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ReN 1869, a novel tricyclic antihistamine, is active against neurogenic pain and inflammation

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Abstract

The tricyclic compound (*R*)-1-(3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5-ylidene)-1-propyl)-3-piperidine carboxylic acid (ReN 1869) is a novel, selective histamine H_1 receptor antagonist. It is orally available, well tolerated, easily enters the central nervous system (CNS) but no adverse effects are seen in mice at 300 mg/kg. ReN 1869 at 0.01–10 mg/kg is antinociceptive in tests of chemical nociception in rodents (formalin, capsaicin, phenyl quinone writhing) but not in thermal tests (hot plate and tail flick). ReN 1869 amplifies the analgesic action of morphine but does not show tolerance after chronic dosing. Moreover, the compound is effective against inflammation of neurogenic origin (antidromic nerve stimulation, histamine-evoked edema) but not in carrageenan-induced inflammation. We suggest that ReN 1869, via H_1 blockade, counteracts the effect of histamine liberated from activated mast cells and inhibits pain transmission in the dorsal spinal cord. ReN 1869 represents a new class of antihistamines with pain-relieving properties that probably is mediated centrally through histamine H_1 receptors but alternative mechanisms of action cannot be excluded.

Keywords: Pain; Inflammation; Histamine; Histamine H_1 receptor; Spinal cord

1. Introduction

Histamine released from mast cells is an established mediator of acute allergic reactions but may also play a role in chronic inflammation. The close connection between mast cells, microvessels and sensory C-fibers has been well demonstrated (Olsson, 1968); hence, histamine, in conjunction with other mast cell products like nerve growth factor (NGF), may contribute to vascular leakage as well as pain sensations (Shu and Mendell, 1999). Histamine is now established as a neurotransmitter and several observations have demonstrated that the histaminergic system plays a role in nociception. Recently, the involvement of histamine was substantiated by the observation that histamine H_1 receptor knockout mice showed decreased sensitivity to nociceptive stimuli (Mobarakeh et al., 2000). Moreover, the highly selective histamine H_1 receptor agonist, 2-(3-trifluoromethylphenyl)-histamine, induces hypernociception following histamine H_1 receptor activation in rodents (Malmberg-Aiello et al., 1998). Altogether, these observations raise the possibility that antihistamines (compounds that are mainly histamine H_1 receptor antagonists) may work as analgesic substances. Antinociception by different chemical classes of antihistamines has indeed been documented in laboratory animals and man (Rumore and Schlichting, 1986; Lamberti et al., 1996; Arrigo-Reina and Chiechio, 1998; Mobarakeh et al., 2000; for review, see Raffa, 2001). However, it has also been observed, that the classical potent and selective histamine H_1 receptor antagonist, mepyramine (pyrilamine), performed less well as an analgesic substance than tricyclic antihistamines like amitriptyline in animal models (Sawynok et al., 1999). This has been explained by superimposing additional effects of the tricyclic compounds on other biogenic amine receptors or uptake mechanism.

In the present investigation, we have pharmacologically characterized a newly discovered compound: (*R*)-1-(3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5-ylidene)-1-

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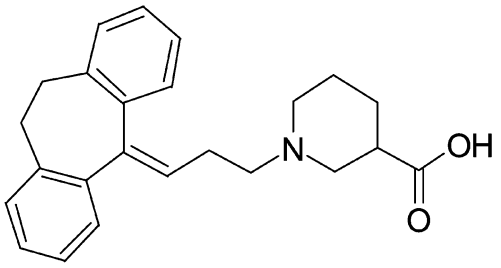

Fig. 1. The chemical structure of ReN 1869 [(*R*)-1-(3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5-ylidene)-1-propyl)-3-piperidine carboxylic acid].

propyl)-3-piperidine carboxylic acid (ReN 1869, Fig. 1). It is a highly selective tricyclic antihistamine that shows functional histamine H_1 receptor antagonism. Binding studies with radioactively labelled ReN 1869 revealed high affinity only for the histamine H_1 receptor in addition to some affinity for a sigma site. No affinity was demonstrated for other receptors including biogenic amine receptors or uptake sites. ReN 1869 is chemically related to amitriptyline, and in animal models, it demonstrates a clear effect against neurogenic inflammation and pain elicited by chemical means.

2. Materials and methods

2.1. In vitro experiments

2.1.1. Membranes

Tissue from Sprague–Dawley rats (200–250 g) or guinea pigs (300–350 g) was dissected and frozen on dry ice. Membranes were prepared by thawing the tissue in ice-cold assay buffer (composition: 120 mM NaCl, 2 mM $MgCl_2$, 0.1 mM dithiotreitol, 10 mM HEPES, pH 6.0) followed by homogenisation for 30 s (Ultra-Torrax homogenizer). The tissue was centrifuged at low speed (1000 × *g*, 10 min, 4 °C) and the pellet, consisting mostly of debris and cell nuclei, was discarded. The membrane-containing fraction was centrifuged at high speed (40 000 × *g*, 30 min, 4 °C), the final pellet resuspended in assay buffer and membrane fractions were frozen at − 80 °C in aliquots. The protein content was estimated in all types of experiments using the BioRad assay with γ-globulin as a standard. Cells from the Chinese hamster ovary (CHO) cell line, expressing the human histamine H_1 receptor (Smit et al., 1996) were harvested from confluent cell culture dishes using calcium-free PBS. The cell suspension was centrifuged at 4 °C (1000 × *g*, 10 min) and the resulting pellet stored at − 80 °C until use. Membranes were then prepared as described above.

2.1.2. [^{3}H]ReN 1869 and [^{3}H]mepyramine binding

ReN 1869 was labelled with ^{3}H in the tricyclic ring system resulting in a specific activity of 40 Ci/mmol (Valsborg and Foged, 2002). Thawed membranes (1 mg protein/tube), test compounds and [^{3}H]ReN 1869 were added to test tubes in a final volume of 0.5 ml. Unless otherwise indicated, the concentration of the radioligand was 5 nM and non-specific binding was defined as the binding in the presence of 10 μM ReN 1869. Samples were incubated for 120 min at 37 °C in a shaking water bath. Free and bound radioactivity was separated by filtration over Whatman GF/F filters that were washed with 25 ml of ice-cold buffer (20 mM Tris–HCl, pH 7.4). Radioligand bound to filters accounted for 5–700 dpm that was subtracted before calculating specific binding.

[^{3}H]mepyramine was purchased from Amersham (UK) with a specific activity of 30 Ci/mmol. Reference compounds were obtained from Sigma (St. Louis, USA) or Research Biochemicals (Natick, USA). [^{3}H]mepyramine binding was performed essentially as described by Chang et al. (1979). Thawed membranes (1 mg protein/tube), buffer, test compounds and 5 nM [^{3}H]mepyramine were added to test tubes to a final volume of 1 ml and placed into a water bath (25 °C) for 60 min. The samples were then placed on an ice bath, filtered over Whatman GF/B filters and washed with 25 ml phosphate buffer (composition: 50 mM Na_2HPO_4/KH_2PO_4, pH 7.5 at 25 °C). Filters were soaked for 2 h in assay buffer supplemented with 0.5% polyethylenimine. Non-specific binding was defined as the binding in the presence of 20 μM of the histamine H_1 receptor antagonist tripolidine. Receptor binding experiments with [^{3}H]mepyramine (3 nM) to membranes from guinea pig brain was performed exactly as described by Hill et al. (1978).

2.1.3. Calcium mobilisation in CHO cells expressing human H_1 receptor

CHO cells expressing the human histamine H_1 receptor were dispersed by Ca^{2+}- and Mg^{2+}-free buffer and allowed to settle on uncoated cover slips in minimum essential medium (MEM) alpha (GibcoBRL) with 10% fetal calf serum and penicillin (10 000 IU/ml)/streptomycin (1000 μg/ml) added for a few hours. The cells were then loaded in the dark with the Ca^{2+}-sensitive fluorescent indicator Fura 2/AM (2.5 μM) in a Krebs/MOPS (morpholino propane sulfonic acid) buffer (in mM: KCl 3, KH_2PO_4 1.2, NaCl 122, $NaHCO_3$ 25, $MgSO_4$ 0.6, MOPS 10, $CaCl_2$ 2, glucose 5.6) for 3/4 h at room temperature. The cover slip was mounted on an upright microscope and the change in [Ca^{2+}] in individual cells (10–20 per cover slip) was measured at room temperature using 340 and 380 nm as excitation wavelengths (Polychromatic, TILL) and 510 nm as emission to a camera (SensiCam®) in combination with Imaging Workbench 2.1 software (Axon Instruments, Foster City, CA).

