Human Opiorphin, a natural antinociceptive modulator of opioid-dependent pathways

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Edited by Susan E. Leeman, Boston University School of Medicine, Boston, MA, and approved October 5, 2006 (received for review July 12, 2006)

Mammalian zinc ectopeptidases play important roles in turning off neural and hormonal peptide signals at the cell surface, notably those processing sensory information. We report here the discovery of a previously uncharacterized physiological inhibitor of enkephalin-inactivating zinc ectopeptidases in humans, which we have named Opiorphin. It is a QRFSR peptide that inhibits two enkephalin-catabolizing ectoenzymes, human neutral ecto-endopeptidase, hNEP (EC 3.4.24.11), and human ecto-aminopeptidase, hAP-N (EC 3.4.11.2). Opiorphin displays potent analgesic activity in chemical and mechanical pain models by activating endogenous opioid-dependent transmission. Its function is closely related to the rat sialorphin peptide, which is an inhibitor of pain perception and acts by potentiating endogenous μ- and δ-opioid receptor-dependent enkephalinergetic pathways. Here we demonstrate the functional specificity in vivo of human Opiorphin. The pain-suppressive potency of Opiorphin is as effective as morphine in the behavioral rat model of acute mechanical pain, the pin-pain test. Thus, our discovery of Opiorphin is extremely exciting from a physiological point of view in the context of endogenous opioidergic pathways, notably in modulating mood-related states and pain sensation. Furthermore, because of its in vivo properties, Opiorphin may have therapeutic implications.

Zinc metal ectopeptidases control the receptor-dependent activity of neural and hormonal mediators involved in the regulation of important physiological functions in mammals. They are located at the surface of cells in nervous and systemic tissues and catalyze postsecretory processing or metabolism of neuropeptides and regulatory peptides (1, 2). Prominent among these neuronal and/or hormonal peptide signals are substance P (SP) and enkephalins, which are implicated in the receptor-dependent modulation of behavioral adaptive responses to stressful or threatening environmental stimuli. They notably regulate spinal processing of nociceptive information and analgesic mechanisms, emotional and/or motivational responses, anxiety, aggression, and immune inflammatory phenomena (3–6).

Because of the physiological importance and the critical role of zinc ectopeptidases in modulating the functional potency of downstream neuronal and hormonal signals, it is essential to focus on what controls their activity and, as a consequence, the overall regulatory cascade. The discovery of upstream regulators of ectopeptidase activity also is exciting from physiopathological and therapeutic points of view because of the potential for developing new candidate drugs.

A brain-specific heptapeptide named spinorphin was isolated and characterized from bovine spinal cord based on its inhibitory activity toward enkephalin-degrading ectoenzymes, such as neutral endopeptidase (NEP; EC 3.4.24.11) and aminopeptidase N (AP-N; EC 3.4.11.2) (7, 8). In addition, we characterized rat sialorphin, a peptide mediator involved in adaptation to environmental changes in rat. Rat sialorphin is an endocrine peptide signal whose expression is activated by androgen regulation and whose secretion is stimulated under adrenergic-mediated response to environmental stress in male rats. It is a physiological inhibitor of the membrane-anchored rat NEP activity and is a powerful inhibitor of pain sensation in rats (9–13). To our knowledge, bovine spinorphin and rat sialorphin are the only identified natural enkephalin catabolism inhibitors inducing antinociception in mammals (8, 13). We therefore asked whether this important inhibitor also is present in humans.

Here, we describe the molecular identification of an endogenous human peptide mediator and demonstrate its functional specificity in vitro and in vivo. The human peptide regulator QRFSR pentapeptide is secreted into human saliva. We call it Opiorphin and demonstrate its dual-inhibitory potency on the enkephalin-inactivating ectopeptidases human NEP (hNEP) and human AP-N (hAP-N). The Opiorphin peptide inhibits chemical- and mechanical-evoked pain behavior by activating endogenous opioid-dependent pathways.

