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Attenuation of the gerbil writhing response by μ-, κ- and δ-opioids, and NK-1, -2 and -3 receptor antagonists

Elizabeth L. Gallantine[a,b], Theo F. Meert[a,*]

[a]*R&D, PRD Johnson & Johnson, Turnhoutseweg 30, B-2340 Beerse, Belgium*

[b]*School of Clinical and Laboratory Sciences, Faculty of Medical Sciences, Medical School, University of Newcastle-Upon-Tyne NE2 4HH, UK*

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Abstract

μ-, κ- and δ-opioid receptor agonists are reported to attenuate the acetic-acid-induced abdominal constriction response in mice. NK-1, -2 and -3 receptor antagonists also display activity in several visceral pain models. As the gerbil NK-1 receptor is comparable to the human receptor, we evaluated the efficacy of NK-1, -2 and -3 receptor antagonists and opioids (both alone and in combination) in the writhing test in this species. The effects of a selective L-type calcium (Ca^{2+}) channel antagonist on the writhing response were also assessed to determine the contribution of Ca^{2+} channel antagonism to the antinociceptive effects of the NK-1 antagonists. Gerbils received subcutaneous injections of either the μ-opioids morphine or fentanyl, the κ-opioid U50,488-H, the δ-opioid SNC80, NK-1 antagonists R116301, CP-96,345 or GR203040, the NK-2 antagonist SR-48968, the NK-3 antagonist SR-142801 or the Ca^{2+} channel antagonist nimodipine. Writhing was evoked 1 h after treatment by intraperitoneal injection of 0.2 ml 1% acetic acid solution and the frequency was recorded. Morphine, fentanyl and U50,488-H attenuated the writhing response dose dependently with complete inhibition occurring at the highest doses. SNC80 did not significantly attenuate the writhing response even at a dose of 40 mg/kg. The tachykinin NK-1 antagonists CP-96,345 and GR203040, the NK-2 antagonist SR-48968 and the NK-3 antagonist SR-142801 reduced the writhing frequency although without complete inhibition. The NK-1 antagonist R116301 displayed limited activity at doses up to 40 mg/kg. Nimodipine did not exhibit any antinociceptive efficacy in this assay. Adding the NK-1, -2 or -3 antagonists to the opioids did not improve the efficacy of the opioids. Selective NK antagonists may therefore be effective in a visceral nociception assay in gerbils but do not modulate opioid action.

Keywords: Opioid; Tachykinin; Writhing; Visceral; Gerbil; NK-1; NK-2; NK-3; Calcium channel antagonist

1. Introduction

Neuroanatomical studies of the neurokinin family have indicated that substance P together with neurokinin A and neurokinin B may be present in many parts of the nociceptive system (Ribeiro-da-Silva and Hokfelt, 2000). The transmission of visceral nociception has been shown to be mediated by these neuropeptides acting on peripheral NK-1, NK-2 and NK-3 receptors (Julia et al., 1999). In rats, a high proportion of visceral afferent neurons (>80%) contain substance P compared to 25% of cutaneous afferents. Furthermore, the highest concentration of NK-1 receptors in the spinal cord can be found in the regions where visceral afferents terminate (laminae I and V; Laird et al., 2000). By combining an NK antagonist with a μ-opioid receptor agonist, it may be possible to simultaneously target two of the systems involved in visceral nociception. This may provide a method of enhancing the efficacy of opioids whilst reducing the dosage and risk of side effects. This can be achieved because NK-1 and μ-opioid receptors often colocalize on primary afferent nerve terminals containing SP (Reichert et al., 2001; Bueno et al., 1997; Lembeck and Donnerer, 1985; Aimone and Yaksh, 1989; Jessell and Iversen, 1977) and because μ-agonists act directly on the primary afferent terminals of SP-containing neurons to

* Corresponding author. Tel.: +32 14 60 32 14; fax: +32 14 60 59 44.
E-mail address: tmeert@prdbe.jnj.com (T.F. Meert).

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presynaptically modulate tachykinin (e.g., SP) release from peripheral nerve endings (Satoh and Minami, 1995; Minami et al., 1995; Aicher et al., 2000). The antinociceptive effects of κ-agonists can partly be attributed to a similar mechanism of action as μ (Millan, 1990) whilst δ-opioids may modulate the release of transmitters other than SP due to the scarce expression of δ-opioid receptor mRNA in SP-containing neurons (Satoh and Minami, 1995; Minami et al., 1995).

Despite the well-proven efficacy of μ-opioid agonists, such as morphine and fentanyl, their use is limited by the undesirable side effects associated with such compounds, including constipation, nausea and vomiting, some risk of respiratory depression, dependence liability, and in some cases, tolerance. There is therefore considerable interest in developing therapeutically useful agonists for other opioid receptor subtypes. Although not entirely free of undesirable properties, κ- and δ-agonists have been shown to mediate analgesia without several of the subjective and physical side effects of μ-opioids (Millan, 1990). However, the intrinsic analgesic potency of these opioids is limited as compared to μ-agonists.

The chemical-induced abdominal constriction assay is a classical model used to evaluate acute visceral pain. An intraperitoneal injection of an irritant induces pain and evokes the writhing response. The μ-agonists morphine and fentanyl block acetic-acid-induced nociception efficiently when given at the site of irritation, suggesting a peripheral opioid component in visceral pain. (Reichert et al., 2001). κ-opioid receptor knockout mice display a heightened response to visceral pain, indicating a role for κ-opioids in visceral antinociception (Simonin et al., 1998). The κ-opioid receptor agonist U50,488-H and the selective, systemically active δ-opioid receptor agonist SNC80 (Bilsky et al., 1995) have also displayed some efficacy in the acetic-acid-induced abdominal constriction assay in mice (Friese et al., 1997; Sora et al., 1999).

NK antagonists are highly species specific. In general, antagonists that bind potently to human NK-1 receptors bind with similar affinity to guinea pig and gerbil NK-1 receptors (Stout et al., 2001; Beresford et al., 1991) due to their comparable receptor sequences. It is for this reason that we considered the gerbil to be a more suitable species than rats or mice for this study. Antagonists of all three neurokinin receptor subtypes have displayed efficacy in visceral pain models. The NK-1 receptor antagonists CP-96,345 and RP-67,580 have been shown to dose dependently inhibit the acetic-acid-induced abdominal stretching in mice (Nagahisa et al., 1992; Garret et al., 1991). SR-48968, a competitive, high-affinity antagonist of the NK-2 receptor (Betancur et al., 1997) produced a dose-dependent inhibition of the jejunal-distension-induced depressor response in rats (Mclean et al., 1998) but failed to inhibit the writhing response in mice (Seguin et al., 1995). The NK-3 antagonist SR-142801 reduced both the rectocolonic inhibitory reflex and the abdominal contractions produced by colorectal distension (Julia et al., 1999).

