Peripheral versus Central Antinociceptive Actions of 6-Amino Acid-Substituted Derivatives of 14-O-Methylxoyomorphine in Acute and Inflammatory Pain in the Rat

Susanna Fürst, Pal Riba, Tamas Friedmann, Julia Timár, Mahmood Al-Khrasani, Ilona Obara, Wioletta Makuch, Mariana Spetea, Johannes Schütz, Ryszard Przewlocki, Barbara Przewlocka, and Helmut Schmidhammer

Department of Pharmacology and Pharmacotherapy, Medical Faculty, Semmelweis University, Budapest, Hungary (S.F., P.R., T.F., J.T., M.A.); Hungarian Academy of Sciences-SE Group of Neuropsychopharmacology, Budapest, Hungary (S.F.); Department of Molecular Neuropharmacology, Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland (I.O., W.M., R.P., B.P.); and Department of Pharmaceutical Chemistry, Institute of Pharmacy and Center for Molecular Biosciences Innsbruck, University of Innsbruck, Innsbruck, Austria (M.S., J.S., H.S.)

Received July 28, 2004; accepted September 21, 2004

ABSTRACT

Opioid analgesics with restricted access to the central nervous system represent a new approach to the treatment of severe pain with an improved safety profile. The objective of this study was to investigate the peripheral and central components of the antinociceptive actions of the 6-amino acid conjugates (glycine, alanine, and phenylalanine) of 14-O-methylxoyomorphine. Their antinociceptive activities were compared with those of the centrally penetrating μ-opioid agonists morphine, fentanyl, and 14-O-methylxoyomorphine. In the tail-flick test in rats, the 6-amino acid conjugates were 45- to 1170-fold more potent than morphine after i.c.v. administration and 19- to 209-fold after s.c. administration. They showed potencies similar to fentanyl after s.c. administration and were more potent after i.c.v. administration. The time course of action was different between s.c. and i.c.v. administration, with significant long-lasting effects after i.c.v. administration. Systemic administration of the peripherally selective opioid antagonist naloxone methiodide antagonized the effects after s.c. but not after i.c.v. administration in the tail-flick test. Subcutaneous 6-amino acid derivatives also elicited antihyperalgesic effects in the formalin test in rats, which were reversed by systemically administered naloxone methiodide. Although morphine exerts its analgesic effects by central and peripheral mechanisms, the investigated new opioids interact primarily with peripheral opioid receptors after s.c. administration. The present data indicate that the 6-amino acid conjugates of 14-O-methylxoyomorphine have limited access to the central nervous system and can mediate antinociception at peripheral sites. Also, they might find clinical application when the central actions of opioids are unwanted.

The potent antinociceptive actions of classical opioids such as morphine are traditionally considered to be mediated centrally through an action at the supraspinal or spinal level (for reviews, see Fürst, 1999; Gutstein and Akil, 2001; Przewlocki and Przewlocka, 2001). Antinociceptive effects have also been demonstrated to result from local application of opioids in the periphery, for example, in mouse writhing (Kolesnikov et al., 1996; Reichert et al., 2001) and in rat models of inflammation (Stein et al., 1989; Perrot et al., 2001) or neuropathic pain (Obara et al., 2004). These effects have been attributed to opioid induced actions mediated by peripheral opioid receptors (Fields et al., 1980; Stein et al., 1995). Neuroanatomical, molecular and electrophysiological studies have shown that such receptors are expressed on peripheral terminals of sensory neurons where they can modulate both afferent and efferent neuronal functions, resulting in antinociception (Schafer et al., 1995; Stein et al., 1996).

