Central and peripheral actions of the novel κ -opioid receptor agonist, EMD 60400

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- 1 The pharmacological characteristics of the κ -opioid receptor agonist, EMD 60400, have been investigated, with particular reference to its central and peripheral sites of action and its ability to influence nociception. The κ agonists ICI 197067 and ICI 204448 were tested for purposes of comparison.
- 2 EMD 60400 and ICI 197067 bind with high affinity (IC_{50} values of 2.8 and 1.5 nM, respectively) and high selectivity to κ -opioid receptors. ICI 204448 has a lower binding affinity (IC_{50} 13.0 nM) and selectivity for κ -opioid receptors.
- 3 EMD 60400, ICI 197067, and ICI 204448 are full and potent agonists in the rabbit vas deferens *in vitro* assay for κ -opioid receptors (IC_{50} values of 41.8, 15.7 and 15 nM, respectively).
- 4 *Ex vivo* binding experiments in mice revealed that EMD 60400 and ICI 197067 were well taken up after s.c. administration. Brain levels of EMD 60400 were lower than those of ICI 197067 at comparable doses, indicating that EMD 60400 does not penetrate into the CNS as well as ICI 197067.
- 5 Haloperidol-induced DOPA accumulation in the nucleus accumbens of the rat was dose-dependently reversed by s.c. application of EMD 60400 and ICI 197067 at doses of and above 3 and 0.3 mg kg⁻¹, respectively. ICI 204448 had no effect on DOPA accumulation at 30 mg kg⁻¹, s.c.
- 6 Prolongation of hexobarbitone-induced sleeping time in mice and motor impairment in the rat rotarod test were observed for EMD 60400 at doses above 3 and 2.5 mg kg⁻¹, s.c., respectively, and for ICI 197067 at doses above 0.3 and 0.25 mg kg⁻¹, s.c., respectively. ICI 204448 was inactive in these tests at doses of 30 and 100 mg kg⁻¹, s.c., respectively.
- 7 EMD 60400 applied s.c. produced dose-dependent naloxone-reversible antinociception in the mouse formalin test (1st and 2nd phase ID_{50} 0.44 and 0.47 mg kg⁻¹, respectively) and rodent writhing test (ID_{50} mouse 0.55 mg kg⁻¹ and rat 0.3 mg kg⁻¹). Furthermore, EMD 60400 was considerably more potent in the rat pressure pain test after the induction of inflammation with carrageenin than under normal analgesic conditions (ID_{50} values 0.1 μ g kg⁻¹ and 4.0 mg kg⁻¹, s.c., respectively). The action of EMD 60400 (50 μ g kg⁻¹, s.c.) in the hyperalgesic pressure pain test was completely antagonized by injection of the κ -opioid antagonist, norbinaltorphimine (100 μ g) into the inflamed tissue, thus demonstrating the peripheral opioid nature of this effect.
- 8 EMD 60400 produced dose-dependent inhibition of neurogenic plasma extravasation elicited by antidromic electrical stimulation of the rat saphenous nerve (ID_{50} value 0.3 mg kg⁻¹, i.v.). This inhibition was completely antagonized by intraplantar injection of norbinaltorphimine (50 μ g).
- 9 EMD 60400, ICI 197067, and ICI 204448 have diuretic effects in rats at doses of and above 0.1, 0.01, and 0.3 mg kg⁻¹, s.c., respectively. An antidiuretic action was also observed with ICI 197067 at very low doses (3 and 6 μ g kg⁻¹, s.c.).
- 10 Pharmacological and biochemical data therefore indicate that the three κ -opioid receptor agonists tested here have different tendencies to elicit centrally-mediated sedation and putative aversion (ICI 197067 > EMD 60400 > ICI 204448) which correspond to their ability to cross the blood-brain barrier. EMD 60400 combines high affinity and selectivity for the κ receptor with a degree of peripheral selectivity. The peripheral actions of systemically-applied EMD 60400 against hyperalgesic pressure pain and neurogenic inflammation are very probably mediated by opioid receptors on the endings of sensory nerve fibres.

Keywords: EMD 60400; ICI 197067; ICI 204448; κ -opioid agonist; antinociceptive; diuretic; antidiuretic; peripherally-selective

Introduction

It has been recognised for a number of years that analgesic actions of opiates can be produced by agonists acting at opioid-receptor subtypes (μ , κ and δ) in the brain and spinal cord. However, this action is often accompanied by undesirable central side effects. In the case of κ -opioid agonists these central side effects include dysphoria and sedation (Pfeiffer *et al.*, 1986), which severely limit the usefulness of these compounds in the clinical setting. Evidence has recently become available that opiates can also have peripheral antinociceptive actions, in particular when inflammation is present (see Barber & Gottschlich, 1992, for recent review). An

opiate agonist which is unable to cross the blood-brain barrier should under inflammatory or hyperalgesic conditions be able to produce a peripheral analgesic effect which is not coupled with unacceptable central side effects.

In this study we have examined some biochemical and pharmacological properties of the novel κ -agonist, EMD 60400 and compared them with those of ICI 204448 and ICI 197067. ICI 204448 (Shaw *et al.*, 1989) is a κ -opioid receptor agonist which has been described as having only limited access to the CNS, though there have been few tests of its antinociceptive effectiveness in animal models of pain (Barber *et al.*, 1992; Rogers *et al.*, 1992). In contrast, ICI 197067 (Costello *et al.*, 1988) is a κ agonist which readily

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penetrates the blood-brain barrier (Shaw *et al.*, 1989). Our aim was to investigate whether EMD 60400 can have peripherally-mediated actions at systemic doses which do not produce centrally-mediated side effects. Some of these results have been presented as a preliminary communication (Barber *et al.*, 1992).

