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

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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 anagelsic 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.

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-
SELDI-TOF MS analysis of the reference synthetic QRFSR peptide. mass) (Fig. 1 corresponding to an acetate salt form (767-Da theoretical molecular mass). Its molecular characteristics are similar to those of the reference synthetic QRFSR peptide (690 Da), which also presents a second molecular form of 769 Da most likely corresponding to the 5 aa residues QRFSR with an experimental molecular mass (determined by surface-enhanced laser desorption ionization–time of flight mass spectrometry (SELDI-TOF MS) analysis) of 690 Da (693-Da theoretical molecular mass). The highest molecular mass corresponds to a salivary basic proline-rich peptide, 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 RATS MR1 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). However, in this biological assay, the Opiorphin maximum inhibitory potency was 62%, demonstrating that, similarly to the synthetic specific NEP-inhibitor thiopran, 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 DPP IV (hDPP IV) 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 hDPP IV 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 exopeptidase. 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 DPP IV 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 DPP IV and therefore would not potentiate SP-mediated nociception 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 ± 14 (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 opiate 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

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**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 endopeptidase 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 endopeptidolysis 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.

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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 Oporphin 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 Oporphin-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 Oporphin 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, Oporphin 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 Oporphin analgesic effect is caused by protection of the endogenous enkephalins released after pain stimuli and potentiation of enkephalin-dependent antinociception.

Our results provide direct evidence for the existence of humans of an endogenous dual inhibitor of enkephalin-degrading ectoenzymes hNEP and hAP-N ectopeptidases. It is a QRFSR peptide named Oporphin because it is an antinociceptive modulator of opioid-dependent pathways. Oporphin 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 p- and δ-opioid receptor-dependent pathways (13). No immunoreactive sialorphin peptide was detected in male human saliva. Oporphin has been found to be a natural inhibitor of enkephalin-inactivating enzymes in humans. This discovery is of crucial importance from a physiological and pathological 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 Oporphin could facilitate adaptive responses to threat-inducing stimuli by potentiating analgesic and antidepressive-like behavior, induced by endogenous enkephalinergetic 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). It is now of interest to explore whether Oporphin is present in various human biological media and to identify human pathological states up-regulating or down-regulating the levels of circulating Oporphin peptide. Oporphin may be, for example, a suitable marker for erectile dysfunction because it recently has been demonstrated that the rat Vcsa1 gene, coding the sialorphin precursor, is strongly down-regulated in the corpora of rats in three distinct models of erectile dysfunction, namely, diabetic, age-related, and neurogenic models (21).

Fig. 3. Oporphin-derived peptide displays potent analgesic activity in vivo in rat pain model. (a–b) Evaluation of the pain response of rats to noxious chemical stimuli after administration of the Oporphin-derived peptide YQRFSR. (a) Effects of YQRFSR peptide (black diamond; 1 mg/kg) compared with morphine (crossed circle; 3 mg/kg i.p. given 15 min before test) and vehicle (white circle) in the absence or presence of the opioid antagonist naloxone (gray diamond; 3 mg/kg s.c. given 30 min before test) on the number of body tremors during the six 10-min periods of the formalin test. (b) Pain index calculated from results shown in a by the AUCI method described in Materials and Methods. The values represent the mean ± SEM of eight animals for each condition: pain index based on paw licking duration (gray-striped bar, vehicle; black-striped bar, YQRFSR peptide; pain index based on body tremor number (white bar, vehicle; black bar, YQRFSR peptide; gray bar, YQRFSR peptide plus naloxone; hatched bar, morphine). *, P < 0.05; **, P < 0.01; ***, P < 0.001 by Dunnett’s test.

Table 1. Human Oporphin behaves as efficiently as morphine in vivo in rat pain model

<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>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>—</td>
<td>3.8 ± 0.9</td>
<td>12</td>
<td>2.0 ± 0.4</td>
</tr>
<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>
</tr>
<tr>
<td>Morphine</td>
<td>6 mg/kg</td>
<td>71.7 ± 25.2***</td>
<td>8</td>
<td>8.5 ± 1.1***</td>
</tr>
<tr>
<td>Oporphin</td>
<td>1 mg/kg of QRFSR peptide</td>
<td>60.5 ± 19.1***</td>
<td>8</td>
<td>10.7 ± 4.3***</td>
</tr>
</tbody>
</table>

Evaluation of the pain response of rats to a noxious mechanical stimulus after administration of Oporphin. The 3-min pin-pain test was performed 5 min after injecting rats either with Oporphin QRFSR 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.