ABBREVIATIONS: M6G, morphine-6-glucuronide; HS-730, 2-[(4α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]acetic acid bis(tetrafluoroborate); HS-731, 2-[(4α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]acetic acid bis(tetrafluoroborate); HS-935, (2S)-2-[(4α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]propanoic acid bis(tetrafluoroborate); HS-936, (2S)-2-[(4α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]propanoic acid bis(tetrafluoroborate); HS-937, (2S)-2-[(4α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6α-yl)amino]-3-phenylpropanoic acid bis(tetrafluoroborate); HS-938, (2S)-2-[(4α-epoxy-3-hydroxy-14β-methoxy-17-methylmorphinan-6β-yl)amino]-3-phenylpropanoic acid bis(tetrafluoroborate); ANOVA, analysis of variance.
Opioids interacting with peripheral opioid receptors without crossing the blood-brain barrier might be used as potent analgesics and should be devoid of centrally mediated side effects. Strategies to restrict the access of opioids to the central nervous system include quaternization and incorporation of highly polar hydrophilic substituents (Brown and Goldberg, 1985; Botros et al., 1989; Portoghese et al., 1995). Generally, it has been found that quaternary compounds have reduced access into the central nervous system, but they have considerably lower affinity and potency to opioid receptors (Herz and Teschemacher, 1971; Brown and Goldberg, 1985). To avoid this problem, opioids with hydrophilic groups attached at the C-6 position of the morphinan structure were synthesized (Botros et al., 1989, Portoghese et al., 1995; Schütz et al., 2003). Loperamide, asimadoline, morphine-6-glucoronide (M6G), and several peptidic κ-opioid agonists are examples of peripherally restricted opioids that have been investigated in experimental and clinical studies (for review, see Stein et al., 2003). Since the numerous side effects (e.g., respiratory depression, sedation, and constipation) and tolerance to morphine, fentanyl, or oxycodone strongly limit their clinical use, there is a continuous search for analgesic drugs with an improved side effect profile (for reviews, see Nicholson, 2003; Stein et al., 2003). Efforts to eliminate or minimize the undesired side effects of morphine led to the synthesis of a significant large number of compounds (for reviews, see Schmidhammer, 1993; Fürst et al., 1995). A derivative of oxymorphone, 14-O-methyloxymorphone, was developed by our group and reported to be about 400- and 40-fold more potent than morphine and oxymorphone, respectively, in the hot-plate test in mice (Schmidhammer et al., 1984; Fig. 1). Further chemical optimization led to 14-methoxymetopon (Schmidhammer et al., 1984; Fig. 1), a highly selective μ-opioid receptor agonist (Fürst et al., 1993; Spetea et al., 2003). This opioid was described as an extremely potent centrally acting analgesic, being 300- to 20,000-fold more potent than morphine and oxymorphone, respectively, in the analgesic paradigm used (Schmidhammer et al., 1990; Fürst et al., 1993; Zernig et al., 2000; King et al., 2003).

Our research efforts in the morphinan series have shifted from the development of highly potent and selective μ-opioid receptor agonists with a central site of action (Fürst et al., 1993; Schmidhammer, 1993) to the development of opioid analgesics that would have limited access to the central nervous system. A family of 6-amino acid-substituted (glycine, alanine, and phenylalanine) derivatives of 14-O-methyloxymorphone (Fig. 2) was developed to obtain opioid agonists that are more hydrophilic than the parent compound and that have peripheral selectivity (Schütz et al., 2003; Spetea et al., 2004). Binding studies revealed that these 6-amino acid conjugates displayed very high affinities to the μ-opioid receptor and were effective in inhibiting the electrically evoked contractions of the mouse vas deferens through the μ-opioid receptor (Spetea et al., 2004). Based on the calculation of blood-brain distribution coefficients, these derivatives were predicted to have restricted access to the central nervous system after systemic administration (Schütz et al., 2003).

In the present study, we aimed to investigate the peripheral and central components of the antinociceptive action of the 6-amino acid conjugates of 14-O-methyloxymorphone after subcutaneous and intracerebroventricular administration in the tail-flick test in rats. In addition, we have examined the dose-response correlations and the site of action of the 6-amino acid derivatives after subcutaneous administration in the formalin test in rats.

Materials and Methods

Materials. The 6-amino acid-substituted derivatives of 14-O-methyloxymorphone, HS-730, HS-731, HS-935, HS-936, HS-937, and HS-938, were prepared as recently described (Schütz et al., 2003; Fig. 2). 14-O-Methyloxymorphone was synthesized as described previously (Schmidhammer et al., 1984; Fig. 1). Morphine hydrochloride, fentanyl, and naloxone hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO) or from Polfa, Kutno, Poland (morphine). Naloxone methiodide (N-methylnaloxonium iodide; quaternary naloxone) was kindly provided by Dr. S. Hosztafi (Alkaloida-ICN, Tiszasvasvari, Hungary) or obtained from Sigma-Aldrich. Formaldehyde was obtained from Odczynniki SA (Lublin, Poland).

All drugs were dissolved in physiological saline and administered to 5 to 10 rats/group. Rats received s.c. injections under the skin of the neck, in a volume of 5 ml/kg body weight in the tail-flick test, or in a volume of 4 ml/kg in the formalin test. Intracerebroventricular (i.c.v.) injections were given directly into the cerebral ventricles in a volume of 5 μl. Control animals received the same volume of s.c. or i.c.v. saline.

Animals. Male Wistar rats (120–150 g) used in the tail-flick test were purchased from Charles River (Budapest, Hungary). Male Wistar rats (250–270 g) used in the formalin test were obtained from the Institute of Pharmacology, Polish Academy of Sciences (Krakow, Poland). Animals were housed in Macrolon cages (six animals per
cage) with food and water ad libitum, in a temperature-controlled room (22 ± 2°C), maintained on a 12-h light/dark cycle (the light on at 8:00 AM). Experiments were carried out in accordance to the Declaration of Helsinki and the Guide for Care and Use of Laboratory Animals. The experimental protocols were approved by the Semmelweis University (Budapest, Hungary) and the Institute of Experimental Animal Care Committee (Institute of Pharmacology, Krakow, Poland).

