British Journal of Pharmacology (1995) 116, 1937-1943

Antinociceptive activity of the tachykinin NK_1 receptor antagonist, CP-99,994, in conscious gerbils

[1]N.M.J. Rupniak, J.K. Webb, A.R. Williams, E. Carlson, S. Boyce & R.G. Hill

Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR

1 The ability of CP-99,994, and its less active enantiomer, CP-100,263, to inhibit spontaneous behaviours and hyperalgesia induced by central infusion of the NK_1 receptor agonist, GR73632 or intraplantar injection of formalin was investigated in rats and gerbils.

2 GR73632 (3 pmol, i.c.v.)-induced foot tapping in gerbils was dose-dependently inhibited by CP-99,994 (0.1-1 mg kg^{-1}, s.c.), but not by CP-100,263 (10 mg kg^{-1}, s.c.) using pretreatment times up to 60 min. The centrally active dose-range for CP-99,994 was increased to 1-10 mg kg^{-1} s.c. with a higher challenge dose of GR73632 (30 pmol, i.c.v.).

3 In gerbils, intrathecal (i.t.) injection of GR73632 (30 pmol) elicited behaviours (licking, foot tapping or flinching and face washing) which closely resembled, but which was less specifically localized than, behaviours seen in animals injected with formalin (0.1-5%) into one hindpaw.

4 In rats, CP-100,263, but not CP-99,994 (up to 30 mg kg^{-1}), inhibited the early phase response to intraplantar injection of 5% formalin (ID_{50} = 13.9 mg kg^{-1}). The late phase was inhibited by both compounds (ID_{50} values 36.3 and 20.9 mg kg^{-1}, respectively). In gerbils, there was marginal evidence for enantioselective inhibition of the early phase induced by formalin (2%). The ID_{50} values were 6.2 mg kg^{-1} for CP-99,994 and 13.4 mg kg^{-1} for CP-100,263.

5 Intrathecal injection of GR73632 (30 pmol) caused thermal hyperalgesia in gerbils which was inhibited enantioselectively by s.c. administration of CP-99,994 (ID_{50} = 2.46 mg kg^{-1}), but not by CP-100,263 (30 mg kg^{-1}).

6 In gerbils, intraplantar injection of formalin (0.1%) caused thermal hyperalgesia which was inhibited by CP-99,994 (ID_{50} = 1.1 mg kg^{-1}, s.c.). There was a nonsignificant trend for an anti-algesic effect of CP-100,236 (estimated ID_{50} = 8.2 mg kg^{-1}, s.c.).

7 These findings support the proposal that NK_1 receptor antagonists may be useful in the clinical management of pain and reinforce the need to dissociate specific and nonspecific antinociceptive effects of available compounds.

Keywords: Formalin paw; gerbil; rat; NK_1 receptor; GR 73632; hyperalgesia; ion channels

Introduction

Electrophysiological studies on anaesthetized or decerebrate animals provide unanimous evidence that NK_1 receptor antagonists may have important clinical utility as analgesic drugs, especially in the management of chronic pain. Systemic administration of CP-96,345 (0.5-2 mg kg^{-1}, i.v.) blocked the excitation of dorsal horn neurones caused by iontophoretic application of substance P, by prolonged noxious thermal stimulation of the cutaneous receptive field, or by intense electrical stimulation of the sensory nerves in cats (Radhakrishnan & Henry, 1991; de Konnick & Henry, 1991). Similarly, RP67580 stereoselectively inhibited the responses of dorsal horn neurones during the early and late phase response to formalin in rats (Chapman & Dickenson, 1993). Several investigations have also shown the ability of NK_1 receptor antagonists to attenuate selectively the facilitation of the flexor reflex (but not the protective reflex response) induced by intrathecal injection of substance P, or by C-fibre conditioning stimuli. These reveal potent antinociceptive effects of CP-96,345 ($\leq$1 mg kg^{-1}, i.v.; Xu *et al.*, 1992) and RP67580 (3-30 μg kg^{-1}, i.v.; Laird *et al.*, 1993) in rats, and of CP-99,994 in rabbits (ID_{50} = 2.7 μg kg^{-1}, i.v.; Boyce *et al.*, 1993).

In contrast to these findings, there is no definitive evidence that these compounds exert NK_1 receptor-mediated antinociceptive effects in conscious animal assays. Although two studies have demonstrated that CP-96,345 can prevent thermal hyperalgesia induced by intrathecal injection of substance P in rats (Malmberg & Yaksh, 1992; Yashpal *et al.*, 1993), antagonism of the effect of exogenously applied substance P does not elucidate the involvement of NK_1 receptors in nociception under physiological conditions.

Antinociceptive effects of NK_1 receptor antagonists in conscious animals have been most extensively investigated with the formalin paw assay since these agents are generally not active in reflex withdrawal tests sensitive to opioid analgesics (Moussaoui *et al.*, 1992; Birch *et al.*, 1992; Rupniak *et al.*, 1993). Injection of formalin into the hindpaw causes a biphasic behavioural response (paw licking and flinching) which is associated with a biphasic increase in substance P levels in the dorsal horn (McCarson & Goldstein, 1990). Several studies have reported antinociceptive, and apparently stereoselective, effects of CP-96,345 ($\leq$10 mg kg^{-1}, s.c.) on the late (but not the early) phase behavioural response to formalin in rats (Yamamoto & Yaksh, 1991; Birch *et al.*, 1992; Yashpal *et al.*, 1993). However, the demonstrated separation in the potency of the isomers was not conclusive ($\leq$2 fold), and in other studies the isomers were found to be equipotent (Nagahisa *et al.*, 1992; unpublished observations). The nonspecific antinociceptive effect of CP-96,345 in the formalin paw assay appears to be attributable to ion channel blockade (Schmidt *et al.*, 1992; Karlsson *et al.*, 1994).

The rat is not the most appropriate rodent species in which to evaluate CP-96,345 or CP-99,994 since these compounds exhibit low affinity for the rat NK_1 receptor compared with that expressed in gerbil and guinea-pig brain (Gitter *et al.*, 1991; Beresford *et al.*, 1991; McLean *et al.*, 1993). An alternative antagonist, RP67580, which has high affinity for the

[1] Author for correspondence.

mouse NK_1 receptor, inhibited both the early and late phase response to formalin in mice (Garrett *et al.*, 1991; Rupniak *et al.*, 1993), but the utility of this compound for nociception assays in conscious animals is compromised by ion channel blocking activity (Rupniak *et al.*, 1993), and by its short plasma half life (Laird *et al.*, 1993).