2.1.4. Patch clamp experiments

Dorsal root ganglion cells were isolated from 5- to 10-day-old Sprague–Dawley rats. After the animals had been decapitated, the vertebral column was quickly removed and opened in ice-cold Hanks' balanced salt solution (HBSS) (GibcoBRL). Ganglia were collected and incubated in a test tube containing HBSS with 2.5 mg/ml collagenase (type IV,

Sigma) for 13 min (37 °C, 95% atmospheric air, 5% CO_2), followed by 7 min with 5 mg/ml Trypsin (type IX, Sigma) in HBSS (37 °C, 95% air, 5% CO_2). The ganglia were washed three times at room temperature and dissociated by gentle tituration with a Pasteur pipette in culture medium consisting of MEM with Earle's salts solution (GibcoBRL) supplemented with 15% heat-inactivated fetal bovine serum (GibcoBRL), penicillin/streptomycin (100 U/ml/100 μg/ml) (GibcoBRL) and DNase 1 mg/ml (Sigma). The neurons were plated on poly-L-ornithine (250 mg/l)-coated cover slips in culture medium and kept at 37 °C (95% atmospheric air, 5% CO_2). Whole-cell patch clamp was performed 3–36 h after plating using an Axopatch 200A amplifier and pCLAMP 6.0.4 (Axon Instruments). Patch pipettes had a resistance of 2–4 MΩ. The data were filtered at 2 kHz and sampled at 4 kHz. The series resistance errors were 60–70% compensated for, linear leakage currents and capacitance transients were electronically compensated for using pCLAMP. Test compounds were applied using a fast superfusion system (DAD12, Adams and List, New York, USA). In experiments on voltage-dependent sodium and Ca^{2+} channels, a depolarizing pulse to +10 mV from holding potentials of −80 mV every 15 s evoked ion currents. The voltage dependence of steady-state inactivation was determined using a test pulse to 0 mV from varying prepulse potentials (−100, −60, −40, −20, −15, −10 mV) of 750-ms duration. In experiments with Ca^{2+} channels, the external solution (in mM) was: tetraethylammoniumCl 130, Glucose 10, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1.2, pH was adjusted to 7.3 with tetraethylammoniumOH. In experiments with sodium and capsaicin channels, the external solution (in mM) was: NaCl 140, KCl 4, Glucose 10, HEPES 10, $CaCl_2$ 2, $MgCl_2$ 1.2, pH adjusted to 7.3 with NaOH. The pipette solution in all cases was (in mM): CsCl 145, HEPES 10, $MgCl_2$ 2, EGTA 11. On the day of use, 1 mM MgATP and 0.3 mM Na_3GTP were added and pH adjusted to 7.2 with CsOH.

2.1.5. Release of calcitonin gene-related peptide (CGRP) from rat spinal cord in vitro

The procedure was essentially as described by Malcangio and Bowery (1993). Spinal cords from male Sprague–Dawley rats (300 g) were placed in ice-cold Krebs buffer (in mM: NaCl 118, KCl 4, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 11). Vibratome slices (thickness 400–500 μm, length 1 cm) from the dorsal part of segment L3–L5 with attached dorsal roots were obtained and placed in a perfusion chamber that allowed bilateral electrical stimulation of the roots. After perfusion with Krebs buffer at room temperature for 1 h, bovine serum albumin (0.1%) and bacitracin (20 μg/ml) were added to the buffer, and 8-ml fractions were collected in 0.1 M acetic acid before and after electrical stimulation of the roots (20 V, 0.5 ms, 10 Hz, 5–15 mA). Test compounds were added to the buffer 5 min before and during stimulation.

Samples were purified on C_{18} columns (Waters) and CGRP immunoreactivity measured using a scintillation proximity assay protein A bead technique (Amersham, Buckinghamshire, UK).

2.2. In vivo experiments

2.2.1. In vivo displacement of [^{3}H]mepyramine binding to mouse central nervous system (CNS)

In situ binding of [^{3}H]mepyramine was measured in awake male NMRI mice (25–35 g) by determination of the amount of bound [^{3}H]mepyramine in cerebellum and spinal cord (Quach et al., 1979). Test substances were given i.p. 55 min before 1 μCi [^{3}H]mepyramine (in 0.2 ml saline) was injected in a tail vein. Five minutes thereafter, the animals were decapitated and blood collected to obtain radioactivity levels in plasma. Spinal cord and cerebellum, respectively, were placed in tarred test tubes and weighed before 5 ml of ice-cold sodium phosphate buffer (50 mM, pH 7.4) was added and the tissue homogenized by Polytron during 10 s. Two aliquots of 2 ml were filtered under vacuum (Filters: GF/B, Whatman). The filters were washed four times with 10 ml cold sodium phosphate buffer and the particulate-bound radioactivity determined by liquid scintillation. The ability of the test substances to displace [^{3}H]mepyramine bound to CNS tissue was assessed by comparing the ratio of the radioactivity in tissue and in plasma in compound-treated and saline-treated mice.

2.2.2. Histamine induced paw edema in rats

The test was performed on 220–250 g Sprague–Dawley male rats in accordance with Amann et al. (1995). In brief, 50 μl of 0.5 μM histamine was injected into the pad of the right hind paw of pentobarbital (50 mg/kg i.p.)-anaesthetised rats. The difference in paw volume, determined by water plethysmography (Ugo Basile, Comerio, Italy) before and 20 min after the histamine injection, represents the edema. Test substances were administered i.p. 30 min before the histamine paw injection.

2.2.3. Carrageenan-induced paw edema in rats

Awake Sprague–Dawley male rats (110–130 g) were given an injection of 0.1 ml carrageenan (2% in saline) into the pad of the hind paw. Water plethysmography before and 1, 2, 3 and 4 h after the injection determined the paw volume.

2.2.4. Formalin-induced pain and paw edema in rats

Fifty microliters of formalin (1% in saline) was injected s.c. into the dorsal part of the right hind paw of female Sprague–Dawley rats (150–180 g) that subsequently were transferred to a transparent observation chamber with a heated 28 °C floor (Hole and Tjolsen, 1993). The behaviour of the animals was observed by a trained individual and scored for a period of 60 min (the number of episodes of flicking or licking the paw). The paw volume was measured by water plethysmography (Ugo Basile) before and 60 min after the formalin injection; the difference represents the paw edema. In some of the animals, mast cell histamine was depleted by

injection of compound 48/80 (50 mg/kg s.c.) daily, 2 days before the test. Test substances were given 30 min before the formalin injection.

2.2.5. C-fos expression in the spinal cord

To determine a possible activation of second-order neurons in the nociceptive pathway during the formalin test, c-*fos* expression was measured in the lumbar (L4–L5) part of the spinal cord following the procedure described by Ingvardsen et al. (1997). In brief, the animals were anaesthetised (pentobarbital, 50 mg/kg i.p.) 2 h after the formalin challenge and killed by transcardiac perfusion fixation with 4% formalin in phosphate buffer at pH 7. The brain and spinal cord were removed, and transverse vibratome sections (50 μm) were made from the lumbar region (from L2 to L5) and processed immunochemically for Fos protein, that is, the product of the c-*fos* gene. Since the majority of Fos-positive cells are found within L4–L5 after formalin injection (Presley et al., 1990), 10 sections from L4–L5 were randomly selected and the number of Fos-positive nuclear profiles was counted on the ipsilateral and contralateral sides. Two regions were defined: superficial dorsal horn (laminae I and II) and nucleus proprius (laminae III–VI).

2.2.6. Capsaicin-induced pain and paw edema in mice

Male NMRI mice (22–25 g) were given 20 μl capsaicin (0.01% in saline) into the pad of the right hind paw where after they were transferred individually into a transparent observation chamber in which the floor was maintained at 28 °C. The pain response was scored during a period of 60 min as the number of episodes of paw licking (Sakurada et al., 1992). The animals were then killed by cervical dislocation and the hind paws were excised at the tarsal joints. The paw edema was measured as the weight difference between the right injected paw and the left untouched paw. Test substances were administered i.p. (0.1 ml/10 g) 30 min or i.t. (10 μl) 5 min before capsaicin.

2.2.7. Neurogenic extravasation in rats

The method was essentially as described by Lembeck and Holzer (1979). In brief, female Sprague–Dawley rats (200–250 g) received guanethidine (20 mg/kg s.c.) the day before the experiments. Then the trachea and a jugular vein were cannulated under pentobarbital sodium anaesthesia (50 mg/kg i.p.) and the animals were placed on a heated table to maintain body temperature. The saphenous nerve on one side was surgically exposed, cut in the thigh, and the distal end placed on a bipolar platinum electrode. Evans blue (1% in saline, 1 ml/kg) was injected i.v. and 5 min later an intense electrical stimulus (10 Hz, 20 V, 1 ms) was given for 1 min. Ten minutes hereafter, the animals were killed by bleeding. The skin innervated by the saphenous nerve was removed, weighed and subsequently, the dye was extracted by formamide and quantitated by colorimetry. Some of the animals were pretreated with compound 48/80, as described above.

2.2.8. Phenylquinone (PQ) writhing

Female NMRI mice were injected with PQ (2 mg/kg i.p.). PQ was dissolved in DMSO and diluted (1:10) in saline containing 5% cremophore. Test substances were given 30 min before PQ. The numbers of writhings were counted in the period 5–10 min after PQ dosing.

2.2.9. Hot plate test

NMRI male mice (30–35 g) were placed on a 57 °C heated plate in a Plexiglas cylinder (20 cm in diameter). Time to the first jump and total number of jumps within 60 s of observation were noted. Test substances were given i.v. 30 min before the test.

