependently, inhibited neurogenic and non-neurogenic acute inflammatory processes and adjuvant-induced chronic oedema and arthritic changes. Endotoxin-evoked myeloperoxidase activity and IL-1β production in the lung, but not IL-1β- or zymosan-induced leukocyte accumulation in the skin were significantly diminished by J-2156.
Conclusions and implications: J-2156 acting on sst_4 receptors inhibits neuropeptide release, vascular components of acute inflammatory processes, endotoxin-induced granulocyte accumulation and IL-1β synthesis in the lung and synovial and inflammatory cells in chronic arthritis. Therefore it might be a promising lead for the development of novel anti-inflammatory drugs.
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Keywords: somatostatin sst_4 receptors; capsaicin-sensitive sensory nerves; neurogenic inflammation; oedema; endotoxin-induced airway inflammation; adjuvant-induced arthritis

Abbreviations: CFA, complete Freund's adjuvant; CGRP, calcitonin gene-related peptide; IL-1β, interleukin 1β; LPS, lipopolysaccharide; MPO, myeloperoxidase; sst_1, somatostatin receptor 1; sst_4, somatostatin receptor 4; SP, substance P; TRPV1, transient receptor potential vanilloid 1

Introduction

Activation of capsaicin-sensitive, transient receptor potential vanilloid 1 (TRPV1) receptor-expressing, sensory nerve terminals results in not only nociception, but the release of several sensory neuropeptides (Szolcsanyi, 1996a, b; Caterina *et al*., 1997). Among these, calcitonin gene-related peptide (CGRP), substance P (SP) and neurokinin A (NKA) elicit arteriolar vasodilatation, plasma protein leakage from venules and leucocyte accumulation locally in the innervated area, effects which are collectively called neurogenic inflammation (Szolcsanyi, 1988; Holzer, 1992; Maggi, 1995;

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Geppetti and Holzer, 1996; Szolcsanyi, 1996a, b). These processes participate in the pathological mechanism of several inflammatory diseases of the skin, joints and airways (Helyes *et al*., 2003) and they cannot be inhibited by the classical nonsteroidal anti-inflammatory agents (Jancso-Gabor and Szolcsanyi, 1970; Helyes *et al*., 2003). Besides these proinflammatory neuropeptides somatostatin is also synthesized in the capsaicin-sensitive subpopulation of primary afferents from where it can be released in response to activation (Gamse *et al*., 1981b; Holzer, 1988; Szolcsanyi *et al*., 1994). Moreover, it has recently been shown that inflammation alters somatostatin mRNA expression in rat sensory neurones (Abd El-Aleem *et al*., 2005).

Somatostatin is widely distributed in the central nervous system (Gamse *et al*., 1981a) and the peripheral tissues in 14 and 28 amino-acid-containing forms (Patel *et al*., 1995; ten Bokum *et al*., 2000). It exerts a wide range of effects like modulation of hormone and neurotransmitter release, cognitive and behavioural processes, the gastrointestinal tract, the cardiovascular system and tumour cell proliferation (ten Bokum *et al*., 2000; Pinter *et al*., 2006). These effects are mediated via five different G-protein-associated somatostatin receptor subtypes which can be divided into two main groups on the basis of their sequence similarities and their binding profile towards synthetic somatostatin analogues: the $SRIF_1$ group comprises the sst_2, sst_3 and sst_5 receptors and the $SRIF_2$ group contains the somatostatin receptor 1 (sst_1) and somatostatin receptor 4 (sst_4) receptors (Reisine and Bell, 1995; Hoyer *et al*., 1995). Octapeptide analogues, such as octreotide (SMS-201995, Sandostatin) or vapreotide (RC-160), have high affinity to sst_2, sst_3 and sst_5 receptors, while low affinity to the other two subtypes. Several data indicate that receptors in the $SRIF_1$ group mediate the endocrine and antiproliferative effects of somatostatin, while our previous data revealed that the $SRIF_2$ group is likely to be responsible for the anti-inflammatory and antinociceptive actions (Helyes *et al*., 2000, 2001; Pinter *et al*., 2002; Szolcsanyi, 2004).

Exogenously administered somatostatin has been shown to inhibit neurogenic inflammation and nociception in several experimental assessments (Lembeck *et al*., 1982; Chrubasik, 1991; Karalis *et al*., 1994; Fioravanti *et al*., 1995). Furthermore, our previous studies provided several lines of evidence for the existence of a novel, somatostatin-mediated neurohumoral counter-regulatory mechanism. Somatostatin released from the activated capsaicin-sensitive sensory nerve terminals reaches the circulation and it is able to elicit systemic anti-inflammatory (Szolcsanyi *et al*., 1998a, b; Helyes *et al*., 2004) and antinociceptive (Helyes *et al*., 2000, 2004) actions (Szolcsanyi, 2004; Pinter *et al*., 2006). Although this function of capsaicin-sensitive afferents is a powerful inhibitory process, the therapeutic value of native somatostatin is limited by its broad range of effects and very short (3 min) plasma half-life (ten Bokum *et al*., 2000). However, potent, stable and selective synthetic somatostatin analogues might be promising for developing novel types of anti-inflammatory/analgesic drugs.

TT-232, a cyclic synthetic heptapeptide analogue (Keri *et al*., 1996) having its highest affinity for the sst_4 receptor (Helyes *et al*., 2005), showed greater inhibitory effects in several inflammatory and nociceptive models than native somatostatin (Helyes *et al*., 2001; Pinter *et al*., 2002; Helyes *et al*., 2005). On the other hand, octapeptide analogues, such as octreotide binding to receptor subtypes of the SRIF1 family and having very low affinity for the other subtypes (Hoyer *et al*., 1995), failed to inhibit neurogenic or non-neurogenic inflammatory responses in our rat experiments (Helyes *et al*., 2001). Therefore, it is likely that the sst_4 and/or the sst_1 receptors mediate the anti-inflammatory effects of somatostatin.

Progress has been made recently developing subtype-selective nonpeptide somatostatin receptor agonists, which could be promising for drug development. A novel sulphonamido–peptidomimetic compound, J-2156 ((1′*S*,2*S*)-4-amino-*N*-(1′-carbamoyl-2′-phenylethyl)-2-(4″-methyl-1″-naphthalenesulphonamino)butanamide), synthesized at Juvantia Pharma (Turku, Finland), belongs to a chemically novel class of somatostatin receptor ligands. J-2156 possesses nanomolar affinity for the human sst_4 and it is over 400-fold more selective for this receptor than any other human somatostatin receptor subtype (Engstrom *et al*., 2005). In a cyclic adenosine monophosphate (AMP) assay the compound acted as a full agonist similar to native somatostatin-14 or somatostatin-28. In a [^{35}S]guanosine-5′-*O*-(3-thio) triphosphate functional assay J-2156 elicited 2–3 times larger response than native somatostatin and increasing concentrations of somatostatin-14 caused a concentration-dependent rightward shift of the concentration–response curves of J-2156 without affecting its maximal effect. J-2156 exerted greater agonism on the sst_4 than its endogenous ligands and it has been defined as a selective, high affinity agonist (Engstrom *et al*., 2005).