Results and Discussion

Isolation of the First hNEP Ectopeptidase Inhibitor in Humans. The strategy for the detection and purification of natural NEP inhibitor(s) in humans was based on the isolation of salivary low-molecular-weight components that inhibit the endoproteolysis of a NEP-sensitive substrate, SP. Indeed, data suggested the existence of low-molecular-weight substance(s) inhibiting NEP ectopeptidase activity in human saliva (14). We combined high-pressure liquid chromatography (HPLC) and models of functional detection (in vitro enzyme assay with human LNCaP epithelial cells expressing membrane-anchored NEP) to screen for putative hNEP ectopeptidase inhibitor(s).

Active molecular populations were isolated from human saliva, according to their methanol solubility and cationic and hydrophobic characteristics. The cation-exchange HPLC (CE-HPLC) of methanol-extracted saliva clearly revealed the presence of one major molecular salivary component, which was eluted in the first-step ammonium acetate gradient profile (10–500 mM) at retention times of 26–28 min and inhibited the endoproteolysis of SP by human ectopeptidases (Fig. 1a, fractions 13 and 14). Fractionation by reverse-phase HPLC (RP-HPLC) of the active and basic molecular form isolated from CE-HPLC showed the presence of two major molecular pop-


The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: SP, substance P; NEP, neutral endopeptidase; hNEP, human NEP; AP-N, aminopeptidase N; hAP-N, human AP-N; pAP-M, porcine aminopeptidase M; CE-HPLC, cation-exchange HPLC; SELDI-TOF MS, surface-enhanced laser desorption ionization-time of flight mass spectrometry; DPPIV, dipeptidylpeptidase IV; hDPPIV, human DPPIV; Ala-pNA, L-alanine-p-nitroanilide.

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SELDI-TOF MS analysis of the reference synthetic QRFSR peptide. Its molecular characteristics are similar to those of the TOF MS analysis of 690 Da (693-Da theoretical molecular mass). The highest molecular mass corresponds to a salivary basic proline-rich peptide. E-P-E (15).

Our data provide direct evidence for the existence of a natural inhibitor of the cell-surface hNEP peptidase, a QRFSR pentapeptide, which is secreted into the human saliva and whose activity is related to the rat sialorphin OHNPR pentapeptide (13) and bovine spinorphin LVVYPWT heptapeptide (7); we named it Opiorphin. Furthermore, it appears that Opiorphin corresponds to the putative mature product of the PRL1 precursor (16). Human PROL1 gene (also known as PRL1 or BPLP gene) is expressed in human salivary glands and belongs to the same multigene family as the sialorphin RATSMR1 precursor (Vcas1 gene; ref. 11). It encodes a secreted polypeptide, predicted from the cDNA (16), that contains in the N-terminal region a putative peptide QRFSR processed by selective cleavage at consensus sites (recognition sites for signal peptidase and paired basic amino acid convertase). Thus, the combined functional biochemical approach and genomic information give the clues to assign a function to the PROL1 gene product.

Human Opiorphin Is a Specific Inhibitor of SP-Degrading hNEP in Vitro.

To characterize the inhibitory potency of Opiorphin on hNEP activity, we used different sources of enzyme: pure recombinant soluble hNEP, LNCaP human prostate epithelial cells constitutively expressing membrane-bound hNEP, and hNEP-transfected HEK293 cells. We used two substrates to determine endopeptidase activity: SP, a physiological NEP-sensitive substrate, and Mca-BK2, a fluorescent synthetic NEP-specific substrate.

In an initial step, the synthetic Opiorphin QRFSR peptide was analyzed for its capacity to inhibit the degradation of SP with human LNCaP cell membranes. It inhibited, in a concentration-dependent manner ($r^2 = 0.89$, $n = 24$), the extracellular endopeptidase activity of SP. The effective concentrations for Opiorphin ranged from 2.5 to 25 µM, being half-maximal (IC$_{50}$) at 11 ± 3 µM (Fig. 2a) (human, in this biological assay, the Opiorphin maximum inhibitory potency was 62%, demonstrating that, similarly to the synthetic specific NEP-inhibitor thiorphan, it is not entirely capable of protecting SP from cleavage by all SP-degrading peptidases expressed at the surface of LNCaP cells.