2.2.10. Data analysis

The results are as a rule presented as mean ± S.E.M. Saturation binding experiments were analysed using an iterative curve-fitting program (EBDA program from G.A. McPherson, Elsevier-Biosoft, UK) to calculate K_d, B_{max} and Hill coefficients. IC_{50} values were calculated from competition binding experiments by a non-linear regression analysis (GraphPad Software, USA). Inhibitory constants (K_i) were calculated from the IC_{50} values using the Cheng–Prusoff equation: $K_i = IC_{50}/(1+[L]/K_d)$. Student's two-tailed *t*-test was used to verify significance between two means. Analysis of variance (ANOVA) was followed by Dunnett's test for multiple comparisons (InStat, GraphPad Software, USA). $P < 0.05$ was considered significant.

2.2.11. Ethics

The animal care and use was made in accordance with the guidelines of the European Community and approved by the National Board for Laboratory Animal Research, "Dyreforsøgstilsynet", Denmark.

3. Results

3.1. In vitro

3.1.1. Selectivity of ReN 1869

ReN 1869 was profiled for activity at 10 μM at various receptors, transporters, enzymes and ion channels at Panlabs (Bothell, WA, USA) in their standard DiscoveryScreen® and ImmunoScreen® test procedure according to the protocols described by the company. ReN 1869 only demonstrated affinity to the histamine H_1 receptor (guinea pig brain, [^{3}H]pyrilamine) with a K_i of 0.19 ± 0.04 μM and the non-selective σ site [guinea pig brain, [^{3}H]1,3-di-tolylguanidine (DTG)] with a K_i of 0.45 μM. Otherwise, no significant affinity was demonstrated. Below are only mentioned the scrutinized receptors and systems of special interest for inflammation and pain. DiscoveryScreen®: adenosine receptors (A_1–A_2), α_1-, α_2-, β_1- and β_2-adrenoceptors, norepinephrine uptake, angiotensin (AT_2), bradykinin (B_2), cholecystokinin (CCK_A and CCK_B), galanin (binding of

[^{125}I]galanin to membranes from whole rat brain), interleukin 1α, tumor necrosis factor α, histamine (H_1 and H_3), muscarinic (M_1–M_2), tachykinin (NK_1), neuropeptide Y (binding of [^{3}H]neuropeptide Y to rabbit kidney medulla), serotonin (5-HT_{1A}, 5-HT_3), vasoactive intestinal peptide, Ca^{2+} channel (L-type), voltage-dependent K^+ channels or Na^+ and Cl channels. Immunoscreen®: arachidonic acid pathway, for example, cyclooxygenase (COX_1 and COX_2), and glucocorticoid receptor. ReN 1869 did not affect binding to glutamate receptors (NMDA, AMPA, kainate and mGlu1 receptor) and did not inhibit transporters for GABA (mouse GAT-1 through GAT-4), glutamate, dopamine, noradrenaline and 5HT (data not shown).

3.1.2. Receptor-binding studies with [^{3}H]ReN 1869

[^{3}H]ReN 1869 showed specific binding to membrane fractions from several rat organs, in particular liver and CNS tissues. In membranes from rat spinal cord, 85–90% specific binding of [^{3}H]ReN 1869 was observed using optimised conditions with an incubation time of 120 min at 37 °C and pH 6.0. No specific binding was observed after the tissue was kept at 95 °C for 30 min (data not shown). The affinity of [^{3}H]ReN 1869 binding was determined by saturation binding experiments followed by Scatchard analysis. For the spinal cord: $K_D = 11 \pm 3$ nM and $B_{max} = 122 \pm 17$ fmol/mg ($n = 6$) (Fig. 2A); for the liver: $K_D = 30 \pm 9$ nM and $B_{max} = 556 \pm 55$ fmol/mg ($n = 3$). In both organs, the Hill coefficient was close to 1. The affinity of [^{3}H]ReN 1869 for histamine H_1 receptors was confirmed by the binding to membranes from CHO cells expressing the human histamine H_1 receptor in a saturable and monophasic manner with a $K_D = 8$ nM and $B_{max} = 1000$ fmol/mg protein, and with a Hill coefficient of approximately 1 (Fig. 2B). CHO cells devoid of the histamine H_1 receptor showed no specific binding (results not shown).

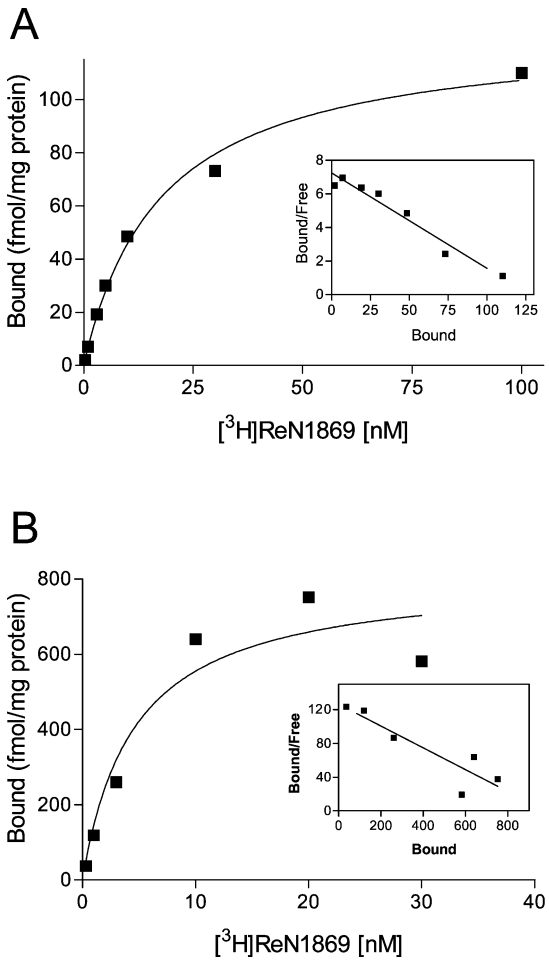

Fig. 2. Saturation binding experiments with [^{3}H]ReN 1869 to membranes prepared from (A) rat spinal cord and (B) CHO cells expressing the human histamine H_1 receptor. Inserts show Scatchard transformations of the saturation curves. The experiments were performed in triplicate as described in Materials and Methods. Non-specific binding was defined as the binding in the presence of 10 μM ReN 1869. No specific binding was observed to membranes prepared from CHO cells expressing vector only (data not shown).

3.1.3. Pharmacological characterisation of [^{3}H]ReN 1869 binding

Numerous selective ligands for various receptors were unable to displace the binding of [^{3}H]ReN 1869 from rat spinal cord membranes; among these, compounds with affinity for opioid, adrenergic, serotonergic, muscarinic, vanilloid, GABA, glutamate, neurokinin receptors or the histamine H_2 receptor. Compounds that were able to dose-dependently displace [^{3}H]ReN 1869 from rat spinal cord membranes included histamine H_1 receptor antagonists, σ receptor ligands and tricyclic antidepressants (Table 1). Full displacement curves were performed for these active compounds. Mepyramine displaced [^{3}H]ReN 1869 binding to rat spinal cord membranes in a monophasic manner with moderate affinity (Table 1, Fig. 3), while several other histamine H_1 antagonists exhibited a biphasic displacement with a high-affinity site (nanomolar range) corresponding to about one third of the specific binding, and a low-affinity site in the micromolar range (Fig. 3, Table 1). The σ receptor ligands, *N*-allylnormetazocine (SKF 10047) and DTG, both of which have negligible affinities for histamine H_1 receptors, displaced [^{3}H]ReN 1869 binding with moderate affinities. The dopamine D_3 receptor agonist, 7-hydroxydipropyl-aminotetralin (7-OH-DPAT), displaced [^{3}H]ReN 1869 probably due to its nanomolar affinity for σ receptors and micromolar affinity for histamine H_1 receptors (Schoemaker, 1993) (Table 1). Tricyclic antidepressants were also able to displace binding of [^{3}H]ReN 1869 to rat spinal cord membranes (Fig. 3, Table 1) most likely due to their high histamine H_1 receptor affinity (Hall and Ogren, 1981).