The aim of the present study was to analyse the effects of J-2156 on sensory neuropeptide release *in vitro* and on acute and chronic inflammatory reactions *in vivo* in rats and mice.

Methods

Animals

Experiments were performed on male Wistar and Lewis rats and Balb/c mice bred and kept in the Laboratory Animal Centre of the University of Pécs under standard pathogen free conditions at 24–25°C and provided with standard rat chow and water *ad libitum*.

Ethics

All experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988) and complied with the recommendations of the International Association for the Study of Pain and the Helsinki Declaration. The studies were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments and licence was given (licence no.: BA 02/2006-2001).

Measurement of electrically evoked sensory neuropeptide release from isolated rat tracheae

The method has been described in detail elsewhere (Helyes *et al*., 1997; Nemeth *et al*., 1998). In brief, rats were exsanguinated in deep anaesthesia (sodium thiobarbital, 50 mg kg^{-1} intraperitoneal (i.p.)), then the whole trachea was removed and cleaned of fat and adhering connective tissues. Tracheae from two rats were placed into the same organ bath to achieve sufficient amount of peptide release and perfused (1 ml min^{-1}) with pH- (7.2) controlled oxygenated Krebs solution for 60 min (equilibration period) at 37°C. After discontinuation of the flow, the solution was changed three times for 8 min to produce prestimulated, stimulated, poststimulated fractions. Electrical field stimulation (40 V, 0.1 ms, 10 Hz for 120 s; 1200 pulses) was performed to elicit neurotransmitter release in the second 8-min period. CGRP, SP and somatostatin concentrations were determined from 200 μl samples of organ fluid of the preparations by means of radioimmunoassay methods developed in our laboratories as described (Nemeth *et al*., 1996; Helyes *et al*., 1997; Nemeth *et al*., 1998). J-2156 was added to the incubation medium at the beginning of each fraction. J-2156 was administered in a concentration range of 10–2000 nM in separate experiments, only one concentration was applied to the same tracheae to avoid neuropeptide depletion. In each group 6 experiments were performed to provide $n = 6$ data per group (12 tracheae per group).

Examination of mustard oil-induced acute neurogenic inflammation in the skin of the rat hindpaw

Both hindlegs of male Wistar rats weighing 180–250 g were acutely denervated (the sciatic and the saphenous nerves were cut 30 min before the induction of inflammation) under sodium pentobarbitone (Nembutal, 40 mg kg^{-1} i.p.) anaesthesia to avoid central reflexes. Acute neurogenic inflammation in the paw skin was evoked by topical application of 1% mustard oil dissolved in paraffin oil. Extravasation of plasma albumin was measured by the Evans blue leakage method. Evans blue (50 mg kg^{-1}) was injected intravenously (i.v.) and neurogenic inflammation was induced 10 min later. Rats were killed by exsanguination 20 min after mustard oil application. The skin of the hindpaws was removed and the extravasated dye was extracted with formamide for 72 h at room temperature for photometric determination at 620 nm. The amount of the accumulated Evans blue, which quantitatively correlates with the intensity of plasma extravasation, was expressed as μg dye g^{-1} wet tissue (Helyes *et al*., 1997, 2001). J-2156 (0.5–100 μg kg^{-1}) was administered i.p. 20 min before the induction of inflammation. For examination of the duration of action, J-2156 was administered i.p. 2 or 6 h before mustard oil smearing. In separate groups, J-2156 was given orally 45 min before the induction of inflammation.

This study was undertaken in blocks with 14–15 rats per occasion. The whole set of data were obtained during 6 days. There were 2 or 3 solvent-treated rats every day and the remaining 12 animals were randomized to receive each treatment. The number of rats was 6–8 in every J-2156-treated group and 14 in the control group.

Measurement of mustard oil-induced neurogenic oedema formation in the mouse ear

Male Balb/c mice (20–25 g) were anaesthesized with ketamine (100 mg kg^{-1}, i.p.) and xylazine (10 mg kg^{-1}, intramuscular (i.m.)) and 10 μl of 1% mustard oil dissolved in paraffin oil was smeared on both sides of the ears. The diameter of the ear was measured with an engineers' micrometre before the treatment and four times during the 3 h-examination period. Oedema was expressed in % compared to the initial control values. J-2156 (10–100 μg kg^{-1}, i.p.) was administered 15 min before mustard oil smearing. Animals of the control group were treated with the same volume of the solvent of J-2156 (saline). There were 8–10 mice in each experimental group. Area under the curve (AUC) values were calculated from percentage ear swelling data.

Examination of non-neurogenic inflammation of the rat paw

Non-neurogenic inflammation was elicited by dextran (100 μl, 5%) or bradykinin (50 μl, 0.25 μg) administered s.c. under the plantar skin of the chronically denervated hindleg of male Wistar rats (180–250 g) under Na-pentobarbital (Nembutal, 40 mg kg^{-1}, i.p.) anaesthesia to produce tissue oedema and plasma extravasation, respectively. The experiments were undertaken using a randomized block design. The hindlimbs were denervated 5 days before dextran or bradykinin injection to elicit degeneration of the leg's nerve supply and therefore exclude the neurogenic part of the inflammatory response. Oedema formation in the rat hindpaw was measured by plethysmometry (Ugo Basile 7140). The transducer of the instrument records small differences in water level caused by volume displacement. The paw volumes were measured prior to s.c. injection of dextran (control value) and 10, 20, 30 min after the treatment. The extent of the oedema was expressed as a percentage of control. AUC values were calculated from percentage paw swelling data.

Non-neurogenic plasma extravasation elicited by bradykinin was determined by the Evans blue technique. J-2156 (1–100 μg kg^{-1}, i.p.) was administered 15 min before the induction of inflammation. Animals of the control groups were pretreated with the same volume of the solvent of J-2156 (saline), there were 6–8 rats in every experimental group.

Examination of carrageenan-induced paw swelling

Carrageenan (3%, 100 μl) was injected into the plantar surface of one hindpaw of male Wistar rats (180–250 g) under Na-pentobarbital (Nembutal, 40 mg kg^{-1}, i.p.) anaesthesia to induce a mixed-type inflammatory response with both neurogenic and non-neurogenic components. The volume of the paw was measured with plethysmometry before and 60, 120, 180 min after carrageenan administration as described above, and oedema was expressed as % of initial control. J-2156 (1–100 μg kg^{-1}, i.p.) was administered 15 min before the induction of inflammation. Animals of the control groups were pretreated with the same volume of the solvent of J-2156 (saline), there were 6–8 rats in every experimental group. AUC values were calculated from percentage paw swelling data.