Methods

In vitro tests

Binding Opioid receptor binding was determined in the presence of 1.5 nM [³H]-U-69593 (κ), 0.5 nM [³H]-PL-017 (μ), or 1.0 nM [³H]-[D-Pen^{2,3}]enkephalin (δ) in 50 mM Tris (2-amino-2-hydroxymethyl-propan-1,3-diol) buffer (pH 7.7) using membranes prepared from guinea-pig cerebellum (κ) or rat cerebrum (μ and δ). Assays were run for 30 min at 25°C and non-specific binding was measured by use of 10 μ M naloxone (κ), 0.3 μ M morphine (μ), or 1 μ M [D-Ala², D-Leu⁵]enkephalin (DADLE; δ).

Rabbit vas deferens This assay was performed in a manner similar to that of Oka *et al.* (1980). Briefly, vasa deferentia were isolated from New Zealand white rabbits (ca. 2.5 kg) and were mounted in organ baths containing 50 ml oxygenated Krebs Henseleit solution at 30°C. The tissue was stimulated electrically with square-wave pulses (0.1 Hz, 2 ms, 40–60 V) via platinum field-stimulating electrodes and contractions were recorded isometrically under a load of 0.5 g. Test substances were dissolved in Krebs Henseleit solution and applied in increasing concentrations at 15 min intervals.

Tests for demonstrating central actions of κ agonists

Ex vivo determination of brain and plasma levels These assays were performed as described by Shaw *et al.* (1989) with a few modifications. Briefly, groups of six female NMRI mice (22–24 g) were injected with vehicle, or test compound dissolved in saline. Thirty minutes later the animals were decapitated, their brains removed and plasma samples collected.

Ex vivo κ -opioid receptor binding was performed in HEPES buffer (pH 7.4) using [³H]-ethylketocyclazocine (0.5 nM). Assays were run for 40 min at 25°C in the presence of 3 μ M DADLE, along with 200 μ l pooled mouse brain homogenate, or the equivalent of 20 μ l pooled blood plasma together with 200 μ l of homogenized guinea-pig brain. Ethylketocyclazocine (10 μ M) was used to define non-specific binding.

Calibration curves were generated by adding the test compound *in vitro* to brain homogenates or plasma samples from control animals. Specific binding values were then obtained for vehicle and treated mice, and the inhibition of binding due to the test compound was used to calculate the concentration of test compound in the brain or plasma of the treated mouse from the corresponding calibration curve. Each estimate of brain and plasma concentration represents mean $\pm$ s.d. from at least six determinations.

Reversal of haloperidol-induced DOPA (3,4-dihydroxyphenylalanine) accumulation This method was adopted with minor modifications from Manzanarez *et al.* (1991). Groups of 4 male Wistar rats (150–200 g) were treated orally with 0.3 mg kg⁻¹ haloperidol suspended in methocel (E. Merck, Darmstadt, Germany). After 1 h the animals were injected s.c. with opiates dissolved in propandiol (E. Merck, Darmstadt, Germany), or propandiol alone. Twenty-five minutes later the animals were injected i.p. with 100 mg kg⁻¹ of the DOPA-decarboxylase inhibitor, NSD 1015, dissolved in physiological saline. After a further 35 min the animals were decapitated, the nucleus accumbens from both brain halves was removed rapidly, weighed, homogenized and centrifuged. Thereafter,

the DOPA content of the samples was analysed by high performance liquid chromatography (Seyfried *et al.*, 1986).

Hexobarbitone-induced sleeping time Groups of 10 male NMRI mice (20–30 g) were injected s.c. with test substances dissolved in distilled water. Thirty minutes later the mice were injected with hexobarbitone (Serva, Heidelberg, Germany; 100 mg kg⁻¹, i.p.) and the duration of narcosis was defined as the time till recovery of the righting reflex.

Rotarod Groups of 3–6 male Wistar rats (160–220 g) were selected for their ability to balance on a rotating rod (diameter 9 cm; rate of rotation 7 revolutions per min). Selected rats were then injected s.c. with test compounds dissolved in distilled water (EMD 60400 and ICI 197067) or 20% dimethylsulphoxide (DMSO; E. Merck, Darmstadt, Germany; ICI 204448). The animals were tested again 30 min after treatment; a rat falling off the bar more than twice within 2 min was considered to be showing motor impairment.

Antinociceptive tests

Formalin test Male NMRI mice (20–30 g) were injected s.c. with test compound dissolved in distilled water (EMD 60400 and ICI 197067), 25% DMSO (ICI 204448) or vehicle. After 30 min 20 μ l of a 3% solution of formalin (E. Merck, Darmstadt, Germany) was injected into a hind paw. The amount of time spent licking the injected paw was measured 0–5 and 20–25 min after the injection of formalin (i.e., in the non-inflammatory first phase and the inflammatory second phase of the reaction, respectively; Hunskaar & Hole, 1987) and expressed as % inhibition compared to the respective vehicle group.

Writhing test Male NMRI mice (20–30 g) and Wistar rats (150–180 g) were injected s.c. with test compound or vehicle (physiological saline or distilled water). Thirty minutes later the animals were injected with a saturated aqueous solution of 2-phenyl-1,4-benzoquinone (E. Merck, Darmstadt, Germany) into the abdominal cavity. The number of writhing movements made in the subsequent 20 min were counted and expressed as % inhibition compared to the respective vehicle group.

Carrageenin-induced hyperalgesia Male Wistar rats (250–370 g) were fitted with a tail cuff and injected with a 1% solution of carrageenin (Marine Colloids Inc., Springfield N.J., U.S.A.) into the base of the tail at the level of the cuff. Three hours later the proportion of freely-moving animals responding to a standard painful pressure (2.5 bar) delivered to the inflamed tissue via a pin built into the cuff was compared in groups of animals receiving different doses of the test substance. The response consisted of vocalisation, turning and/or biting the cuff. The κ -opioid receptor agonists were dissolved in distilled water and applied s.c. at the same time as carrageenin. Peripheral antinociceptive effects of systemically-applied EMD 60400 were demonstrated by applying 100 μ g norbinaltorphimine dissolved in 25 μ l saline into the inflamed portion of the tail.

Experiments were also carried out on freely-moving rats which had not been injected with carrageenin. In this case the pain response to a pressure of 4 bar was tested 30 min after the s.c. application of the opiates.