The doses of NK_1 receptor antagonists employed in behavioural experiments (mg kg^{-1} range) are substantially higher than those required to demonstrate antinociception in anaesthetized animal preparations ($\mu g\ kg^{-1}$ range). The behavioural assays may have been confounded by the use of compounds which are rapidly eliminated from the plasma, or which have relatively low affinity for the NK_1 receptor expressed in the species under investigation. An alternative interpretation is that, for ethical reasons, the intensity and duration of the noxious stimuli which can be applied to conscious animals in reflex withdrawal assays are not of sufficient severity to recruit the NK_1 receptor-mediated facilitation of nociceptive transmision demonstrated in unconscious animals (see Hill, 1994).

In order to clarify these issues, we now compare the antinociceptive activity of CP-99,994 in conscious rats and gerbils by use of behavioural assays. This compound was chosen for investigation since it has the lowest affinity of currently available NK_1 receptor antagonists for the L-type calcium channel *in vitro* (McLean *et al.*, 1993). Moreover, CP-99,994 exerts centrally-mediated effects on NK_1 agonist-induced behaviour in gerbils in the mg kg^{-1} dose range, with greater than 1,000 fold higher potency than its less active enantiomer, CP-100,263 (Rupniak & Williams, 1994).

Methods

Subjects

The subjects were male Sprague-Dawley rats (100–300 g, Bantin and Kingman), and male and female Mongolian gerbils (50–70 g, Leeds University), housed in groups of 4–6 under standard laboratory conditions. Experiments conformed to ethical guidelines for investigation of pain in conscious animals (Zimmerman, 1983). Each animal was used once only and was humanely killed immediately aftter completion of testing.

Inhibition of GR73632-induced foot tapping in gerbils

The centrally active dose-range and approximate duration of action of CP-99,994 following s.c. injection was defined by the inhibition of foot tapping induced by central infusion of the highly selective tachykinin NK_1 receptor agonist, GR73632. In gerbils, activation of central NK_1 receptors causes a species-specific, repetitive hindfoot tapping response which is virtually continuous throughout a 5 min observation period with a dose of 3 pmol i.c.v. of GR73632 (Rupniak & Williams, 1994). Therefore, in experiments designed to establish the duration of central activity of CP-99,994 following different pretreatment times, a dose of 3 pmol i.c.v. of GR73632 was employed. The effect of increasing the dose of GR73632 to 30 pmol i.c.v. on the ID_{50} for CP-99,994 was also determined since this dose of GR73632 was employed in other experiments to induce hyperalgesia following intrathecal injection (see below).

CP-99,994 (0.03–10 mg kg^{-1}), the less active enantiomer CP-100,263 (10 mg kg^{-1}) or water were administered s.c. in the neck 5, 30 or 60 min prior to i.c.v. infusion of GR73632. Animals were then briefly anaesthetized by inhalation of an isoflurane/oxygen mixture and an incision made in the midline of the scalp. GR73632 (3 or 30 pmol in 5 μl) was infused into the cerebral ventricles by vertical insertion of a cuffed 27 gauge needle to a depth of 4.5 mm below bregma. Immediately following recovery of the righting reflex, animals were placed in an individual observation box (25 cm × 20 cm × 20 cm). The duration of foot tapping was recorded for 5 min with a stopclock, giving a maximum possible duration of 300 s.

Intrathecal injection of GR73632 in gerbils

(i) Spontaneous behaviours Intrathecal injections were performed under brief isoflurane/oxygen anaesthesia using the method described by Hylden & Wilcox (1980). A caudal skin incision was made to enable visualisation of the lumbar vertebrae. A 30 gauge hypodermic needle (Needleworks, U.K.) attached to a 10 μl Hamilton syringe was inserted into the intervertebral space between L5 and L6 and GR73632 (3–30 pmol), or vehicle, injected in a volume of 5 μl. The vehicle was artificial CSF, pH 7.0, comprising in mM: NaCl 122.6, $NaHCO_3$ 26.2, KCl 5.4, $MgSO_4$ 2.0, NaH_2PO_4 1.2 and $CaCl_2$ 2.0. For observation of spontaneous behaviours, animals were placed in individual observation boxes following recovery of the righting reflex as described above, and behaviours recorded continuously for 30 min with a hand-held keypad interfaced with a BBC microcomputer.

(ii) Induction of thermal hyperalgesia The effect of intrathecal injection of GR73632 on thermal nociception was investigated by a modified paw flick test (Hargreaves *et al.*, 1988). Gerbils were habituated on glass tables (1 m × 2 m) under individual, clear perspex boxes (21 cm × 13 cm × 10 cm) for at least 3 h before determination of the baseline paw flick latency. Up to 24 animals could be accommodated on each table. A mobile radiant heat source, located 2.5 cm beneath the glass surface, and focused on the plantar surface of the animals' hind foot, was pre-calibrated to give a baseline paw withdrawal latency of approximately 12 s. Response latencies were determined for both hind feet on three occasions and the mean from these six measurements recorded as the baseline for each animal. Any subjects with a mean response latency of less than 9 or greater than 16 s were removed from the experiment. Paw flick latencies were again recorded 1 h following intrathecal injection of GR73632 (30 pmol) as described above. CP-99,994 (0.1–10 mg kg^{-1}), CP-100,263 (30 mg kg^{-1}) or water was administered s.c. in the neck immediately prior to intrathecal injection of GR73632 or artificial CSF.

Formalin paw experiments

(i) Spontaneous behaviours in rats and gerbils Animals were habituated to individual observation boxes (25 cm × 20 cm × 20 cm) for at least 1 h before subcutaneous injection of formalin into one hind paw (0.1–5% in 50 μl). Spontaneous behavious were recorded for up to 60 min immediately thereafter to include early and late phase responses. For evaluation of the antinociceptive effect of CP-99,994 and CP-100,263, test compounds (3–30 mg kg^{-1}) or water were administered either i.p 10 min before 5% formalin (rats), or s.c. into the midscapular region 30 min prior to 2% formalin (gerbils). The duration of licking directed at the formalin-injected paw was recorded for 0–5 min (early phase) and 10–60 min (late phase) in rats, and for 0–10 min (early phase) in gerbils.