SP is primarily inactivated in vivo not only by the endopeptidase NEP but also by dipeptidylpeptidase IV (DPPIV; EC 3.4.13.11), which also is located at the human LNCaP cell surface (17, 18). The inhibitory specificity of Opiorphin therefore was assessed by measuring the endopeptidase activity of SP in an enzyme assay by using pure recombinant hNEP or human DPPIV (hDPPIV) in soluble ectodomain forms. Opiorphin prevented hNEP-mediated endopeptidase activity of SP by 90%. Its inhibitory potency was strictly concentration-dependent ($r^2 = 0.90$, $n = 18$), ranging from 5 to 50 µM, and was half-maximal at 29 ± 1 µM (Fig. 2b). In contrast, the breakdown of SP by hDPPIV was not prevented by 25 or 50 µM Opiorphin (Fig. 2c). The native membrane-anchored hNEP-specific inhibitory potency of Opiorphin was confirmed by using the synthetic NEP-specific substrate, Mca-BK2, and LNCaP cell membranes; the IC$_{50}$ value was 25 µM.

These data demonstrate that the inhibitory potency of Opiorphin on the SP-catabolizing cell-surface enzymes in vitro is specifically due to its functional interaction with hNEP endopeptidase. Opiorphin has been found to be a physiological hNEP inhibitor in humans. Studies monitoring the in vivo metabolism of SP indicate that brain NEP and DPPIV are both involved in its primary cleavage (18); according to our in vitro data, it appears that Opiorphin cannot entirely protect SP, notably, from breakdown by DPPIV and therefore would not potentiate SP-mediated nociception in vivo.
Human Opiorphin Is a Dual Inhibitor of Enkephalin-Degrading hNEP and hAP-N in Vitro. In an initial step, the inhibitory potency of Opiorphin on both hNEP and hAP-N activities was assessed in an enzyme assay by using membrane preparations of human HEK293 transfected cells selectively expressing either membrane-anchored hNEP or hAP-N. Cell-membrane endoectopeptidase and aminopeptidase activities were assayed in vitro by measuring the breakdown of artificial selective substrates, Mca-BK2 and L-alanine-p-nitroanilide (Ala-pNA), respectively. Under initial velocity conditions, the breakdown of Mca-BK2 by the cell-surface hNEP-HEK was inhibited by 83% in the presence of the synthetic specific NEP-inhibitor thiorphan (0.5 μM). We found that inhibition by the Opiorphin of Mca-BK2 endoproteolysis by the cell-surface recombinant hNEP was concentration-dependent ($r^2 = 0.88$, $n = 29$ determination points), with an IC$_{50}$ value of 33 ± 6 μM. The breakdown of Ala-pNA by the hAP-N HEK cell membranes was inhibited by 89% by the aminopeptidase inhibitor bestatin (50 μM). Opiorphin inhibits the Ala-pNA cleavage by hAP-N at 10 to 90 μM effective doses ($r^2 = 0.93$, $n = 22$ determination points) with an IC$_{50}$ value of 65 ± 9 μM (Fig. 4, which is published as supporting information on the PNAS web site). Thus, our data indicate that the human Opiorphin is a dual inhibitor of hNEP and hAP-N ectopeptidase activities in vitro.

Because of the complementary role of NEP and AP-N in enkephalin inactivation, we thus explored the effect of Opiorphin on the breakdown of the Met-enkephalin physiological substrate by using purified porcine aminopeptidase M (pAP-M; EC 3.4.11.2) and recombinant soluble hNEP in vitro. We found that Opiorphin prevented, in a concentration-dependent manner, the Met-enkephalin cleavage mediated by the aminopeptidase pAP-M ($r^2 = 0.66$, $n = 18$ determination points; Fig. 2d) and by the endopeptidase hNEP ($r^2 = 0.66$, $n = 22$ determination points). Its inhibitory potency was half-maximal at 36 ± 12 μM for pAP-M and at 33 ± 11 μM for hNEP.