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 aprotinin (1,000...
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, Deerfield, IL). 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 a 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 Synergi Max-RP column (Phenomenex, AIT-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; Wiesner, Fontenay-sous-Bois, France) at 1-min intervals, and the fractions were analyzed after lyophilization for their inhibitory potency of the hNEP ectopeptidase activity.

**SELDI-TOF MS.** Ciphergen (Fremont, CA) ProteinChip array technology and N-terminal sequence analyses were performed in the platform of Analyses and Protein Microsequencing, Pasteur Institute. After freeze-drying, the major active fractions of the solution were concentrated in the pellet, which was washed with cold Tris buffered HCl, pH 6.5 (NEP) or pH 7.3 (AP-N). After centrifugation at 1,200 g for 5 min, the resulting supernatant was gently sonicated (20 s at 4°C). A second centrifugation at 100,000 g of pure hDPPIV, of which 94% was recovered in ultra-pure water (60–100 μl). A 2- to 5-μl spot of sample was deposited on an Au or NP stick, and SELDI-TOF MS analysis was performed after the addition of 0.8 μl of matrix (α-cyano-4-hydroxycinnamic acid saturated in 50% acetonitrile/0.5% TFA then diluted 10 or 50 times in the same solution). N-terminal sequence analysis was carried on the rest of the sample.

**Functional Characterization: Biochemical Assays. Sources of ectopeptidases NEP, AP-N, and DPPIV. LNCaP cell line.** The prostate epithelial cell line LNCaP (adenocarcinoma, catalog no. CRL-1740; ATCC, Manassas, VA) is one of several human cell lines expressing NEP (17, 22). The LNCaP cells expressed membrane-bound NEP in defined medium culture conditions (i.e., RPMI medium 1640 containing insulin, transferrin, and selenium; Gibco-Invitrogen, Carlsbad, CA) and after a 48-h induction by 10−9 M dihydrotestosterone. The experimental model of incubation of membrane preparations originating from these cells allowed the analysis of hNEP-mediated endoproteolysis of SP under conditions of initial velocity measurement, i.e., 98 ± 10 pM/min per μg of cell-membrane proteins (n = 12). The LNCaP membrane activity was inhibited in the presence of specific synthetic NEP inhibitors, such as thiorphan (Bachem, Bubendorf, Switzerland) (58 ± 7%, n = 13 for maximum inhibitory potency at 1 μM), or in the presence of a specific synthetic DPPIV inhibitor, such as DPPIV-inh2 (Calbiochem, San Diego, CA) (42 ± 5%, n = 3 for maximum inhibitory potency at 10 μM). In contrast, bestatin (25 μM) and captopril (10 μM), which block the aminopeptidase and angiotensin-converting enzyme activities, respectively, did not significantly inhibit SP hydrolysis by cell-surface peptidases. This finding indicates that in our experimental conditions, the extracellular breakdown of SP was caused mainly by hNEP and hDPPIV endopeptidase activities located at the surfaces of these cells.

**hNEP or hAP-N transformed Hek293 cell line (catalog no. CRL-1573; ATCC, Manassas, VA).** An experimental model of incubation of membrane preparations originating from HEK293 cells, devoid of constitutive NEP or AP-N expression, and transfected with hNEP cDNA or hAP-N cDNA has been developed. The transfection of pcMV-hNEP, pcDNA3-hAP-N constructs, or empty vectors in HEK293 cells was performed by using the jetPEI cationic polymer transfection reagent (Qiogene, Inc., Irvine, CA) according to the manufacturer’s instructions.

The SP72-hAP-N construct was generously provided by L. Vogel (University of Copenhagen, Copenhagen, Denmark) (23). After digestion with Xhol and EcoRV, the hAP-N DNA insert was purified and ligated into the pcDNA3 eukaryote expression vector (Invitrogen, Carlsbad, CA) to generate the pcDNA3-hAP-N plasmid.