**Tail-Flick Test.** The tail-flick test in the rat was performed according to the described procedure (Fürst et al., 1993). A beam light was focused on the tip of the tail, and the latency required for the rat to remove its tail was determined before (baseline) and after drug administration, using an arbitrary cut off time of twice the control reaction time and expressed as percentages. The antinociceptive activity was assessed 30, 60, 120, and 180 min after s.c. drug administration, and 10, 20, 30, 60, 120, 180, 240, 300, 360, 420, and 480 min after i.c.v. administration.

To evaluate the site of action (peripheral or central) of the opioid agonists, the effect of naloxone, the tertiary opioid antagonist that readily crosses the blood-brain barrier, and that of the peripherally selective opioid antagonist naltrexone methiodide on the antinociceptive action in the tail-flick test was assessed after s.c. and i.c.v. administration. In experiments when the antagonist effect was assessed, i.c.v. naloxone or naltrexone methiodide was given 10 min before the s.c. administration of the opioid agonist. Antagonists were coadministered with the respective agonist, when they were given by the same route of administration, s.c. or i.c.v. Measurements were performed 30 min after s.c. and 20 min after i.c.v. administration of the opioid agonists.

**Formalin Test.** The formalin test was used as a model of tonic inflammatory pain. Pain-related behavior induced by local administration of formalin is characterized by the occurrence of two characteristic phases of increased pain sensitivity in rats. The first phase is related to a direct stimulation of nociceptors, primarily owing to the increased secretion of substance P, bradykinin, and stimulatory amino acids. In the second phase, there is an increase in the levels of histamine, prostaglandins, 5-hydroxytryptamine, and bradykinin, which leads to the development of a localized inflammatory response and progressive functional changes in the dorsal horn and the central nervous system.

Fifteen minutes after s.c. administration of the opioid agonists, rats were lightly anesthetized by inhalation of halothane (2–3% (v/v/oxygen mixture), 5 l/min) for 2 to 3 min in a Plexiglas chamber. Rats were then s.c. injected with 100 μl of 12% formalin into the dorsal part of the right hind paw as described previously (Dorazil-Dudzik et al., 2004). After formalin injection, rats were placed in a single wire cage for observation. Pain-related behavior was quantified by counting the incidence of spontaneous flinches, shakes, and jerks of the formalin-injected paw. Pain reactions were continuously counted for 90 min for each animal and then totaled over two characteristic periods: 5 to 15 min (first phase) and 35 to 40, 40 to 45, 45 to 50, 75 to 80, 80 to 85, and 85 to 90 min (second phase) after formalin administration. Naloxone methiodide (2131 nmol/kg s.c.) was injected simultaneously with the highest dose of each opioid agonist, 15 min before formalin injection.

**Data Analysis.** The differences between the results obtained both by the tail-flick test or the formalin test were statistically determined by analysis of variance (ANOVA). For the significant differences between the groups, we used Student's t test with Bonferroni correction as post hoc test. The ED_{50} values and 95% confidence limits were given in nanomoles. A p value <0.05 was considered statistically significant.

**Results**

**Antinociceptive Effects in the Tail-Flick Test in the Rat**

**Subcutaneous Administration.** In the tail-flick test in the rat, morphine, fentanyl, 14-O-methyloxymorphone, and five of the six 6-amino acid-substituted derivatives of 14-O-methyloxymorphine elicited dose- and time-dependent antinociceptive effects after s.c. administration. The values of ED_{50} are listed in Table 1. The peak ED_{50} values of the amino acid conjugates were ranging between 29 and 315 nmol/kg. One compound, HS-938, failed to produce 50% antinociception between 30 and 120 min after s.c. administration of 3600 nmol/kg (Table 1). The calculated ED_{50} values of morphine, fentanyl, and 14-O-methyloxymorphine at the peak of action (30 min) were 6053, 38.6, and 14.9 nmol/kg, respectively.

As shown in Table 1, morphine, fentanyl, and 14-O-methyl-oxymorphine produced their maximum antinociceptive effect 30 min after s.c. administration, whereas the new opioid agonists reached their peak of action at 60 min. The antino- ciceptive action of morphine, fentanyl, 14-O-methyloxymorphine, HS-731, and HS-935 lasted about 120 min, whereas the effect of HS-730, HS-936, and HS-937 lasted for 3 h after a single s.c. administration. A 2-fold higher dose of morphine was required to produce the same effect at 2 h after s.c. administration compared with the ED_{50} dose at the peak of action. In the case of HS-731, HS-935, and HS-937, only a 1.0- to 1.5-fold ED_{50} dose, as determined at 60 min, was required to produce antinociception at 120 min after s.c. administration. Notably, dose-dependent antinociceptive effects were still observed at 3 h after s.c. HS-730, HS-936, and 14-O-methyloxymorphine elicited dose- and time-dependent antinociceptive effects after s.c. administration.