We postulate that Opiorphin is an authentic physiological dual inhibitor of enkephalin-inactivating NEP/AP-N ectopeptidases and that it potentiates enkephalin-mediated antinociception in vivo.

Human Opiorphin Displays Analgesic Activity in Vitro. To evaluate the effects of Opiorphin on pain responses in vivo, we used a behavioral rat pain model, the formalin test, described in ref. 13. The antinociceptive potency of its derivative YQRFSR peptide was investigated in the behavioral rat model of chemical-induced pain because, unlike the native Opiorphin, it exhibits a relatively similar inhibitory efficacy toward both human and rat NEP ectopeptidases in vitro (IC$_{50}$ at 30 and 38 μM, respectively).

Systemic administration of YQRFSR peptide inhibited, in a concentration-dependent manner, the pain behavior exhibited by rats during the early phase (first 20 min after formalin injection, $P = 0.025$ by ANOVA) and the late phase (40–60 min after formalin injection corresponding to inflammatory pain phase, $P = 0.0001$ by ANOVA) of the formalin test. It significantly reduced the time spent by treated rats in paw licking of the formalin-injected hind paw: from 144 ± 17 s, $n = 8$ (vehicle) to 97 ± 14 s, $n = 8$ (0.5 mg/kg, $P = 0.05$) and to 84 ± 13 s, $n = 8$ (1 mg/kg, $P = 0.02$ by Dunnett’s $t$ test) for early test period; and from 63 ± 13 s (vehicle) to 9 ± 3 s (1 mg/kg, $P = 0.001$) for late phase. It also significantly decreased the number of body tremors exhibited by rats during this last test period from 126 ± 17 (vehicle) to 104 ± 14 (0.5 mg/kg) and 61 ± 5 (1 mg/kg) ($P = 0.002$ by ANOVA; Fig. 5, which is published as supporting information on the PNAS web site). These data clearly indicate that the Opiorphin-derived peptide inhibits nociception induced by acute and long-acting chemical stimuli.

A second series of studies was undertaken to determine whether the endogenous opioidergic pathway is required for its antinociceptive effect. The antinociceptive potency of 1 mg/kg Opiorphin-derived peptide was confirmed. Hence, it significantly reduced the number of body spasms exhibited by treated rats throughout the 60-min test period ($P < 0.0001$ by ANOVA; Fig. 3a) and also the time spent by treated rats in paw licking ($P < 0.001$ versus controls by Dunnett’s $t$ test; Fig. 3b). Interestingly, the spasm index (Fig. 3b) indicated that its analgesic potency during this period was almost as efficient as a 3 mg/kg morphine dose; i.e., 100 ± 10, $n = 8$ (vehicle) versus 43 ± 8, $n = 8$ (YQRFSR-derived peptide) and 23 ± 11, $n = 8$ (morphine). Furthermore, the effect of the Opiorphin-derived peptide was abolished by pretreatment with the broad-spectrum opioid receptor antagonist naloxone, i.e., spasm index: 112 ± 13, $n = 8$ (Fig. 3b), indicating that the opioid receptors are required for full hypoalgesia induced by the peptide. We conclude that the Opiorphin-derived peptide produces its pain-suppressive effects by activating endogenous opioid-dependent pathways, which are essential for the spinal and supraspinal control of nociceptive inputs (4). Hence, we propose that the pharmacological effect of Opiorphin-derived peptide leads to potentiate inhibitory control of...
nociceptive inputs, involving the endogenous opioid agonists that interact with opioid receptors.

Pharmacokinetic monitoring of the in vivo metabolism of the YQRFSR peptide revealed that the peptide is cleaved in the rat bloodstream <1 min after i.v. injection. Cleavage at the level of the peptide bond formed with the N-terminal Y residue suggests that its pain-suppressive action mainly is attributable to the QRFSR peptide activity. Thus, the antinociceptive potency of the native Opiorphin QRFSR peptide was investigated in the behavioral rat model of acute mechanical pain, i.e., the pin-pain test, described in ref. 13. As shown in Table 1, compared with controls, the Opiorphin-treated rats (1 mg/kg) spent significantly more time in peripheral aversive pin areas and crossed a higher number of pin-overlaid squares during the test period (P = 0.0002 by Mann–Whitney U test). These data indicate that Opiorphin at 1 mg/kg dose inhibits the perception of sharp painful stimuli and is as efficient in its pain-suppressive potency as morphine at 6 mg/kg dose.

