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L-733,060, a novel tachykinin NK_1 receptor antagonist; effects in $[Ca^{2+}]_i$ mobilisation, cardiovascular and dural extravasation assays

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Abstract

This study investigated the properties of a novel piperidine ether-based tachykinin NK_1 receptor antagonist L-733,060, ((2*S*,3*S*)-3-((3,5-bis(trifluoromethyl)phenyl)methyloxy)-2-phenyl piperidine and its 2*R*,3*R*-enantiomer L-733,061 on $[Ca^{2+}]_i$ mobilisation in Chinese hamster ovary cells transfected with human tachykinin NK_1 receptors, compared to their effects in rodent cardiovascular and neurogenic plasma extravasation assays. Using FURA-2-imaging techniques, L-733,060 inhibited substance P-induced $[Ca^{2+}]_i$ mobilisation with an estimated affinity of 0.8 nM whereas L-733,061 (30–300 nM) did not. No significant effects of L-733,060 were observed on mean arterial blood pressure or heart rate in conscious or anaesthetised rats at doses of < 3000 μg kg^{-1} i.v. L-733,060 also stereoselectively inhibited neurogenic plasma extravasation in rat dura produced by electrical stimulation of trigeminal nerves with an ID_{50} of 212 ± 19 μg kg^{-1} i.v. Thus, L-733,060 is a novel antagonist of human tachykinin NK_1 receptors which stereoselectively inhibits neurogenic plasma extravasation at doses that do not cause adverse cardiovascular effects.

Keywords: Substance P; Tachykinin NK_1 receptor; Plasma extravasation; Migraine; Pain

1. Introduction

The peptide substance P is an endogenous mammalian neuromodulator that acts preferentially on tachykinin NK_1 receptors. Substance P is thought to be involved in the perception of pain since it is released in response to tissue damage/injury from sensory nerve terminals in the periphery as well as within the dorsal horn of the spinal cord (Hökfelt et al., 1975; Duggan et al., 1988; Malcangio and Bowery, 1995). Substance P has also been implicated in the pathogenesis of migraine as it is thought to be released from trigeminal sensory nerves which innervate pain producing cranial structures that when activated experimentally can produce neurogenic inflammation within the dura mater (Moskowitz, 1993).

Since the original identification of the quinuclidine CP-96,345 as a high-affinity non-peptide antagonist of the human tachykinin NK_1 receptor subtype (Snider et al., 1991), it has been shown that such antagonists can have non-specific actions on voltage-dependent Na^+ (Caeser et al., 1993) and Ca^{2+} channels (Schmidt et al., 1992; Guard et al., 1993). In particular, CP-96,345 is 70-fold more effective at inhibiting neuronal sodium currents than the local anaesthetic lignocaine (Caeser et al., 1993). Some of the analgesic-like properties of CP-96,345 are also seen with its relatively inactive 2*R*,3*R*-enantiomer (Nagahisha et al., 1992) and this probably reflects non-specific block of cation channels. Consequently, the significance of tachykinin NK_1 receptors in some in vivo anti-nociceptive and anti-inflammatory assays has remained ambiguous. Block of ion channels by tachykinin NK_1 receptor antagonists may also result in adverse cardiovascular side-effects (Lembeck et al., 1992; Constantine et al., 1994). Despite this, we have recently shown that neurogenic plasma extravasation in the dura of rats (Shepheard et al., 1993) and the stimulus-induced facilitation of spinal cord nociceptive reflexes (Laird et al., 1993) are inhibited by RP 67580, a non-peptide antagonist which has high affinity for rodent tachykinin NK_1 receptors. Furthermore, this activity was not seen with the 3*S*,7*S*-enantiomer of RP 67580 which binds with low affinity to tachykinin NK_1 receptors. Extravasation in the dura mater caused by application of