Measurement of lung myeloperoxidase activity and interleukin-1β concentration in endotoxin-induced airway inflammation

Subacute airway inflammation was evoked in female CD1 mice (20–25 g) by 60 μl *Escherichia coli* (serotype: 083) lipopolysacharide (LPS) applied intranasally to produce a subacute peribronchial/perivascular pulmonary inflammation (Okamoto *et al*., 2004). Animals were killed by anaesthetic overdose and exsanguinated 24 h later. Lungs were cut into half to be able to perform both myeloperoxidase (MPO) and interleukin-1β (IL-1β) measurements from the same lung. The wet weight of each sample was measured, then they were frozen in liquid nitrogen and stored at −80°C.

Accumulation of granulocytes, especially neutrophils was determined by assessment of MPO activity. Lung pieces were thawed and chopped into small pieces then homogenized in 4 ml 20 mM potassium-phosphate buffer (pH 7.4). The homogenate was centrifuged at 10 000 *g* at 4°C for 10 min and the pellet was resuspended in 4 ml 50 mM potassium-phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide (pH 6.0) and centrifuged again. Neutrophil accumulation was assessed by comparing MPO enzyme activity of the samples to a human standard MPO preparation. MPO activity was assayed from the supernatant using H_2O_2-3,3′,5,5′-tetramethyl-benzidine (TMB/H_2O_2). Reactions were performed in 96-well microtitre plates in room temperature. The optical density (OD) at 620 nm was measured at 5 min intervals for 30 min, using a microplate reader (Labsystems, Finland) and plotted. The reaction rate (ΔOD/time) was derived from an initial slope of the curve. A calibration curve was then produced with the rate of reaction plotted against the standard samples and calculated as units per g wet lung.

To measure the concentration of the inflammatory cytokine IL-1β, lung samples were homogenized in 450 μl Rosewell's Park Memorial Institute media (RPMI) 1640 buffer containing 50 μl phenylmethylsulphonyl fluoride (PMSF) with a polytrone homogenizer (Kika Lab Techniques, Germany) at 13.500 r.p.m. for 2 min. The homogenates were centrifuged for 10 min at 10 000 r.p.m. and 4°C. The concentration of IL-1β was determined by a specific enzyme-linked immunosorbent assay (ELISA) technique and expressed as ng cytokine per g wet lung.

J-2156 (10 μg kg^{-1} i.p.) was administered three times during the development of the inflammatory reaction: 20 min before LPS, 6 h and 22 h after LPS. Mice in the control group were treated the same way with the same volume of saline, there were 10–14 mice in each experimental group.

Examination of non-neurogenic neutrophil cell accumulation in the back skin of the mouse induced by IL-1β or zymosan

Experiments were performed on male Balb/c mice (20–25 g) anaesthesized with ketamine (100 mg kg^{-1}, i.p.) and xylazine (10 mg kg^{-1}, i.m.). Leucocyte accumulation in the back skin was determined on the basis of MPO activity using spectrophotometry at the end of the experiment to quantify the cellular phase of the inflammatory reaction.

The cytokine, mouse recombinant IL-1β (3 pmol), or zymosan (0.01%) was injected in 100 μl volume intradermal (i.d.) into the dorsal skin. In control, noninflamed mice sterile Tyrode solution was applied in the same volume. J-2156 in 10 or 100 μg kg^{-1} doses or its solvent (saline) was injected i.p. 10 min before the induction of inflammation. The study was performed in a randomized block design ($n = 6$–8 per group). At the end of the 3 h accumulation period the animals were killed by anaesthetic overdose and exsanguinated, then 8 mm diameter dorsal skin pieces were punched out. Neutrophil accumulation was determined from the frozen skin samples by assessment of MPO activity as described above.

Investigation of adjuvant-induced chronic inflammatory oedema formation and arthritic changes

Complete Freund's adjuvant (CFA; killed Mycobacteria suspended in paraffin oil; 0.1 ml, 1 mg ml^{-1}) was injected intraplantarly and into the root of the tail of male Lewis rats. In order to enhance systemic effects, an additional injection was given into the tail on the following day. The volume of the paws was measured by plethysmometry (Ugo Basile Plethysmometer 7140, Comerio, Italy) before the experiment and 2, 5, 8, 12, 15, 18 and 21 days after CFA administration. Paw oedema was expressed as percentage of initial control values. AUC values were calculated from percentage paw swelling data were analysed with one-way Analysis of Variance (ANOVA) followed by Bonferroni's modified *t*-test.

The left tibio-tarsal joints were excised *in toto* after killing the animals by Nembutal (pentobarbital sodium; May and Baker, UK) overdose (100 mg kg^{-1} i.p.) on the 21st experimental day. The specimens were fixed in 4% formaldehyde for 8 h, decalcified in a demineralizing solution containing 7 w/v% $AlCl_3$, 5 v/v% formic acid and 8.5 v/v% HCl for 8 h at 4°C and dehydrated at 4°C for 8 h in 5 w/v% in saccharose followed by immersion into 10 and 15 w/v% saccharose for subsequent two periods of 8 h. Then the samples were embedded in paraffin, sectioned with a microtome (5 μm) and stained with haematoxylin and eosin. (Helyes *et al*., 2004). Arthritic changes were scored by a pathologist without knowledge of the treatments received, using a grading scale of 0–3 according to the proportion of areolar tissue that was densely infiltrated with mononuclear cells. Synovial lining cell hyperplasia and the number of histiocytes observed in the synovial tissue were graded similarly on the same scale. Cartilage destruction was scored on a scale of 0–3, ranging from no damage to fully destroyed cartilage layers. Bone erosion scores were obtained on the following features: 0 = normal, 1 = mild loss of cortical bone at a few sites, 2 = moderate loss of cortical trabecular bone, 3 = marked loss of bone at many sites (Weinberger *et al*., 1997; Helyes *et al*., 2004). From every joint (6–8 rats in each group) 4–5 sections were taken in different depths to give a representative appreciation of the whole joint. Mean scores were determined from the different sections of the individual animals and composite score values of the different experimental groups were calculated from these mean scores.

J-2156 (1 and 10 $\mu g\,kg^{-1}$) or in the control group, saline was injected i.p. three times every day throughout the whole experimental period ($n=6$–8 per group).

Statistical analysis

For statistical evaluation of the *in vivo* plasma extravasation values and skin MPO data analysis of variance (one-way ANOVA) followed by Bonferroni's modified *t*-test were used. In the oedema studies (mustard oil-induced ear swelling; dextran-, carrageenan- and adjuvant-evoked paw oedema) percentage swelling compared to the initial ear thickness and paw volume was calculated, area under the % oedema curves (AUC) were determined from the whole data set and analysed with one-way ANOVA followed by Bonferroni's modified *t*-test. Histological inflammatory score values were expressed as medians with ranges and evaluated with Kruskal–Wallis followed by Dunn's test. In the *in vitro* peptide release experiments data were evaluated by one-way ANOVA followed by Bonferroni's modified *t*-test to compare the effect of different J-2156 concentrations to the control peptide outflow values. MPO and IL-1β results in the endotoxin-induced lung inflammation model were evaluated by Student's *t*-test unpaired comparison. In all cases $P<0.05$ was considered significant.