Other in vivo tests

Neurogenic inflammation Male Wistar rats (270–330 g) were anaesthetized with pentobarbitone sodium (Sanofi, Paris; 60 mg kg⁻¹, i.p.) and their saphenous nerves were exposed and sectioned at the level of the thigh, the right nerve being hung in paraffin over a pair of stimulating electrodes, the

other serving as an internal control. Evans blue (E. Merck, Darmstadt, Germany; 50 mg kg⁻¹, i.v.) and κ agonists were dissolved in a 0.9% solution of NaCl and applied simultaneously through a cannula in a tail vein. After 15 min the saphenous nerve was stimulated antidromically with square-wave electrical pulses (5 mA, 1 ms, 2 Hz) for 10 min, following which the animal was killed with an overdose of anaesthetic. The skin of both hindpaws was then removed and incubated for 90 h in formamide (E. Merck, Darmstadt, Germany) at room temperature. Extracted dye was measured with an LKB Ultrospec spectrophotometer at 620 nm. Comparisons were made of the mean amounts of extracted dye (expressed as μ g dye per 100 mg skin) on stimulated and control sides for groups of animals treated with saline (vehicle) or test compound. Alternatively, the mean of differences between stimulated and control sides were used for analysis.

Local inhibitory actions of systemically-applied EMD 60400 were tested for by injecting 50 μ g norbinaltorphimine dissolved in 25 μ l saline into the paw (intraplantar, i.pl.) 10 min after the application of Evans blue and agonist and 5 min before the beginning of nerve stimulation. The control side was injected with 25 μ l saline at the same time.

In other experiments substance P (10 μ g) was dissolved in 25 μ l saline and injected into the paw 15 min after the injection of Evans blue and EMD 60400. It was allowed to act for 10 min before the experiment was terminated. Investigations into the role of the adrenal glands were performed on rats adrenalectomized immediately prior to preparation of the saphenous nerves.

Urinary volume in conscious rats Male Wistar rats (180–250 g) received water *ad libitum* and were deprived of food for 18 h before experiments. Groups of three rats had their urinary bladders emptied by light pressure and were placed in metabolism cages for 6 h. The volume of urine produced in this time was expressed as ml per 100 g body weight. In additional experiments with volume-loaded rats, a 0.6% solution of NaCl (10 ml per 100 g, i.p.) was injected just before the beginning of the experiment. Agonists were dissolved in distilled water and injected s.c. in a volume of 0.1 ml per 100 g immediately before the start of the measurements.

Substances

The following substances were used: [D-Ala², D-Leu⁵]enkephalin acetate (Bachem), EMD 60400 (N-methyl-N-[(1S)-1-phenyl-2-((3S)-3-hydroxypyrrolidine-1-yl)-ethyl]-2-aminophenylacetamide 2HCl, E. Merck, Darmstadt, Germany; Figure 1), ethylketocyclazocine methanesulphonate (Sterling Winthrop), haloperidol HCl (E. Merck, Darmstadt, Germany), ICI 197067 ((2S)-N-[2-(N-methyl-3, 4-dichlorophenylacetamido)-3-methylbutyl]-pyrrolidine HCl (E. Merck, Darmstadt, Germany; Cambridge Research Biochemicals; Figure 1), ICI 204448 (2-[3-(1-(3,4-dichlorophenyl)-N-methylacetamido)-2-pyrrolidonoethyl]-phenoxy]acetic acid HCl; E. Merck, Darmstadt, Germany; Figure 1), morphine HCl (E. Merck, Darmstadt, Germany), naloxone HCl (Sigma), norbinaltorphimine 2HCl (Research Biochemicals Inc.), NSD 1015 (*m*-hydroxybenzylhydrazine 2HCl; Sigma), substance P tetratrifluoroacetate (Bachem) and U 69593 ((5 α ,7 α ,8 β)-(–)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro (4,5)-dec-8-yl)-phenylacetamide; Upjohn, Kalamazoo, U.S.A.). The ligands [³H]-U 69593, [³H]-PL 017 ([N-MePhe³, D-Pro⁷]morphiceptin), [³H]-[D-Pen^{2,3}]enkephalin, [³H]-[D-Ala², D-Leu⁵]enkephalin acetate and [³H]-ethylketocyclazocine were from NEN Research Products, Dreieich.

Statistics

Tests of statistical significance were made with the Dunnett test for multiple comparisons with control; 95% confidence intervals were calculated by the method of Litchfield & Wilcoxon (1949), except in the case of the rotarod and pressure

pain tests, where the method of Dixon & Mood (1948) was used.

Results

In vitro tests

Binding Experiments with radiolabelled ligands revealed that EMD 60400 and ICI 197067 bind potently to κ -opioid receptors, with IC₅₀ values (mean $\pm$ s.d.) of 2.8 $\pm$ 1.3 and 1.5 $\pm$ 0.2 nM, respectively; ICI 204448 had an IC₅₀ value for binding to κ -opioid receptors of 13.0 $\pm$ 5.7 nM (all *n* = 3). The corresponding figures for μ and δ binding were 500 $\pm$ 115, 100 $\pm$ 57 and 233 $\pm$ 60 nM, and 333 $\pm$ 88, 1200 $\pm$ 400 and 167 $\pm$ 33 nM, respectively (all *n* = 3).

Electrically-stimulated rabbit vasa deferentia EMD 60400, ICI 197067, and ICI 204448 were full and potent κ -opioid receptor agonists which were able to produce concentration-dependent inhibition of electrically-induced contractions of rabbit isolated vasa deferentia. IC₅₀ values (means with 95% confidence intervals) were 41.8 (35.1–49.9; *n* = 4), 15.7 (5.2–47; *n* = 4), and 15 (12–19; *n* = 3) nM, respectively.

Central actions of systemically-applied κ agonists

Ex vivo determination of brain and plasma levels Results from this test indicated that EMD 60400 is absorbed well into blood plasma following its s.c. administration (see Table 1 for comparison with ICI 197067). The concentration of EMD 60400 in the brains of treated mice increased from a threshold between 1.0 and 10 mg kg⁻¹, whereas for ICI 197067 this threshold lay between 0.1 and 1.0 mg kg⁻¹ (Table 1). The brain concentration of ICI 204448 was below 2 pg mg⁻¹ after s.c. application at a dose of 10 mg kg⁻¹.