In this study, we also studied the effects of the selective NK-1 receptor antagonists GR203040 and R116301 in the acetic-acid-induced writhing assay. Both compounds gain rapid access to the CNS following peripheral administration and have relatively long durations of action (Beattie et al., 1995; Romerio et al., 1999; Challet et al., 2001). Preclinical data obtained with R116301 have demonstrated its potency and selectivity for NK-1 receptors in a range of assays (Megens et al., 2002).

CP-96,345 has been shown to antagonise L-type calcium (Ca^{2+}) channels at high doses (Schmidt et al., 1992). GR203040 produces only a weak effect on L-type Ca^{2+} channels (Beattie et al., 1995). R116301 has a low affinity for rat Ca^{2+} ion channel ligand binding sites (Megens et al., 2002). Ca^{2+} channel antagonists have been shown to exert an antinociceptive effect in some animal models (Zharkovsky et al., 1999). We studied the effects of the selective L-type Ca^{2+} channel antagonist nimodipine in the writhing assay to determine if the antinociceptive effects of the NK-1 antagonists could be partially attributed to the Ca^{2+} channel antagonism.

The present study was undertaken to (1) determine the potency of μ-, δ-, and κ-opioids, and NK-1, -2 and -3 receptor antagonists in the acetic-acid-induced abdominal constriction assay in gerbils; (2) to investigate the effect of coadministration of opioids and NK antagonists; (3) to assess the effects of Ca^{2+} channel antagonism in the writhing test and (4) to validate the use of gerbils as a suitable species in the acetic-acid-induced abdominal constriction assay.

2. Materials and methods

2.1. Animals

Experiments were performed on adult female gerbils (*Meriones unguiculatus*, *Crl(MON)BR*, Charles River, Sulzfeld, Germany) weighing 50–60 g at the time of testing. Prior to testing, animals were housed for at least 1 week in communal cages (5–10 animals per cage) with food and water available ad libitum. The environment was maintained at a constant temperature of 22±1 °C in a 12-h light/dark cycle. Each animal was used only once. The guidelines for animal research issued by the International Association for the Study of Pain (Zimmerman, 1983) were adhered to. Approval from the Institutional Ethical Committee was obtained to perform the described experiments.

2.2. Acetic-acid-induced abdominal constriction assay

We adapted the acetic-acid-induced abdominal constriction assay (Collier et al., 1968; Liu et al., 2003). The gerbils were placed in individual rubber-floored Plexiglas observation cages that held a back mirror to facilitate observation. Different doses of the test compounds were administered subcutaneously 1 h prior to an intraperitoneal injection of 0.2

ml 1% acetic acid (+trypan blue) solution. A volume of 0.2 ml acetic acid was selected as this volume was shown to produce a highly reproducible baseline compared to lower volumes (unpublished data). Scoring of the writhing displayed by the animals began 5 min after intraperitoneal acetic acid injection as the writhing movements were too vigorous to score each writhe accurately immediately after injection. The frequency of the writhing movements was then noted from 5 to 15 min and from 50 to 60 min after acetic acid injection. Two observation periods were used to assess the effects of the test compounds on the acute and tonic components of the writhing response. A clear reduction in the number of writhes was scored as antinociception. After testing, the site of injection was examined for the presence of blue coloration in the peritoneum. Incorrectly injected animals were disregarded (<3%).

Writhing itself is characterized by a lengthways stretch of the abdomen accompanied by arching of the back and extension of the hindlimbs. One writhe was considered to have occurred with the adoption of this posture and to have terminated upon the reassumption of a 'normal position' (Seguin et al., 1995). Licking or scratching of the abdomen may precede a writhe.

2.3. Testing scheme

Dose–response curves were constructed for morphine (in water; 0.08–10 mg/kg), fentanyl (in $1H_2T$; 0.005–0.16 mg/kg), U50,488-H (in water; 0.16–40 mg/kg), SNC80 (in $1H_2T$; 10–40 mg/kg), R116301 (in water with 10% cyclodextrin; 2.5, 10 and 40 mg/kg), CP-96,345 (in $2H_2T$; 2.5, 5, 10 and 40 mg/kg), GR203040 (in water with $1H_2T$ and 20% cyclodextrin; 2.5, 5, 10, 20 and 40 mg/kg), SR-48968 (in water; 1.25, 2.5, 5, 10 and 40 mg/kg) and SR-142801 (in water with $1H_2T$ and 10% cyclodextrin; 2.5, 5, 10, 20 and 40 mg/kg; n=5–7 animals per dose).

Dose–response relationships were then reestablished for morphine (0.16–5 mg/kg) and fentanyl (0.01–0.08 mg/kg) in combination with fixed doses of R116301, CP-96,345, GR203040, SR-48968 and SR-142801 (2.5 or 10 mg/kg; n=5 animals per condition). Dose–response relationships were also reestablished for U50,488-H (0.16–2.5 mg/kg) and SNC80 (40 mg/kg only) following simultaneous administration of GR203030, SR-48968 and SR-142801 at fixed doses of either 2.5 or 10 mg/kg.

2.4. Effects of Ca^{2+} channel antagonism in the writhing response

The selective L-type Ca^{2+} channel antagonist nimodipine was used to assess the effect of Ca^{2+} channel antagonists in the acetic-acid-induced abdominal constriction assay. Animals received a subcutaneous injection of either vehicle (water with tween) or nimodipine (2.5, 5, 10 or 40 mg/kg; n=7 animals per dose) 1 h prior to the intraperitoneal acetic acid injection.