3.1.4. Species differences

The characterisation of [^{3}H]ReN 1869 binding to rat spinal cord membranes suggested that the compound is a high-affinity histamine H_1 receptor ligand/moderate affinity σ receptor ligand. Possible species differences were examined because ReN 1869 initially showed a relatively low affinity for the histamine H_1 receptor (guinea pig brain) at Pan-

Table 1
Profile of [^{3}H]ReN 1869 binding to rat spinal cord membranes

Compound	Receptor affinity (K_i)	K_i (1.site) [nM]	Percentage in 1.site affinity state	K_i (2.site) [nM]
Mepyramine[a]	H_1 (4.5 nM)	172 ± 11	100	
Astemizole[a]	H_1 (5.2 nM)	1.1–2.9	26–31	2422–2465
Diphenhydramine[a]	H_1 (17 nM)	36–264	31–33	4318–
Triprolidine[a]	H_1 (5.6 nM)	4.2–9.6	36– 39	5077–9215
Promethazine[a]	H_1 (2.9 nM)	2–22	35–100	53–
Thioperamide[b]	H_3 (σ)	62–91	68–74	
DTG[c]	σ (32 nM), H_1>10 μM	1950	100	
SKF 10047[c]	σ (115 nM), H_1>10 μM	103	45	15474
7-OH-DPAT[c]	σ (48 nM), D_3/H_1 = 7.9 μM	58–185	62–71	5471–
(*S*)-sulpiride[d]	D_2/D_3, no σ affinity	–	–	>10000
Amitriptyline[e]	H_1 (4.1 nM)	0.6–0.6	24–37	88–197
Chlorpromazine[e]	H_1 (36 nM); σ (180 nM)	26	70	426
Clomipramine[e]	H_1 (31 nM); σ (546 nM)	75–76	100–100	
Doxepin[e]	H_1 (0.7 nM)	1.24 ± 0.34	43 ± 3	1846 ± 197
Mianserin[e]	H_1 (0.9 nM)	1.89 ± 0.99	37 ± 2	6570 ± 3018

For details, see Materials and Methods. Means ± S.E.M. (n = 1–5). Sigma receptor affinities are from Tam and Cook (1984), Wu et al. (1991), Shirayama et al. (1993), Schoemaker (1993) and Zabetian et al. (1994). Histamine H_1 receptor affinities from Chang et al. (1979), Hall and Ogren (1981), Schoemaker (1993) and Ter Laak et al. (1993).

[a] Displacement of specific [^{3}H]ReN 1869 binding to membrane preparation from rat spinal cord by various compounds including reference histamine H_1 receptor antagonists.

[b] Histamine H_3 receptor antagonists.

[c] σ receptor agonists.

[d] Dopamine receptor antagonist.

[e] Tricyclic antidepressants.

labs (K_i = 190 nM). This result was confirmed for the guinea pig brain histamine H_1 receptor using [^{3}H]mepyramine (Table 2); however, large species differences of the ability of ReN 1869 to displace [^{3}H]mepyramine/[^{3}H]ReN 1869 binding were observed (Table 2).

3.1.5. Histamine-induced Ca^{2+} mobilisation in CHO cells expressing human histamine H_1 receptors

Exposure of CHO cells, expressing human histamine H_1 receptors, to histamine leads to an increase in the intracellular Ca^{2+} activity via an activation of phospholipase C (Smit et al., 1996). In our study, histamine caused a concentration-dependent increase in intracellular Ca^{2+} activity with an ED_{50} of 39 ± 24 nM and a maximal effect at 350 nM. Comparable, repetitive increases of $[Ca^+]_i$ were provoked by 30-s exposures to 300 nM histamine repeated every 5 min. Mepyramine or ReN 1869 dose-dependently reduced the responses (Fig 4). IC_{50} for mepyramine was 0.011 ± 0.0004 μM while ReN 1869 was approximately 100-fold less potent with an IC_{50} of 1.70 ± 0.002 μM.

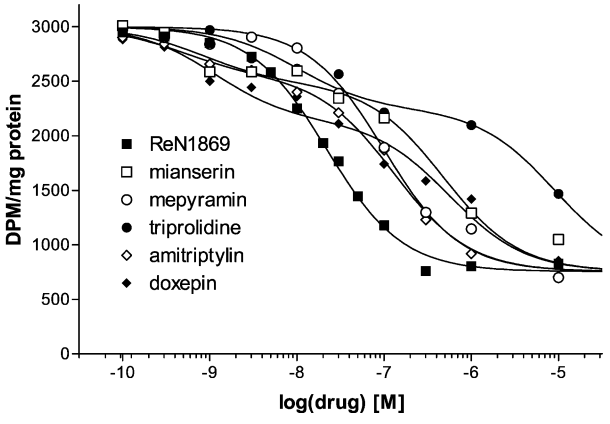

Fig. 3. Displacement of specific [^{3}H]ReN 1869 binding to rat spinal cord membranes by histamine H_1 receptor antagonists and tricyclic antidepressants. Data from ReN 1869 and mepyramine could be fitted to a one-site while the rest to a two-site competition-binding model using the GraphPad Prism program. For further details, see text and Table 1.

Table 2
Species differences between specific [^{3}H]ReN 1869 or [^{3}H]mepyramine binding

	ReN 1869 K_1 (nM)	Mepyramine K_1 (nM)
[^{3}H]mepyramine binding to guinea pig brain	189 ± 14	1.2 ± 0.2
[^{3}H]mepyramine binding to rat spinal cord	68 ± 14	8.5 ± 1.3
[^{3}H]mepyramine binding to human H_1 clone	11	0.6
[^{3}H]ReN 1869 binding to guinea pig brain	69	4660
[^{3}H]ReN 1869 binding to rat spinal cord	26.3 ± 1.5	172 ± 11
[^{3}H]ReN 1869 binding to human H_1 clone	4.7–9.3	13.4–47

Displacement studies of specific [^{3}H]ReN 1869 or [^{3}H]mepyramine binding by ReN 1869 or mepyramine were performed on membranes from guinea pig forebrain, rat spinal cord and CHO cells expressing the human histamine H_1 receptor. Means ± S.E.M. (n = 1–4).

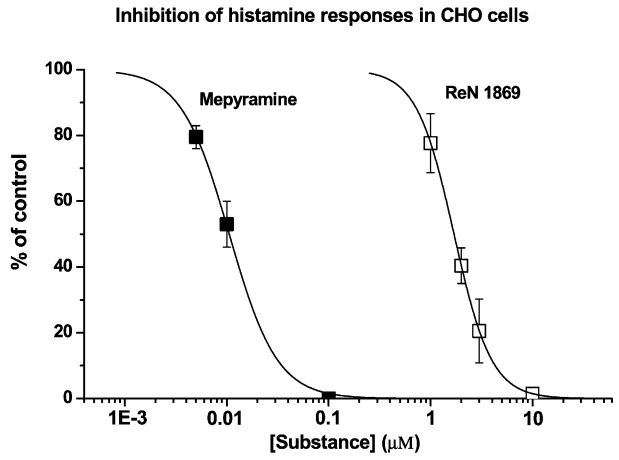

Fig. 4. Intracellular [Ca^{2+}] was determined by the Fura-2 technique. Ca^{2+} levels in CHO cells expressing the human histamine H_1 receptor were increased by exposure to 300 nM histamine. Mepyramine blocked the response with an IC_{50} of 0.01 μM while ReN 1869 exhibited an IC_{50} of 1.7 μM. Means ± S.E.M. for trials ($n = 2–6$) each comprising of 10–20 cells.

3.1.6. Voltage-dependent currents

Calcium currents were elicited by depolarising pulses every 15 s before, during and after exposure to ReN 1869. The current was almost completely (95%) inhibited by Cd^{+} (100 μM); two thirds of the Ca^{2+} current in these cells is carried by N-type channels (blocked by ω-conotoxin GVIA, 1 μM) and 20% by of L-type channels (nimodipine, 5 μM). Concentrations up to 100 μM ReN 1869 had no significant inhibitory effects. Voltage-dependent steady-state inactivation was not significantly changed during exposure to ReN 1869 (results not shown). Likewise, the sodium currents, which are mainly carried by the tetrodotoxin-insensitive type, were unaffected by the ReN 1869 (results not shown). In approximately 75% of the cells, capsaicin (0.3 μM) induced an inward current which gradually diminished after repeated dosages due to tachyphylaxis (Docherty et al., 1996). Pretreatment of the cells for 5 min with 10 μM ReN 1869 and the administration of capsaicin together with ReN 1869 had no influence on the magnitude of the current (results not shown).

3.1.7. CGRP release from dorsal spinal cord

Slices of rat dorsal spinal cord (L3–L5) containing the dorsal roots were constantly perfused with oxygenated Krebs buffer. Fractions of the outflow, representing periods of 8 min, were collected and the CGRP content measured. The average CGRP level of the first three fractions represented the basal release. ReN 1869 was added to the perfusion fluid in the third period and did not influence the basal CGRP release. The electrical stimulation during the fourth period typically induced a 3- to 5-fold increase that reached normal levels after the two next periods. ReN 1869 dose-dependently reduced this release. During stimulation, the CGRP release was (in percentage of basal level): control: 389 ± 63%; ReN 1869: 0.01 μM 298 ± 50%, 0.1 μM 183 ± 40% ($P < 0.05$), 1.0 μM 147 ± 34% ($P < 0.01$).

3.2. In vivo

3.2.1. In vivo displacement of [^{3}H]mepyramine binding to mouse CNS

The in vivo binding of [^{3}H]mepyramine to mouse spinal cord and cerebellar histamine H_1 receptors was dose-dependently inhibited by ReN 1869 and mepyramine (Fig. 5). The amount of radioactivity in the group that received mepyramine (10 mg/kg) was considered as non-specific binding. Hence, the magnitude of specific binding was for the spinal cord: mepyramine (1 mg/kg) 35.5%, (10 mg/kg) 0%; ReN 1869 (1 mg/kg) 32.5%, (10 mg/kg) 11% and for the cerebellum: mepyramine (1 mg/kg) 19.3%, (10 mg/kg) 0%; ReN 1869 (1 mg/kg) 12.5%, (10 mg/kg) 13%.