Figure 1 Chemical structures of EMD 60400, ICI 197067 and ICI 204448.

Table 1 Estimation of brain and plasma concentrations (mean $\pm$ s.d.) by *ex vivo* binding in the mouse

	Dose (mg kg ⁻¹ s.c.)	Brain level (pg mg ⁻¹)	Plasma concentration (ng ml ⁻¹)
EMD 60400	0.1	< 1	3.1 $\pm$ 0.2
	1.0	2.6 $\pm$ 3.5	20.4 $\pm$ 5.9
	10	120.0 $\pm$ 18.0	403.0 $\pm$ 35.0
	50	470.0 $\pm$ 166.0	1200.0 $\pm$ 117.0
ICI 197067	0.1	21.5 $\pm$ 3.5	23.6 $\pm$ 5.0
	1.0	167.4 $\pm$ 37.0	175.6 $\pm$ 8.4
	10	848.0 $\pm$ 27.0	626.8 $\pm$ 44.5

Haloperidol-induced DOPA accumulation Levels of DOPA in haloperidol-treated control groups (mean $\pm$ s.e.mean) were 4,547 $\pm$ 126 (n = 13), 5,258 $\pm$ 238 (n = 14), and 4,089 $\pm$ 247 (n = 3) ng g⁻¹ for EMD 60400, ICI 197067, and ICI 204448, respectively. Statistically significant inhibition of haloperidol-induced DOPA accumulation was obtained with EMD 60400 at doses of and above 3.0 mg kg⁻¹, s.c. and with ICI 197067 at doses of and above 0.3 mg kg⁻¹, s.c. (see Table 2 for ID₅₀ values). ICI 204448, on the other hand, produced no significant reduction in DOPA accumulation at a dose of 30 mg kg⁻¹, s.c. (Table 2).

Sleeping time Sleeping times in the vehicle-treated groups (mean $\pm$ s.e.mean) were 73.6 $\pm$ 3.6 (n = 30), 71.9 $\pm$ 3.2 (n = 29), and 82.0 $\pm$ 5.9 (n = 15) min for EMD 60400, ICI 197067, and ICI 204448, respectively. EMD 60400 prolonged hexobarbitone-induced sleeping in mice at doses above 3.0 mg kg⁻¹, s.c., whereas narcosis-potential after pretreatment with ICI 197067 was observed at doses above 0.3 mg kg⁻¹ (see Table 2 for ID₅₀ values). ICI 204448 was ineffective in this test at a dose of 30 mg kg⁻¹, s.c. (Table 2).

Rotarod The threshold dose for sedation 30 min after treatment with EMD 60400 lay between 2.5 and 5.0 mg kg⁻¹, s.c. and for ICI 197067 between 0.25 and 0.55 mg kg⁻¹, s.c. (see Table 2 for ID₅₀ values). ICI 204448 was completely inactive in this test at doses of 50 and 100 mg kg⁻¹, s.c. (Table 2).

Antinociceptive actions of the κ agonists

Mouse formalin-induced paw licking All three compounds produced dose-dependent antinociception against the non-inflammatory (1st) and inflammatory (2nd) phases of the nociceptive response to formalin (Figure 2; Table 3). When applied at a high enough dose these compounds were able to abolish completely the response to inflammatory and non-inflammatory pain.

The effect of naloxone on the opiate action was also tested. Licking was inhibited (mean $\pm$ s.e.mean; 1st phase/2nd phase) by 53 $\pm$ 9.6/52 $\pm$ 16.7% (n = 6) in mice treated with 1.0 mg kg⁻¹ EMD 60400, s.c., by -13 $\pm$ 16.7/21 $\pm$ 15.5% (n = 10) in mice injected 20 min before formalin with 1.0 mg kg⁻¹ naloxone, s.c., and by -4 $\pm$ 14.6/15 $\pm$ 25.8% (n = 6) in mice injected with both EMD 60400 and naloxone.

Mouse and rat writhing ICI 197067, EMD 60400 and ICI 204448 also had dose-dependent antinociceptive effects in

mouse and rat writhing tests, with each substance being equieffective in the two species (Figure 3; Table 3). The antinociceptive action of EMD 60400 in this test in mice was inhibited by the s.c. application of naloxone given 30 min prior to phenylbenzoquinone. The number of pain symptoms (mean $\pm$ s.e.mean) in the vehicle-treated group was 53.3 $\pm$ 4.1

Figure 2 Effects of κ agonists on formalin-induced paw licking observed 0–5 (open symbols) and 20–25 (closed symbols) min after the intraplantar injection of formalin. The κ agonists, EMD 60400 ($\square$, $\blacksquare$), ICI 197067 (Δ , $\blacktriangle$) and ICI 204448 ($\circ$, $\bullet$) were applied s.c. 30 min before formalin. Absolute licking times (mean $\pm$ s.e.mean; 1st phase/2nd phase) in the vehicle-treated groups were 51.3 $\pm$ 2.2/53.5 $\pm$ 12.4 (n = 12), 49.7 $\pm$ 4.1/57.5 $\pm$ 5.4 (n = 15), and 51.6 $\pm$ 5.1/26.3 $\pm$ 6.7 (n = 19) s for EMD 60400, ICI 197067, and ICI 204448, respectively. Each point in the figure represents the mean $\pm$ s.e.mean of measurements from 5–11 mice.