Thus, Opiorphin displays potent analgesic activity in chemical and mechanical rat pain models by activating endogenous opioid-dependent transmission. Its demonstrated in vitro inhibitory potency on the enkephalin-inactivating ectopeptidases NEP and AP-N leads us to propose that the Opiorphin analgesic effect is caused by protection of the endogenous enkephalins released after pain stimuli and potentiation of enkephalin-dependent antinociception.

Our data provide direct evidence for the existence in humans of an endogenous dual inhibitor of enkephalin-degrading ectoenzymes hNEP and hAP-N ectopeptidases. It is a QRFSR peptide named Opiorphin because it is an antinociceptive modulator of opioid-dependent pathways. Opiorphin is the human functional homologue of the rat sialorphin, previously identified by a post-genomic approach as an inhibitor of pain perception that acts by potentiating endogenous μ- and δ-opioid receptor-dependent pathways (13). No immunoreactive sialorphin peptide was detected in male human saliva. Opiorphin has been found to be a natural inhibitor of enkephalin-inactivating enzymes in humans. This discovery is of crucial importance from a physiological and pathophysiological point of view when the extent of the functions mediated by the endogenous opioidergic pathways are considered. Indeed, the endogenous opioid peptides, in particular the enkephalins, have a pivotal role in the control of pain perception and mood-related states, including modulation of emotional and/or motivational responses (5, 6, 19). Endogenous Opiorphin could facilitate adaptive responses to threat-inducing stimuli by potentiating analgesic and antidepressive-like behavior, induced by endogenous enkephalinergic systems, in humans. This finding is consistent with our observations showing significant antidepressant-like effects of rat sialorphin (100 μg/kg) in the forced-swim behavioral despair test (C.R. and M.M., unpublished data) in addition to its analgesic activity and facilitative effect on socio-sexual motivation in male rats (13, 20).

Finally, because of its in vivo properties, Opiorphin may have therapeutic implications as a potential initiator of molecular pathways that could be exploited to develop new candidate drugs for the clinical management of pain relief and the alleviation of emotional disorders.

Materials and Methods

Molecular Characterization. The study was designed to search for the natural NEP inhibitor(s) in human salivary secretions. The protocol of clinical research established with the Centre of Biomedical Research of the Pasteur Institute received the agreement of the Consultant Committee for Protection of Persons in Biomedical Research (CCPPRB; accession no. 2045, Paris-Cochin), and human saliva was collected from 10 healthy male volunteers. The saliva was collected into previously chilled tubes containing aproitinin (1,000

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Time spent in pin-areas, s</th>
<th>No. of rats</th>
<th>Pin-squares crossed, n</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>—</td>
<td>3.8 ± 0.9</td>
<td>12</td>
<td>2.0 ± 0.4</td>
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<tr>
<td>YQRFSR peptide</td>
<td>1 mg/kg</td>
<td>41.1 ± 8.4***</td>
<td>8</td>
<td>7.5 ± 1.9**</td>
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<td>Morphine</td>
<td>6 mg/kg</td>
<td>71.7 ± 25.2***</td>
<td>8</td>
<td>8.5 ± 1.1***</td>
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<tr>
<td>Opiorphin</td>
<td>1 mg/kg</td>
<td>60.5 ± 19.1****</td>
<td>8</td>
<td>10.7 ± 1.4***</td>
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Evaluation of the pain response of rats to a noxious mechanical stimulus after administration of Opiorphin. The 3-min pin-pain test was performed 5 min after injecting rats either with Opiorphin YQRFSR peptide or its derivative, YQRFSR peptide (1 mg/kg) or vehicle via the tail vein or with morphine (6 mg/kg i.p. given 15 min before test). Results are expressed as means ± SEM of 8–12 animals. **, P < 0.01 and ***, P < 0.001 by Mann–Whitney U test.
kallikrein inhibitor units/ml; Sigma-Aldrich, St. Louis, MO), Pefabloc (0.4 mM; Roche Molecular Biochemicals, Indianapolis, IN), and HCl (0.1 M final concentration) and then stored at −80°C. Then, the three-step purification procedure (methanol acid extraction, CE-HPLC, and RP-HPLC) was used to isolate human salivary components. All of the extracts and chromatographic fractions were analyzed for their capacity to inhibit the hydrolysis of SP by human cell membranes containing NEP (LNCaP cell line; ATCC, Manassas, VA).