(ii) Induction of thermal hyperalgesia in gerbils The effect of intraplantar injection of formalin (0.01–1%) on the latency to withdraw the foot from a noxious thermal stimulus was determined by the paw flick procedure described above. CP-99,994 (0.3–10 mg kg^{-1}), CP-100,263 (3–10 mg kg^{-1}) or saline was administered s.c. into the midscapular region immediately prior to intraplantar injection of formalin or saline. Baseline and 1 h post-formalin response latencies were each recorded on 4 occasions from the injected foot and were meaned.

Test compounds

GR73632 ((+)-Ava [L-Pro^9, Me-Leu^{10}]substance P-(7-11); Cambridge Biochemicals Inc. U.S.A.) was dissolved in distilled water and frozen at −70°C in aliquots at the required concentration until use. CP-99,994 ((**2S,3S**)-*cis*-3-(2-methoxybenzylamino)-2-phenyl piperidine) and CP-100,263 ((**2R,3R**)-

cis-3-(2-methoxybenzylamino)-2-phenyl piperidine) were synthesized as the dihydrochloride salts by the Department of Medicinal Chemistry, MSD, Harlow, Essex. All doses are expressed as the free base equivalent.

Statistical analysis

Hyperalgesia was determined by subtracting the response latency after intrathecal injection of GR73632 or intraplantar injection of formalin from the pretreatment baseline value for each individual animal. Where necessary, data were subjected to logarithmic or square root transformation to achieve normality and homogeneity of variance prior to one or two-way analysis of variance (ANOVA) followed, where appropriate, by Dunnett's or Newman-Keuls multiple comparison *t* tests using BMDP statistical software (BBN Software Products Corporation, U.S.A.). The dose of CP-99,994 and CP-100,263 inhibiting behaviour or hyperalgesia by 50% (ID_{50}) was calculated by non-linear least squares regression analysis using GraFit (Erithacus Software, U.K.).

Results

Determination of centrally active dose-range of CP-99,994 in gerbils

Subcutaneous injection of CP-99,994 (0.1 – 1 mg kg^{-1}) either 5, 30 or 60 min before i.c.v. infusion of GR73632 (3 pmol) caused a dose-dependent inhibition of foot tapping (5 min: $F_{4,13}=34.80$, $P<0.01$; 30 min: $F_{4,10}=76.48$, $P<0.01$; 60 min: $F_{4,10}=32.78$, $P<0.01$; Table 1). The ID_{50} values were 0.38 mg kg^{-1} at 5 min, 0.15 mg kg^{-1} at 30 min and 0.12 mg kg^{-1} at 60 min. There was no other observable effect of CP-99,994 on spontaneous behaviour or motor function. In contrast, no inhibition of GR73632-induced foot tapping was observed after treatment with the less active enantiomer, CP-100,263 (10 mg kg^{-1}, s.c.) either 5, 30 or 60 min previously (Table 1).

There was a greater than 6 fold shift to the right in the inhibition curve for CP-99,994 when the dose of GR73632 was increased to 30 pmol i.c.v. ($F_{3,8}=234.83$, $P<0.01$) using a 5 min pretreatment (Table 1). The ID_{50} value for CP-99,994 was increased to 2.52 mg kg^{-1}, s.c.

Intrathecal injection of GR73632 in gerbils

(i) Spontaneous behaviours A behavioural syndrome, in which grooming predominated, was elicited by intrathecal injection of GR73632 (10 or 30 pmol) in gerbils. Especially prominent was licking of both hind feet, hind legs and the tail (caudal licking; $F_{3,26}=4.27$, $P=0.01$), and grooming of the face and forepaws (face washing; $F_{3,26}=7.20$, $P<0.01$). At the highest dose examined (30 pmol, i.t.), a discontinuous repetitive hind foot tapping or flinching response was observed, which appeared qualitatively similar to that induced by i.c.v. administration of GR73632, but which was considerably less vigorous and less frequent ($F_{3,26}=7.05$, $P<0.01$). These behaviours were not notable in animals receiving intrathecal injections of artificial CSF (Table 2).

(ii) Induction of thermal hyperalgesia In separate experiments, the effect of intrathecal injection of GR73632 (30 pmol, a dose producing robust behavioural effects) on thermal nociception was examined. The intensity of the radiant heat source was adjusted to give a paw flick latency in normal animals of approximately 12 s; 1 h following intrathecal injection, GR73632 caused a reduction in the mean withdrawal latency of 6.1 ± 0.9 s for both hind paws compared with the pretreatment baseline latency. In contrast, intrathecal injection of artificial CSF did not cause hyperalgesia to radiant heat

Table 1 Inhibition of GR73632-induced foot tapping by CP-99,994 in gerbils

Treatment and dose (mg kg^{-1}, s.c.)		*% inhibition and pretreatment time* (min) 5	30	60
3 pmol GR73632 i.c.v.				
CP-99,994	0.1	7.2 ± 7.9	20.0 ± 7.9	52.4 ± 5.7*
	0.3	34.8 ± 13.4*	89.4 ± 9.4*	70.5 ± 15.8*
	1.0	93.8 ± 6.2*	100 ± 0*	100 ± 0*
CP-100,263	10.0	0	3.6 ± 0.6	2.7 ± 1.8
30 pmol GR73632 i.c.v.				
CP-99,994	1.0	2.6 ± 2.2	ND	ND
	3.0	66.5 ± 6.0*	ND	ND
	10.0	100 ± 0*	ND	ND

Values are the mean ± 1 s.e.mean for 3 or 4 gerbils expressed as a percentage inhibition of the duration of foot tapping observed in vehicle-treated animals. For both doses of GR73632, foot tapping was virtually continuous throughout the 5 min observation period in control subjects. Raw data were subjected to one-way ANOVA followed by Dunnett's *t* test.
*$P<0.05$ compared with vehicle-treated animals; ND = not determined.

Table 2 Behaviours elicited by intrathecal injection of GR73632 in gerbils

Treatment and Dose (pmol i.t.)	*Duration* (s/30 min) *Caudal licking*	*Foot tapping/ flinching*	*Face washing*
Vehicle	21.4 ± 6.3	16.7 ± 4.5	41.3 ± 5.7
3	143.6 ± 49.6	54.9 ± 12.7	99.0 ± 23.4
10	272.0 ± 98.6*	43.9 ± 9.9	196.4 ± 55.6
30	264.0 ± 51.1*	135.3 ± 33.9*	346.5 ± 79.4*

Values are the mean ± 1 s.e.mean durations for groups of 7 to 8 animals during a 30 min observation period. Data were subjected to one-way ANOVA followed by Dunnett's *t* tests.
*$P<0.05$ compared with injection of artificial CSF.