Table 3 Summary of ID₅₀ values (with 95% confidence intervals) in antinociceptive and related tests

Test	EMD 60400	ICI 197067	ICI 204448
Formalin			
1st phase	0.44 (0.17–1.1)	0.04 (0.02–0.1)	18.9 (7.3–49.1)
2nd phase	0.47 (NC)	0.02 (0.01–0.04)	12.6 (8.4–18.9)
Writhing			
Mouse	0.55 (0.41–0.75)	0.02 (0.01–0.03)	1.9 (0.6–5.9)
Rat	0.3 (0.2–0.5)	0.04 (0.01–0.11)	4.5 (0.6–31.5)
Pressure pain			
Hyperalgesic	0.1 μ g kg ⁻¹ (0.02–0.47)	4.4 μ g kg ⁻¹ (1.3–14.9)	0.8 (0.3–2.4)
Normalgesic	4.0 (2–8)	77 μ g kg ⁻¹ (24–246)	> 30
Neurogenic inflammation	0.3* (0.1–1.0)	1.8* (0.64–5.0)	1.5* (0.4–4.9)

ID₅₀ values (mg kg⁻¹, s.c. or i.v.*). NC = not calculable.

Table 2 Summary of ID₅₀ values (with 95% confidence intervals) in tests of central activity

Test	EMD 60400	ICI 197067	ICI 204448
DOPA accumulation	5.0 (0.7–35)	2.0 (0.02–168)	> 30
Hexobarbitone sleeping	16 (10.0–25.6)	3.3 (1.5–7.3)	> 30
Rotarod	6.1 (4.3–8.7)	0.71 (0.45–1.12)	> 100

ID₅₀ values (mg kg⁻¹, s.c.).

Figure 3 Effects of κ agonists on writhing movements observed 0–20 min after the i.p. injection of 2-phenyl-1,4-benzoquinone. The κ agonists EMD 60400 ($\square$, $\blacksquare$), ICI 197067 (Δ , $\blacktriangle$) and ICI 204448 ($\circ$, $\bullet$) were applied s.c. 30 min before 2-phenyl-1,4-benzoquinone. The numbers of writhing movements (mean $\pm$ s.e.mean) in the vehicle-treated groups in mice were 51.6 ± 4.5 , 36 ± 2.2 and 41.1 ± 1.1 (all $n = 15$) and rats were 53.6 ± 4.3 ($n = 12$), 43.4 ± 2.8 ($n = 16$), and 48.2 ± 4.6 ($n = 11$) for EMD 60400, ICI 197067, and ICI 204448, respectively. Each point in the figure represents the mean $\pm$ s.e.mean of measurements from 4 rats (closed symbols) or 10 mice (open symbols).

($n = 15$), in the group treated with 1.0 mg kg^{-1} naloxone, s.c., was 53.6 ± 4.1 ($n = 10$), in the group injected with 0.6 mg kg^{-1} EMD 60400 was 12.0 ± 1.7 ($n = 10$), and in the group given both EMD 60400 and naloxone was 37.5 ± 4.8 ($n = 10$; there was no difference between this group and the vehicle-treated group, $P > 0.05$).

Carrageenin-induced hyperalgesia Both EMD 60400 and ICI 197067 proved extraordinarily effective at inhibiting pressure pain under conditions of inflammatory hyperalgesia, with ID_{50} values in the $\mu\text{g kg}^{-1}$ range (Figure 4; Table 3). ICI 204448 was not as potent, but nevertheless had a dose-dependent antinociceptive effect in this test (Figure 4; Table 3). The antinociceptive effectiveness (number of rats not responding to painful pressure) was four out of six animals treated 30 min before testing with EMD 60400 $0.5 \mu\text{g kg}^{-1}$, s.c., 0 out of three animals injected with naloxone 0.2 mg

Figure 4 Effects of the κ agonists EMD 60400 ($\square$, $\blacksquare$), ICI 197067 (Δ , $\blacktriangle$) and ICI 204448 ($\circ$, $\bullet$) on pressure pain in the rat. Measurements were either made 30 min after the application of the κ agonist in rats without inflammation (open symbols), or 3 h after the injection of carrageenin into the tail (closed symbols), in which case the κ agonists were applied at the same time as carrageenin. Each point represents the % of animals ($n = 3$ –6) which did not respond to a standard noxious press.

kg^{-1} , i.p. 20 min before testing, and 0 out of six animals treated with EMD 60400 and naloxone.

The opiate action against pressure pain was weaker in normalgic rats, i.e. animals which had not been injected with carrageenin (Figure 4). In these animals EMD 60400 and ICI 197067 were 40,000 and 17.5 times less effective, respectively, than under conditions of inflammatory hyperalgesia, whereas ICI 204448 was ineffective at doses of 10 and 30 mg kg^{-1} , s.c. (Figure 4; Table 3).

Other in vivo effects

Neurogenic inflammation Neurogenic plasma extravasation in anaesthetized rats was dose-dependently inhibited by the i.v. administration of all three κ -opioid receptor agonists (Figure 5; Table 3), whereby EMD 60400 was most effective

Figure 5 Effects of κ agonists on neurogenic plasma extravasation evoked by 10 min antidromic electrical stimulation of the saphenous nerve. The κ agonists, EMD 60400 ($\blacksquare$), ICI 197067 ($\blacktriangle$) and ICI 204448 ($\bullet$) were applied i.v. 15 min before the beginning of nerve stimulation. Neurogenic inflammation was defined as the difference between dye levels in stimulated and control paws. Dye levels (mean $\pm$ s.e.mean) in paws from vehicle-treated rats were 3.4 ± 0.3 ($n = 21$), 3.7 ± 0.4 ($n = 23$), and 3.9 ± 0.5 ($n = 22$) $\mu\text{g dye per } 100 \text{ mg skin}$ for EMD 60400, ICI 197067, and ICI 204448, respectively. Each point in the figure represents the mean $\pm$ s.e.mean from 7 animals.

Figure 6 Effects of κ agonists on urinary volume in non-hydrated rats in the 6 h following s.c. application of EMD 60400 ($\blacksquare$), ICI 197067 ($\blacktriangle$) and ICI 204448 ($\bullet$). The amount of urine (mean $\pm$ s.e. mean) in the vehicle-treated groups were 0.56 ± 0.05 ($n = 30$), 0.49 ± 0.03 ($n = 20$) and 0.44 ± 0.03 ($n = 30$) $\text{ml } 100 \text{ g}^{-1} \text{ body weight}$ for EMD 60400, ICI 197067, and ICI 204448, respectively. Each point in the figure represents the mean $\pm$ s.e.mean of 5 or 10 measurements.

and ICI 204448 and ICI 197067 were equipotent.