The saliva samples (45 ml altogether) were treated according to the following protocol.

**Extraction of low-molecular-weight components in methanol acid at 4°C.** First, 4 vol of methanol containing 0.1% trifluoroacetic acid (TFA) solution were added to 1 vol of saliva. This step inactivates and precipitates high-molecular-weight proteins and allows the solubilization of the low-molecular-weight salivary constituents. The methanol mixture quickly was homogenized and then centrifuged at 4°C and 12,000 × g for 15 min. The methanol was removed from the supernatant after lyophilization.

**CE-HPLC.** The methanol-extracted saliva was solubilized in solvent A (10 mM ammonium acetate, pH 4.3) and injected into a HEMA-IEC BIO-1000 carboxymethyl column (Alltech, AT-France, Houilles, France). Components were eluted and isolated according to their cationic characteristics in a two-step linear gradient of 10–500 mM and 500–900 mM ammonium acetate (pH 4.7), successively at 1 ml/min flow rate. Fractions of 2 ml were collected, and the solvent was removed after lyophilization.

**RP-HPLC.** The active fractions of the previous CE-HPLC were solubilized in solvent A (0.1% TFA in H2O) and injected into a HEMA-IEC BIO-1000 carboxymethyl column (Alltech, AT-France, Houilles, France). After a 10-min equilibrium period under isocratic conditions (solvent A, 1 ml/min), sample components were eluted with a linear gradient of 1–99% solvent B (100% acetonitrile/0.1% TFA, by vol) at 1 ml/min flow rate. Fractions of 1 ml were collected, and the solvent was removed after lyophilization.

The active fractions underwent a further purification procedure on a new Synergi Max-RP column through elution with a linear gradient of 1–99% solvent B (100% methanol/0.1% TFA). Column eluates were collected (microsorb tubes, Nunc; VWR, Fontenay-sous-Bois, France) at 1-min intervals, and the fractions were analyzed after lyophilization for their inhibitory potency of the hNEP ectopeptidase activity.

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NEP- and DPPIV-sensitive substrates, and we used native Met-enkephalin, YGGFM (Bachem, Bubendorf, Switzerland), as NEP- and APN-sensitive substrate.

**Measurement of ectopeptidase activity.** Hydrolysis of substrates was measured by monitoring their metabolism rate by the ectopeptidase activities in the presence and absence of the selective synthetic peptidase inhibitors to assess the specificity of each enzyme assay. These inhibitors were added to the preincubation medium. According to conditions of initial velocity measurement, time and temperature of incubation and protein concentrations of cell membranes or soluble enzymes were defined for each assay.

**Measurement of hNEP and hDPPIV activities by using SP substrate.** In microsorb tubes, the standard reaction mixture consisted of cell membranes or soluble enzymes in 50 mM Tris-HCl, pH 6.5 (hNEP) or pH 7.5 (hDPPIV), containing 0.1% BSA (200 μl final volume). The SP substrate (60 nM final concentration containing 100 nCi 3H-SP) was added after preincubation for 10 min, and the hydrolysis was carried out for 20 min (membrane-bound ectoenzyme), 30 min (soluble hNEP), or 45 min (soluble hDPPIV) at 25°C in a constantly shaken water bath. The reaction was terminated by cooling to 4°C and adding HCl (0.3 M final concentration). The reaction tubes then were centrifuged (4,500 × g for 15 min at 4°C), and the products of the reaction were isolated by using C-18 Sep-Pak cartridges (Waters, Milford, MA) as described in ref. 13.