(difference in response latency compared with baseline = 0.6 ± 0.4 s; $F_{1,10} = 30.74$, $P < 0.01$ compared with GR73632-treated animals). Administration of CP-99,994 (0.1–10 mg kg^{-1}, s.c.) immediately before intrathecal injection of GR73632 (30 pmol) caused a dose-dependent inhibition of hyperalgesia ($F_{9,59} = 4.67$, $P < 0.01$; Figure 1). The ID_{50} value, calculated on the basis that the highest dose examined (10 mg kg^{-1}) caused 100% inhibition of hyperalgesia, was 2.46 mg kg^{-1}. In contrast, treatment with CP-100,263 (30 mg kg^{-1}) did not prevent the induction of hyperalgesia by GR73632 (Figure 1).

Injection of formalin into the paw

(i) Spontaneous behaviour in rats and gerbils In rats, intraplantar injection of formalin (5%) caused two discrete periods of paw licking, the first occurring during the first 5 min after the injection (early phase), followed by a sustained and intense period 10–60 min after the injection (late phase).

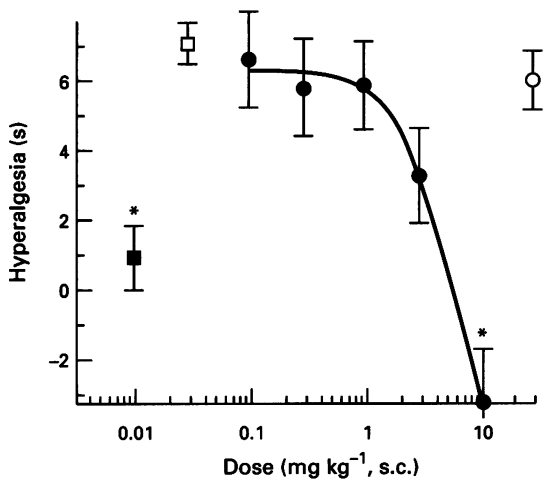

Figure 1 Inhibition of GR73632 (30 pmol i.t.)-induced thermal hyperalgesia by CP-99,994 (●) in gerbils; (□) GR73632; (○) CP-100,263; (■) CSF. Values are the mean ± 1 s.e.mean difference in paw flick latency determined 1 h after injection of GR73632 compared with pretreatment baseline values: ID_{50} for CP-99,994 = 2.46. Groups of 4 to 9 animals received each treatment. Data were subjected to analysis of variance followed by Newman-Keuls or Dunnett's *t* tests. *$P < 0.05$ compared with GR73632 plus vehicle treatment.

Pretreatment with the lowest dose of CP-99,994 (3 mg kg^{-1}, 10 min before formalin) caused a potentiation of licking ($F_{3,18} = 5.11$, $P < 0.01$), whilst higher doses (up to 30 mg kg^{-1}) tended to attenuate licking during the early phase, although this failed to reach significance. CP-100,263 (10 or 30 mg kg^{-1}) caused a dose-dependent inhibition of licking during the early phase ($F_{3,20} = 4.93$, $P = 0.01$). The less active enantiomer, CP-100,263, appeared slightly more potent ($ID_{50} = 13.9$ mg kg^{-1}) than CP-99,994 (estimated $ID_{50} = 25.9$ mg kg^{-1}; Figure 2).

Both enantiomers caused a dose-dependent inhibition of paw licking during the late phase in rats ($F_{3,18} = 3.16$, $P = 0.05$ for CP-99,994 and $F_{3,20} = 3.72$, $P = 0.03$ for CP-100,263). Unlike the early phase, CP-99,994 was slightly more potent than CP-100,263, but the degree of enantiomeric separation was marginal ($ID_{50} = 20.9$ mg kg^{-1} for CP-99,994, compared with 36.6 mg kg^{-1} for CP-100,263; Figure 2).

In gerbils, intraplantar injection of formalin (0.1–5%) also caused two discrete periods of behavioural activation, but the late phase was considerably less intense than that seen in rats (duration of paw licking approximately 10 times lower in gerbils). The early phase lasted for up to 20 min after injection of formalin, followed by the late phase between 20 and 45 min post injection. The most prominent behaviour was licking of the injected foot and limb (early phase: $F_{4,22} = 7.69$, $P < 0.01$; late phase: $F_{4,22} = 22.58$, $P < 0.01$), and repetitive flinching of the injected leg (early phase: $F_{4,24} = 10.42$, $P < 0.01$; late phase: $F_{4,24} = 6.22$, $P < 0.01$). During the early phase, there was a nonsignificant trend for a decrease in face and forepaw grooming ($F_{4,24} = 2.64$, $P = 0.06$). In contrast, in the late phase, there was a marked increase in face washing following intraplantar injection of 0.3% formalin ($F_{4,24} = 8.37$, $P < 0.01$). Face washing was not increased by higher concentrations of formalin during the late phase, when grooming was focused on the injected paw (Table 3).

A concentration of 2% formalin was selected in order to produce a robust paw licking response in the early phase. The antinociceptive activity of CP-99,994 and CP-100,263 was determined by recording the duration of paw licking for 10 min after administration of formalin. Pretreatment with either CP-99,994 or CP-100,263 (3–30 mg kg^{-1}, s.c., 30 min before formalin) caused a dose-dependent inhibition of paw licking ($F_{6,56} = 9.45$, $P < 0.01$; Figure 3). In this assay, a dose of 30 mg kg^{-1} of CP-99,994 was required to abolish licking completely; this dose of CP-100,263 also completely blocked the response to formalin. Across this dose-range there was marginal enantioselectivity of approximately 2 fold (ID_{50} for CP-99,994 = 6.2 mg kg^{-1}, compared with 13.4 mg kg^{-1} for CP-100,263).