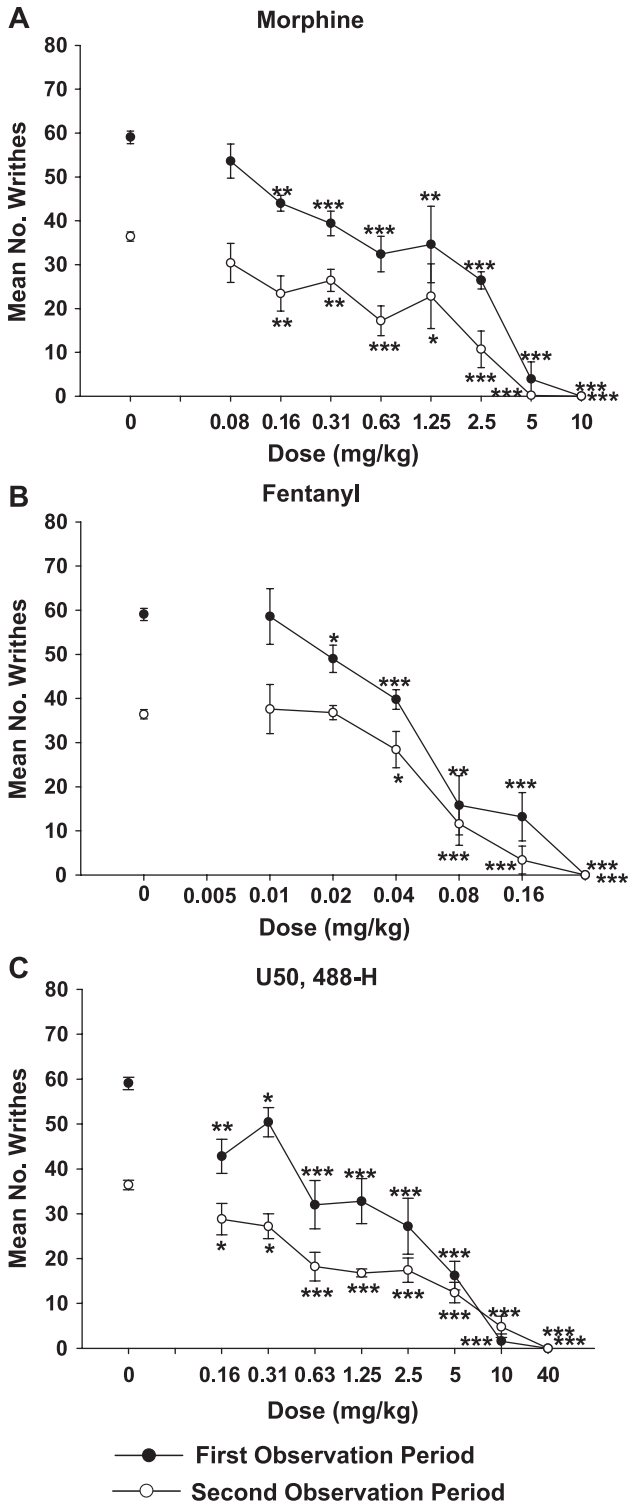

Fig. 1. Effects of μ-, δ- and κ-opioids in the writhing test in gerbils. The mean±S.E.M. values of the number of writhes in the first and second observation periods are shown. Significant, dose-dependent reductions in the writhing frequency were observed with (A) morphine, (B) fentanyl and (C) U50,488-H following intraperitoneal acetic acid injection. The Mann–Whitney U Test (two-tailed) was used to determine statistical significance (*P<.05, **P<.01, ***P<.001).

Table 1
Comparative activity of morphine alone and in combination with a NK-1, -2 or -3 receptor antagonist

	Dose (mg/kg)	First observation period		Second observation period	
		Mean no. of writhes	ID_{50} (mg/kg)	Mean no. of writhes	ID_{50} (mg/kg)
Morphine	(0.16)	44.0 (1.8)		23.4 (4.0)	
	(0.31)	39.4 (2.8)		26.4 (2.5)	
	(0.63)	32.4 (4.1)	1.03 (0.60–1.76)	17.2 (3.4)	1.78 (1.11–2.88)
	(1.25)	34.6 (8.7)		22.8 (7.4)	
	(2.5)	28.6 (1.9)		14.4 (5.0)	
	(5)	6.4 (3.9)		0.2 (0.2)	
R116301 (2.5 mg/kg)	+Morphine (0.16)	56.2 (5.3)		32.0 (4.9)	
	(0.31)	46.8 (5.0)		29.8 (2.9)	
	(0.63)	41.2 (6.1)	1.55 (0.90–2.66)	27.4 (3.1)	2.04 (1.51−2.76)
	(1.25)	42.6 (4.0)		22.4 (2.7)	
	(2.5)	25.2 (4.2)		4.8 (3.4)	
	(5)	5.2 (5.2)		2.2 (0.9)	
R116301 (10 mg/kg)	+Morphine (0.16)	52.4 (5.4)		41.8 (4.1)	
	(0.31)	37.0 (6.2)		20.2 (6.2)	
	(0.63)	29.2 (3.4)	0.89 (0.52–1.53)	16.2 (4.0)	1.02 (0.60–1.76)
	(1.25)	46.6 (2.8)		26.4 (2.7)	
	(2.5)	32.0 (4.9)		3.4 (3.2)	
	(5)	2.0 (1.2)		1.8 (1.0)	
CP-96,345 (2.5 mg/kg)	+Morphine (0.16)	47.4 (3.1)		34.2 (4.3)	
	(0.31)	48.4 (4.2)		26.4 (2.6)	
	(0.63)	48.2 (5.0)	1.55 (1.04–2.32)	31.4 (2.8)	2.35 (1.57–3.51)
	(1.25)	29.0 (3.9)		25.2 (4.2)	
	(2.5)	28.0 (8.0)		8.0 (3.6)	
	(5)	0.6 (0.4)		4.2 (2.8)	
CP-96,345 (10 mg/kg)	+Morphine (0.16)	51.0 (2.8)		27.0 (2.8)	
	(0.31)	46.4 (3.9)		23.2 (4.0)	
	(0.63)	47.0 (4.4)	1.55 (1.14–2.10)	24.0 (3.6)	1.03 (0.64–1.65)
	(1.25)	37.4 (2.5)		12.6 (2.2)	
	(2.5)	10.2 (5.3)		1.6 (1.4)	
	(5)	7.4 (2.2)		2.2 (0.7)	
GR203040 (2.5 mg/kg)	+Morphine (0.16)	55.8 (2.6)		31.2 (4.1)	
	(0.31)	44.8 (1.4)		27.0 (2.1)	
	(0.63)	48.2 (4.6)	1.18 (0.87–1.59)	27.0 (3.2)	1.55 (1.03–2.32)
	(1.25)	31.6 (9.8)		14.4 (4.4)	
	(2.5)	17.8 (4.6)		5.6 (4.0)	
	(5)	0.6 (0.4)		2.6 (1.5)	
GR203040 (10 mg/kg)	+Morphine (0.16)	48.4 (3.3)		25.4 (2.2)	
	(0.31)	49.0 (5.6)		32.2 (5.7)	
	(0.63)	42.8 (4.6)	0.78 (0.52–1.17)	29.4 (2.3)	0.89 (0.72–1.11)
	(1.25)	14.6 (6.4)		2.4 (1.3)	
	(2.5)	0.8 (0.4)		0 (0)	
	(5)	1.0 (0.5)		1.6 (1.0)	
SR-48968 (2.5 mg/kg)	+Morphine (0.16)	38.0 (4.1)		26.6 (3.3)	
	(0.31)	51.2 (4.1)		23.8 (2.7)	
	(0.63)	48.4 (3.2)	2.69 (1.57–4.62)	27.6 (3.2)	2.35 (1.73–3.17)
	(1.25)	51.2 (3.7)		25.4 (2.3)	
	(2.5)	43.4 (4.5)		15.4 (9.1)	
	(5)	1.4 (1.0)		0.8 (0.4)	
SR-48968 (10 mg/kg)	+Morphine (0.16)	64.0 (9.0)		33.2 (7.4)	
	(0.31)	54.0 (3.2)		24.2 (2.1)	
	(0.63)	63 (6.6)	2.04 (1.55–2.76)	36.2 (6.3)	1.78 (1.43–2.20)
	(1.25)	47.7 (6.7)		30.6 (3.4)	
	(2.5)	21.8 (6.5)		3.4 (2.0)	
	(5)	12.6 (4.3)		1.2 (0.8)	
SR-142801 (2.5 mg/kg)	+Morphine (0.16)	61.6 (4.4)		34.0 (4.0)	
	(0.31)	52.0 (2.0)		35.4 (2.2)	
	(0.63)	49.6 (6.2)	1.78 (1.19–2.66)	25.2 (2.5)	1.55 (1.15–2.10)
	(1.25)	43.4 (3.4)		18.0 (2.7)	
	(2.5)	17 (11.4)		0.6 (0.4)	
	(5)	1.4 (0.6)		1.0 (0.8)	