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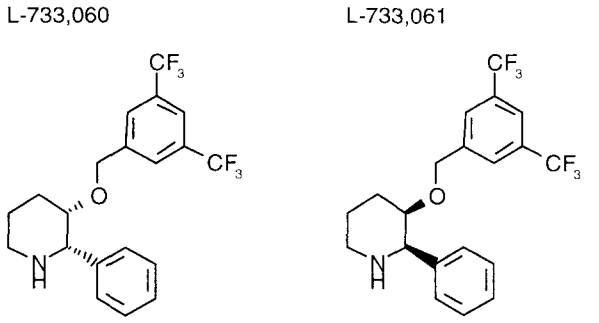

Fig. 1. Chemical structure of L-733,060 and its 2*R*,3*R*-enantiomer L-733,061.

exogenous substance P is also blocked by tachykinin NK_1 receptor antagonists (Moussaoui et al., 1993). Thus, tachykinin NK_1 receptors may, indeed, have a functional role in some forms of pain and inflammation.

In this study, we describe the ability of a novel stereoselective antagonist of human tachykinin NK_1 receptors, L-733,060 ((2*S*,3*S*)-3-((3,5-bis(trifluoromethyl) phenyl)methyloxy)-2-phenyl piperidine; Harrison et al., 1994), shown together with its 2*R*,3*R*-enantiomer L-733,061 in Fig. 1, to inhibit Ca^{2+} mobilisation in human tachykinin NK_1 receptor-transfected Chinese hamster ovary (CHO) cells and dural extravasation in rats. To determine whether L-733,060 and L-733,061 had any adverse cardiovascular properties, their effects on heart rate and blood pressure were examined in anaesthetised rats and gerbils as well as in conscious rats. Conscious rats were studied to eliminate the possibility that effects were altered due to the anaesthesia. Here, we show that L-733,060 is a highly effective tachykinin NK_1 receptor antagonist which inhibits neurogenic plasma extravasation at doses that do not cause adverse cardiovascular effects in rodents.

2. Materials and methods

2.1. Ca^{2+} mobilisation in CHO cells transfected with human tachykinin NK_1 receptors

The mobilisation of $[Ca^{2+}]_i$ in Chinese hamster ovary cells transfected with human tachykinin NK_1 receptors was studied using FURA-2-imaging techniques (e.g., Seabrook and Fong, 1993). Cells were plated onto poly-D-lysine-coated coverslips and were loaded immediately prior to experimentation with the Ca^{2+}-sensitive fluorescent dye FURA-2. Cells were bathed in the membrane permeant acetoxymethyl form of FURA-2 (2 μM) for 40 min and were then washed in extracellular solution that contained (in mM): NaCl (150), KCl (3), Hepes (10), glucose (10), sucrose (20), $CaCl_2$ (2) and $MgCl_2$ (2) at pH 7.4. Cells were treated with L-733,060, L-733,061 or vehicle (0.1% dimethylsulfoxide; DMSO), for 60 min and then the ability of a maximally effective concentration of substance P (100 nM) to mobilise $[Ca^{2+}]_i$ was examined. The fluorescence intensity at 510 nm caused by alternate excitation with 340- and 380-nm wavelength light was used to estimate the mean intracellular $[Ca^{2+}]_i$ levels according to the methods of Grynkiewicz et al. (1985). To determine the functional affinity of L-733,060 for human tachykinin NK_1 receptors, the ability of this ligand to displace the substance P concentration-effect curve was also examined. The EC_{50} for substance P was determined in control conditions (1 h in 0.1% DMSO) and then in a separate set of experiments to avoid tachyphylaxis was determined after 1 h equilibration in L-733,060. The estimated antagonist affinity, assuming competitive antagonism, was calculated from the shift in the concentration-effect curve using the following equation, $K_A = [\text{antagonist}]/(\text{concentration ratio} - 1)$.