Drugs and chemicals

Sodium pentobarbitone was obtained from Serva (Heidelberg, Germany), mustard oil (allyllisothiocyanate) and dextran from Fluka (Buchs, Switzerland), Evans blue dye, carrageenan, zymosan, IL-1β, human MPO standard preparation, H_2O_2-3,3′,5,5′-tetramethyl-benzidine, bradykinin, LPS, PMSF and CFA from Sigma (St Louis, MO, USA), RPMI 1640 buffer from Biochrom Ltd (Berlin, Germany), rat α-CGRP, [Tyr^1]somatostatin-14 and Tyr-α-CGRP(23–37) from Bachem (Bubendorf, Switzerland), SP RIA-tracer from Amersham (Amersham, UK) and IL-1β ELISA kit from BD Sciences Eastern Europe (Heidelberg, Germany). J-2156 was synthesized at Juvantia Pharma (Turku, Finland) and dissolved in isotonic saline. SP antiserum was kindly provided by Professor DJ Dockray, University of Liverpool and C-terminal-sensitive somatostatin antiserum and CGRP antiserum by Dr T Görcs, Semmelweis University Medical School of Budapest. ^{125}I-labelled Tyr-α-CGRP(23–37) (specific activity 75.6TBq $mmol^{-1}$ and ^{125}I-labelled [Tyr^1]somatostatin-14 (specific activity 74.3TBq $mmol^{-1}$) were prepared in our laboratory (Nemeth *et al*., 2002).

Results

Effect of J-2156 on electrically evoked sensory neuropeptide release from isolated rat tracheae

Release of SP, CGRP and somatostatin increased from 1.77 ± 0.03 to 5.96 ± 0.15 fmol mg^{-1}, from 0.17 ± 0.01 to 0.69 ± 0.05 fmol mg^{-1} and from 0.18 ± 0.01 to 0.51 ± 0.21 fmol mg^{-1}, respectively, in response to electrical field stimulation. J-2156 (10–2000 nM) significantly inhibited the stimulation-evoked release of all the three measured sensory neuropeptides in a concentration-dependent manner, although it did not influence basal, nonstimulated peptide release. When analyzing the experimental data as sigmoidal concentration–response curves via a nonlinear least square curve fitting procedure (see Figure 1), maximal effects of about 50% inhibition were obtained for SP and CGRP, while the maximal effect on the release of somatostatin appeared to be somewhat lower. The EC_{50} values with 95% confidence intervals for SP, CGRP and somatostatin amounted to 11.6 nM (1.96–64.42 nM), 14.3 nM (2.69–74.53 nM) and 110.7 nM (43.24–284.63 nM), respectively (Figure 1).

Effect of J-2156 on acute neurogenic inflammation in the rat hindpaw skin

Mustard oil applied to the paw skin induced a marked extravasation of plasma protein, assessed by leakage of Evans blue dye. As shown in Figure 2a, this leakage was inhibited significantly, but not dose-dependently, by J-2156 (1–100 $\mu g\,kg^{-1}$, i.p.). A similar, nondose-dependent, inhibition was induced by native somatostatin (Figure 2b). The inhibitory action of 10 $\mu g\,kg^{-1}$ J-2156 lasted for 6 h (Figure 2a). Oral administration of 10 or 100 $\mu g\,kg^{-1}$ J-2156 also significantly diminished this neurogenic inflammatory response, 60 min after dosing, but both doses exerted about 50% inhibition (10 $\mu g\,kg^{-1}$, 79 ± 10.6 and 100 $\mu g\,kg^{-1}$, 83 ± 13.6 versus control, 175 ± 14.2 μg Evans blue dye (g wet weight tissue)$^{-1}$).

Effect of J-2156 on acute neurogenic oedema of the mouse ear

In the control group ($n=10$), ear thickness increased from 262 ± 17 μm to 381 ± 26 μm within 3 h in response to topical application of 1% mustard oil. J-2156 (10, 50 and 100 $\mu g\,kg^{-1}$

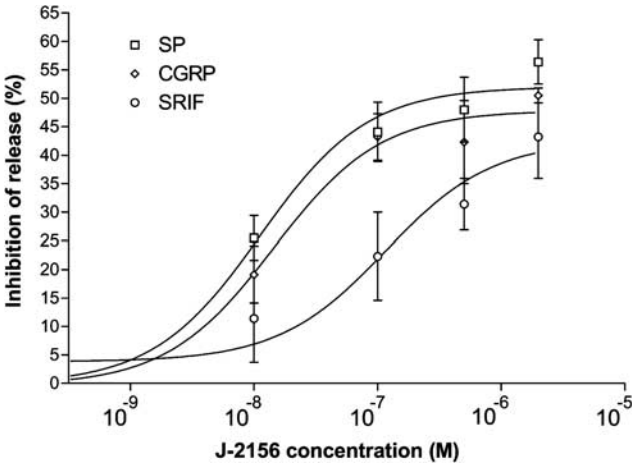

Figure 1 Concentration–response curves demonstrating the inhibitory effect of J-2156 on electrically evoked release of SP, CGRP and somatostatin (SRIF) from isolated rat tracheae. Concentration of the peptide in the basal, prestimulation fraction was subtracted from both the respective stimulated and poststimulated fractions and then these values were added. Data points representing mean ± s.e.m. inhibitory effects (%) were calculated by comparing the results of the studies with J-2156 to the control experiments, $n=6$.

i.p.; $n=8$ in each group) diminished mustard oil-induced ear swelling, the inhibitory effects of all three doses on oedema formation were significant after 2 h, whereas only 50 $\mu g\,kg^{-1}$ reached the level of statistical significance already at 20 min (Figure 3). The AUC value calculated on the basis of the time course of percentage ear swelling was 99 ± 9.7 units in the control, solvent-treated group. In comparison to control, the corresponding data in mice treated with J-2156 (10, 50 and 100 $\mu g\,kg^{-1}$) were 76 ± 11.3, 47 ± 6.7 and 57 ± 10.3, respectively. The two higher doses of J-2156 significantly reduced the oedema response, in terms of the AUC values and there was no significant difference in the magnitude of the inhibitory effects of these two doses.

Effect of J-2156 on acute non-neurogenic inflammatory reactions of the rat hindpaw

J-2156 significantly decreased bradykinin-evoked Evans blue accumulation in the plantar skin of the chronically denervated rat hindpaw in all the three applied doses (1–100 $\mu g\,kg^{-1}$ i.p.), but dose–response correlation was not observed. The greatest inhibition, about 56%, was achieved after pretreatment with the smallest dose of 1 $\mu g\,kg^{-1}$ (Figure 4).