3.2.2. Histamine-induced paw edema

ReN 1869 (in doses as low as 10 μg/kg i.p.) significantly inhibited the histamine-evoked paw edema (Fig. 6). The ED_{50} was approximately 300 μg/kg. Interestingly, even a high dose of mepyramine (10 mg/kg) was unable to inhibit significantly this type of edema (0.29 ± 0.06 versus 0.34 ± 0.05 in controls, $n = 7$).

3.2.3. Carrageenan-evoked rat paw edema

ReN 1869 (1 mg/kg s.c.) was administered 30 min before paw injection with carrageenan and had no effect on the development of the paw edema. Dexamethasone (1 mg/kg s.c.) was given 1 h before carrageenan and expectedly diminished the edema. This effect was not affected by the simultaneous administration of 1 mg/kg ReN 1869 (Fig. 7).

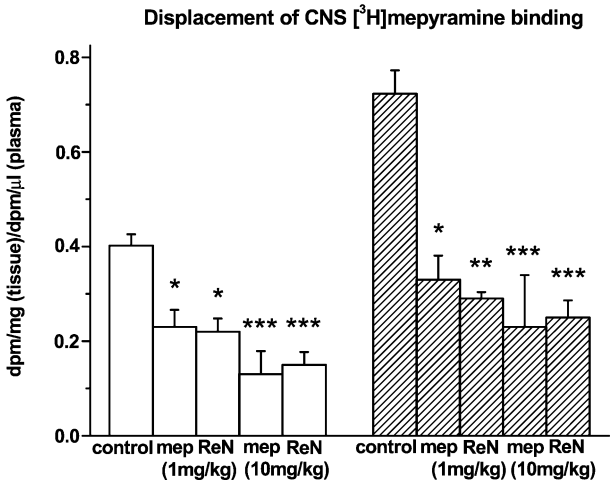

Fig. 5. Inhibition of in vivo [^{3}H]mepyramine binding to mouse spinal cord (open bars) and cerebellum (hatched bars) by i.p. administration of mepyramine (mep) or ReN 1869 (ReN). For details, see text. Means ± S.E.M. ($n = 17$ for controls, other groups $n = 3–4$). Asterisks indicate significance compared to untreated mice at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

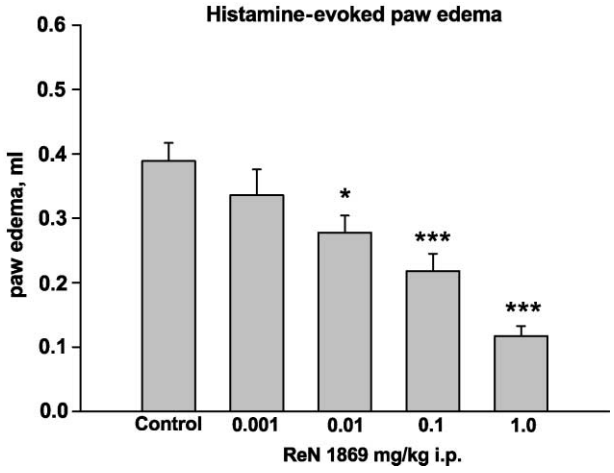

Fig. 6. Swelling of rat hind paw following histamine injection into paw pad. ReN 1869 was administered 30 min before the histamine injection and the edema was evaluated 30 min thereafter. Means ± S.E.M. ($n = 6–9$). Asterisks indicate significance compared to controls at * $P < 0.05$ and *** $P < 0.001$.

3.2.4. Formalin test

The injection of 1% formalin into the paw of conscious rats induced a characteristic biphasic pain response with flicking and licking of the affected paw: an early phase of 5–10 min duration followed by a quiescent period of additional 5–10 min before a more sustained (tonic or late) phase of approximately 30 min duration evolved. ReN 1869 significantly inhibited both phases. The maximal inhibition of the early phase was 40% at 0.1 mg/kg, while a more pronounced dose dependency was seen in the late phase with 50% inhibition after 0.1 mg/kg (Fig. 8). Pretreatment with nalox-

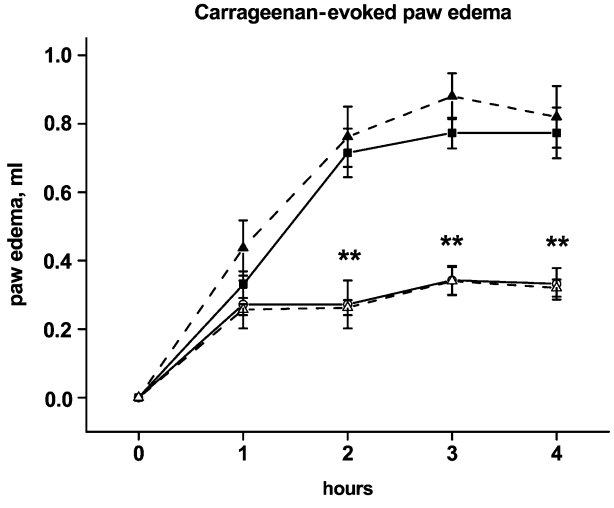

Fig. 7. Development of rat paw edema following paw injection of carrageenan. Controls are shown by (■) and the rats given ReN 1869 (1 mg/kg s.c.) by (▲). The edema was significantly counteracted by dexamethasone (1 mg/kg s.c.) (○). No further effect was observed with a combination of dexamethasone and ReN 1869 (△). Means ± S.E.M. ($n = 5$). Asterisks indicate significance compared to 0 h at ** $P < 0.01$.

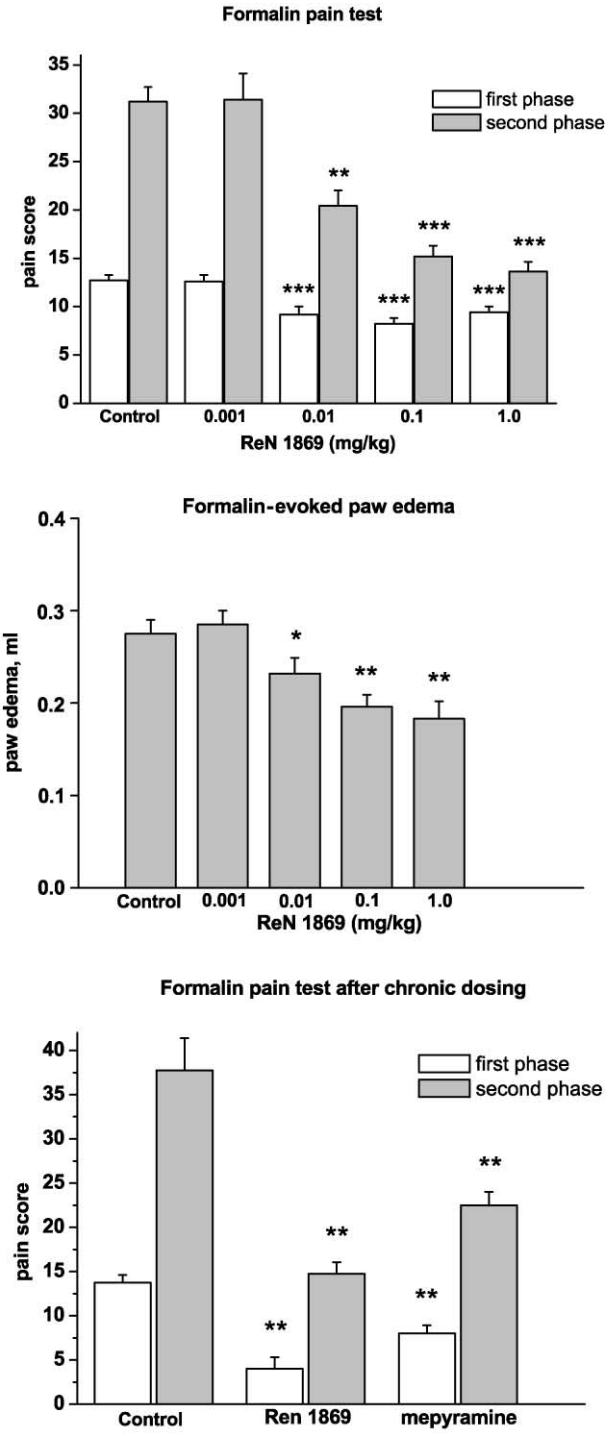

Fig. 8. Upper panel shows the pain behaviour in rats during the two phases following injection of formalin (1%) in the dorsal part of the paw with or without prior (− 30 min) administration of ReN 1869. Middle panel shows the paw edema in these rats and the effect of ReN 1869. Lower panel shows that no tolerance developed in the formalin pain test in rats after 5 days of continuous administration of ReN 1869 or mepyramine, respectively. Compounds were administered via the drinking water (2–3 mg/kg/day). Means ± S.E.M. ($n = 4–7$). Asterisks indicate significance compared to controls at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Table 3
The effect of ReN 1869 and compound 48/80 in the rat formalin test

	Early-phase pain (score)	Late-phase pain (score)	Paw edema (ml)
Control	12.4 ± 0.50	28.0 ± 1.4	0.26 ± 0.01
ReN 1869	7.0 ± 0.67[a]	13.0 ± 0.83[a]	0.18 ± 0.02[a]
Compound 48/80	6.3 ± 0.53[a]	13.8 ± 1.3[a]	0.16 ± 0.01[a]
Compound 48/80 + ReN 1869	6.4 ± 0.64[a]	12.3 ± 0.84[a]	0.19 ± 0.02[b]

The formalin test was performed on female Sprague–Dawley rats as described in Materials and Methods. ReN 1869 (0.1 mg/kg i.p.) was given 30 min before formalin. Compound 48/80 was administered daily (50 mg/kg s.c.) for 2 days before the experiment.