2.2. Cardiovascular effects in rats and gerbils

The effects of administration of L-733,060 and L-733,061 on blood pressure and heart rate were examined in conscious rats and in anaesthetised rats and gerbils. For conscious rat studies, male Sprague-Dawley rats (250–300 g) were anaesthetised by inhalation of isoflurane and a tail artery and vein were cannulated for measurement of arterial blood pressure and heart rate, and i.v. administration of drugs respectively. Animals were placed in a quiet room in open restraining cages that allow movement, but prevent turning to gain access to the vascular cannulae, and were allowed 2 h to recover from surgery before experimentation. For anaesthetised animal studies, male Sprague-Dawley rats (250–300 g) or male Mongolian gerbils (70–100 g) were anaesthetised with pentobarbitone sodium (60 mg kg^{-1}, i.p.) and a carotid artery cannulated for measurement of arterial blood pressure and heart rate. A jugular vein was cannulated for i.v. administration of drugs and supplementary doses of anaesthetic (pentobarbitone sodium, 10 mg kg^{-1}) as required. Rectal temperature was monitored and maintained between 37–38°C using a homeothermic blanket system. In all experiments, an i.v. injection of vehicle (distilled water) followed by sequential rising doses (300–3000 μg kg^{-1}) of either L-733,060 or L-733,061 ($n = 3$–5/group) were given as an infusion over 30 s at 15-min intervals. There were no effects of repeated injection of vehicle (distilled water) on blood pressure or heart rate in either conscious or anaesthetised rats. Measurements of heart rate and blood pressure were made as the mean value in the minute before vehicle injection (baseline), as the maximum effect immediately (0–30 s) after injection and as the mean value at 1–2, 5–6, 10–11 and 14–15 min after each injection. Data is presented as actual heart rate and blood pressure or as the percentage change from vehicle. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with repeated measures design and within-group contrast analysis.

2.3. Neurogenic plasma extravasation

In male Sprague-Dawley rats (200 g) anaesthetised with pentobarbitone sodium (60 mg kg^{-1} i.p.), dural plasma extravasation produced by electrical stimulation of the right trigeminal ganglion was measured using [^{125}I]bovine serum albumin as a plasma marker as described by Shepheard et al. (1995). The effects of i.v. L-733,060 (10–1000 μg kg^{-1} i.v.), L-733,061 (1000 μg kg^{-1} i.v.) or distilled H_2O vehicle (n = 8–10/group) on this dural extravasation