**Measurement of hNEP ectopeptidase and hAP-N ectopeptidase activities with Mca-BK2 and Ala-pNA synthetic substrates (25, 26).** In 96-well microplates, the standard reaction consisted of cell membranes in 50 mM Tris-HCl, pH 6.5 (hNEP) or pH 7.3 (hAP-N) (200 μl final volume). The Mca-BK2 substrate (5 μM final concentration) or Ala-pNA substrate (100 μM final concentration) was added after preincubation for 10 min, and the kinetic of appearance of the signal was analyzed directly for 20 min at 25°C (hNEP) or 90 min at 37°C (hAP-N) with a multim wavelength fluorimeter (320 nm excitation and 405 nm emission filters) or spectrophotometer (405 nm) reader. In the conditions of initial velocity measurement, the hNEP-mediated endopeptidase of Mca-BK2 was 894 ± 221 relative fluorescent units (RFU)/min per μg of hNEP-HEK membrane protein, and the membrane-bound hAP-N-mediated exopeptidase of Ala-pNA was 0.40 ± 0.02 milliL/min per μg of hAP-N-HEK membrane protein.

**Measurement of NEP and AP-N activities with Met-enkephalin substrate (13).** In microsorb tubes, the standard reaction mixture consisted of soluble pure enzymes in 50 mM Tris-HCl, pH 6.5 (hNEP), 50 mM Tris-HCl, pH 7.3 (hAP-N) or pH 7.5 (hAP-M) as described in ref. 13. The formalin test was used to assess the activity of Opioidin-derived peptide, YORFSR, on chemical pain response (13). Male Wistar rats (350–400 g body weight; Charles River Breeding Laboratories, France) were experimentally tested once. Then, 50 μl of a 2.5% formalin solution was injected under the surface of the hind paw 15 min after i.v. injection of the tested compound. The duration of paw licking and the number of body spasms were recorded for a period of 60 min after formalin administration. The pain-pain test was used to assess the activity of native Opioidin ORFSR peptide and its derivative YORFSR peptide on mechanical pain response (13). The rat was placed in the central square of the experimental device, an open field divided into nine equal squares (150 × 150 mm), eight of them peripheral and overlaid with stainless steel pins and one central and without pins. The rat’s behavior was recorded for a 3-min test. Each rat was placed in the test compartment without pins for 30 min during the 2 days before exposure to the pain test; control rats spent 75% of time in peripheral surfaces when not exposed to pin-prick pain and only 2% under pin-induced pain conditions. Results are expressed as means ± SEM. The significance of differences between groups was evaluated by using ANOVA followed, when significant, by Dunnett’s t test (formalin pain) or Mann–Whitney U test (pin pain) to compare each treated group to the control (vehicle). For all statistical evaluations, the level of significance was set at P < 0.05. The pain index for formalin test, based on paw licking duration and body tremor number, was calculated by the AUCt method as follows: area under the curve of treated rat/mean area under the curve of control rats. All statistical analyses were carried out by using the Statview 5 statistical package (SAS Institute Inc., Cary, NC). In all experiments, the care and euthanization of study animals were in accordance with the European Community standards on the care and use of laboratory animals.

We thank B. Lefevre-Ladoie and J. Weitzman for critical review of the manuscript and helpful comments. We also thank Dr. C. Sadorge, N. Joly (Centre of Biomedical Research, Institut Pasteur), and V. Mellon (ICAré, Institut Pasteur) for help in the clinical research investigations. This research was in large part funded by the “Direction de la Valorisation et des Partenariats Industriels,” Institut Pasteur.
Figure 4

A

Relative fluorescent Unit (RFU)
RFU/min/µg membrane protein

[QRFSR-peptide], µM

B

mAbsorbance at 405 nm (mA405)
mA405/min/µg membrane protein

[QRFSR-peptide], µM
Figure 5

A. Duration of paw licking, sec

B. Number of body tremors

Time after formalin injection, min