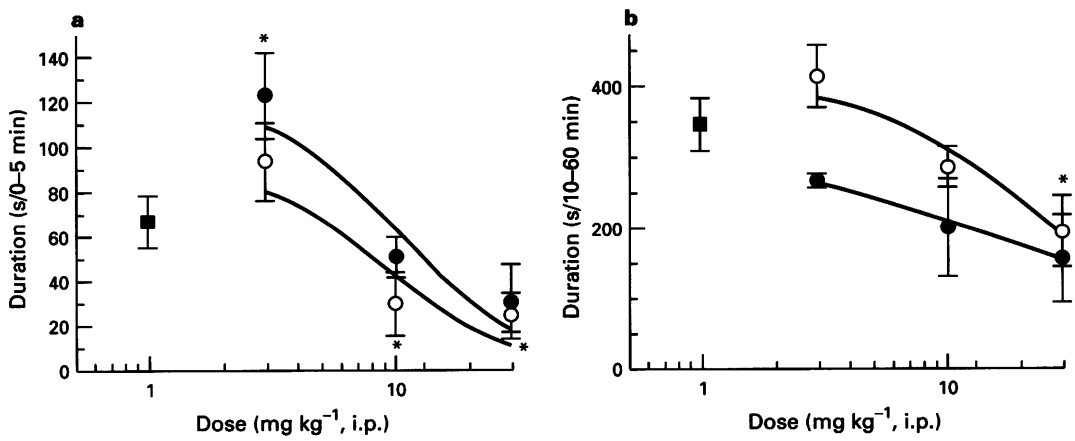

Figure 2 Inhibition of formalin (5%)-induced paw licking by CP-99,994 and CP-100,263 during the early (0–5 min) (a) and late (10–60 min) (b) phase in rats. (■) Vehicle; (●) CP-99,994; (○) CP-100263; In (a) ID_{50} for CP-99,994 = 25.9 and for CP-100,263 = 13.9. In (b) ID_{50} for CP-99,994 = 20.9 and for CP-100,263 = 36.6. Values are the mean ± 1 s.e.mean for groups of 3 to 7 animals. Data were subjected to analysis of variance followed by Newman-Keuls or Dunnett's *t* tests. *$P < 0.05$ compared with formalin plus vehicle treatment.

Table 3 Behaviours elicited following intraplantar injection of formalin (0.1–5%) in gerbils

Early phase	*Duration* (s) *or number* (0–20 min)		
Concentration of formalin (%)	*Licking injected paw*	*Flinching*	*Face washing*
Vehicle	26.9±8.2	57.0±15.0	50.6±7.0
0.1	46.3±14.7	69.0±20.0	59.5±14.0
0.3	50.2±14.0*	178.0±40.0*	31.6±4.1
1.0	102.9±16.5*	281.0±26.0*	34.8±6.8
5.0	125.6±14.4*	114.0±18.0*	31.1±6.4
Late phase	*Duration* (s) *or number* (20–45 min)		
Vehicle	1.9±1.2	5.0±3.0	27.6±9.4
0.1	4.6±1.9	22.0±13.0	19.0±9.8
0.3	35.1±5.9*	101.0±46.0*	91.9±11.9*
1.0	25.6±6.7*	126.0±25.0*	28.0±11.0
5.0	51.8±7.2*	53.0±21.0*	47.9±7.9

Values are the mean±1 s.e.mean durations of paw licking or face washing, or the number of flinches of the injected limb, recorded during each observation period. Groups of 5 or 6 animals received each concentration of formalin. Data were subjected to square root transformation prior to one-way ANOVA followed by Dunnett's *t* test.
*$P<0.05$ compared with vehicle treatment.

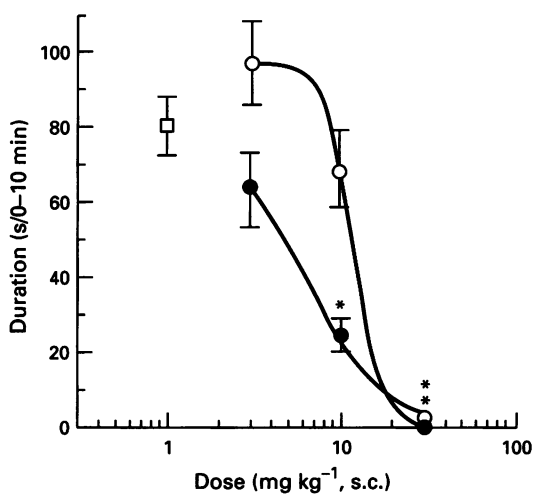

Figure 3 Inhibition of formalin (2%)-induced paw licking by pretreatment with CP-99,994 (●) or CP-100,263 (○) (3–30 mg kg^{-1}, s.c. 30 min previously) in gerbils; (□) vehicle. Values are the mean±1 s.e.mean durations for groups of 9 or 10 animals during a 10 min obsevation period. ID_{50} for CP-99,994=6.2 and for CP-100,263=13.4. Data were subjected to logarithmic transformation prior to analysis of variance and Newman-Keuls or Dunnett's *t* tests. *$P<0.05$ compared with formalin plus vehicle treatment.

Table 4 Induction of thermal hyperalgesia by intraplantar injection of formalin in gerbils

Concentration of formalin (%)	*Hyperalgesia* (s)
Saline	0.82±0.68
0.01	0.52±0.49
0.03	1.29±0.49
0.1	3.38±0.82*
0.3	3.14±0.79*
1.0	5.97±0.62*

Hyperalgesia was defined as the reduction in paw flick latency after administration of formalin compared with the pretreatment baseline values; 4 separate determinations were made for each foot before and after injection of formalin. Values are the mean±1 s.e.mean for 8–13 gerbils. Data were subjected to one-way ANOVA prior to Dunnett's *t* test.

(ii) Induction of thermal hyperalgesia in gerbils Intraplantar injection of formalin (0.1–1%, 1 h previously) caused a reduction in paw flick latency of up to 6 s compared with pretreatment baseline values ($F_{5,55}=7.26$, $P<0.01$; Table 4). From these experiments a concentration of 0.1% formalin was selected as the minimum dose causing robust hyperalgesia with which to investigate the anti-algesic effects of CP-99,994 and CP-100,263. Administration of CP-99,994 (0.3–10 mg kg^{-1}, s.c.) immediately before intraplantar injection of formalin caused a dose-dependent and complete inhibition of hyperalgesia ($F_{8,69}=3.84$, $P<0.01$). There was also a nonsignificant trend for inhibition of hyperalgesia with the same dose-range of CP-100,263, the inhibition curve lying to the right and parallel with that for CP-99,994. Two-way ANOVA revealed a statistically significant difference between the dose-response curves for the two enantiomers ($F_{1,41}=6.48$, $P=0.02$). Calculation of the ID_{50} values indicated an enantiomeric separation of approximately 8 fold in this assay (ID_{50} for CP-99,994=1.1 mg kg^{-1} compared with an estimated ID_{50} of 8.2 mg kg^{-1} for CP-100,263; Figure 4).