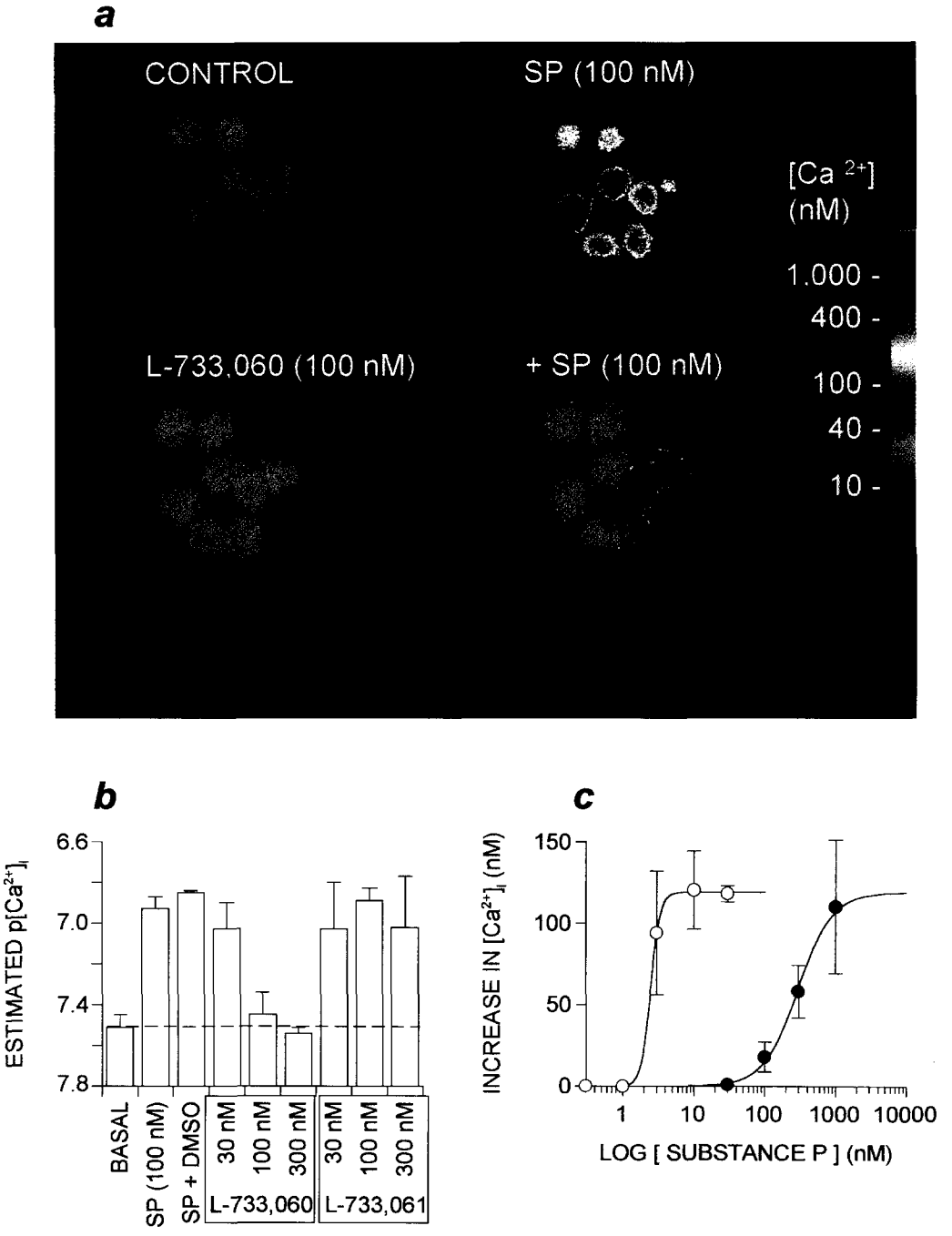

Fig. 2. Inhibition of substance P-induced Ca^{2+} mobilisation in human tachykinin NK_1 receptor-transfected Chinese hamster ovary cells. (a) FURA-2 fluorescence ratio signal (see Section 2) from a field of eight cells in control conditions and 40 s after application of substance P (100 nM), compared to that after 1 h equilibration in L-733,060 (100 nM). (b) Histogram comparing the effects of substance P (100 nM) on estimated intracellular Ca^{2+} concentrations in vehicle (0.1% dimethysulfoxide, DMSO) or cells treated with L-733,060 (30–300 nM) or L-733,061 (30–300 nM). Dotted line represents the negative logarithm of the estimated intracellular Ca^{2+} concentration, $p[Ca^{2+}]_i$, under control conditions which was 7.51, equivalent to 31 nM. (c) Rightward displacement of the control concentration-effect curve for substance P (EC_{50} = 2.4 nM, open circles) by 100 nM L-733,060 (EC_{50} = 295 nM; filled circles). Data represent the mean ± S.E.M. of 19–23 cells.

were examined. An extravasation ratio, i.e., the ratio of the extravasation in the stimulated to unstimulated sides was calculated. The half-maximally effective dose (ID_{50}) of L-733,060 was determined using Grafit (Erithacus Software). Statistical analysis was carried out using one-way ANOVA and Students *t*-test.

2.4. Drugs

L-733,060 ((2*S*,3*S*)-3-((3,5-bis(trifluoromethyl)phenyl)methyloxy)-2-phenyl piperidine and its 2*R*,3*R*-enantiomer L-733,061 were synthesised at Merck Sharp and Dohme Research Laboratories as previously described (Harrison et al., 1994) and both compounds were used as their hydrochloride salts. Substance P was purchased from Calbiochem and the acetoxymethyl form of FURA-2 from Molecular Probes. Doses refer to the base weight and compounds were dissolved in distilled H_2O and were administered at a volume of 1 ml kg^{-1}. For Ca^{2+}-imaging studies, L-733,060 was dissolved in DMSO.