**TABLE 1**

Antinociceptive potencies of 6-amino acid conjugates of 14-O-methyloxymorphine against radiant heat-induced nociception in the tail-flick test in the rat after s.c. administration as a function of time

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time After s.c. Administration (min)</th>
<th>ED_{50} (nmol/kg s.c.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>MORPHINE</td>
<td>6053^{b}</td>
<td>7626</td>
</tr>
<tr>
<td>FENTANYL</td>
<td>38.6^{b}</td>
<td>62.4</td>
</tr>
<tr>
<td>14-O-METHYLOXYMORPHINE</td>
<td>14.9^{a}</td>
<td>24.0</td>
</tr>
<tr>
<td>HS-730</td>
<td>137</td>
<td>58.5^{b}</td>
</tr>
<tr>
<td>HS-731</td>
<td>50.8</td>
<td>29.0^{b}</td>
</tr>
<tr>
<td>HS-935</td>
<td>150</td>
<td>68.9^{b}</td>
</tr>
<tr>
<td>HS-936</td>
<td>&gt;850</td>
<td>53.4^{a}</td>
</tr>
<tr>
<td>HS-937</td>
<td>641</td>
<td>315^{b}</td>
</tr>
<tr>
<td>HS-938</td>
<td>&gt;3600</td>
<td>&gt;3600</td>
</tr>
</tbody>
</table>

<sup>a</sup> — no dose-response relationship.<br>
<sup>b</sup> Peak of effect.
HS-937. The shortest duration of action was elicited by fentanyl, and a 10-fold higher dose was required to induce 50% analgesia at 120 min after s.c. administration than the dose determined at the peak effect (Table 1).

Antinociceptive potencies in the tail-flick test of α- versus β-epimers (Table 1; Fig. 2) did not differ significantly. The ratios are between 1.3 and 2.0 except for HS-938, the 6β-phenylalanine derivative, which proved to be ineffective up to 3600 nmol/kg, whereas its α-counterpart HS-937 showed a dose-dependent effect after s.c. administration (ED50 = 315 nmol/kg, at 60 min; Table 1).

**Intracerebroventricular Administration.** All tested opioid agonists produced dose- and time-related antinociceptive effects in the tail-flick test in rats after i.c.v. administration (Table 2). The time-response curves of higher i.c.v. doses corresponding to the calculated ED95 of the test opioids are shown in Fig. 3. The onset of the antinociceptive response elicited by fentanyl, morphine, and 14-O-methylmorphine was rapid, with a peak of action at 10 and 20 min, respectively (Fig. 3A). This effect rapidly declined and returned to the preinjection value about 1 to 2 h after i.c.v. drug injection. In contrast, the 6-amino acid conjugates of 14-O-methylmorphine exhibited a significantly increased duration of the antinociceptive effect (Fig. 3B). Their antinociceptive action showed a somewhat slower onset of action, which peaked at 30 to 120 min and lasted about 4 to 7 h. Whereas HS-938 failed to induce antinociception by s.c. administration up to 3600 nmol/kg, it produced a time- and dose-dependent response when applied i.c.v. (Table 2; Fig. 3B).

**Relative Antinociceptive Potency after s.c. and i.c.v. Administration in the Tail-Flick Test**

The peak antinociceptive potencies of the 6-amino acid derivatives of 14-O-methylmorphine relative to morphine and fentanyl are shown in Table 3. The differences in potency of the novel opioid agonists compared with morphine and fentanyl were much more remarkable after i.c.v. than after s.c. administration. Although they were slightly less potent or equipotent compared with fentanyl, they were about 19- to 209-fold more potent than morphine when administered s.c. (Table 3). However, the 14-O-methylmorphine derivatives proved to be 45- to 1170-fold more potent than morphine when given i.c.v. Furthermore, HS-938, which failed to produce antinociception by s.c. administration, was 45-fold more potent than morphine after i.c.v. injection (Tables 2 and 3). In contrast to s.c. administration, whereby the difference observed between the new opioids and fentanyl was negligible, the 6-amino acid conjugates of 14-O-methylmorphine were about 2- to 55-fold more potent analgesics than fentanyl after i.c.v. administration.