The i.v. application of EMD 60400 did not reduce the mean ($\pm$ s.e.mean) amount of dye measured in the paw after i.pl. injection of 10 μ g substance P (control animals had 18.8 ± 1.74 μ g dye per 100 mg skin, $n = 13$; animals treated with 1 mg kg^{-1} EMD 60400 had 18.4 ± 2.72 μ g dye per 100 mg skin, $n = 7$). Furthermore, the action of EMD 60400 was not blocked by acute adrenalectomy. EMD 60400 at a dose of 1.0 mg kg^{-1} produced a $33 \pm 4.7\%$ (mean $\pm$ s.e.mean) inhibition of neurogenic extravasation in intact animals (Figure 4) and a $40 \pm 6.3\%$ inhibition in adrenalectomised animals ($n = 7$).

Diuresis All three κ -opioid receptor agonists were able to produce large (between 3 and 9 fold) increases in total urinary volume (Figure 6) after s.c. application in non-hydrated conscious rats. ICI 197067 was the most potent of these compounds, with a diuretic effect at doses of and above 10 μ g kg^{-1} (Figure 6). EMD 60400 produced diuresis at doses of and above 100 μ g kg^{-1} (Figure 6). Diuresis was increased following doses of ICI 204448 of and above 0.3 mg kg^{-1} , though it was noticeable that this compound was not able to raise urinary volume to the extent of ICI 197067 or EMD 60400.

ICI 197067 at very low doses elicited antidiuresis in hydrated rats. Vehicle-treated animals produced 3.5 ± 0.09 ml urine per 100 g (mean $\pm$ s.e.mean), whereas rats treated with 1, 3 and 6 μ g kg^{-1} ICI 197067, s.c., produced 3.7 ± 0.09 , 3.0 ± 0.04 and 3.1 ± 0.08 ml urine per 100 g (all $n = 5$), of which the latter two values are significantly less than control ($P < 0.01$).

Peripheral actions of systemically-applied EMD 60400

The antinociceptive action of systemically-applied EMD 60400 in the hyperalgesic pressure pain test was completely

Figure 7 Reversal of EMD 60400-induced inhibition of neurogenic extravasation by intraplantar (i.pl.) norbinaltorphimine. Shown are mean amounts of dye ($\pm$ s.e.mean) measured in skin from paws in which the saphenous nerve was stimulated antidromically (solid columns) and from control paws which were all injected with 25 μ l NaCl solution or 50 μ g norbinaltorphimine (BNI), while rats were treated with either NaCl or 1 mg kg^{-1} EMD 60400 i.v. The number of animals in each group is shown in the corresponding open column. The amount of dye measured on the stimulated side of rats injected with EMD 60400 was significantly different from control (** $P < 0.01$), otherwise differences from control were not statistically significant.

antagonized by small doses of the κ -opioid receptor antagonist, norbinaltorphimine, applied directly into the inflamed portion of the tail. Six out of six rats responded to painful pressure 30 min after receiving vehicle s.c. and 5 min after an injection of 100 μ g norbinaltorphimine into the tail, two out of six rats responded after the application of 50 μ g kg^{-1} EMD 60400 s.c. and vehicle into the tail, whereas six out of six animals responded to pressure after receiving EMD 60400 s.c. and norbinaltorphimine in the tail. There was no evidence that norbinaltorphimine at this dose can produce systemic inhibition of κ antinociception. Two out of six animals responded to pressure 30 min after the injection of EMD 60400 0.5 μ g kg^{-1} , s.c. and vehicle i.p., while one out of six rats responded after the application of EMD 60400 s.c. and norbinaltorphimine 100 μ g, i.p.

The cutaneous locus of action of EMD 60400 against neurogenic inflammation was demonstrated by the i.pl. application of norbinaltorphimine. The inhibitory action of EMD 60400 (1 mg kg^{-1} , i.v.) in the skin of the hind paw could be completely antagonized by the i.pl. application of 50 μ g norbinaltorphimine (Figure 7). This dose of norbinaltorphimine had no significant effect on neurogenic plasma extravasation on its own (Figure 7) and also did not antagonize the opiate action when given by the i.v. route of administration. The amount of dye in the skin (mean $\pm$ s.e. mean of the difference between stimulated and unstimulated paws expressed as μ g dye per 100 mg skin) was 6.3 ± 0.7 ($n = 5$) in vehicle-treated animals, 5.7 ± 0.7 ($n = 4$) after 50 μ g norbinaltorphimine i.v., 2.6 ± 0.8 ($n = 5$) after 1.0 mg kg^{-1} EMD 60400 i.v., and 2.8 ± 1.1 ($n = 5$) after EMD 60400 and norbinaltorphimine.

Discussion

There have been a number of reports that centrally-active κ -opioid receptor agonists can have direct, peripheral antinociceptive actions in inflamed tissue (see Barber & Gottschlich, 1992, for recent review). These findings, along with the recognition that the systemic application of such agonists in humans has unacceptable central side effects (Pfeiffer *et al.*, 1986), have led to interest in the development of 'peripherally-selective' κ -opioid receptor agonists for the treatment of inflammatory hyperalgesia. EMD 60400 (Barber *et al.*, 1992), ICI 197067 (Costello *et al.*, 1988) and ICI 204448 (Shaw *et al.*, 1989) are κ agonists which were expected to have different degrees of access to the CNS following their systemic application.