3. Results

3.1. Ca^{2+} mobilisation in Chinese hamster ovary cells transfected with human tachykinin NK_1 receptors

L-733,060 inhibited the $[Ca^{2+}]_i$ mobilisation caused by substance P (100 nM) in a concentration-dependent manner (Fig. 2). In control conditions, substance P (100 nM) caused a mean increase in estimated $[Ca^{2+}]_i$ levels from 31 to 118 nM ($n = 17$ cells from 3 experiments; Fig. 2b). After 1 h incubation in vehicle (0.1% DMSO), this response was unchanged but after treatment with L-733,060 the effects of substance P were significantly attenuated and almost entirely abolished at a concentration of 300 nM. In contrast, the substance P-induced $[Ca^{2+}]_i$ mobilisation in the presence of L-733,061 (30–300 nM) was unaffected (Fig. 2). The half-maximally effective concentration (EC_{50}) of substance P under control conditions (0.1% DMSO) was 2.4 ± 0.7 nM ($n = 19$ cells). In the presence of L-733,060 (100 nM), this concentration-effect curve was shifted to the right in a surmountable manner giving an EC_{50} of 295 ± 17 nM ($n = 23$; Fig. 2c). This shift in the concentration-effect curve was equivalent to an estimated affinity of 0.8 nM (estimated $pK_A = 9.10$).

Table 1
Cardiovascular effects of L-733,060 and L-733,061 in barbiturate-anaesthetised rats and gerbils

Species	Dose (μg kg^{-1} i.v.)	L-733,060		L-733,061	
		BP (%)	HR (%)	BP (%)	HR (%)
Rat	300	−4 ± 3	0 ± 2	−9 ± 5	−3 ± 2
	1000	−20 ± 6	−5 ± 3	−31 ± 4 [a]	−8 ± 4
	3000	−51 ± 3 [a]	−14 ± 4	−65 ± 2 [a]	−17 ± 7
Gerbil	300	−1 ± 1	−7 ± 2	−3 ± 1	−5 ± 5
	1000	−3 ± 3	−11 ± 4	−2 ± 2	−4 ± 7
	3000	−16 ± 5	−18 ± 4	−17 ± 6	−9 ± 8

Data are mean ± S.E.M. [a] $P < 0.05$ from vehicle (one-way ANOVA within-group comparison).