**Antinociceptive s.c./i.c.v. Potency Ratios in the Tail-Flick Test**

As shown in Table 4, the difference between the doses required to elicit 50% antinociceptive effect after peripheral systemic (s.c.) versus central (i.c.v.) administration (ED50 nanomoles per kilogram, s.c./ED50 nanomoles per rat, i.c.v.) was 23 in the case of fentanyl, 172 for morphine, and 87 for 14-O-methylmorphine, the parent molecule. The s.c./i.c.v. potency ratios of the 6-amino acid conjugates were found to be much higher compared with morphine, fentanyl, and 14-O-methylmorphine, 569, 651, 967, 1887, >4600, and

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time after i.c.v. Administration (min)</th>
<th>ED50 (nmol/rat i.c.v.)</th>
<th>Time after i.c.v. Administration (min)</th>
<th>ED50 (nmol/rat i.c.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>10 20 30 60 120 180 240</td>
<td>68.2 (22.1–56.3)</td>
<td>35.1 (35.4–91.1)</td>
<td>6.0 (4.16–11.1)</td>
</tr>
<tr>
<td>Fentanyl</td>
<td></td>
<td>1.68 (1.00–2.75)</td>
<td>1.72 (1.00–2.70)</td>
<td>0.39 (0.18–0.51)</td>
</tr>
<tr>
<td>14-O-Methylmorphine</td>
<td></td>
<td>0.205 (0.121–0.348)</td>
<td>0.101 (0.052–0.152)</td>
<td>0.048 (0.027–0.082)</td>
</tr>
<tr>
<td>HS-730</td>
<td></td>
<td>0.122 (0.065–0.238)</td>
<td>0.059 (0.030–0.106)</td>
<td>0.038 (0.018–0.082)</td>
</tr>
<tr>
<td>HS-731</td>
<td></td>
<td>0.289 (0.193–0.463)</td>
<td>0.157 (0.102–0.264)</td>
<td>0.067 (0.034–0.128)</td>
</tr>
<tr>
<td>HS-936</td>
<td></td>
<td>0.105 (0.065–0.184)</td>
<td>0.056 (0.034–0.110)</td>
<td>0.027 (0.016–0.063)</td>
</tr>
<tr>
<td>HS-937</td>
<td></td>
<td>0.234 (0.124–0.445)</td>
<td>0.136 (0.072–0.233)</td>
<td>0.072 (0.038–0.130)</td>
</tr>
<tr>
<td>HS-938</td>
<td></td>
<td>1.05 (0.567–1.94)</td>
<td>0.126 (0.077–0.208)</td>
<td>0.070 (0.034–0.138)</td>
</tr>
</tbody>
</table>
5000 for HS-935, HS-936, HS-731, HS-730, HS-938, and HS-937, respectively. These ratios were observed to be remarkably high in the case of HS-938 and HS-937, the phenylalanine-substituted /H9252- and /H9251-epimers, respectively (s.c./i.c.v. ED50 ratios 4600 and 5000, respectively) (Table 4).

**Antagonist Effect on the Antinociceptive Action in the Tail-Flick Test**

Among the new opioid agonists, HS-731 was selected for a more detailed investigation of the site of action and compared with the action of morphine (Fig. 4). Although systemically (s.c.) administered naloxone methiodide did not alter the effect of s.c. morphine (7769 nmol/kg), except in a very high dose (21,308 nmol/kg), it antagonized the s.c. antinociceptive effect of HS-731 (183 nmol/kg) (Fig. 4, A and B). A similar degree of antagonism (34–42%), as determined for HS-731, was observed in rats after s.c. coadministration of equipotent antinociceptive doses of HS-730, HS-935, HS-936, and HS-937, with 320 nmol/kg naloxone methiodide (data not shown).

**TABLE 3**

Relative antinociceptive (peak) potencies of 6-amino acid conjugates of 14-O-methylxoxymorphone derivatives compared with morphine and fentanyl in the tail-flick test in the rat after s.c. and i.c.v. administration.

<table>
<thead>
<tr>
<th>Compound</th>
<th>14-O-Methylxoxymorphone</th>
<th>HS-730</th>
<th>HS-731</th>
<th>HS-735</th>
<th>HS-935</th>
<th>HS-936</th>
<th>HS-937</th>
<th>HS-938</th>
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<tr>
<td>Relative Potency</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Morphine = 1</td>
<td>Fentanyl = 1</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>i.c.v.</td>
<td>s.c.</td>
<td>i.c.v.</td>
<td>s.c.</td>
<td>i.c.v.</td>
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<td>i.c.v.</td>
</tr>
<tr>
<td>14-O-Methylxoxymorphone</td>
<td>406</td>
<td>204</td>
<td>2.56</td>
<td>9.65</td>
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<tr>
<td>HS-730</td>
<td>103</td>
<td>1132</td>
<td>0.66</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HS-731</td>
<td>209</td>
<td>1170</td>
<td>1.33</td>
<td>55</td>
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<tr>
<td>HS-735</td>
<td>88</td>
<td>290</td>
<td>0.56</td>
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<tr>
<td>HS-935</td>
<td>113</td>
<td>428</td>
<td>0.72</td>
<td>20</td>
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<td>HS-936</td>
<td>19</td>
<td>557</td>
<td>0.12</td>
<td>26</td>
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<td>45</td>
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</tr>
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</table>