On the other hand, J-2156 dose-dependently inhibited non-neurogenic oedema formation of the rat paw 10, 20 and 30 min after intraplantar dextran injection (Figure 5). The minimal effective dose (MED) was 10 $\mu g\,kg^{-1}$. The 100 $\mu g\,kg^{-1}$ dose inhibited paw swelling by about 40% (Figure 5), as determined from the AUC values (control, 1313 ± 85.4 units; J-2156, i.p. 1 $\mu g\,kg^{-1}$, 1435 ± 75.5; 10 $\mu g\,kg^{-1}$; 916 ± 48.5: 100 $\mu g\,kg^{-1}$, 785 ± 62.3). Only the two higher doses of J-2156 induced a significant inhibition

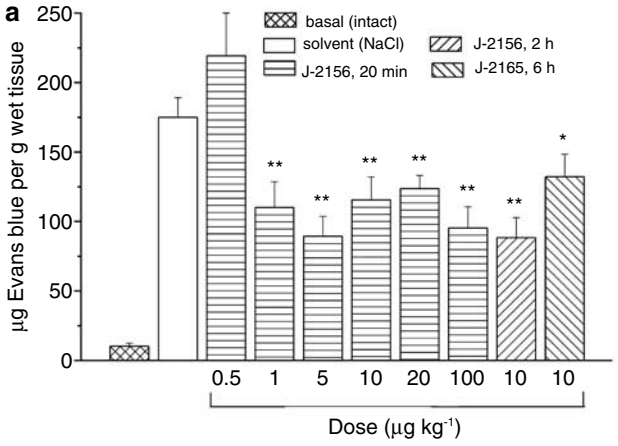

Figure 2 Effect of i.p. (**a**) J-2156 and (**b**) native somatostatin-14 on 1% mustard oil-induced Evans blue accumulation in the skin of the acutely denervated rat hindpaw. In the control group, saline (solvent) was applied i.p. in the same volume. Each column shows the mean of $n=6$–14 experiments $\pm$ s.e.m. $^{**}P<0.01$ J-2156-treated versus control (one-way ANOVA followed by Bonferroni's modified *t*-test).

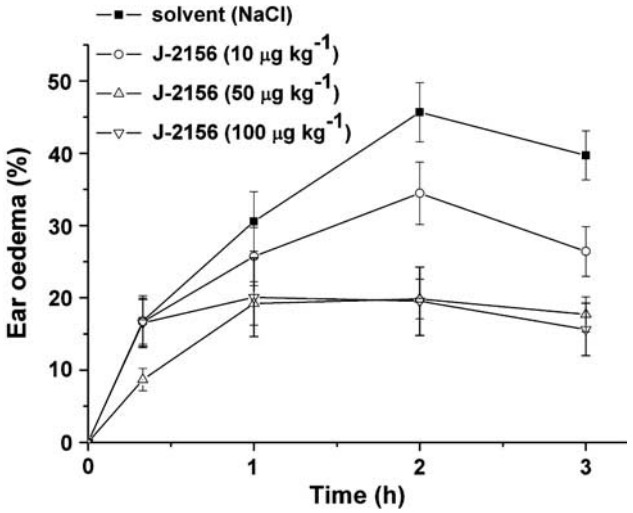

Figure 3 Effect of i.p. J-2156 on 1% mustard oil-induced, neurogenic ear oedema of the mouse. Results are expressed in % swelling as compared to the solvent-treated control group. Each data point shows the mean of $n=8$–10 experiments $\pm$ s.e.m.

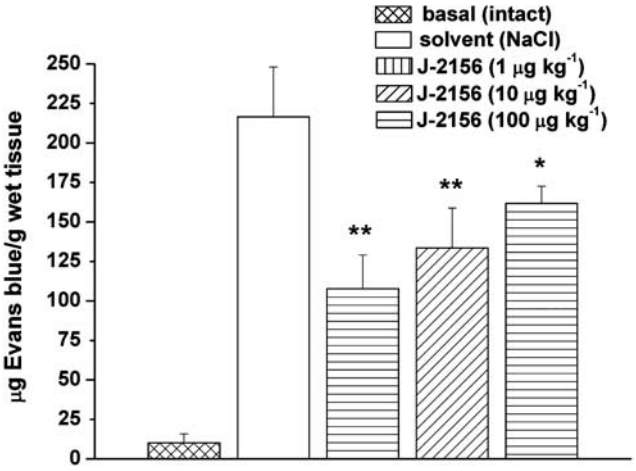

Figure 4 Effect of i.p. J-2156 on bradykinin-induced, non-neurogenic, Evans blue accumulation in the skin of the acutely denervated rat hindpaw. In the control group, saline (solvent) was applied i.p. in the same volume. Each column shows the mean of $n=6$–8 experiments $\pm$ s.e.m. $^{*}P<0.05$; $^{**}P<0.01$ J-2156-treated versus control (one-way ANOVA followed by Bonferroni's modified *t*-test).

of oedema formation, but there was no significant difference between the magnitude of the inhibitory effects of these doses.

Effect of J-2156 on carrageenan-evoked paw oedema of the rat

Intraplantar injection of carrageenan induced 30 and 32% paw swelling in control, saline-treated rats at 60 and 120 min, respectively and this oedema was inhibited by i.p. pretreatment with 10 and 100 $\mu g\,kg^{-1}$ J-2156 at both time points (Figure 6). The control AUC value was 78 ± 4.3 units and the corresponding values in rats treated with J-2156 (1, 10 and 100 $\mu g\,kg^{-1}$ i.p.) were 61 ± 3.7, 44 ± 2.8 and 51 ± 2.9 units, respectively. As observed in the other oedema experiments, only the two higher doses of J-2156 induced a significant inhibition and no dose–response correlation was found.

Effect of J-2156 on endotoxin-induced granulocyte accumulation and IL-1β production in the lung

Intranasal LPS administration resulted in a 5.5-fold-increase of MPO activity reflecting the number of accumulated leucocytes and a 6.2-fold-increase of the concentration of the inflammatory cytokine IL-1β in the lung after 24 h, compared to untreated lung. These cellular inflammatory responses were clearly inhibited after three i.p. injections of 10 $\mu g\,kg^{-1}$ J-2156 during the development of the inflammatory reaction (Figure 7a, b).

Effect of J-2156 on IL-1β- or zymosan-induced non-neurogenic leucocyte accumulation in the back skin of mice

Within 3 h following the i.d. injection of IL-1β (3 pmol) or zymosan (0.01%), MPO activity in the back skin of the mouse increased about 3- or 4-fold relative to that in samples of skin injected with Tyrode solution (Figure 8). This cellular response to either inflammatory agent, however, was not influenced by i.p. pretreatment with 10 or 100 $\mu g\,kg^{-1}$ J-2156 (Figure 8).