The mean number of writhes (±S.E.M.) are shown for all doses of morphine (0.16–5 mg/kg) simultaneously administered with an NK antagonist. The ID_{50}s values (mg/kg; with 95% confidence limits) are also indicated.

2.5. Drugs

Morphine–HCl was purchased from Belgopia (Louvain-La-Neuve, Belgium). Fentanyl–HCl, R116301, CP-96,345, SR-48,968, SR-142801 and GR203040 were obtained from Janssen Pharmaceutica (Beerse, Belgium). U50,488-HCl, SNC80 and nimodipine were supplied by Tocris Cookson (Bristol, UK). All drugs were freshly prepared as aqueous solutions prior to experimentation. All doses refer to base equivalents. Drugs were administered in a volume of 10 ml/kg volumes per injection. Control animals received an equivalent volume of sterile physiological saline (NaCl 0.9%, Baxter) or vehicle. The appropriate vehicle for each compound tested was administered (n=5–6 animals per vehicle group).

2.6. Data analysis

All data are expressed as mean±S.E.M. values. Differences between experimental conditions were evaluated using a Mann–Whitney U test (two-tailed) corrected for repeated measures. Asterisks indicate statistical significance (*P<.05,

Table 2
Comparative activity of fentanyl and combination with an NK-1, -2 and -3 receptor antagonist

	Dose (mg/kg)	First observation period		Second observation period	
		Mean no. of writhes	ID_{50} (mg/kg)	Mean no. of writhes	ID_{50} (mg/kg)
Fentanyl	(0.01)	49.0 (3.1)		36.8 (1.6)	
	(0.02)	39.8 (2.2)	0.049 (0.036–0.067)	28.4 (4.1)	0.065 (0.048–0.088)
	(0.04)	40.4 (7.5)		32.2 (2.7)	
	(0.08)	13.2 (5.4)		3.4 (3.2)	
R116301 (2.5 mg/kg)	+Fentanyl (0.01)	47.6 (2.9)		40.2 (2.4)	
	(0.02)	58.4 (1.9)	0.057 (0.046–0.070)	26.8 (5.6)	0.057 (0.035–0.091)
	(0.04)	45.4 (4.2)		28.4 (3.0)	
	(0.08)	10.8 (5.1)		9.0 (4.1)	
R116301 (10 mg/kg)	+Fentanyl (0.01)	51.6 (5.5)		33.8 (6.0)	
	(0.02)	44.8 (3.5)	0.028 (0.016–0.049)	29.0 (5.0)	0.049 (0.033–0.074)
	(0.04)	18.4 (7.4)		22.7 (5.3)	
	(0.08)	18.8 (6.7)		11.4 (4.4)	
CP-96,345 (2.5 mg/kg)	+Fentanyl (0.01)	53.6 (6.6)		24.8 (3.7)	
	(0.02)	59.4 (7.0)	0.043 (0.032–0.058)	30.0 (4.0)	0.057 (0.038–0.085)
	(0.04)	34.6 (8.6)		18.4 (6.1)	
	(0.08)	4.2 (1.7)		9.4 (4.9)	
CP-96,345 (10 mg/kg)	+Fentanyl (0.01)	56.8 (4.3)	0.025 (0.017–0.036)	35.0 (4.2)	0.040 (0.030–0.053)
	(0.02)	37.8 (3.6)		23.0 (3.1)	
	(0.04)	25.3 (3.8)		19.0 (4.8)	
	(0.08)	2.0 (1.5)		1.3 (0.9)	
GR203040 (2.5 mg/kg)	+Fentanyl (0.01)	54.4 (4.5)		32.4 (3.7)	
	(0.02)	40.4 (3.0)	0.037 (0.025–0.056)	41.6 (3.2)	0.049 (0.036–0.067)
	(0.04)	35.8 (9.2)		29.4 (7.0)	
	(0.08)	4.0 (2.3)		3.6 (1.7)	
GR203040 (10 mg/kg)	+Fentanyl (0.01)	59.2 (4.4)		28.4 (4.9)	
	(0.02)	43.4 (3.4)	0.037 (0.25–0.056)	31.4 (5.3)	0.043 (0.025–0.074)
	(0.04)	33.8 (7.5)		33.4 (5.9)	
	(0.08)	1.0 (0.8)		6.8 (5.9)	
SR-48968 (2.5 mg/kg)	+Fentanyl (0.01)	50.2 (2.4)	0.037 (0.25–0.056)	25.8 (2.4)	0.075 (0.055–0.101)
	(0.02)	45.2 (9.0)		27.0 (3.2)	
	(0.04)	49.2 (8.5)		37.6 (1.9)	
	(0.08)	15.4 (4.3)		8.4 (5.2)	
SR-48968 (10 mg/kg)	+Fentanyl (0.01)	54.0 (11.3)	0.049 (0.030–0.080)	27.4 (3.5)	0.065 (0.043–0.097)
	(0.02)	48.0 (3.9)		27.4 (1.8)	
	(0.04)	41.4 (7.1)		26.6 (6.7)	
	(0.08)	17.6 (7.9)		9.8 (4.8)	
SR-142801 (2.5 mg/kg)	+Fentanyl (0.01)	71.0 (2.4)	0.057 (0.046–0.070)	43.0 (2.7)	0.065 (0.048–0.088)
	(0.02)	64.0 (3.4)		41.4 (3.9)	
	(0.04)	60.0 (4.3)		40.0 (5.3)	
	(0.08)	10.8 (1.8)		7.0 (4.7)	
SR-142801 (10 mg/kg)	+Fentanyl (0.01)	48.8 (4.3)	0.049 (0.030–0.080)	38.0 (5.0)	0.075 (0.055–0.101)
	(0.02)	35.8 (5.8)		33.8 (3.1)	
	(0.04)	47.4 (4.2)		31.0 (2.8)	
	(0.08)	23.8 (6.5)		13.0 (6.8)	

The mean number of writhes (±S.E.M.) are shown for all doses of fentanyl (0.01–0.08 mg/kg) simultaneously administered with an NK antagonist. The ID_{50}s values (mg/kg; with 95% confidence limits) are also indicated.