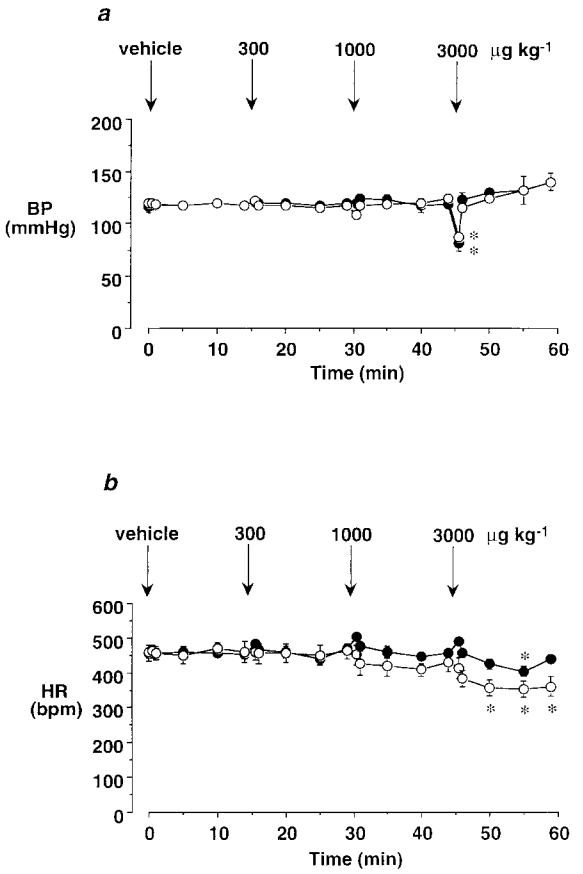

Fig. 3. Cardiovascular effects of L-733,060 and L-733,061 in conscious rats. (a) Time course of changes in mean arterial blood pressure (BP) and (b) in mean heart rate during i.v. administration of vehicle (distilled H_2O) and increasing doses of L-733,060 (filled circles) and L-733,061 (open circles). Significant differences from vehicle heart rate and blood pressure were observed with L-733,060 and L-733,061 only at a dose of 3000 μg kg^{-1} i.v. ($P < 0.05$ denoted by asterisks; one-way ANOVA). Data are expressed as mean ± S.E.M. of 3 animals/group.

3.2. Cardiovascular effects in rats and gerbils

In conscious rats, administration of L-733,060 had no significant effects at doses of < 3000 μg kg^{-1} i.v.; however, at this dose, there was an immediate but transient decrease in mean blood pressure from 116 ± 5 to 82 ± 8 mm Hg (−30 ± 5%) which returned to baseline within 1 min (Fig. 3). This was accompanied by a decrease in mean heart rate from 457 ± 23 to 403 ± 16 beats min^{-1} (−12 ± 2%) which began to return towards baseline within 15 min. The same profile of effects were observed following administration of L-733,061 since there were no significant effects at doses of < 3000 μg kg^{-1} but, at this dose, there was a transient decrease in mean blood pressure from

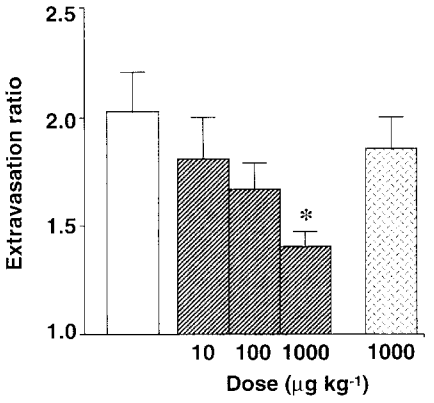

Fig. 4. Inhibition of electrically stimulated plasma extravasation in dura mater of rat by L-733,060. Plasma extravasation was elicited by electrical stimulation of the right trigeminal ganglion (see Section 2) and the extravasation ratio is expressed as the fraction of extravasation on stimulated vs. unstimulated sides. Histogram shows the effect of pre-treatment (i.v.) with distilled H_2O vehicle (open bar), L-733,060 (10–1000 μg kg^{-1}; hatched bars) or with L-733,061 (1000 μg kg^{-1}; stippled bar). Data are expressed as mean ± S.E.M. Asterisks denote $P < 0.05$ relative to vehicle (unpaired Student's t-test). $n = 8–10$ animals for each group.

120 ± 7 to 87 ± 9 mm Hg (−27 ± 5%) and a decrease in mean heart rate from 460 ± 19 to 353 ± 23 beats min^{-1} (−23 ± 2%) (Fig. 3). Similarly, in anaesthetised rats, no significant hypotensive or bradycardic effects were observed with L-733,060 at doses of < 3000 μg kg^{-1} i.v. although significant hypotension was observed with L−733,061 at 1000 μg kg^{-1} (Table 1). No significant hypotensive or bradycardic effects were observed with L-733,060 or L-733,061 in anaesthetised gerbils at doses up to 3000 μg kg^{-1} i.v.

3.3. Neurogenic plasma extravasation

Following electrical stimulation of the trigeminal ganglion in vehicle-treated rats, the extravasation ratio in dura was 2.03 ± 0.18. Pretreatment with L-733,060 (10–1000 μg kg^{-1} i.v.) produced a statistically significant dose-related inhibition of this extravasation with an ID_{50} of 212 ± 19 μg kg^{-1} (Fig. 4). In contrast, L-733,061 (1000 μg kg^{-1} i.v.) had no significant effects on plasma extravasation.