The hNEP coding sequence was cloned from a human placental cDNA library (BD Biosciences, Le Pont de Claix, France). The purified cDNA insert was subcloned in the pcMV eukaryote expression vector according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Sequence verification of the resulting plasmid, called pcMV-hNEP, demonstrated that the amplified NEP sequence corresponded exactly to the published one (24).

**Cell-membrane preparations.** The cell pellet was collected and harvested in 10 vol (vol/wt) of ice-cold 50 mM Tris-HCl buffered at pH 6.5 (NEP) or pH 7.3 (AP-N). After centrifugation at 1,200 g for 5 min, the resulting supernatant was gently sonicated (20 s at 4°C). A second centrifugation at 100,000 g of 30 min concentrated the cell membranes in the pellet, which was washed with cold Tris-HCl buffer, resuspended in fresh buffer, aliquoted, and stored at ~80°C. The proteins in cell-membrane suspensions were determined by using the Bio-Rad (Marnes-la-Coquette, France) DC protein assay.

**Recombinant hNEP and hDPPIV.** Soluble ectoenzymes (devoid of N-terminal cytosol and transmembrane segment) were purchased from R&D Systems (Minneapolis, MN). Pure enzyme was resuspended in 50 mM Tris-HCl, pH 6.5 (hNEP), or 25 mM Mes, pH 4.9 (hDPPIV), aliquoted, and stored at ~80°C. Under experimental conditions of initial velocity measurement, the SP-lyophilizing activity was 5,770 ± 170 pM/min per μg of pure hNEP, of which 99% was inhibited by 0.5 μM thiorphan, and 1,925 ± 74 pM/min per μg of pure hDPPIV, of which 94% was inhibited by 10 μM DPPIV-inh2.

**Purified kidney pAP-M.** Purified kidney pAP-M was purchased from Roche Applied Science (Indianapolis, IN). Purified enzyme was dialyzed against Tris-HCl, pH 7.3, aliquoted, and stored at ~80°C. Under experimental conditions of initial velocity measurement, the SP-lyophilizing activity was 5,770 ± 170 pM/min per μg of pure hNEP, of which 99% was inhibited by 0.5 μM thiorphan, and 1,925 ± 74 pM/min per μg of pure hDPPIV, of which 94% was inhibited by 10 μM DPPIV-inh2.

**Substrates. Synthetic selective substrates.** We used Mca-RPPGF-SAFK (Dnp)-OH, a bradykinin analog named Mca-BK2, which is an internally quenched fluorescent substrate selective for NEP and ECE endopeptidases (R&D Systems (Minneapolis, MN), and Ala-pNA, a colorimetric substrate for aminopeptidase activities (Bachem, Bubendorf, Switzerland).

**Natural substrates.** We used modified triitated SP, [(3,43H)Pro2,Sar1-Met(O2)11]-SP (PerkinElmer-NEN, Wellesley, MA), and native SP, RPKPQQFFGLM (Bachem, Bubendorf, Switzerland), as

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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 ectopeptidases 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. Miflurpiril (100 µl) was added 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 multiwell spectrophotometer (320 nm excitation and 405 nm emission filters) or spectrophotometer (405 nm) reader. In the conditions of initial velocity measurement, the hNEP-mediated endopeptidolytic cleavage of Mca-BK2 was 894 ± 221 relative fluorescent units (RFU)/min per µg of NEP-HEK membrane protein, and the membrane-bound hAP-N-mediated exopeptidolytic cleavage of Ala-pNA was 0.40 ± 0.02 milliRFU/min per µg of AP-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 HCl, pH 7.3 (hAP-N) or pH 7.0 for hAP-M) as described in ref. 13. The reaction was terminated by cooling to 4°C and adding HCl (0.3 M final concentration). The products of the reaction were isolated and quantified by RP-HPLC (C-18 Synergi polar-RP, 2 µm, 4.6 × 250 mm, Phenomenex, St. Quenix, France) coupled to a spectrophotodetector (Surveyor LC system and ChromQuest analyzer; Thermo Electron Corp., Waltham, MA).

Figure 4

(A) Relative fluorescent Unit (RFU) RFU/min/μg membrane protein vs. [QRFSR-peptide], μM

(B) mAbsorbance at 405 nm (mA405) mA405/min/μg membrane protein vs. [QRFSR-peptide], μM
Figure 5

A: Duration of paw licking, sec

B: Number of body tremors

Time after formalin injection, min