The results from the present *in vitro* experiments indicate that EMD 60400 and ICI 197067 are potent, selective and full κ -opioid receptor agonists. We were able to confirm the low κ binding affinity of ICI 204448 relative to ICI 197067 reported on the basis of inhibition of [3 H]-bremazocine binding by Shaw *et al.* (1989; 33.2 and 1.19 nM, respectively). Under the present experimental conditions the binding affinities of ICI 204448 and ICI 197067 for the κ -opioid receptor were 13.0 and 1.5 nM, respectively. The present binding studies further indicate that ICI 204448 is not particularly selective in its affinity for the κ -opioid receptor, with a $\kappa:\mu:\delta$ binding ratio of 1:17.9:12.8 (this ratio was 1:179:119 and 1:67:800 for EMD 60400 and ICI 197067, respectively). Our IC_{50} value for the agonist potency of ICI 204448 in the rabbit vas deferens preparation (15 nM) is also similar to the 10.8 nM found by Shaw *et al.* (1989). On the other hand, the $\kappa:\mu$ selectivity of ICI 197067 calculated in the present experiments on the basis of [3 H]-U-69593:[3 H]-PL-017 binding (1:67) is much less than that reported by Costello *et al.* (1988) using [3 H]-bremazocine:[3 H]-naloxone binding (1:1900).

We have tried to obtain a measure of the ability of EMD 60400, ICI 197067 and ICI 204448 to induce the centrally-mediated side effects, aversion and sedation. The increase in DOPA accumulation in the nucleus accumbens induced by the block of inhibitory D_2 receptors with halo-

peridol is known to be reversed by centrally-active κ -opioid receptor agonists (Manzanares *et al.*, 1991) because dopamine release in the accumbens is also subject to presynaptic inhibition from κ agonists (Smith *et al.*, 1992). Since the κ agonists used here have only very poor affinity for dopamine receptors (C.A. Seyfried, unpublished observations), results with this method can be taken as a measure of activity at central κ -opioid receptors in that neurotransmitter system and area of the brain in which κ agonists are believed to mediate their aversive effects (Di Chiara & Imperato, 1988; Spanagel *et al.*, 1990). The sedative effects of the κ -opioid receptor agonists were investigated in the mouse hexobarbitone narcosis and rodent rotarod tests, in which centrally-active κ agonists are known to be active (e.g. Hayes *et al.*, 1990; Fürst, 1991).

These methods have produced similar results. Systemically-applied ICI 204448 gave no sign of activating central κ -opioid receptors in rats and mice even at high doses. Thus ICI 204448 was not detected in *ex vivo* binding experiments after s.c. application of 10 mg kg⁻¹. Thirty mg kg⁻¹ ICI 204448 s.c. produced no reduction in DOPA accumulation in the nucleus accumbens (Table 2) or prolongation of hexobarbitone-induced sleeping (Table 2), and 100 mg kg⁻¹ ICI 204448 s.c. did not impair motor performance in the rotarod test (Table 2). Since ICI 204448 is as potent in the rabbit vas deferens assay as the other κ agonists used here it is likely that these results are indicative of poor penetration into the CNS due to the polar zwitterionic structure of ICI 204448 (Shaw *et al.*, 1989).

ICI 197067 displays a greater ability to penetrate into the CNS than ICI 204448 (Shaw *et al.*, 1989). Our results in the *ex vivo* binding test (Table 1) are comparable with those of Shaw *et al.* (1989), who found a brain concentration of 119 pg mg⁻¹ tissue after the s.c. application of ICI 197067 at a dose of 0.23 mg kg⁻¹. In our experiments ICI 197067 began to increase its concentration in the brain substantially at s.c. doses between 0.1 and 1.0 mg kg⁻¹ (Table 1). This compound also produced significant inhibition of DOPA accumulation at doses of 0.3 mg kg⁻¹, s.c. and above, prolonged hexobarbitone-induced sleeping at doses above 0.3 mg kg⁻¹, s.c., and impaired performance on the rotarod at doses above 0.25 mg kg⁻¹, s.c. The data therefore suggest that ICI 197067 penetrates the blood-brain barrier in sufficient amounts to elicit the centrally-mediated side effects, sedation and putative aversion, at a threshold dose of around 0.3 mg kg⁻¹, s.c.

The threshold dose for these effects with EMD 60400 is in the order of 10 fold higher since inhibition of DOPA accumulation, prolongation of sleeping time and impaired rotarod performance were all observed at doses beginning around 3.0 mg kg⁻¹, s.c. Results from the *ex vivo* experiments also indicate that the concentration of EMD 60400 in the brain began to increase markedly only after doses between 1 and 10 mg kg⁻¹, s.c. (Table 1). We interpret these findings as being due to a reduced tendency of EMD 60400 to cross the blood-brain barrier, as compared to ICI 197067. The structural characteristics of EMD 60400, namely the inclusion of 2 polar groups (NH₂ and OH; Figure 1), therefore appear to allow the combination of high affinity and selectivity for the κ -opioid receptor with a relatively high threshold for producing central side effects.

Centrally-acting κ -opioid receptor agonists generally induce sedative effects at higher doses than central antinociceptive actions (Hayes *et al.*, 1990; Hunter *et al.*, 1990). The three κ agonists we tested were effective in the rodent writhing test (Figure 3), and the present ID₅₀ values for ICI 197067 and ICI 204448 in the mouse writhing test (0.02 and 1.9 mg kg⁻¹, s.c., respectively) are in good agreement with those published previously by others (0.05 (0.02–0.06) and 4.4 (2.5–7.4) mg kg⁻¹, s.c., respectively; Costello *et al.*, 1988; Rogers *et al.*, 1992).

EMD 60400, ICI 197067 and ICI 204448 were also effective in the mouse formalin test against both non-inflammatory (1st phase) and inflammatory (2nd phase) pain, although we,

in contrast to Rogers *et al.* (1992) who used the rat, saw no evidence of more pronounced inhibition of the 2nd than the 1st phase by the peripherally-selective compound ICI 204448 (Figure 2; Table 3). The dose-response relationships indicate that all three κ agonists produce their antinociceptive actions in this test in dose-ranges in which antinociceptive effects can be found in the writhing test.

The sites of action of the κ agonists in these tests are still unclear. Rogers *et al.* (1992) concluded on the basis of their experiments with s.c. and i.c.v. application that systemic antinociceptive effects of the peripherally-selective κ agonist, GR 94839, in the mouse writhing test were probably due to penetration of this compound into the CNS. Moreover, the fact that the antinociceptive action of GR 94839 after systemic application in the formalin test was only partially antagonized by i.p.l. application of the opiate antagonists, naltrexone (1 μ g) or norbinaltorphimine (100 μ g; Rogers *et al.*, 1992) may indicate that GR 94839 is having central and peripheral actions in this test. Both central and peripheral inhibitory effects of κ agonists have been reported previously in the formalin test (Haley *et al.*, 1990; Pelissier *et al.*, 1990).