**TABLE 4**

Antinociceptive s.c./i.c.v. potency ratios of 6-amino acid conjugates of 14-O-methylxoxymorphone derivatives compared with morphine and fentanyl in the tail-flick test in the rat

s.c./i.c.v. was calculated as the ratio of ED50 (nanomoles per kilogram s.c.)/ED50 (nanomoles per rat i.c.v.).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s.c.</td>
</tr>
<tr>
<td>Morphone</td>
<td>6053</td>
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<tr>
<td>Fentanyl</td>
<td>38.6</td>
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<tr>
<td>14-O-Methylxoxymorphone</td>
<td>14.9</td>
</tr>
<tr>
<td>HS-730</td>
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<td>HS-936</td>
<td>53.4</td>
</tr>
<tr>
<td>HS-937</td>
<td>315</td>
</tr>
<tr>
<td>HS-938</td>
<td>&gt;3600</td>
</tr>
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</table>

Fig. 3. Time course of the antinociceptive effects produced by i.c.v. administration of morphine (78 nmol/rat), fentanyl (6 nmol/rat), and 14-O-methylxoxymorphone (0.25 nmol/rat) (A) and HS-730 (0.18 nmol/rat), HS-731 (0.18 nmol/rat), HS-935 (0.34 nmol/rat), HS-936 (0.34 nmol/rat), HS-937 (0.29 nmol/rat), and HS-938 (1.44 nmol/rat) (B) in the tail-flick test in the rat. Each point represents the mean ± S.E.M. n = 5 to 8.
the action of s.c. HS-731 (183 nmol/kg) (Fig. 4, A and B). The same experiments were performed with HS-730, HS-935, HS-936, HS-937, and HS-938, and similar results were obtained (data not shown).

Antinociceptive Effects in the Formalin Test in the Rat after Subcutaneous Administration

Subcutaneous administration of morphine and 6-amino acid conjugates of 14-<sup>O</sup>-methyloxymorphone, HS-730, HS-731, HS-937, and HS-938, produce a dose-dependent antinociceptive action in both phases of the formalin test in rats (Figs. 5A, left, and 6). The ED<sub>50</sub> values of the 6-amino acid derivatives in the first and second phase of the formalin response were ranging between 72 and 171 nmol/kg and 110 and 292 nmol/kg, respectively (Table 5). The antinociceptive potencies of the new opioid agonists in the formalin test were 10- to 22- and 5- to 13-fold higher in the first and second phase, respectively, compared with morphine (Fig. 6). Their inhibition of pain behavior seems to be somewhat higher in the first phase than in the second phase, whereas morphine produced comparable inhibition of formalin-induced behavior in both phases. Although s.c.-administered 68-phenylalanine epimer HS-938 was ineffective in eliciting antinociception in the tail-flick test up to 3600 nmol/kg (Table 1), it produced antihyperalgesic effects in the formalin-injected paw after s.c. administration (ED<sub>50</sub> first phase 79 nmol/kg and second phase 109 nmol/kg; Table 5).

To establish the site of action of the 6-amino acid derivatives and morphine, the peripherally selective opioid antagonist naloxone methiodide was s.c. coadministered with the opioid agonists, and the response in the formalin-injected paw was assessed. As shown in Fig. 5B (right), the antihyperalgesic effects of s.c. 366 nmol/kg HS-730 or HS-731 were significantly antagonized by s.c. 2131 nmol/kg naloxone methiodide. Similar inhibitory effects of naloxone methiodide were observed after coinjection with the other tested amino acid derivatives (data not shown). The antinociceptive activity of s.c. 5278 nmol/kg morphine in the formalin test was also significantly reversed by s.c. naloxone methiodide (Fig. 5B, right).

Discussion

The major finding of the present study is that the 6-amino acid conjugates (glycine, alanine, and phenylalanine) of 14-<sup>O</sup>-methyloxymorphone produce potent antinociceptive effects in acute nociceptive and inflammatory pain in rats. These effects were demonstrated to be mediated preferentially via activation of opioid receptors located in the periphery after systemic (s.c.) administration.