Discussion

These studies show that CP-99,994 can enantioselectively inhibit thermal hyperalgesia induced either by spinal application of an exogenous NK_1 receptor agonist, or by intraplantar injection of formalin, in conscious animals. These findings are consistent with *in vivo* electrophysiological studies showing that facilitated nociceptive reflexes are inhibited by NK_1 receptor antagonists (Laird *et al.*, 1993), and with other evidence for an involvement of NK_1 receptors in nociception obtained with anaesthetized rats (Yashpal *et al.*, 1993; Chapman & Dickenson, 1993).

Unlike formalin-induced thermal hyperalgesia, use of a more conventional behavioural endpoint, the duration of licking directed at the injected paw, did no permit a clear separation in the antinociceptive effects of CP-99,994 and CP-100,263. In agreement with previous reports using CP-96,345 in rats (Yamamoto & Yaksh, 1991; Nagahisa *et al.*, 1992; Yashpal *et al.*, 1993), there was a suggestion of enantioselectivity for the inhibition of formalin-induced licking in the late, but not the early phase by CP-99,994 in rats. However, this was marginal (1.5 fold difference in the ID_{50} for CP-99,994 and CP-100,263), indicating a substantial nonspecific antinociceptive effect of both compounds in the dose-range greater than 10 mg kg^{-1}, where the inhibition curves for both com-

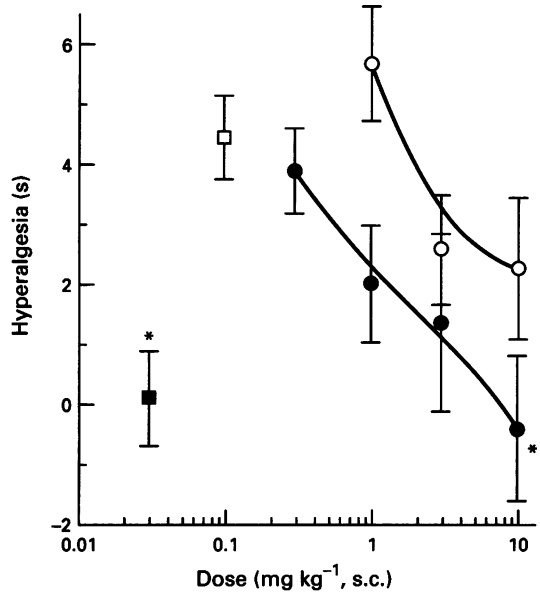

Figure 4 Inhibition of formalin (0.1%)-induced thermal hyperalgesia by administration of CP-99,994 or CP-100,263 (0.3–10 mg kg^{-1}, s.c., administered immediately before formalin). (□) Formalin; (■) vehicle; (○) CP-100,263; (●) CP-99,994. ID_{50} for CP-100,263 = 8.2 and for CP-99,994 = 1.1. Paw flick latencies were determined before and 1 h after intraplantar injection of formalin and the difference in response time used as a measure of hyperalgesia. Values are the mean ± 1 s.e.mean latencies for groups of 6 or 12 animals. Data were subjected to analysis of variance and Newman-Keuls or Dunnett's *t* tests. *$P<0.05$ compared with formalin plus vehicle treatment.

pounds converged. Similar findings have been reported recently by Smith *et al.* (1994). This profile is indistinguishable from that observed previously with CP-96,345 (Nagahisa *et al.*, 1992), and is perhaps surprising in view of the low affinity of CP-99,994 compared with CP-96,345 for calcium channels labelled by [^{3}H]-desmethoxyverapamil reported by McLean *et al.* (1993). However, a more recent study indicates that unlike CP-96,345 and RP67580, CP-99,994 has no appreciable affinity for the dihydropyridine binding site, but that these compounds all have similar affinity for the phenylalkylamine site (Lombet & Spedding, 1994). Moreover, blockade of other ion channels, notably an anaesthetic-like interaction with sodium channels, by such compounds might cause antinociception by producing a conduction block of the peripheral nerve (Karlsson *et al.*, 1994).

There is conflicting evidence concerning the involvement of NK_1 receptors in the early and late phase behavioural response to formalin. In mice, peptide and nonpeptide NK_1 receptor antagonists appear to block both the early and the last phase (Garrett *et al.*, 1991; Rupniak *et al.*, 1993; Sakurada *et al.*, 1994). However, in rats, only the late phase of paw licking was attenuated by CP-96,345 (Yamamoto & Yaksh, 1991; Birch *et al.*, 1992; Yashpal *et al.*, 1993). In the present study, CP-99,994 also attenuated only the late phase in rats. Surprisingly, this compound potentiated the early phase at a single dose. This phenomenon is unlikely to be mediated via NK_1 receptor blockade, since a potentiation of the response of dorsal horn neurones to intraplantar injection of formalin has been reported previously in rats treated with RP67581, the stereoisomer of RP67580 (Chapman & Dickenson, 1993), and may be related to the nonspecific proconvulsant activity observed in previous conscious animal studies (Rupniak & Jackson, 1994). It is possible that an unidentified pharmacological action of nonpeptide NK_1 receptor antagonists may negate or confound their antinociceptive activity in the formalin paw test.

Since, CP-99,994 has low affinity for the rat NK_1 receptor, it would be expected that inhibition of formalin-induced paw licking should be demonstrable with lower doses of CP-99,994 in gerbils. However, inhibition of the early phase again revealed only marginal (2 fold) enantiomeric separation, with complete convergence of the inhibition curves at doses greater than 10 mg kg^{-1}. This observation is in agreement with the report by Smith *et al.* (1994). The late phase behavioural response to formalin in gerbils was not sufficiently robust to permit evaluation of antagonists.