[a] Indicates significance compared to controls at $P<0.05$.

[b] $P=0.07$. Means ± S.E.M. ($n=8$).

one (0.3 mg/kg i.p.) did not influence the antinociceptive effect of ReN 1869 (results not shown).

The effects of ReN1869 and mepyramine were compared in groups consisting of four rats. Mepyramine (1 mg/kg s.c.) reduced the pain behaviour in the early phase marginally but only significantly ($P<0.01$) in the late phase. ReN 1869 (0.1 mg/kg i.p.) showed more pronounced effects in both phases ($P<0.01$) comparable to those shown in Fig. 8. As expected, morphine (3 mg/kg i.v., $n=5$) markedly decreased the pain behaviour; the early-phase pain by 75% ($P<0.001$) and the late phase by 65% ($P<0.05$). These rats were also scrutinized for c-*fos* expression in the lumbar spinal cord (see below).

Development of tolerance was assessed in rats that were dosed with ReN 1869 (or mepyramine) for 5 days via the drinking water (equivalent to 2–3 mg/kg/day). The formalin pain test performed on the fifth day revealed that ReN 1869 and mepyramine still retained their analgesic capabilities (Fig. 8).

The formalin-evoked paw edema was also inhibited by ReN 1869 dose dependently, and a 30% inhibition was achieved at 1.0 mg/kg (Fig. 8). A comparable inhibition of the pain behaviour and the paw edema was observed in animals in which mast cells had been degranulated following administration of compound 48/80 for 2 days before the experiment. In these animals, there was no additional anti-edema effect of ReN 1869 (Table 3).

3.2.5. C-fos expression

The number of Fos-positive nuclear profiles was significantly increased in the dorsal horn of L4–L5 ipsilateral to the

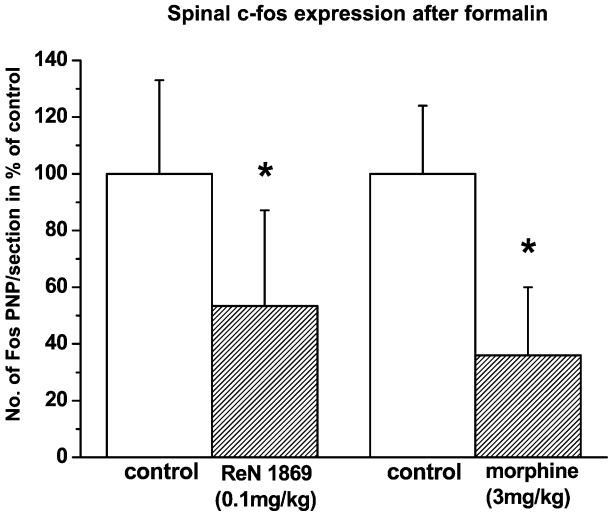

Fig. 9. The influence of ReN 1869 and morphine administered 30 min before injection of formalin in the hind paw on the number of Fos-positive nuclear profiles (FosPNP) in rat lumbar spinal cord laminae I, II. The four bars represent: control $n=7$, ReN 1869 $n=4$, and control $n=4$ and morphine $n=5$. Fos-positive nuclear profiles are expressed relative to untreated controls in which the number of Fos-positive nuclear profiles (ipsilateral–contralateral side) varied between 100 and 200 per section. Means ± S.D. Asterisks indicate significance compared to control at * $P<0.05$ (unpaired *t*-test).

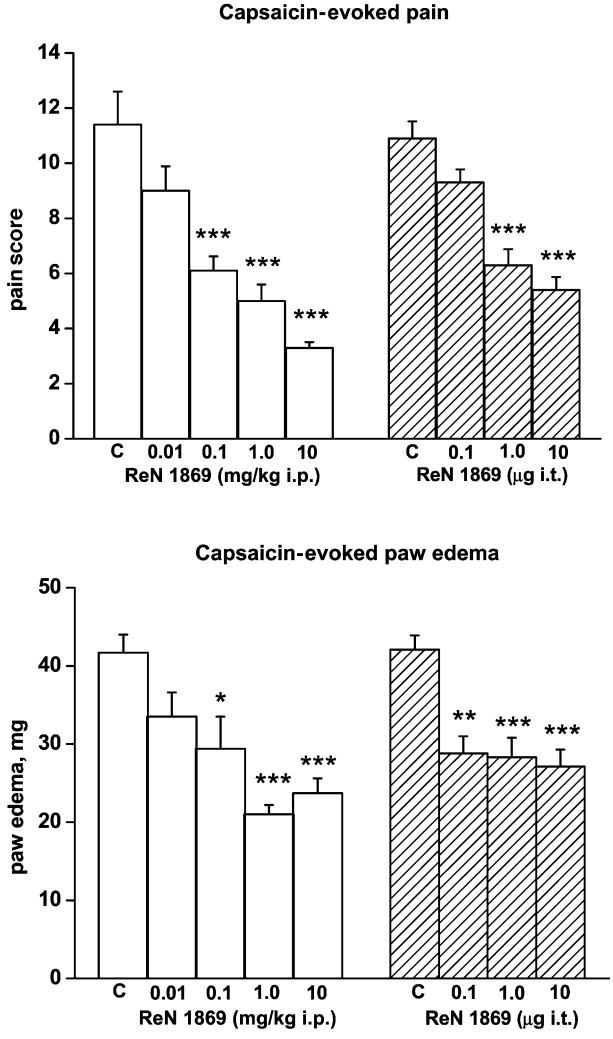

Fig. 10. Upper panel demonstrates a dose-dependent effect of ReN 1869 on the pain behaviour in mice elicited by the injection of 20 μl of 0.01% capsaicin in saline into the pad of a hind paw. Lower panel shows that ReN 1869 dose dependently attenuates the capsaicin-evoked paw edema even when the compound is given i.t. Means ± S.E.M. ($n=4–7$). Asterisks indicate significance compared to untreated mice at * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

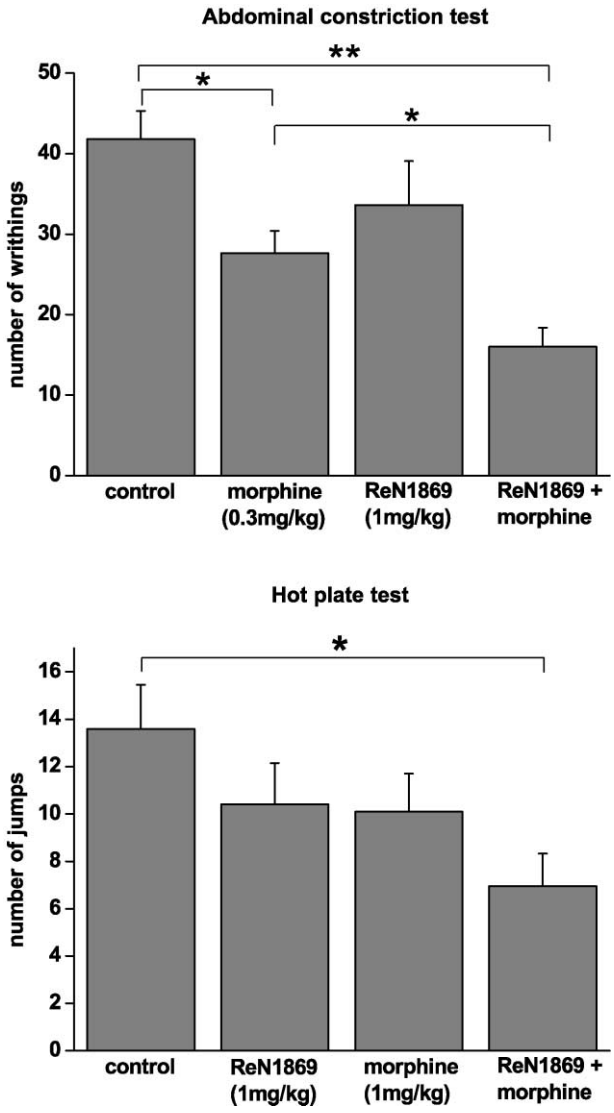

Fig. 11. Effect of ReN 1869 and morphine on abdominal constrictions elicited by i.p. administration of phenylquinone (upper panel) or on the "hot plate test" (57 °C) (lower panel). In the phenylquinone writhing test, morphine, but not ReN 1869 had significant effects but the combination of both compounds caused an augmented effect. A similar phenomenon was observed in the hot plate test. Means ± S.E.M. ($n = 4-7$). Asterisks indicate significance at * $P < 0.05$ and ** $P < 0.01$.

formalin-injected paw in all animals. The majority of the Fos-positive nuclear profiles were observed medially in laminae I and II. By contrast, the Fos response in laminae III–VI was distributed uniformly across the spinal cord gray matter. Morphine (3 mg/kg i.p., $n = 5$) reduced the number of Fos-positive nuclear profiles in laminae I and II by 64% (Fig. 9) and in laminae III–VI by 66% as compared to a control group of four rats. ReN 1869 (0.1 mg/kg i.p., $n = 4$) also decreased the number of Fos-positive nuclear profiles in laminae I and II significantly (47%) in relation to the control group ($n = 7$) (Fig. 9), while the 30% reduction in laminae III–VI was insignificant. Results from laminae III–VI are not shown.