The 6-amino acid derivatives elicited antinociception in a dose-dependent manner after s.c. administration. The peripherally selective opioid antagonist naloxone methiodide reversed the antinociceptive effects of these opioid agonists. Typically, the pain ceased very rapidly upon s.c. and i.c.v.
administration of morphine, fentanyl, and 14-O-methyloxymorphine, but in the case of the new opioids, there was some delay in the onset of the effect after s.c. administration. Extremely long-lasting effects were measured in the tail-flick test after i.c.v. administration. In the tail-flick test, potencies of the 6-amino acid derivatives did not significantly differ between the \( \beta \) and \( \alpha \)-epimers, in agreement with in vitro biological data whereby also no major changes in opioid binding and agonist activity were detected (Spetea et al., 2004). In the case of the phenylalanine conjugates, the \( \beta \)-epimer HS-938 failed to elicit antinociception up to 3600 nmol/kg after s.c. administration. However, its \( \alpha \)-counterpart HS-937 produced dose- and time-dependent antinociceptive action. In contrast to the inactivity of the \( \beta \)-epimer HS-938 determined after s.c. administration, it proved to be a potent antinociceptive agent after i.c.v. administration. The 6\( \beta \)-phenylalanine derivative was reported to have some preference for \( \delta \)-over \( \mu \)-opioid receptors in binding and \( \delta \)-opioid agonism in mouse vas deferens bioassays (Spetea et al., 2004). The increased activity at \( \delta \)-opioid receptors might explain the unusual ineffectiveness detected in the tail-flick test after systemic administration. However, a potent antihyperalgesic effect of s.c. HS-938 was also found in the formalin pain model. These observations corroborate with earlier reports on the lack of antinociceptive effects of other \( \delta \)-opioid agonists after s.c. application in the tail-flick test, which are, however, more active in visceral pain models such as the writhing test (Quock et al., 1999). Antinociceptive effects of \( \delta \)-opioid agonists are enhanced in hyperalgesia and allodynia after spinal and supraspinal administration (Desmeules et al., 1993; Mika et al., 2001).

The potent antinociceptive effect of this class of opioids might be attributed to the substitution pattern in position C-6 of the morphinan skeleton. It was reported that numerous opioids bearing various substituents in position C-6, e.g., azido (Knoll et al., 1975) and 6-O-glucuronide in morphine (Abbott and Palmour, 1988; Frances et al., 1992), or amino in oxymorphone (Botros et al., 1989), induce strong analgesic actions. As recently established in structure-activity relationship studies, the substitution at position C-6 with ioniz-

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**Fig. 5.** Effect of the 6-amino acid conjugates of 14-O-methylxymorphine in the formalin test in the rat. Opioid agonists were administered 15 min before the formalin injection, and the number of paw flinches was counted during a 5-min observation period over the first (5–15 min) and second phase (35–90 min) of formalin-induced behavior. A, left, time-effect curves of s.c. morphine, HS-730, and HS-731 in the formalin test in the rat. B, right, antagonist effect of s.c.-administered naloxone methiodide (QNX; 2131 nmol/kg) on the antinociceptive effects induced by morphine, HS-730, and HS-731 in the first and second phase of the formalin test in rats. All data are presented as means ± S.E.M. \( n = 8 \) to 10. The asterisk (*) denotes significance versus saline treated group, \( +, p < 0.05; ++, p < 0.01; +++, p < 0.001 \) (ANOVA with Bonferroni test). The + denotes significance between group treated with agonist alone versus group treated with agonists and antagonist; \( +, p < 0.05; ++, p < 0.01; +++, p < 0.001 \) (ANOVA with Bonferroni test).
Antinociceptive effect (ED$_{50}$) of morphine and 6-amino acid conjugates of 14-O-methyloxymorphone after s.c. administration in the first (5–10 min) and second phase (40–45 min) of the formalin test. Rats were given the respective opioid agonist s.c. 15 min before intraplantar injection of formalin. Data are presented as percentage of inhibition of pain behavior after formalin injection. Each point represents as means ± S.E.M. n = 8 to 10.

### Table 5

Antinociceptive effect (ED$_{50}$) of morphine and 6-amino acid conjugates of 14-O-methyloxymorphone after s.c. administration on formalin-induced pain behavior in the rat observed in the first (5–10 min) and second (40–45 min) phase

<table>
<thead>
<tr>
<th>Compound</th>
<th>First Phase (nmol/kg s.c.)</th>
<th>Second Phase (nmol/kg s.c.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>1613</td>
<td>1472</td>
</tr>
<tr>
<td>HS-730</td>
<td>72</td>
<td>110</td>
</tr>
<tr>
<td>HS-731</td>
<td>125</td>
<td>204</td>
</tr>
<tr>
<td>HS-937</td>
<td>171</td>
<td>292</td>
</tr>
<tr>
<td>HS-938</td>
<td>79</td>
<td>109</td>
</tr>
</tbody>
</table>

able groups such as amino acid residues does not have any detrimental effect on opioid activity at the receptor level (Spetea et al., 2004) and on antinociceptive potency as described here. The parent molecule 14-O-methyloxymorphone proved to be a more potent antinociceptive agent after s.c. administration than the new 6-amino acid-substituted derivatives, whereas it was observed to produce similar potency after i.c.v. administration.