**$P<.01$, ***$P<.001$). ID_{50} values (mg/kg) and 95% confidence limits were calculated by linear regression from the proportion of animals fulfilling fixed criteria for activity at <35 writhes in the first observation period and <15 writhes in the second observation period. These all or none criteria are based on a large control population. Only 2.7% and 0.9% of control animals fulfilled the criteria in the first and second observation periods, respectively. Differences in ID_{50} values were evaluated by means of the Student's *t* test (two-tailed) for independent samples using the differences between log ID_{50} values (method of Sack, 1982).

3. Results

3.1. Opioids

Injection of 0.2 ml 1% acetic acid into the intraperitoneal cavity of gerbils produced writhing characterized by a lengthways stretch of the abdomen and extension of the hindlimbs, commencing immediately after the injection. Control animals pretreated with vehicle or saline (total n=120) produced an average of 59.1±1.2 writhes within the first observation period and 38.0±1.1 writhes in the second period. In total, only 2.8% and 0.9% of the control/vehicle animals had <35 writhes and <15 writhes, during the first and second observation period, respectively.

Morphine (Fig. 1A) and fentanyl (Fig. 1B) injected 1 h prior to acetic acid injection attenuated the number of writhes in a dose-dependent manner, producing a complete inhibition of writhing during both observation periods at 10 and 0.16 mg/kg, respectively. The ID_{50} values (with 95% confidence limits) of morphine (Table 1) were 1.03 (0.60–1.76) and 1.78 (1.11–2.88) mg/kg in the first and second periods, respectively. The corresponding ID_{50} values for fentanyl (Table 2) were 0.049 (0.036–0.067) and 0.065 (0.048–0.088) mg/kg in the first and second

Table 3
Comparative activity of U50,488-H alone and in combination with an NK-1, -2 or -3 receptor antagonist

	Dose (mg/kg)	First observation period		Second observation period	
		Mean no. of writhes	ID_{50} (mg/kg)	Mean no. of writhes	ID_{50} (mg/kg)
U50,488-H	(0.16)	42.8 (3.8)		28.8 (3.5)	
	(0.31)	50.4 (3.2)		27.2 (2.8)	
	(0.63)	32.0 (5.4)	1.03 (0.60–1.77)	18.2 (3.2)	1.03 (0.60–1.77)
	(1.25)	32.8 (5.0)		16.8 (0.9)	
	(2.5)	27.2 (6.3)		17.4 (2.7)	
GR203040 (2.5 mg/kg)	+U50,488-H (0.16)	50.0 (3.9)		24.6 (4.1)	
	(0.31)	44.0 (3.4)		28.8 (5.4)	
	(0.63)	47.6 (4.8)	1.17 (0.69–2.01)	29.6 (5.3)	2.33
	(1.25)	40.0 (5.3)		19.8 (3.9)	
	(2.5)	39.0 (6.6)		25.6 (1.6)	
GR203040 (10 mg/kg)	+U50,488-H (0.16)	45.0 (5.6)		23.6 (3.3)	
	(0.31)	38.8 (5.4)		23.0 (1.5)	
	(0.63)	41.8 (5.8)	0.51 (0.32–0.83)	29.6 (3.0)	1.76
	(1.25)	27.4 (1.7)		28.0 (2.3)	
	(2.5)	30.2 (4.6)		27.0 (4.2)	
SR-48968 (2.5 mg/kg)	+U50,488-H (0.16)	42.4 (7.3)		26.8 (3.7)	
	(0.31)	44.6 (5.7)		30.0 (2.4)	
	(0.63)	37.8 (4.0)	1.55	21.7 (6.7)	3.07
	(1.25)	29.0 (5.2)		19.9 (2.5)	
	(2.5)	40.8 (4.3)		24.2 (1.8)	
SR-48968 (10 mg/kg)	+U50,488-H (0.16)	44.0 (5.0)		27.8 (3.9)	
	(0.31)	45.4 (3.8)		32.6 (2.8)	
	(0.63)	46.2 (7.0)	0.89 (0.49–1.62)	31.0 (10.0)	3.53
	(1.25)	31.4 (4.0)		20.0 (1.7)	
	(2.5)	42.4 (6.9)		26.8 (3.9)	
SR-142801 (2.5 mg/kg)	+U50,488-H (0.16)	60.0 (4.9)		33.6 (5.6)	
	(0.31)	49.8 (4.5)		31.2 (3.0)	
	(0.63)	45.0 (5.3)	2.03	29.6 (4.5)	3.53
	(1.25)	37.2 (2.3)		25.4 (1.6)	
	(2.5)	40.0 (7.1)		30.6 (4.5)	
SR-142801 (10 mg/kg)	+U50,488-H (0.16)	61.4 (5.1)		36.6 (3.4)	
	(0.31)	36.8 (3.6)		25.4 (1.7)	
	(0.63)	42.2 (4.8)	1.02 (0.60–1.75)	25.2 (2.3)	3.07
	(1.25)	41.2 (4.4)		32 (3.3)	
	(2.5)	22.4 (4.7)		19.2 (5.2)	

The mean number of writhes (±S.E.M.) are shown for all doses of U50,488-H (0.16–2.5 mg/kg) simultaneously administered with an NK antagonist. The ID_{50}s values (mg/kg; with 95% confidence limits) are also indicated.