3.2.6. Capsaicin test

The pain behaviour elicited by the injection of capsaicin into the mouse paw was inhibited dose dependently by ReN 1869 administered i.p. or i.t. (Fig. 10). The ED_{50} for the i.p. route was 0.3 mg/kg and for the i.t. route 10 μg. ReN 1869 attenuated the ensuing development of the paw edema measured 15 min after capsaicin injection. Interestingly, ReN 1869 also inhibited the paw edema when given i.t. (Fig. 10). The effects of ReN 1869 and mepyramine were compared in groups consisting of four mice. Administration of ReN 1869 (1 and 10 μg i.t.) 5 min before the capsaicin challenge reduced pain behaviour by 25% and 54%, respectively ($P < 0.05$) which is comparable to the results obtained after i.p. administration, whereas mepyramine (1 and 10 μg i.t.) insignificantly decreased the pain behaviour by 10% and 19%, respectively.

3.2.7. Phenylquinone writhing

The Panlabs profile suggested an analgesic potential of ReN 1869 by demonstrating that the compound inhibited phenylquinone-induced writhings in mice by 77% at 100 mg/kg p.o. It should be noted that the profiling showed no toxicity in mice after 100 mg/kg i.p. or 300 mg/kg p.o.

No effect on phenylquinone-induced writhings was seen at lower doses: control: 40 ± 4 (number of writhings/5 min); ReN 1869: 0.1mg/kg: 43 ± 5, 1.0mg/kg: 46 ± 5, 10 mg/kg: 40 ± 3. When morphine (0.3 mg/kg s.c.), which by itself only had a small effect, was combined with ReN 1869 (1 mg/kg i.v.), the response of either single drug was potentiated (Fig. 11).

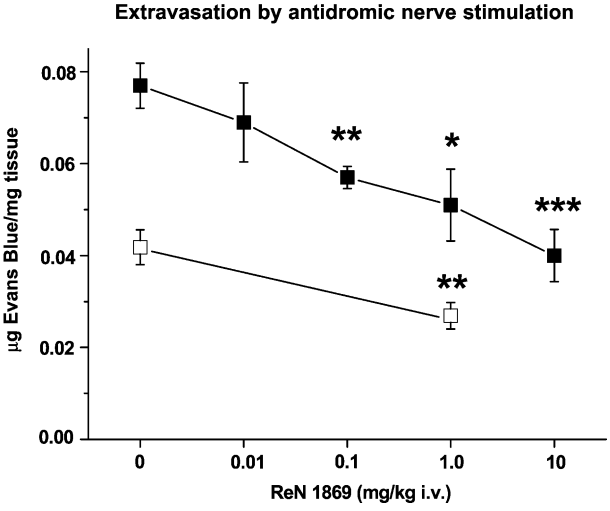

Fig. 12. The effect of ReN 1869 on the extravasation of Evans blue in rat skin following electrical stimulation of the exposed saphenous nerve in normal rats (closed symbols) or in rats pretreated with compound 48/80 (open symbols). Means ± S.E.M. ($n = 5-7$). The asterisks indicate significant differences compared to rats given no compound at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Both values from the rats given compound 48/80 are significantly different from normal rats ($P < 0.001$).

3.2.8. Tail flick and hot plate test

The test substance did not produce analgesia in these models of acute pain. ReN 1869 (30 mg/kg i.p.) was without effect in the tail flick test (performed at Panlabs) or in the hot plate test at 10 mg/kg (results not shown). But when a low dose of ReN 1869 (1 mg/kg i.v.) was combined with a low dose of morphine (1 mg/kg s.c.), there was a significant effect (Fig. 11).

3.2.9. Neurogenic extravasation

Fig. 12 shows that ReN 1869 dose-dependently inhibited the cutaneous protein extravasation induced by antidromic stimulation of the saphenous nerve. The amount of Evans blue dye was reduced by approximately 40% at 10 mg/kg i.v. In animals pre-treated with compound 48/80, the dye extravasation associated with electrical stimulation was reduced by 33%. In this situation, ReN 1869 (1 mg/kg i.v.) was still able to inhibit the residual response by additional 30% (Fig. 12).

4. Discussion

The novel tricyclic compound, ReN 1869, shows strong affinity and selectivity to histamine H_1 receptors and exhibits pronounced antinociceptive and anti-inflammatory effects. These properties are traditionally not linked to the pharmacology of H_1 receptor antagonists (meaning non-selective or selective histamine H_1 receptor antagonists) although there are several reports that support this notion (e.g. Raffa, 2001). Below, we show that the reported analgesic and anti-inflammatory effects of ReN 1869 are due mainly to histamine H_1 receptor antagonism.

4.1. Receptor binding

The receptor characterisation revealed that ReN 1869 had no other significant binding sites than the histamine H_1 and σ receptors. There were, however, large species differences of the affinities of mepyramine or ReN 1869 as determined by homologous or heterologous binding (Table 2). This does not imply that ReN 1869 and mepyramine bind to different receptors. Displacement binding studies with the cloned tachykinin NK_1 receptors also showed large differences in the potency of compounds. The potency was dependent on the particular radioligand although these radioligands apparently label the same site at the receptor (Hastrup and Schwartz, 1996). The difference in affinity of ReN 1869 and mepyramine ranged up to 100-fold in guinea pig brain membranes, whereas the two drugs had comparable, high affinities to the human histamine H_1 receptor (Table 2). The fact that [^{3}H]ReN 1869, like [^{3}H]mepyramine (Smit et al., 1996) exhibited specific and high-affinity binding to CHO cells expressing the human histamine H_1 receptor, but not to native cells, is a strong evidence for a potent histamine H_1 receptor ligand. The observation that ReN 1869 had a much lower affinity than mepyramine in the functional test involving the human histamine H_1 receptor (Fig. 4) may reflect different allosteric interactions of the antagonists with the histamine H_1 receptor molecule.

The high H_1 affinity of ReN 1869 (and low affinity to the σ site) was corroborated by the ability of selective compounds to displace [^{3}H]ReN 1869 binding (Table 1). The biphasic displacement curves generated by some histamine H_1 receptor antagonists and tricyclic antidepressants (Fig. 3) imply that ReN 1869 has two binding sites. Biphasic displacement curves were shown for the binding of [^{3}H]doxepin to rodent CNS in a study by Tran et al. (1981) who reported the high-affinity site to be the histamine H_1 receptor, whereas the nature of the low affinity was unknown. The values in Table 1 suggest that binding of ReN 1869 to rat spinal cord comprises a high-affinity H_1 site and possibly a low-affinity σ site.

4.2. The role of histamine in pain and inflammation

Although the σ receptor ligand DTG has been shown to be antinociceptive (Kest et al., 1995a), it augments formalin-induced pain behaviour (Kest et al., 1995b). We therefore focus our discussion on the histamine H_1 receptor and its natural agonist, histamine.

Histamine is able to excite a subpopulation of sensory neurons, that is, mechanoinsensitive C-fibers (Schmelz et al., 1997) and polymodal nociceptors, as well as potentiate their response to other stimuli (e.g. heat) (Koda et al., 1996). This action is mediated via the histamine H_1 receptors which normally are present on 5–10% of small sensory neurons (Ninkovic et al., 1982; Kashiba et al., 1999). The main sensation induced by an application of histamine to the skin is itch (Simone et al., 1991), but pain may also be elicited in the diseased state (Birklein et al., 1997) or when high doses are used (Juan and Lembeck, 1974).

It is established that C-fibers, besides conveying pain sensations, also have an efferent role that serves regulatory and trophic functions (Maggi and Meli, 1988). The fibers are pivotal for neurogenic inflammation in which vasodilatation and plasma extravasation is elicited by vasoactive neuropeptides (e.g. substance P and CGRP) released from activated nerve endings (Jancsó et al., 1967; Lembeck and Holzer, 1979). Histamine may have a role in this inflammatory response since it stimulates some of the C-fibers and increases capillary permeability via histamine H_1 receptors (Owen et al., 1984).

In the periphery, mast cells are the main source of histamine and various other mediators (e.g. 5-HT and NGF) that excite neurons and increase capillary permeability (Leon et al., 1994). Vice versa, the neurons can release substances (e.g. SP) that provoke mast cell exocytosis (Fewtrell et al., 1982) and increase capillary permeability allowing the entry of other algogenic substances (e.g. bradykinin). Thus, histamine and histamine H_1 receptors are part of feedback systems between nerves, mast cells and capillaries, which constitute the basis for neurogenic inflammation as well as neuronal sensitisation that is important for pain conditions.