4. Discussion

This study describes the stereoselective effects of the novel tachykinin NK_1 receptor antagonist, L-733,060 on human tachykinin NK_1 receptors and upon plasma extravasation in rat dura. These data provide further evidence to support the concept that tachykinin NK_1 receptors may be involved in mediating the plasma extravasation component of neurogenic inflammation in the dura mater of rodents and, therefore, may be involved in the pathogenesis of migraine (Moskowitz, 1993).

L-733,060 displaces substance P binding to human tachykinin NK_1 receptors with an affinity (0.8 ± 0.5 nM) 400-fold higher than its weaker enantiomer L-733,061 (340 nM; Harrison et al., 1994). The effects of L-733,060 are selective for human tachykinin NK_1 over NK_2 and NK_3 receptors (binding affinities at tachykinin NK_2 and NK_3 receptors > 1000 nM) although some affinity for benzothiazepine-binding sites on L-type Ca^{2+} channels is observed at high concentrations (IC_{50} vs. [^{3}H]diltiazem binding in rabbit smooth muscle = 760 nM; Harrison et al., 1995). Like some other classes of non-peptide tachykinin NK_1 receptor antagonists, L-733,060 had a lower affinity for rat cloned tachykinin NK_1 receptors expressed in Chinese hamster ovary cells (550 nM; M.A. Cascieri et al., unpublished observations). These variations in binding affinity across species are partly due to the non-peptide antagonist interacting with different epitopes to which the peptide agonists bind on the receptor (Fong et al., 1992).

As predicted from the ability of L-733,060 to displace substance P binding to cloned tachykinin NK_1 receptors, L-733,060 inhibited the Ca^{2+} mobilisation caused by receptor activation in tachykinin NK_1 receptor-transfected Chinese hamster ovary cells. Using FURA-2-imaging techniques, this effect was shown to be selective for the 2*S*,3*S*-enantiomer which preferentially binds to tachykinin NK_1 receptors and, thus, was unlikely to involve non-selective effects, such as inhibition of Ca^{2+} entry, which is known to contribute to Ca^{2+} mobilisation in these cells (Seabrook and Fong, 1993). From the rightward displacement of the substance P concentration-effect curve, the functional affinity of L-733,060 for human tachykinin NK_1 receptors was estimated to be 0.8 nM, which was identical to that measured in binding studies.

Tachykinin NK_1 receptor antagonists that block cation channels have been shown to cause bradycardia and hypotension (Lembeck et al., 1992; Constantine et al., 1994). In the present study, bradycardia and hypotension was seen with both L-773,060 and L-773,061 at a dose of 3000 μg kg^{-1}. Both of these enantiomers have similar affinity for Ca^{2+} channels but differ some 300-fold in their affinity for tachykinin NK_1 receptors. Since the cardiovascular effects showed no enantiomeric selectivity, it is unlikely that they were due to antagonism of tachykinin NK_1 receptors but more probably as a consequence of their affinity for Ca^{2+} channels. In addition, it has been shown that tachykinin NK_1 receptors in gerbils have a more similar pharmacology to human tachykinin NK_1 receptors than the rat (Beresford et al., 1991). Therefore, if the cardiovascular effects of L-733,060 involved tachykinin NK_1 receptors we would expected them to be more pronounced in the gerbil. However, less bradycardia and hypotension occurred in gerbils than in rats, further indicating that it was unlikely to involve tachykinin NK_1 receptors.