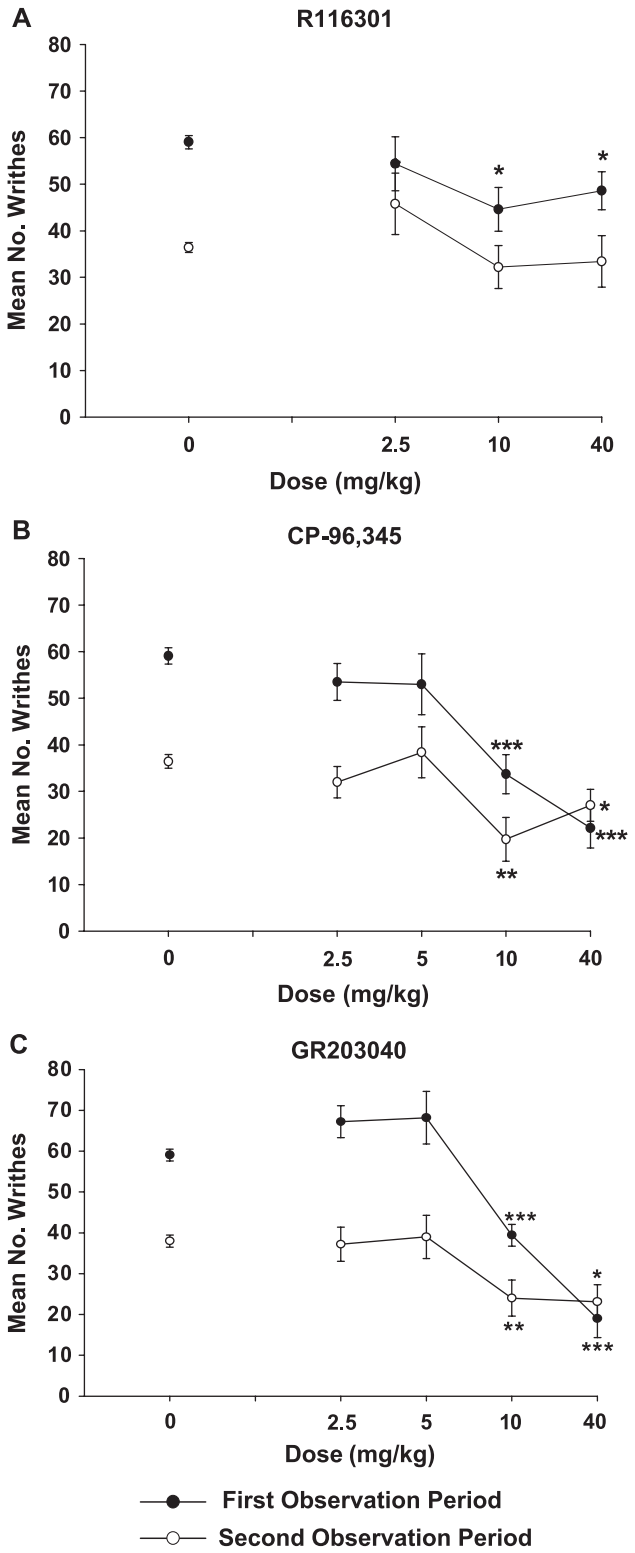

Fig. 2. Effects of the NK-1 receptor antagonists GR203040, R116301 and CP-96,345 on the writhing response in gerbils. The mean±S.E.M. values of the number of writhes in the first and second observation periods are shown. Significant reductions in the writhing frequency were observed at doses of 10 and 40 mg/kg (A) GR203040, (B) R 116301 and (C) CP-96,345 after intraperitoneal acetic acid injection. The Mann–Whitney *U* Test (two-tailed) was used to determine statistical significance (**P*<.05, ***P*<.01, ****P*<.001).

observation periods, respectively. U50,488-H (Fig. 1C) dose dependently reduced the writhing starting at 0.16 mg/kg in both the first (*P*<.001) and second observation periods (*P*<.01). Complete inhibition of the writhing response was observed at 40 mg/kg, but at this dose, the animals showed signs of sedation. The ID_{50} values of U50,488-H (Table 3) were 1.03 (0.60–1.77) mg/kg in the first observation period and 2.35 (1.37–4.04) mg/kg in the second. SNC80 did not significantly alter the writhing frequency even at doses up to 40 mg/kg.

3.2. NK-1, -2 and -3 antagonists

The tachykinin NK-1 antagonist R116301 (Fig. 2A) produced only a limited inhibition of writhing at 40 mg/

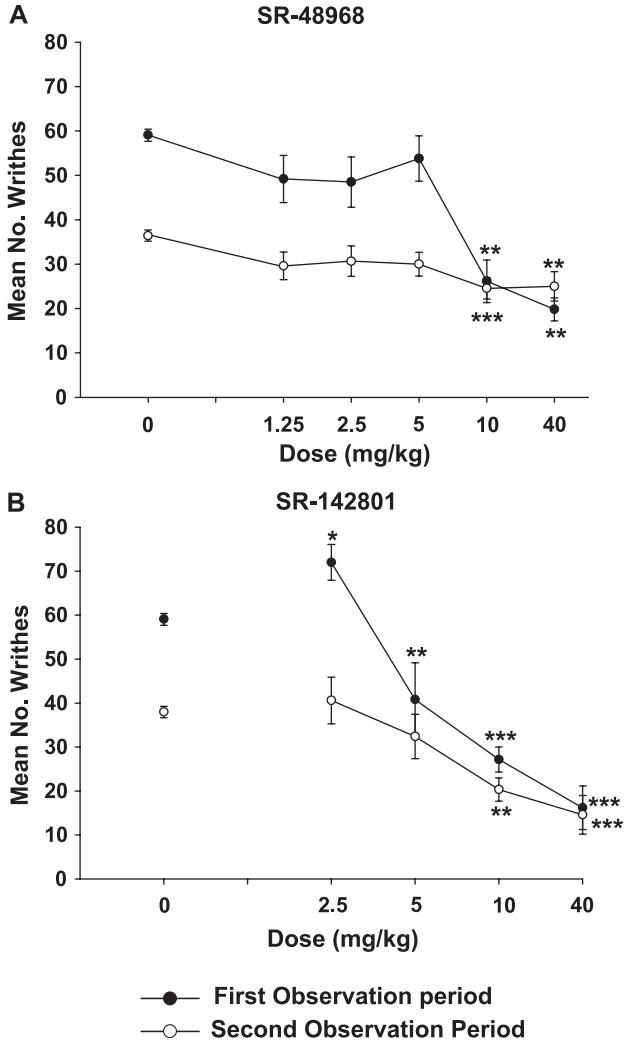

Fig. 3. Effects of the NK-2 receptor antagonist SR-48,968 and the NK-3 receptor antagonist SR-142801 in the acetic-acid-induced abdominal constriction assay in gerbils. The mean±S.E.M. values of the number of writhes invoked by acetic acid injection in the first and second observation periods are shown. Significant reductions in the number of writhes were observed at doses of 10 and 40 mg/kg SR-48,968, and at doses of 2.5, 5, 10 and 40 mg/kg SR-142801. The Mann–Whitney *U* Test (two-tailed) was used to determine statistical significance (**P*<.05, ***P*<.01, ****P*<.001).

kg in both the first (48.6±4.1 writhes) and second (33.4±5.5 writhes) observation periods. In contrast, the tachykinin NK-1 antagonists CP-96,345 (Fig. 2B) and GR203040 (Fig. 2C) both displayed significant inhibition of the writhing in both observation periods compared with the vehicle-treated group. However, neither compound fully inhibited the writhing. The NK-2 antagonist SR-48968 (Fig. 3A) significantly reduced the frequency of writhing ($P < 0.001$) from doses 10 mg/kg onward during both observation periods. Administration of the tachykinin NK-3 antagonist SR-142801 (Fig. 3B) resulted in significant reduction of writhing at 10 and 40 mg/kg in both the first and second observation periods. However, at a lower dose of 2.5 mg/kg, SR-142801 resulted in an increase in the writhing response in the first observation period (72±4.1). As with NK-1 antagonists, complete inhibition of writhing could not be produced with neither SR-48968 nor SR-142801.