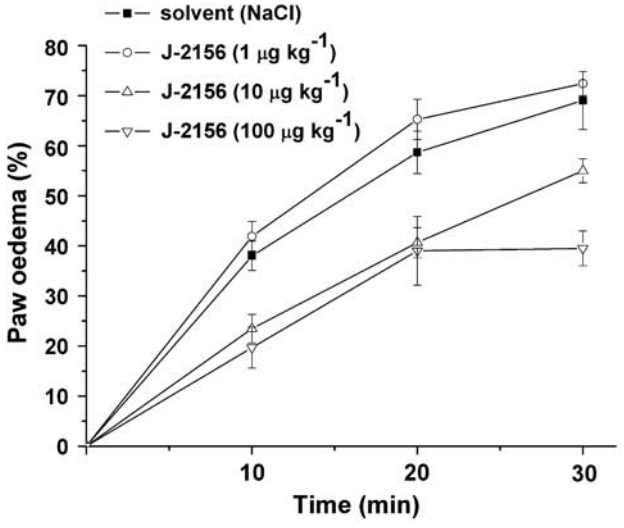

Figure 5 Effect of i.p. J-2156 on non-neurogenic oedema induced by subplantar injection of dextran (100 μl 5%) in the chronically denervated hindleg 10, 20 and 30 min after the induction of inflammation. In the control group of rats the same volume of saline was given. Symbols indicate % increase of the volume of the hindpaws as compared to the initial values measured with plethysmometry before dextran injection. Each data point represents the mean of $n = 6$–8 experiments $\pm$ s.e.m.

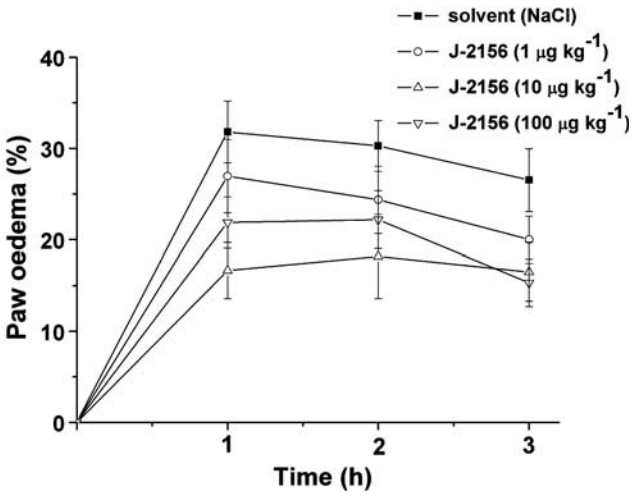

Figure 6 Effect of i.p. J-2156 on carrageenan (100 μl; 1%)-induced paw oedema of the rat 1, 2 and 3 h after the induction of inflammation. In the control group of rats the same volume of saline was given. Symbols indicate % increase of the volume of the hindpaws as compared to the initial values measured with plethysmometry before carrageenan injection. Each data point represents the mean of $n = 6$–8 experiments $\pm$ s.e.m.

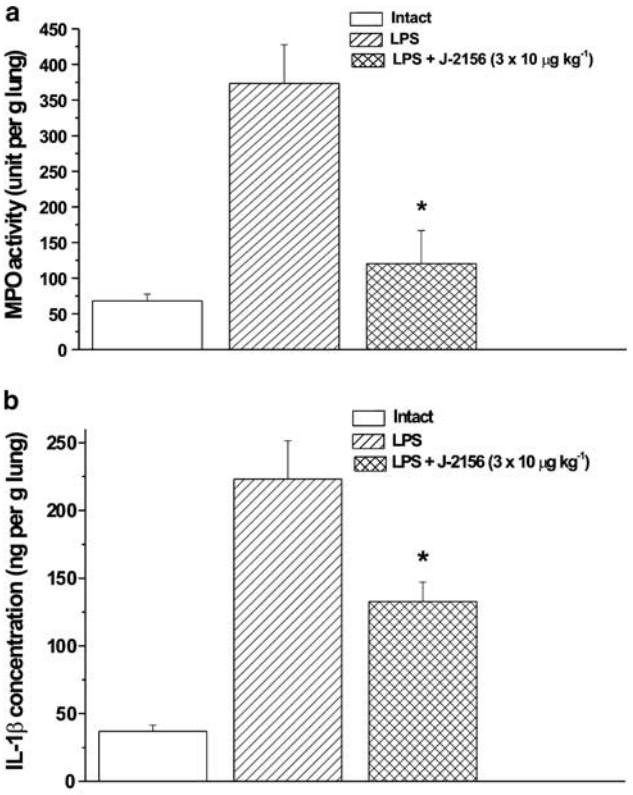

Figure 7 Effect of i.p. J-2156 ($3 \times 10\,\mu g\,kg^{-1}$, i.p.) on LPS-induced (**a**) MPO enzyme activity and (**b**) IL-1β production in the mouse lung. Columns show the mean of $n = 10$–14 experiments $\pm$ s.e.m. *$P < 0.01$ J-2156-treated versus solvent-treated control (Student's *t*-test for unpaired comparison).

Effect of J-2156 on adjuvant-induced chronic paw oedema and inflammatory changes in the tibiotarsal joint

In control, solvent-treated animals ($n=8$) the volume of the CFA-injected paw gradually increased from 0.79 ± 0.007 to $1.28\pm0.075\,cm^3$ reaching a maximal swelling of 60% 8 days after the induction of inflammation (Figure 9a). The AUC value, calculated from percentage paw oedema data, was 1108 ± 104.1 units in the control group, while the corresponding AUC in rats treated with J-2156 (3×1 and $10\,\mu g\,kg^{-1}$ per day i.p.; $n=6$ in each group) throughout the 21-day experimental period were 738 ± 47.5 and 827 ± 59.5, respectively. Statistical analysis revealed that the lower J-2156 dose inhibited significantly oedema formation (as AUC units) compared to the control group, but the effect of the higher dose did not prove to be statistically significant.

The unilateral tibiotarsal joints of the adjuvant-treated rats were damaged by expanding synovial pannus. Widening of the synovial cavity, mononuclear cell infiltration, thickening of the synovial membrane, disruption of the cartilagineous tissue and bone erosion were apparent. Treatment with 3×1–$10\,\mu g\,kg^{-1}$ per day J-2156 i.p. markedly reduced inflammatory changes such as synovial swelling, lymphocyte accumulation and cartilage erosion and signs of bone destruction were not seen. Interestingly, the smaller dose proved to be more effective as shown by the composite inflammatory score values (Figure 9b).

Discussion and conclusions

The present results demonstrate that the selective sst_4 receptor agonist peptidomimetic compound, J-2156, was able to significantly inhibit electrically induced sensory neuropeptide release from rat tracheae *in vitro* and both neurogenic and non-neurogenic vascular inflammatory reactions *in vivo* in rats and mice.