Using a functional assay for CNS penetration of NK_1 receptor antagonists (inhibition of GR73632-induced foot tapping), it was clear that central NK_1 receptors in gerbils remained occupied for more than 60 min after subcutaneous administration of CP-99,994. The ID_{50} value of 0.12 mg kg^{-1}, s.c. determined using a 60 min pretreatment is consistent with previous data obtained using an intravenous bolus injection of CP-99,994 (ID_{50} = 0.06 mg kg^{-1}; Rupniak & Williams, 1994). The μg kg^{-1} potency and enantioselectivity of CP-99,994 demonstrated in this way is compatible with antinociceptive activity determined by use of electrophysiological, but not behavioural, endpoints (see Hill, 1994, for review). Hence, the distribution and duration of action of this compound in the plasma and CNS does not appear to explain why doses approximately 1,000 times higher than those needed to occupy central NK_1 receptors are required to inhibit formalin-induced behaviour in gerbils.

The absolute potency of CP-99,994 *in vivo* is at least partly determined by the concentration of agonist at the NK_1 receptor, since there was a marked shift to right in the inhibition curve for foot tapping when the dose of GR73632 was increased from 3 to 30 pmol i.c.v. Under this condition, the ID_{50} for CP-99,994 (2.52 mg kg^{-1}) was in good agreement with the doses needed to inhibit GR73632 and formalin-induced thermal hyperalgesia (ID_{50} values = 2.46 and 1.10 mg kg^{-1}, respectively). Therefore, during hyperalgesia, the local concentration of substance P, and/or the NK_1 receptor reserve in the spinal cord may require that relatively high doses of NK_1 receptor antagonists are administered in conscious animal assays.

The greater potency and more convincing enantioselectivity exhibited by CP-99,994 for inhibition of hyperalgesia, rather than spontaneous behaviours, induced by formalin is consistent with the view based on electrophysiological studies that facilitated, but not protective reflexes are modulated by spinal NK_1 receptors (Laird *et al.*, 1993). The striking similarity between the behaviours elicited by intrathecal injection of GR73632 and intraplantar injection of formalin implies that stimulation of spinal NK_1 receptors is aversive. Flicking or tapping of the feet, intense licking of the feet and legs, and face washing were prominent features of both syndromes, but in the case of the formalin paw assay these behaviours were focused predominantly on the injected foot. The ability to block hyperalgesia with lower doses of CP-99,994 than are required to inhibit overt behaviours induced by formalin may be attributable to the use of a much lower concentration of formalin (0.1 rather than 2%) which causes a less profound activation of endogenous substance P release.

The anti-algesic effects of CP-99,994 appear to be mediated at least partly via NK_1 receptors located in the spinal cord since CP-100,263 failed to inhibit the hyperalgesia caused by intrathecal infusion of GR73632. The poorer enantiomeric separation achieved against formalin-induced hyperalgesia (and behaviour) suggests that both compounds have other nonspecific actions in the periphery (the paw itself, and/or the nerve). It would therefore be of interest to compare the effect of intrathecal application of CP-99,994 and CP-100,263 on formalin-induced behaviour. This experiment was not performed in the present studies because anaesthesia interfered with the response to formalin. A preliminary experiment on cannulated conscious rats showed that intrathecal injection of CP-96,345, but not CP-96,344, (200 μg) inhibited the late phase response to formalin (Yamamoto & Yaksh, 1991). A detailed investigation using a wider dose-range of both compounds and a more appropriate species whose NK_1 receptor has high affinity for CP-96,345, has not yet been undertaken.

Collectively, the preclinical findings provide support, which is not yet conclusive, for the potential of NK_1 receptor antagonists to treat clinical pain, especially to prevent the development of hyperalgesia during the initial stages of nerve or tissue injury. A recent study examining rats with a sciatic nerve constriction injury, which caused guarding behaviours and persistent thermal hyperalgesia, showed an induction of preprotachykinin gene expression by large A cells in the dorsal root ganglion which normally transmit non-noxious sensory stimuli (Marchand *et al.*, 1994). However, preliminary studies have failed to demonstrate an anti-algesic effect of acute administration of either CP-96,345 (400 μg intrathecally) in rats with sciatic nerve ligation (Yamamoto & Yaksh, 1992), or CP-99,994 (up to 100 μg kg^{-1}, i.v.) in patients with painful peripheral neuropathy and allodynia (Suarez *et al.*, 1994). Neither of these studies was optimised with respect to the time of initiation, duration of treatment, or dose of the compound under investigation. More aggressive treatment regimes will clearly require carefully controlled studies in order to dissociate specific and nonspecific analgesic effects of currently available compounds.

References

BERESFORD, I.J.M., BIRCH, P.J., HAGAN, R.M. & IRELAND, S.J. (1991). Investigation into species variants in tachykinin NK_1 receptors by use of the non-peptide antagonist, CP-96,345. *Br. J. Pharmacol.*, **104,** 292–293.

BIRCH, P.J., HARRISON, S.M., HAYES, A.G., ROGERS, H. & TYERS, M.B. (1992). The non-peptide NK_1 receptor antagonist, (±)-CP-96,345, produces antinociceptive and anti-oedema effects in the rat. *Br. J. Pharmacol.*, **105,** 508–510.

BOYCE, S., LAIRD, J.M.A., TATTERSALL, F.D., RUPNIAK, N.M.J., HARGREAVES, R.J. & HILL, R.G. (1993). Antinociceptive effects of NK1 receptor antagonists: comparison of behavioural and electrophysiological tests. *I.A.S.P. Publications Congress Abstracts*, p 231.

CHAPMAN, V. & DICKENSON, A.H. (1993). The effect of intrathecal administration of RP67580, a potent neurokinin 1 antagonist, on nociceptive transmission in the rat spinal cord. *Neurosci. Letts.*, **157,** 149–152.

DE KONINCK, Y. & HENRY, J.L. (1991). Substance P-mediated slow excitatory postsynaptic potential elicited in dorsal horn neurones *in vivo* by noxious stimulation. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 11344–11348.

GARRET, C., CARRUETTE, A., FARDIN, V., MOUSSAOUI, S., PEYRONEL, J.-F., BLANCHARD, P.-C. & LADURON, P.M. (1991). Pharmacological properties of a potent and selective nonpeptide substance P antagonist, RP67580. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 10208–10212.

GITTER, B.D., WATERS, D.C., BRUNS, R.F., MASON, N.R., NIXON, J.A. & HOWBERT, J.J. (1991). Species differences in affinities of non-peptide antagonists for substance P receptors. *Eur. J. Pharmacol.*, **197,** 237–238.

HARGREAVES, K., DUBNER, R., BROWN, F., FLORES, C. & JORIS, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*, **32,** 77–88.

HILL, R.G. (1994). Role of receptors in nociception. In *The Tachykinin Receptors*. ed. Buck, S.H., pp. 471–498. N.J.: Humana Press Inc.