3.3. Coadministration of opioids and NK antagonists

The simultaneous administration of μ-, δ- or κ-opioids with an NK-1, -2 or -3 receptor antagonist produced no changes in the writhing response compared to opioids alone (Tables 1–4).

The ID_{50} values for morphine, fentanyl and U50,488-H in combination with different doses of the NK antagonists were found not to differ from the ID_{50} values for the opioids alone (Tables 1–3).

3.4. The effects of Ca^{2+} channel antagonism on the writhing response

Control animals pretreated with vehicle (n=12) exhibited an average of 51.3±4.8 and 21.9±2.5 writhes in the first and second observation periods, respectively. The number of writhes produced by each individual control animal fell within the range of writhes obtained in the control group in the opioid and NK antagonist study. Administration of nimodipine, a selective L-type Ca^{2+} channel antagonist, did not significantly affect the number of writhes observed following the intraperitoneal injection of acetic acid even at the highest dose tested (40 mg/kg; Fig. 4).

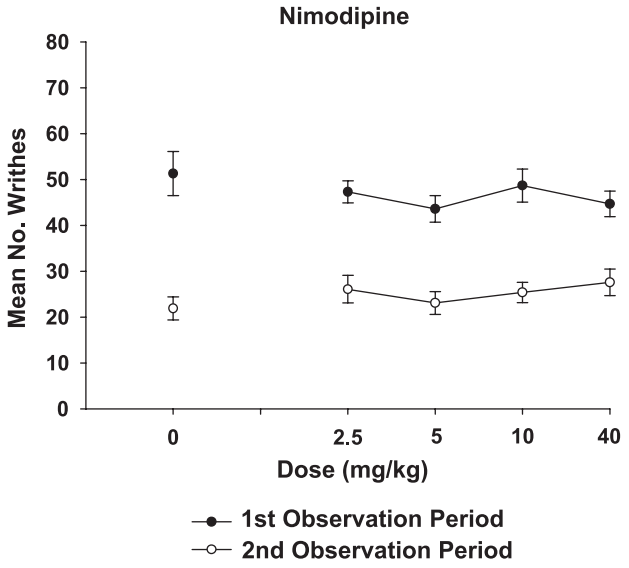

Fig. 4. Effects of the L-type Ca^{2+} channel nimodipine on the writhing response in gerbils. The mean±S.E.M. values of the number of writhes in the first and second observation periods are given. No significant effects on the writhing response were observed following administration of nimodipine.

4. Discussion

In the present study, the acetic-acid-induced abdominal constriction assay was used to evaluate the effects of μ-, κ- and δ-opioids, and tachykinin antagonists alone and in combination in a model for visceral nociception. The writhing test is most commonly performed in mice or rats, but in this study, gerbils were thought to be more suitable as the binding affinity of the NK-1 receptor antagonist CP-96,345 to the gerbil NK receptor is very similar to those seen with human and guinea pig receptors (Beresford et al., 1991; Gitter et al., 1991) but very different to those of rats and mice. Furthermore, the tachykinin receptor sequences of humans, gerbils and guinea pigs are very homologous (Stout et al., 2001). Our choice of species is further supported by the comparable results seen in our study to

Table 4
Comparative activity of SNC80 alone and in combination with an NK-1, -2 and-3 receptor antagonist

	Dose (mg/kg)	First observation period Mean no. of writhes	Second observation period Mean no. of writhes
SNC80	(10)	51.0 (5.8)	37.2 (4.1)
	(40)	51.6 (3.2)	39.0 (2.6)
GR203040 (2.5 mg/kg)	+SNC80 (40)	45.6 (7.2)	24.6 (2.6)
GR203040 (10 mg/kg)	+SNC80 (40)	30.0 (4.2)	24.2 (5.0)
SR-48968 (2.5 mg/kg)	+SNC80 (40)	24.0 (2.5)	19.6 (1.9)
SR-48968 (10 mg/kg)	+SNC80 (40)	33.0 (4.2)	23.0 (3.3)
SR-142801 (2.5 mg/kg)	+SNC80 (40)	36.0 (4.0)	23.8 (3.0)
SR-142801 (10 mg/kg)	+SNC80 (40)	50.8 (1.1)	26.8 (2.6)

The mean number of writhes (±S.E.M.) values are indicated.

those obtained in earlier studies using different species. The μ-opioids morphine and fentanyl, and the κ-opioid U50,488-H attenuated the gerbil writhing response to intraperitoneal acetic acid injection in a significant, dose-dependent manner. At relatively high doses, they were able to completely inhibit the writhing. This activity was present directly (during the first observation period) and 1 h after the acetic acid injection (in the second period). These data are consistent with previous results (Friese et al., 1997; Sora et al., 1999; Schmauss et al., 1983). Sora et al. (1999) reported a clear reduction of the writhing response in mice pretreated with a subcutaneous injection of 10 mg/kg morphine and 10 mg/kg U50,488-H. Other authors also observed dose-dependent reductions in the writhing frequency of mice treated with low doses of intraperitoneal morphine (60 to 120 μg/0.3 ml; Reichert et al., 2001; Bentley et al., 1981). In the present study, the δ-opioid SNC80 was less potent than the μ- and κ-opioids as no significant effects were observed even at doses of 40 mg/kg SNC80. These data are in accord with Sora et al. (1999) who reported that SNC80 was less active than both morphine and U50,488-H in reducing the writhing responses in mice. κ- and δ-opioids also show efficacy in other visceral pain models. They are reported to attenuate the visceromotor and cardiovascular responses to noxious colorectal distension responses (Ozaki et al., 2000).

Of the NK-1 antagonists tested in the writhing model, only CP-96,345 and GR203040 produced significant, dose-dependent reductions in the writhing response during both observation periods. They displayed similar activities as both first showed significant activity at 10 mg/kg. CP-96,345 has also been reported to be active in the acetic-acid-induced writhing test in mice (Nagahisa et al., 1992; Garret et al., 1991). However, neither GR203040 nor CP-96,345 were as effective as the μ- or κ-opioids in attenuating the writhing frequency as they did not fully inhibit the response in either observation period. It is possible that the antinociceptive effects of CP-96,345 may be partially attributed to its antagonism of L-type Ca^{2+} channels at high doses (Schmidt et al., 1992). Ca^{2+} channel antagonists have been reported to have antinociceptive effects in the rat hot plate and tail withdrawal tests (Zharkovsky et al., 1999), and the gerbil formalin test (Rupniak et al., 1993). Nimodipine, a selective L-type Ca^{2+} antagonist, produced antinociceptive effects in the mouse writhing test following both intracerebroventricular and intraperitoneal administration (Miranda et al., 1992, 1993). However, our results indicate that nimodipine is ineffective in the gerbil writhing test. Furthermore, neither GR203040 nor R116301 possess significant activity at Ca^{2+} channels (Beattie et al., 1995; Megens et al., 2002). We therefore assume that the effects of CP-96,345 in this study are not due to its effects at Ca^{2+} channels.