Characteristic differences occurred in the time course of the antinociceptive action of the new opioids depending on the route of application, s.c. or i.c.v., in the tail-flick test. The rapid onset with a maximum effect early, which waned very shortly after drug administration, is a typical characteristic of the time course of action of opioid agonists (e.g., morphine, fentanyl, and etorphine) that mediate analgesia via central mechanisms and were described under both normal and inflammatory conditions (Millan et al., 1987; Aceto et al., 1997). In contrast, the new opioids administered s.c. acted longer with some delay in onset. Intracerebroventricular administration of the 6-amino acid conjugates produced a different time course of action in the tail-flick test than the centrally acting opioids, with extremely prolonged effects. Our observations are consistent with previous findings on the effect of i.c.v. morphine and its active metabolite M6G in different pain tests (Abbott and Palmour, 1988; Frances et al., 1992). Significantly long-lasting analgesia was reported for i.c.v. M6G, a hydrophilic molecule, which poorly penetrates the blood-brain barrier (Abbott and Palmour, 1988; Frances et al., 1992; Tegeder et al., 2003). One possible explanation of the long-lasting action might be a slow elimination of opioids containing hydrophilic groups from the brain based upon their reduced lipid solubility. It was observed that the differences in the duration of action of various opioids, after i.c.v. administration, are largely determined by their hydrophilic/lipophilic character and the time that the compound remains in the ventricular system (Herz and Teschemacher, 1971).

That the presence of hydrophilic groups is indeed important in limiting penetration into the central nervous system was demonstrated by comparing the activity ratios of the peripheral (s.c.) versus central (i.c.v.) potencies in the tail-flick test of the 6-amino acid conjugates with those of centrally penetrating μ-opioid agonists morphine, fentanyl, and 14-O-methyloxymorphone. There was a marked enhancement of potency of the new agonists after i.c.v. in comparison with s.c. administration. Very good correlations between hydrophilicity and the i.v./i.c.v. activity quotients were earlier described for various opioids (Herz and Teschemacher, 1971; Botros et al., 1989; Frances et al., 1992; Portoghese et al., 1995).

To further explore the peripheral and central components of antinociceptive actions of the 6-amino acid conjugates of 14-O-methyloxymorphone after s.c. and i.c.v. administration, the antagonist effects of naloxone methiodide, which has restricted access to the central nervous system, were investigated in the tail-flick test. Antinociceptive effects of peripherally administered HS-731 were dose dependently antagonized by s.c. naloxone methiodide. However, an approximately 10-fold higher dose of antagonist was required to antagonize the effects of morphine to the same extent as the s.c. HS-731. A low dose of naloxone methiodide (320 nmol/kg s.c.) also significantly attenuated the s.c. action of the other 6-amino acid conjugates, in contrast to s.c. morphine, indicating that these ionizable opioids have a peripheral site of action. The lack of antagonism by low doses of naloxone methiodide on the antinociception elicited by s.c. morphine, and the attenuation in nociception with significantly higher antagonist dose in the tail-flick test corroborate with previous findings in hyperalgesia and inflammation (Kayser et al., 1991; Perrot et al., 2001; Reichert et al., 2001; Shannon and Lutz, 2002). Subcutaneous naloxone methiodide was ineffective in antagonizing the effects of the i.c.v. administered agonists. In addition, even a 10-fold higher dose of naloxone methiodide failed to block the antinociceptive effects of i.c.v. HS-731.

On the other hand, antinociceptive effects of HS-731 after i.c.v. administration were antagonized by i.c.v. naloxone, demonstrating an opioid mechanism of action. Although i.c.v.-injected naloxone inhibited almost completely the effects of systemically administered morphine, it failed to antagonize s.c. HS-731. Moreover, i.c.v. coadministration of the agonists with naloxone methiodide also resulted in signifi-
Opioid agonists in this series can find clinical application where system and can mediate antinociception at peripheral sites. That such opioids have limited access to the central nervous system after systemic administration. Injection of opioids (Stein et al., 2003). Systemic rather than peripheral analgesic effects were achieved after local injection. The peripheral nature of antinociception produced by the new opioids is supported by the calculated blood-brain distribution coefficients (Schütz et al., 2003), which indicate that they have a limited ability to enter the central nervous system after systemic administration. In most of the published experimental and clinical reports, peripheral analgesic effects were achieved after local injection of opioids (Stein et al., 2003). Systemic rather than local administration of opioids with exclusive or predomi-
nant peripheral action would be more convenient in many cases.

In conclusion, the pharmacological profile described for the 6-amino acid conjugates of 14-O-methyloxymorphine indicate that such opioids have limited access to the central nervous system and can mediate antinociception at peripheral sites. Opioid agonists in this series can find clinical application where the central actions of opioids are unwanted.

Acknowledgments

We acknowledge the excellent technical assistance of I. Wachtl and K. Parina.

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Address correspondence to: Prof. Dr. Susanna Fürst, Department of Pharmacology
and Pharmacotherapy, Medical Faculty, Semmelweis University, Nagyvadi ter
4, P.O. Box 370, H-1445 Budapest, Hungary. E-mail: furzsu@pharma.sote.hu