Centrally, histamine H_1 receptors are located in regions which suggest a role in nociception, for example, mesensephalic periaqueductal gray matter and dorsal spinal cord CNS (Palacios et al., 1981). The location in the spinal cord, where most of the C-fibers make their first synapse, is of special interest in this connection. Histamine H_1 receptors are concentrated in layers I and II in the spinal dorsal horn (Ninkovic et al., 1982), mostly on primary afferents (Ninkovic and Hunt, 1985). Furthermore, nociceptive neurons in the dorsal horn of the spinal cord respond to an intradermal injection of histamine (Jinks and Carstens, 2000).

4.3. Antinociceptive effects of ReN 1869

The observed antinociceptive effect of ReN 1869 or mepyramine in the formalin test was not as prominent as that of morphine but it is comparable to that induced by, for example, the cyclooxygenase inhibitors (Malmberg and Yaksh, 1992). Importantly, prolonged administration of ReN 1869 was not associated with the development of tolerance (Fig. 8). The involvement of histamine H_1 receptors in this test is shown by the comparable effect of mepyramine and ReN 1869. Furthermore, Mobarakeh et al. (2000) showed in knockout mice, that the lack of histamine H_1 receptors caused a 34% and 46% inhibition of the early- and late-phase pain responses, respectively, which is comparable to the maximal effect of ReN 1869 and the results obtained with compound 48/80-pretreated rats (Table 3). The latter suggests that mast cell histamine plays a role. Finally, Parada et al. (2001) recently showed that co-administration of formalin and mepyramine (or another histamine H_1 receptor antagonist, meclizine) in the rat paw significantly and dose-dependently attenuated the pain response of both phases without affecting heat-elicited pain or mechanical hyperalgesia ruling out possible local anaesthetic effects.

No significant effect of ReN 1869 (< 10 mg/kg) was seen in models of acute pain like the hot plate test and PQ writhing. However, ReN 1869 was able to amplify significantly the effect of a subliminal dose of morphine (Fig. 11). This potentiation of the effect of opioids has also been observed with other antihistamines (Carr et al., 1985).

The fact that ReN1869 and mepyramine counteracted capsaicin-evoked pain when given i.t. suggests that a central site is involved. This was corroborated by the observation that ReN 1869, like morphine, attenuated Fos protein expression of neuronal cell bodies in layers I and II of the dorsal spinal cord in the formalin test. In this connection, it is interesting that ReN 1869 dose-dependently attenuated the CGRP release from dorsal spinal cord since the source of CGRP is the C-fibers (Pohl et al., 1990). Neuropeptides are implicated in central pain processing (Wiesenfeld-Hallin et al., 1984; Mantyh et al., 1997) and hence inhibition of their release should attenuate pain sensation. This is the case in the flexor reflex model that is employed for testing centrally acting analgesic compounds (Woolf and Wiesenfeld-Hallin, 1986). In this model, ReN 1869 indeed counteracted the evoked reflex activity dose dependently confirming a spinal action of the compound (Xu et al., unpublished).

4.4. Anti-inflammatory effect of ReN 1869

The paw edema provoked by the injection of histamine and the extravasation elicited by antidromic nerve stimulation are part of neurogenic inflammation since they are counteracted by prior inactivation of the C-fiber system (Amann et al., 1995; Jancsó et al., 1967; Lembeck and Holzer, 1979). The same applies for the edema elicited by capsaicin and to some extent that by formalin (Wheeler-Aceto and Cowan, 1991; Damas and Liegeois, 1999), but not for the edema elicited by carrageenan (Gamillscheg et al., 1984). Hence, ReN 1869 seems to be preferentially active against neurogenic inflammation.

The observation that ReN 1869, but not mepyramine, inhibited the histamine paw edema (Fig. 6) is puzzling since Barnett and Kreutner (1991) reported that several other H_1 antagonists counteracted the histamine-evoked paw edema in mice. The role of histamine H_1 receptors for the extravasation elicited by nerve stimulation is also not clear. In the original paper by Jancsó et al. (1967), the H_1 antagonist chloropyramine was unable to inhibit the extravasation, whereas Lembeck and Holzer (1979) partly could inhibit the extravasation by systemic administration of a combination of H_1/H_2 receptor antagonists, and significantly (80–90%), by prior mast cell histamine depletion with compound 48/80. However, the observation that the extravasation provoked by antidromic stimulation of the trigeminal nerve was blocked by a combination of mepyramine and cimetidine (histamine H_2 receptor antagonist) but not by cimetidine alone (Couture and Cuello, 1984) suggests that the histamine H_1 receptor plays a significant role.

The importance of neuropeptides for this type of inflammation is well established (Lembeck and Holzer, 1979; Newbold and Brain, 1993) and hence, the subsequent mast cell activation and capillary extravasation. The fact that ReN 1869 was able to further diminish the extravasation in mast cell-depleted animals (Fig. 12) suggests that the compound also acts directly on the C-fiber.

In the formalin test, prior mast cell degranulation by compound 48/80 showed an effect similar to that observed with ReN 1869 (Table 3) implying that histamine indeed is involved. But also here is the role of H_1 antagonists unclear. Damas and Liegeois (1999) found that mepyramine (3 mg/kg i.p.) significantly inhibited the rat paw edema when provoked by a strong formalin solution (5%) but not by one of the lower strength (1.75%) that is comparable to the formalin solution used in the present study.

The paw edema elicited by capsaicin was almost completely inhibited by ReN 1869 and mepyramine (Fig. 10) demonstrating the importance of histamine H_1 receptors. This observation combined with the finding that ReN 1869 had no influence on capsaicin-mediated responses of isolated sensory neurons implies that histamine is involved. This is prob-

ably mediated by the action of capsaicin on C-fibers that releases substances (e.g. SP) that subsequently cause mast cells to release histamine. It is noteworthy that ReN 1869 and mepyramine were able to counteract the paw edema even when administered intrathecally. The lowest effective dose of ReN 1869 was equivalent to 0.005 mg/kg excluding a systemic effect. Thus, central mechanisms exist that contribute to the development of peripheral neurogenic inflammation in which histamine H_1 receptors are important. These mechanisms may involve spinal substance P release and/or dorsal root reflexes which are important for the development of neurogenic inflammation because they control the peripheral release of neuropeptides (Jacques and Couture, 1990; Rees et al., 1994; Lin et al., 1999).

4.5. Mode of action of ReN 1869

The discussion above strongly suggests that ReN 1869 exerts its antinociceptive and anti-inflammatory actions via histamine H_1 receptor blockade peripherally as well as centrally. The fact that ReN 1869 inhibited the binding of [^{3}H]mepyramine in the CNS as potently as mepyramine (Fig. 5) demonstrates that ReN 1869 easily crosses the blood–brain barrier like mepyramine (Yamazaki et al., 1994). Stimulation of histamine H_1 receptor activity is, in the periphery, mediated by histamine released from mast cells, as discussed above, and centrally by the extensive histaminergic system that originates from the posterior hypothalamus and which give rise to histaminergic fibers in the spinal cord, for example, in laminae I and II (Panula et al., 1989). Currently, it is unclear to what extent histamine is released under various pain conditions. However, there is another possibility for activating the histamine H_1 receptor that does not require an agonist. It has been shown that the histamine H_1 receptor exhibits constitutive activity and that H_1 antagonists are able to depress this activity (Bakker et al., 2000). Hence, they should in fact be named inverse histamine H_1 receptor agonists. Histamine H_1 receptor signalling stimulates phospholipase C, increases intracellular Ca^{2+} levels and the production of diacylglycerol that subsequently activates protein kinase C. This enzyme has a pivotal role in pain sensation since it activates and sensitises the primary afferents (Ahlgren and Levine, 1994; Malmberg et al., 1997). Morphine, on the other hand, inhibits the nociceptive pathway via inhibition of neuronal Ca^{2+} channel activity and augmentation of K^+ channel activity through the cAMP pathway (Moises et al., 1994). Thus, the existence of two separate biochemical nociceptive pathways is the basis for ReN 1869 to augment the antinociceptive effect of morphine.

Although ReN 1869 was active in several pain tests, it was apparently less efficacious than morphine. This may be due to a low-activation/low-expression level of histamine H_1 receptors during normal conditions. Recently, it was found that local nerve injury induced a 3- to 4-fold upregulation of histamine H_1 receptors in the dorsal root ganglion cells supplying this region. The response involved mainly small-sized neurons containing substance P and CGRP (Kashiba et al., 1999). The observation suggests that H_1 antagonists may be especially effective in chronic pain conditions related to nerve injury. In this connection, it should be noted that amitriptylin, which is extensively used for chronic pain (Onghena and Van Houdenhove, 1992), is a potent H_1 antagonist, and that several other H_1 antagonists have antinociceptive actions (Raffa, 2001).

ReN 1869 has already been shown to be effective in humans; not only to counteract histamine-evoked wheal, flare and itch but also as an antinociceptive agent with a central mode of action (Thomsen et al., unpublished). The fact that the compound is a very selective histamine H_1 receptor antagonist (or inverse histamine H_1 receptor agonist), exhibits very few side effects despite the fact that it easily penetrates the blood–brain barrier, is orally available and does not exhibit tolerance, makes it a potential new candidate for the treatment of chronic pain and inflammatory conditions of neurogenic origin.

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