R116301, a recently developed NK-1 receptor antagonist, displayed limited activity in the writhing test even at high doses. This is despite binding experiments with human NK-1 receptors illustrating comparable potencies with CP-96,345 and GR203040 (Beattie et al., 1995; Megens et al., 2002; Beresford et al., 1991). However, following subcutaneous administration, R116301 was found to be 31 times less potent than GR203040 but equipotent to CP-96,345 in the inhibition of extravasation in guinea pigs (Megens et al., 2002). R116301 displayed comparable activity to other NK-1 antagonists in a variety of inflammatory and behavioural models (Megens et al., 2002) and also dose dependently reduced the duration of paw lifting in a formalin test in gerbils (unpublished data). Systemic administration of R116301 has recently been shown to attenuate SP-induced paw lifting in gerbils with a chronic constriction injury (Meert et al., 2003). NK-1 antagonists are also active on the rat jejunal distension response. CP-99,994 inhibited the cardiovascular depressor response, a marker indicative of nociception. This NK-1 antagonist also reduced the sensitivity to distension (Mclean et al., 1998).

The tachykinin NK-2 receptor antagonist SR-48968, and the NK-3 receptor antagonist SR-142801 both revealed some activity in the writhing test starting at doses of 10 mg/kg. As with the NK-1 antagonists, the NK-2 and NK-3 receptor antagonists were less effective than the μ- and κ-opioids due to their failure to completely inhibit the writhing response in both observation periods. NK-2 and NK-3 antagonists also display activity in other models of visceral pain such as noxious colorectal and jejunal distension (Mclean et al., 1998; Julia et al., 1999) in rats.

Conflicting results have been reported about the efficacy of NK antagonists in visceral pain models. Seguin et al. (1995) found SR-48968 to be inactive in the writhing response in mice. However, the NK-2 antagonist attenuated the writhing response in rats (Julia and Bueno, 1997). Although there is considerable evidence to support the effectiveness of NK-1 antagonists in the writhing test in mice (Nagahisa et al., 1992; Garret et al., 1991) and rats (Seguin et al., 1995), other authors have reported the NK-1 antagonist RP-67,580 to have no effect on the abdominal constriction response to acetic acid. It is such contradictory evidence as this that highlights the need for a suitable species in which to study response to NK antagonists.

The differences in the analgesic efficacies of the opioids and tachykinin antagonists may be related to their respective mechanisms of action. Evidence from NK-1 receptor knockout mice has indicated that the NK-1 receptor is required for the induction of the neurogenic inflammation, normal pain and hyperalgesia evoked by intraperitoneal acetic acid injection. Mice without a functional NK-1 receptor or SP signalling display impaired nociception (Laird et al., 2000; Zimmer et al., 1998). By preemptively blocking the NK-1 receptor with an NK-1 antagonist before acetic acid injection, we may partially prevent the initiation of inflammation and neurogenic inflammation and therefore contribute to the impaired nociception observed. However, it must be acknowledged that other inflammatory mediators and signalling pathways will remain functional.

The analgesic efficacy of tachykinin receptor antagonists may also be limited because SP is only a cotransmitter of visceral nociceptive transmission. SP is colocalized with glutamate and acts to potentiate the postsynaptic nociceptive effects of glutamate (Liu et al., 1998). It has previously been shown that local infusion of glutamate can invoke abdominal contractions (Bueno and Fioramonti, 2002) Furthermore, a novel glutamate receptor antagonist was reported to be effective in attenuating the writhing response (Chen et al., 2000). However, in a similar fashion to the NK receptor antagonists, it could not completely inhibit the writhing response. These findings suggest that it is insufficient to target only one neurotransmitter when trying to achieve an effective blockade of visceral afferent transmission. Only by simultaneously antagonising the major cotransmitters of primary sensory neurons and inflammatory mediators can afferent transmission and visceral inflammation be inhibited (Holzer, 2001).

In contrast to NK receptor antagonists, opioids cause a full blockade of neurotransmitter release by their effects on presynaptic membrane excitability. μ-opioid receptors are expressed both presynaptically and postsynaptically to SP-containing primary afferent neuron terminals. Following the occupation of the receptors, the opioids can produce their analgesic effects by altering membrane potassium conductance to cause a hyperpolarisation to attenuate the excitability of nociceptive input terminals or to inhibit the propagation of action potentials. This hyperpolarisation also diminishes the calcium-dependent release of transmitters, including SP, calcitonin gene-related peptide (CGRP) and glutamate, which are colocalized in the terminals of primary afferent neurons (Reichert et al., 2001; Bueno et al., 1997; Lembeck and Donnerer, 1985; Aimone and Yaksh, 1989; Jessell and Iversen, 1977). κ-opioids manifest their effects by a similar mechanism of action through peripheral visceral receptors. They prevent calcium channel opening on SP neurons and therefore attenuate the calcium-dependent release of neurotransmitters. In comparison to the expression of μ- and κ-opioid mRNA (90% and 30%, respectively) within SP-containing neurons, the distribution of δ-opioid receptor mRNA is very scarce (Minami et al., 1995; Satoh and Minami, 1995). However, δ-opioid receptors are up-regulated when inflammation is present and there may be increased efficacy of δ-opioid agonists at the later stages of inflammation (Zhou et al., 1998). The activity of the δ-agonists may be limited by the use of preemptive administration in an acute setting, such as the writhing test. The availability of δ-opioid receptors to the agonist will be limited in early stages of inflammation.

The distribution of opioid receptor mRNA within the different laminae of the spinal dorsal horn may also be of interest when explaining the relative efficacies of the opioid agonists tested. Visceral primary afferent neurons terminate in laminae I and V. μ-opioids receptor expression is very intense in laminae I and II where nociceptive C and Aδ fibres principally terminate. Similarly, κ-opioid mRNA is intensely expressed in laminae I and II but also in lamina V. In contrast, there is only a low to moderate expression of δ-opioid receptors throughout laminae I–VI. (Satoh and Minami, 1995). This evidence indicates that μ- and κ-opioids may be of more significance than δ-opioids in the postsynaptic modulation of nociceptive input from visceral primary afferent neurons.

In conclusion, this study has indicated that the μ-opioids morphine and fentanyl and the κ-opioid U50,488-H are active in attenuating acetic-acid-induced visceral nociception in gerbils. However, the δ-opioid SNC80 did not display activity in this assay. NK receptor antagonists also display some efficacy, although they are less effective than the μ- and κ-opioids in this model. Coadministration of μ-, δ- or κ-opioids with NK-1, NK-2 or NK-3 receptor antagonists does not appear to cause any potentiation of the analgesic effects produced by the opioids alone in acute conditions.

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