In rat lung mRNA of two somatostatin receptor subtypes, predominantly sst_4 and a lesser extent sst_1, have been identified (Schloos *et al*., 1997). A concentration-dependent inhibition of the release of proinflammatory neuropeptides, SP and CGRP, from the peripheral terminals of capsaicin-sensitive sensory nerve endings of isolated tracheae was observed in the presence of J-2156 (10–2000 nM). This observation implies that prejunctional sst_4 activation is responsible – at least partially – for the ability of J-2156 to diminish neurogenic inflammation. Although J-2156 was able to diminish electrically induced release of the anti-inflammatory peptide somatostatin as well, the outflow of this peptide was much less sensitive to the inhibitory effect of J-2156. For the same extent of inhibition, 10–20 times higher concentrations of J-2156 were required to inhibit somatostatin release than to inhibit release of CGRP and SP, although the maximal inhibition was about 40% for all three peptides. This ability of J-2156 to inhibit somatostatin release at higher concentrations than SP and CGRP release might provide an explanation for the lack of dose–response correlations in some of the *in vivo* inflammation models.

Previous studies in the same *in vitro* model revealed that other agents acting at opioid-like or opioid receptors exerted

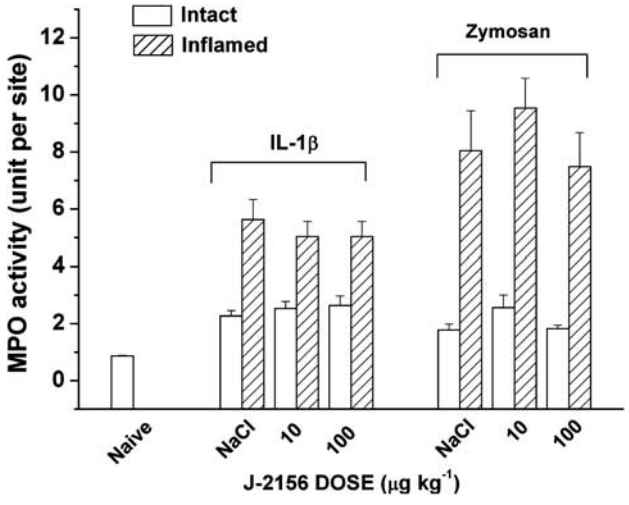

Figure 8 No effect of i.p. J-2156 on IL-1β or zymosan-induced neutrophil accumulation in the back skin of mice as measured by MPO enzyme activity. Columns show the mean of n = 6–8 experiments ± s.e.m.

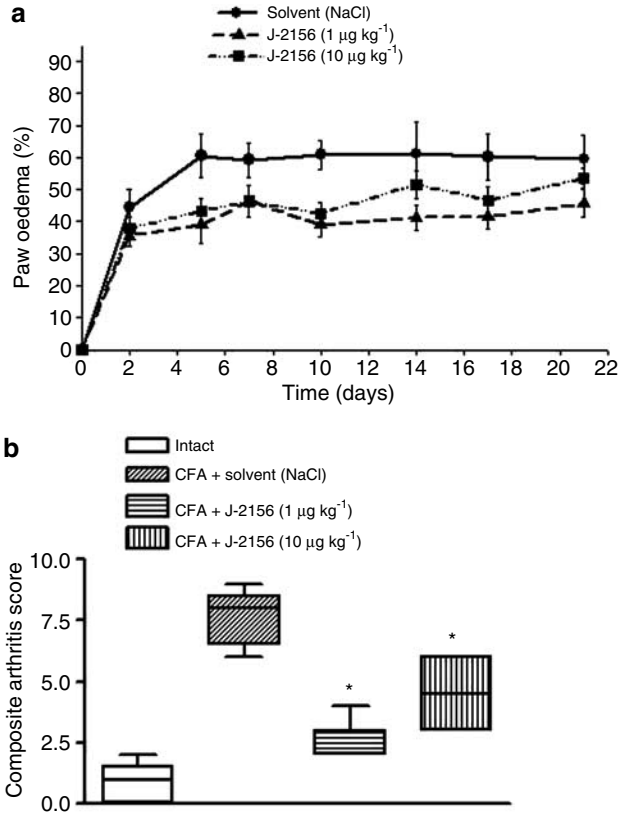

Figure 9 (**a**) Effect of J-2156 (3×1–$10\,\mu g\,kg^{-1}$ per day i.p.) on CFA-induced paw oedema throughout the 21-day-experimental period. Results are expressed as percentage swelling compared to the initial paw volumes. Data points represent the mean of $n=6$–8 experiments ± s.e.m.; (**b**) effect of J-2156 on inflammatory changes in the tibiotarsal joint 21 days after CFA administration. Data, presented as box and whisker plots with the medians shown, are the composite inflammatory scores obtained from $n=6$–8 animals per group calculated on the basis synovial proliferation/enlargement, inflammatory cell infiltration, articular cartilage breakdown and bone destruction. These parameters were graded from 0 to 3 and added; *$P<0.05$ (Kruskal–Wallis followed by Dunn's *post hoc* test).

similar efficacy (similar maximal inhibitory effect) on neuropeptide release from sensory nerve terminals. Nociceptin at 100 nM diminished electrically evoked SP and CGRP release by 63 and 44%, respectively (Helyes *et al*., 1997), while the corresponding data for 500 nM endomorphin-1 were 43 and 42% (unpublished results). Based on these effects nociceptin was able to diminish neurogenic inflammatory reactions (Helyes *et al*., 1997).

Mustard oil in concentrations under 5% causes pure neurogenic inflammation without the involvement of mast cells (Inoue *et al*., 1997; Szolcsanyi *et al*., 1998b) by selectively stimulating the capsaicin-sensitive sensory nerve endings (Jancso *et al*., 1967). In our experiments, we used 1% mustard oil to induce plasma protein extravasation and inhibition of this neurogenic response by J-2156 was about 40–45%, which was similar to the effect of native somatostatin after i.p. administration (Helyes *et al*., 2001). J-2156 similarly diminished Evans blue leakage after oral administration, implying gastrointestinal absorption and its effect lasted for 6 h. In mice, neurogenic inflammation, assessed by ear swelling was diminished by 50% after pretreatment with 50 and 100 $\mu g\,kg^{-1}$ J-2156 from 1 h onwards, while at 20 min, only the effect of 50 $\mu g\,kg^{-1}$ was significant. The 10 $\mu g\,kg^{-1}$ dose appeared to be close to the ED_{50} value for this response, as its effect between 1 and 3 h amounted to about half of the higher doses.