The antinociceptive actions of the κ agonists in the normalgesic pressure pain test reported here (Figure 4) also correspond well to their effectiveness in the writhing test (with the exception of ICI 204448; Figure 3). However, EMD 60400 and ICI 204448 become much more potent antinociceptive agents in tissue inflamed by the injection of carrageenin, as do GR 94839 and ICI 204448 in the zymosan-induced hyperalgesia model in the rat paw (Rogers *et al.*, 1992). It seems very probable that under these conditions EMD 60400 is producing antinociception in the periphery rather than the CNS since the action of this compound can be completely inhibited by injection of the κ -opioid receptor antagonist, norbinaltorphimine, directly into the tail at a dose (100 μ g) which produces no systemic inhibition of κ -mediated antinociception.

Independent of where it is acting, the selectivity of EMD 60400 for κ -opioid receptors means that its antinociceptive actions in the writhing and formalin tests are most probably mediated by these receptors. We found that the action of EMD 60400 in these tests could be reversed by pretreatment with naloxone (1 mg kg⁻¹, s.c.), like Hunter *et al.* (1990) who found that this dose of naloxone completely antagonized antinociception produced by CI-977, PD 117302, U 69593, U 50488 and other κ -agonists in the rat paw pressure test. The observation that the action of EMD 60400 in the hyperalgesic pressure pain test can be completely antagonized by 0.2 mg kg⁻¹ naloxone s.c. may, however, indicate that this compound can also have activity at μ -opioid receptors under some *in vivo* experimental conditions.

In the present study we have shown that systemically-applied EMD 60400 also inhibits neurogenic inflammation (Figure 5). This is probably achieved via a purely peripheral opioid mechanism in the skin because the inhibitory action of this compound can be completely reversed by 50 μ g norbinaltorphimine applied directly into the paw (Figure 7). This dose of norbinaltorphimine produces no systemic inhibition of the opiate effect. Similar effects have been obtained with ICI 204448 using 10 μ g naloxone as the antagonist (A. Barber, unpublished observations) and previous studies have shown that the inhibitory action of systemically-applied ethylketocyclazocine in this test can be antagonized by the systemic application of the quaternised opiate antagonist, N-methyl nalorphine (Smith & Buchan, 1984).

The diuretic effects of EMD 60400, ICI 197067 and ICI 204448 described here are typical for κ -opioid receptor agonists (Vonvoightlander *et al.*, 1983; Leander *et al.*, 1983). Antidiuretic effects of κ agonists at high doses (e.g. 10 mg kg⁻¹ U 69593, s.c.; Hayes *et al.*, 1990; 10 mg kg⁻¹ U 50488, i.p.; Bianchi, 1991) and in the 1st hour following the blockade of α_2 -adrenoceptors with idazoxan or yohimbine (Bianchi, 1991) have also been observed. But antidiuretic actions of substances of this class at such very low doses relative to

effective doses in the mouse writhing test have not been documented previously.

Diuretics have been described as having weak antinociceptive effects in rats in the hot plate test (Poggioli *et al.*, 1985) but it is unclear to what extent the diuretic action of the κ agonists contributes to the antinociception reported here, for instance, by reducing swelling. Antinociception and diuresis following the application of EMD 60400 and ICI 197067 occur in the same dose-range in the formalin, writhing and normalgesic pressure pain tests (Figures 2,3,4 and 6). However, these compounds are effective in the hyperalgesic pressure pain test at doses too low to cause diuresis (Figure 4), which therefore rules out the possibility of a pseudo-antinociceptive effect through loss of body fluid.

An important mechanism of the peripheral antinociceptive action of κ -opioid agonists in the hyperalgesic pressure pain model is likely to be the reduction in the flow of tonic sensory information from the painful area (Russell *et al.*, 1987; Haley *et al.*, 1990). Such a peripheral inhibitory action of systemically-applied EMD 60400 on the spontaneous and evoked activity of slowly-conducting afferents from the inflamed knee joint of the cat has recently been demonstrated (Meblinger *et al.*, 1993).

In the present study it was found that systemically-applied EMD 60400 did not reduce the effectiveness of i.pl. substance P in eliciting plasma extravasation in rat skin. Since substance P is the main putative neurotransmitter mediating neurogenic inflammation (Holzer, 1988), this indicates that the opiates have a pre- rather than a postjunctional mechanism of action in this test. The results with adrenalectomized animals also demonstrated that EMD 60400 does not exert an indirect action via the adrenal glands (Carey *et al.*, 1987). The most likely mechanism involves the presynaptic inhibition of the release of substance P from primary afferent fibres in the skin (Yonehara *et al.*, 1992). Peripheral opiate receptors on small diameter sensory neurones capable of mediating inhibition of impulse flow and substance P release have been documented (Stein *et al.*, 1990).

The results of our experiments therefore indicate that EMD 60400 and ICI 197067 are potent and selective κ -opioid receptor agonists which display different tendencies to cross the blood-brain barrier, as measured by *ex vivo* binding. This is reflected in their relative abilities to elicit the centrally-mediated side effects, sedation and putative aversion (ICI 197067 > EMD 60400 > ICI 204448). Furthermore, EMD 60400 inhibits hyperalgesic pressure pain and neurogenic inflammation through a peripheral mechanism of action. This could involve inhibition of the flow of tonic and evoked sensory information from inflamed areas to the CNS, and inhibition of the peripheral release of the inflammatory mediator substance P, presumably through the activation of peripheral κ -opioid receptors on primary sensory neurones. Systemic application of these agonists also produced antinociception in the writhing, normalgesic pressure pain, and formalin tests, though the relative contribution of central and peripheral antinociceptive components in these models remains to be determined. The development of peripherally-selective κ agonists may allow novel treatment of inflammatory pain in man which is not accompanied by unacceptable central side effects.

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