Neurogenic inflammation in the meninges has been proposed (Moskowitz, 1993) to contribute to the pathogen-

esis of migraine and this may, in part, be mediated via activation of tachykinin NK_1 receptors (Shepheard et al., 1993). Compounds with clinical efficacy in the treatment of migraine, e.g., 5-HT receptor agonists, have been shown to block plasma extravasation produced in the dura mater of rodents by electrical stimulation of trigeminal ganglia (Moskowitz, 1993; Shepheard et al., 1995). Consequently, we examined the ability of L-733,060 to inhibit plasma extravasation in rat dura. Despite a lower affinity for rat vs. human tachykinin NK_1 receptors, L-733,060 inhibited dural plasma extravasation in the rat produced by electrical stimulation of the trigeminal ganglion at a dose (ID_{50} of 212 $\mu g\ kg^{-1}$ i.v.) at least 10-fold lower than that producing significant hypotension. Therefore, it is unlikely that the reduction in extravasation was a consequence of functional antagonism due to a reduction in tissue perfusion pressure. The inhibition of neurogenic plasma extravasation by L-733,060 is likely to be due to a specific action on tachykinin NK_1 receptors since L-733,061, the enantiomer of L-733,060 that is less active at tachykinin NK_1 receptors, was inactive at a dose of 1000 $\mu g\ kg^{-1}$ i.v.

The present finding that L-733,060 inhibits electrically evoked dural plasma extravasation in the rat is in line with our previous studies using the rat tachykinin NK_1 receptor-selective antagonist RP 67580 and the prototypic human tachykinin NK_1 receptor antagonist CP 99,994 (Shepheard et al., 1993, 1995). The relatively modest activity of L-733,060 ($ID_{50} = 212\ \mu g\ kg^{-1}$ i.v.) in the dural extravasation assay compared to RP 67580 ($ID_{50} = 0.6\ \mu g\ kg^{-1}$ i.v.) is consistent with its weaker affinity at rat tachykinin NK_1 receptors (L-733,060 $IC_{50} = 550$ nM and RP 67580 $IC_{50} = 5$ nM). Similar comparisons of the activity of L-733,060 in the dural extravasation assay with another human tachykinin NK_1-selective antagonist, CP-99,994 shows that their activities are again broadly in line with the difference in their affinity for rat tachykinin NK_1 receptors (CP 99,994 $IC_{50} = 200$ nM; ID_{50} for inhibition of dural extravasation $= 52\ \mu g\ kg^{-1}$ i.v.; Shepheard et al., 1995). The activity ratio of test compounds in vivo will be influenced by differences in their pharmacokinetic characteristics unlike their activity in radioligand-binding studies in vitro. Thus, it is not surprising that there are some small discrepancies between the IC_{50} ratio of compounds in vitro compared to their in vivo activity.

The present results with L-733,060 provide further evidence for the involvement of tachykinin NK_1 receptors, activated by neurokinins released from perivascular trigeminal sensory nerves, in mediating the plasma extravasation component of neurogenic inflammation in the dura mater of rodents. If this peripheral inflammatory mechanism is involved in the pathogenesis of migraine, then compounds, like L-733,060, may be useful in its treatment with the advantage that they may have a reduced risk of cardiovascular side-effects relative to earlier tachykinin NK_1 receptor antagonists and exisiting therapies.

In addition to L-733,060, a number of other second generation non-peptide tachykinin NK_1 receptor antagonists have recently been described which also have reduced ion channel activity. These include CP-99,994 (McLean et al., 1993), SR 140333 (Emons-Alt et al., 1993), LY 303870 (Hipskind et al., 1995) and GR 203040 (Beattie et al., 1995). The availability of such a diverse array of non-peptide tachykinin NK_1 receptor antagonists will inevitably facilitate the characterisation of the functional role of tachykinin NK_1 receptors in pain and inflammation and may also provide the basis for novel analgesics and anti-inflammatory agents. Indeed, L-733,060 has recently been shown to have centrally mediated antinociceptive effects in conscious gerbil formalin paw tests (Rupniak et al., 1996) and to attenuate spinal cord nociceptive responses (Cumberbatch et al., 1995). Clinical evaluation of non-brain penetrant and brain penetrant tachykinin NK_1 receptor antagonists (see Shepheard et al., 1995) are required to determine whether peripheral NK_1 receptor-mediated meningeal inflammatory mechanisms are involved in migraine headache pain and to further understand the pathophysiology of this disorder.

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