Furthermore, in the rat, J-2156 inhibited non-neurogenic inflammatory processes like dextran-induced paw swelling and bradykinin-evoked plasma protein extravasation in the chronically denervated hindpaw. Dextran exerts oedema formation through mast cell degranulation, bradykinin induces plasma protein leakage via acting directly on venules. It is well established that bradykinin exerts plasma protein leakage through the activation of B2 receptors on postcapillary venules. However, there are sst_4 receptors as well on these vascular endothelial cells (Curtis *et al*., 2000) and activation of these receptors by J-2156 might mediate an inhibitory action via signal transduction mechanisms and second messenger systems. Furthermore, B2 receptors are also present on mast cells and mediators released from mast cells, such as histamine, contribute to the plasma extravasation. Mast cells also express sst receptors (Renold *et al*., 1987) and J-2156 could inhibit mediator release as suggested by its inhibitory effect on dextran-induced oedema formation. Thus, these data indicate that the sst_4 agonist J-2156 elicits its acute anti-inflammatory effect via its targets on mast cells and/or venules besides inhibiting neuropeptide release from sensory nerve terminals. Molecular biological evidence supporting this concept has been recently provided for the expression of sst_1 and sst_4 receptors in human blood vessels (Curtis *et al*., 2000).

J-2156 exerted significant inhibition of adjuvant-induced chronic paw swelling and histological changes in the tibiotarsal joint. Immunoreactivity for sst_4 receptors has been detected in about 40% of rat dorsal root ganglion neurones and some satellite cells. The number of sst_4-expressing neural profiles was significantly higher at 21 days than at 3 days in the adjuvant-induced arthritis model (Bar *et al*., 2004). Data indicating that sst_4 receptors exist on synovial cells, lymphocytes and macrophages (ten Bokum *et al*., 1999; Taniyama *et al*., 2005) also underlie these results which reveal the functional significance of these receptors in the development of chronic arthritis in the rat.

J-2156 also inhibited the cellular phases of inflammation (granulocyte accumulation as determined by the MPO activity and production of the inflammatory cytokine IL-1β) in the endotoxin-induced murine pneumonitis model. LPS is a primary activator of monocytes/macrophages that operate via cytokine release with consequent granulocyte activation (Lefort *et al*., 2001). All these inflammatory and immune cells release several other inflammatory mediators, which can activate airway sensory nerve endings (Kraneveld and Nijkamp, 2001). The released neuropeptides, for example, SP in turn influence the inflammatory process by acting at receptors localized on inflammatory cells such as granulocytes. J-2156 might indirectly inhibit granulocyte function in this model by decreasing SP release from nerve endings. This concept is supported by our other finding, that zymosan or IL-1β-induced leucocyte accumulation in the back skin of mice was not influenced by J-2156. This is likely to be due to the fact that granulocytes themselves do not express sst_4 receptors, which is in accordance with previous data showing the absence of sst receptors on granulocytes (Hiruma *et al*., 1990; Lichtenauer-Kaligis *et al*., 2000). IL-1β directly evokes the accumulation of leucocytes at the site where it is injected (in this model into the back skin) without involvement of neurogenic inflammatory components such as SP, since previous data revealed that the NK1 receptor antagonist SR140333 had no effect on i.d. IL-1β-induced neutrophil accumulation (Pinter *et al*., 1999).

In some of the *in vivo* models such as carrageenan-induced oedema formation or adjuvant-induced chronic inflammation in which neurogenic components take part besides the non-neurogenic inflammatory factors, higher doses of J-2156 evoked a smaller inhibitory effect. This might be explained – at least partially – by the fact that high doses of this compound is able to decrease the release of somatostatin from sensory nerve terminals, therefore, inhibit an endogenous anti-inflammatory mechanism. Receptor desensitization is not likely to be involved in this process, since J-2156 has been shown a low propensity to cause desensitization (Engstrom *et al*., 2006). Furthermore, sst_4 receptors in general have been reported not to undergo desensitization (Csaba and Dournaud, 2001).

The octapeptide somatostatin analogue, octreotide, which has been reported to inhibit hormone secretion and tumour cell proliferation through sst_2, sst_3 and sst_5 receptors (Hofland *et al*., 1995; Siehler and Hoyer, 1999) had no influence on inflammatory processes in our previous experiments (Helyes *et al*., 2001). In contrast, the heptapeptide agonist TT-232 (Keri *et al*., 1996), which is a less selective and lower affinity agonist on the sst_4 receptor than J-2156 (Helyes *et al*., 2005), also inhibited simultaneously the release of sensory neuropeptides and mast cell degranulation (Helyes *et al*., 2001, 2005). Although the potency of J-2156 was greater than that of TT-232 *in vivo* (in the rat their minimal effective doses were 1 and 10 $\mu g\,kg^{-1}$ i.p., respectively), its efficacy was smaller. The maximal inhibition exerted by J-2156 was 39% on mustard oil-evoked neurogenic inflammation and 30% on dextran-induced non-neurogenic oedema formation. The

corresponding values for TT-232 were 64 and 52%, respectively (Pinter *et al*., 2002; Helyes *et al*., 2005). As TT-232 binds also to sst_1 as well as to sst_4 with affinities of 1300 nM and 200 nM, respectively (Helyes *et al*., 2005), and it also exerts strong tyrosine kinase inhibitory action (Vantus *et al*., 2001), these additional activities of TT-232 might give a potential explanation for its greater efficacy. However, the major advantage of J-2156 over TT-232 is in its peptidomimetic structure, which, as suggested by the effects of p.o. applied J-2156 on mustard oil-induced plasma extravasation, might make oral administration possible. Haemodynamic measurements revealed that J-2156 did not influence mean arterial blood pressure and heart rate in the rat up to 100 $\mu g\,kg^{-1}$ i.p. dose (unpublished observation).

The classical nonsteroidal anti-inflammatory agents, diclofenac and the selective COX-2 inhibitor meloxicam (Engelhardt *et al*., 1995) diminished non-neurogenic oedema in a dose-dependent manner, but did not influence neurogenic plasma extravasation (Jancso-Gabor and Szolcsanyi, 1970; Helyes *et al*., 2003). High doses of corticosteroids acting mainly by suppressing gene expression of proinflammatory factors and by upregulation of anti-inflammatory proteins (e.g. lipocortin-1), are also able to attenuate neurogenic inflammation partly through lipocortin 1-dependent mechanisms, but postjunctional effects upon SP activity/binding and prejunctional components might contribute to their action (Ahluwalia *et al*., 1995). However, the many serious side effects of the corticosteroids limit their use in clinical practice.

Evidence has been provided for the presence of somatostatin sst_4 (ten Bokum *et al*., 2000) receptors on sensory nerve terminals, blood vessels (Meyerhof, 1998; Torrecillas *et al*., 1999) and inflammatory/immune cells (ten Bokum *et al*., 1999; Lichtenauer-Kaligis *et al*., 2000; Taniyama *et al*., 2005). The present results provide biochemical and functional evidence for the involvement of sst_4 receptors in the inhibition of acute neurogenic/non-neurogenic inflammatory reactions and chronic arthritis. Based on these data, J-2156, a potent and sst_4 selective agonist that inhibited inflammation even after oral administration could provide interesting perspectives for the development of a novel class of anti-inflammatory agent.

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Conflict of interest

Dr Mia Engstrom and Dr Siegfried Wurster were employed by Juvantia Pharma Ltd (Turku, Finland) at the time when these studies were performed.

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