HYLDEN, J.L.K. & WILCOX, G.L. (1980). Intrathecal morphine in mice: a new technique. *Eur. J. Pharmacol.*, **67,** 313–316.

KARLSSON, U., NASSTROM, J. & BERGE, O.-G. (1994). (±)-CP-96,345, an NK_1 receptor antagonist, has local anaesthetic-like effects in a mammalian sciatic nerve preparation. *Regul. Pept.*, **52,** 39–46.

LAIRD, J.M.A., HARGREAVES, R.J. & HILL, R.G. (1993). Effect of RP67580, a non-peptide neurokinin$_1$ receptor antagonists, on facilitation of a nociceptive, spinal flexion reflex in the rat. *Br. J. Pharmacol.*, **109,** 713–718.

LOMBET, A. & SPEDDING, M. (1994). Differential effects of non-peptidic tachykinin receptor antagonists on Ca^{2+} channels. *Eur. J. Pharmacol.*, **267,** 113–115.

MALMBERG, A.B. & YAKSH, T.L. (1992). Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. *Science*, **257,** 1276–1278.

MARCHAND, J.E., WURM, W.H., KATO, T. & KREAM, R.M. (1994). Altered tachykinin expression by dorsal root ganglion neurones in a rat model of neuropathic pain. *Pain*, **58,** 219–231.

MCCARSON, K.E. & GOLDSTEIN, B.D. (1990). Timecourse of the alteration in substance P levels in the dorsal horn following formalin: blockade by naloxone. *Pain*, **41,** 95–100.

MCLEAN, S., GANONG, A., SEYMOUR, P.A., SNIDER, R.M., DESAI, M.C., ROSEN, T., BRYCE, D.K., LONGO, K.P., REYNOLDS, L.S., ROBINSON, G., SCHMIDT, A.W., SIOK, C. & HEYM, J. (1993). Pharmacology of CP-99,994; a nonpeptide antagonist of the tachykinin NK_1 receptor. *J. Pharmacol. Exp. Ther.*, **267,** 472–479.

MOUSSAOUI, S.M., CARRUETTE, A., MONTIER, F. & GARRET, C. (1992). RP67580, a non-peptide substance P antagonist, inhibits neurogenic inflammation and possesses antinociceptive activities in rodents. *Neuropeptides*, **22,** 26.

NAGAHISA, A., ASAI, R., KANAI, Y., MURASE, A., TSUCHIYA-NAKAGAKI, M., NAKAGAKI, T., SHIEH, T.-C. & TANIGUCHI, K. (1992). Non-specific activity of (±)-CP-96,345 in models of pain and inflammation. *Br. J. Phamacol.*, **107,** 273–275.

RADHAKRISHNAN, V. & HENRY, J.L. (1991). Novel substance P antagonist, CP-96,345, blocks responses of cat spinal dorsal horn neurons to noxious cutaneous stimulation and to substance P. *Neurosci. Letts.*, **132,** 39–43.

RUPNIAK, N.M.J., BOYCE, S., WILLIAMS, A.R., COOK, G., LONGMORE, J., SEABROOK, G.R., CAESER, M., IVERSEN, S.D. & HILL, R.G. (1993). Antinociceptive activity of NK_1 receptor antagonists: non-specific effects of racemic RP67580. *Br. J. Pharmacol.*, **110,** 1607–1613.

RUPNIAK, N.M.J. & JACKSON, A. (1994). Non-specific inhibition of dopamine receptor agonist-induced behaviour by the tachykinin NK_1 receptor antagonist CP-99,994 in guinea-pigs. *Eur. J. Pharmacol.*, **262,** 171–175.

RUPNIAK, N.M.J. & WILLIAMS, A.R. (1994). Differential inhibition of foot tapping and chromodacryorrhoea in gerbils by CNS penetrant and non-penetrant tachykinin NK_1 receptor antagonists. *Eur. J. Pharmacol.*, **267,** 215–223.

SAKURADA, T., KATSUMATA, K., YOGO, H., TANONO, K., SAKURADA, S., OHBA, M. & KISARA K. (1994). The neurokinin-1 receptor antagonist, sendide, exhibits antinociceptive activity in the formalin test. *Pain*, **60,** 175–180.

SCHMIDT, A.W., MCLEAN, S. & HEYM, J. (1992). The substance P receptor antagonist CP-96,345 interacts with Ca^{2+} channels. *Eur. J. Pharmacol.*, **219,** 491–492.

SMITH, G., HARRISON, S., BOWERS, J., WISEMAN, J. & BIRCH, P. (1994). Non-specific effects of the tachykinin NK1 receptor antagonist, CP-99,994, in antinociceptive tests in rat, mouse and gerbil. *Eur. J. Pharmacol.*, **271,** 481–487.

SUAREZ, G.A., OPFER-GEHRKING, T.L., MACLEAN, D.B. & LOW, P.A. (1994). Double-blind, placebo-controlled study of the efficacy of a substance P (NK_1) receptor antagonist in painful peripheral neuropathy. *Neurology*, **44,** (Suppl 2), A220.

XU, X.-Y., DALSGAARD, C.J. & WIESENFELD-HALLIN, Z. (1992). Intrathecal CP-96,345 blocks reflex facilitation induced in rats by substance P and C-fibre conditioning stimulation. *Eur. J. Pharmacol.*, **216,** 337–344.

YAMAMOTO, T. & YAKSH, T.L. (1991). Stereospecific effects of a nonpeptidic NK1 selective antagonist, CP-96,345: antinociception in the absence of motor dysfunction. *Life Sci.*, **49,** 1955–1963.

YAMAMOTO, T. & YAKSH, T.L. (1992). Effects of intrathecal capsaicin and an NK-1 antagonist, CP-96,345, on the thermal hyperalgesia observed following unilateral constriction of the sciatic nerve in the rat. *Pain*, **51,** 329–334.

YASHPAL, K., RADHAKRISHNAN, V., CODERRE, T.J. & HENRY, J.L. (1993). CP-96,345, but not its stereoisomer, CP-96,344, blocks the nociceptive responses to intrathecally administered substance P and to noxious thermal and chemical stimuli in the rat. *Neuroscience*, **52,** 1039–1047.

ZIMMERMANN, M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain*, **16,** 109–110.

(Received March 21, 1995
Revised June 5, 1995
Accepted June 12, 1995)