Isolation of a Specific \( \mu \)-Opiate Receptor Peptide, Morphiceptin, from an Enzymatic Digest of Milk Proteins*

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Specific radioimmunoassays have been developed for the measurement of naturally occurring morphiceptin and \( \beta \)-casomorphin. These peptides and related exorphins were isolated from an enzymatic digest of caseins by chromatographic techniques including gel filtration, hydrophobic column and multiple-step high pressure liquid chromatography. Three exorphins were purified and characterized in their radioimmunological, biological, and chemical properties. They were identified as morphiceptin, \( \beta \)-casomorphin, and \( \delta \)-prolyl-\( \beta \)-casomorphin. Since morphiceptin is a highly specific \( \mu \)-agonist and can be derived from a milk protein, it is possible that morphiceptin is an exogenous opioid ligand specific for \( \mu \)-receptors in the brain and gastrointestinal tract.

We have recently reported that a synthetic tetrapeptide amide fragment (Tyr-Pro-Phe-Pro-NH\(_2\)) of a milk protein, \( \beta \)-casein, is a potent and specific agonist for \( \mu \)-opioid receptors (1). This peptide was named morphiceptin. It has potent analgesic and cataleptic activities in rats when administered intracerebroventricularly (2, 3) and produces bradycardia by intravenous administration (4). These effects are blocked by naloxone, suggesting \( \mu \)-opioid receptor-mediated effects. Brantl et al. (5) first isolated \( \beta \)-casomorphin, a heptapeptide (Tyr-Pro-Phe-Pro-Gly-Pro-Ile), from a peptone digest of \( \beta \)-casein and showed its opioid-like activity in inhibiting the electrically stimulated muscle contraction of isolated guinea pig ileum (5, 6). Morphiceptin is an amino-terminal tetrapeptide fragment of \( \beta \)-casomorphin, but the former is about 50 to 100 times more active than the latter in receptor binding assays (1). Although the physiological significance of morphiceptin is not known, the peptide may be important if proven to exist naturally. The present studies establish a possible natural existence of morphiceptin. It can be isolated from an enzymatic digest of casein. The isolated material was characterized as morphiceptin from its radioimmunofactivity, receptor binding specificity, and by mass spectrometry. In addition, \( \beta \)-casomorphin and \( \delta \)-prolyl-\( \beta \)-casomorphin were isolated from the same digest.

**MATERIALS AND METHODS**

Synthetic morphiceptin, \( \beta \)-casomorphin, and other related peptides were synthesized by the solid-phase method and were available from previous studies (1, 2). Enzymatic casein hydrolysate (lot 40F-0560) was purchased from Sigma.

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1 The abbreviations used are: RIA, radioimmunoassay; HPLC, high pressure liquid chromatography.
reverse-phase high pressure liquid chromatography (HPLC) was employed for further purification. The residues were dissolved in 0.5 ml of 20 mM ammonium acetate (pH 4.2) and applied to a Magnum 9 column (Whatman, Partisil PXL 10/50 ODS) and eluted with a linear acetonitrile gradient (10 to 50%) in 20 mM ammonium acetate buffer (pH 4.2) for 2 h at 2 ml/min. Active fractions were pooled, lyophilized separately, and reapplied to the same column. These materials were further purified by reverse-phase HPLC on an analytical column (Partisil PXL 10/25 ODS) with a 0 to 50% acetonitrile gradient (30 min) and on ion-exchange HPLC columns (Partisil 10 SAX/25). The identity of each peptide was confirmed using synthetic standards. These final homogeneous materials were subjected to gel filtration (Bio-Gel P-2, 1 x 22-cm column), receptor binding assay, and mass spectrometry.

Opiate receptor binding assays were performed with rat (male Sprague-Dawley) brain membrane preparations using [3H]-FK 33824 (0.05 nM, 2 Ci/µmol) and [D-Ala², D-Leu⁵]enkephalin (0.05 nM, 2 Ci/µmol) as labeled µ-agonist and δ-agonists, respectively (9, 10).

The purified peptides were hydrolyzed in 6 M HCl at 130 °C for 16 h and subjected to amino acid analysis. The results are expressed as ratios relative to phenylalanine content. The nearest integral values are also shown.

Mass spectra were recorded on a Finnigan triple-stage quadrupole mass spectrometer (11) equipped with a Saddle-Field neutral beam gun from Ion Tech Ltd., Teddington, England. The gun was operated at approximately 10 mA and the beam current was 10 pA. Argon was employed as the collision gas in the middle quadrupole of this instrument. Reported experiments were run on two separate peptide preparations. In the first run, a copper target was employed, while in the second run stainless steel was used. No differences were observed with the different target materials.

RESULTS

RIA of Morphiceptin and β-Casomorphin—Specific antibodies to morphiceptin and β-casomorphin were raised in rabbits. The specificity of the anti-morphiceptin antibody is shown in Fig. 1. Morphiceptin binds to this antibody with a high affinity. A median inhibitory concentration (IC₅₀ value) of 3 nM in displacing ¹²⁵I-morphiceptin from the antibody was obtained under standard conditions. This same antibody shows less than 0.1% cross-reactivity with other related peptides such as β-casomorphin, β-casomorphin-5 (Tyr-Pro-Phε-Pro-Gly), Des-Tyr-β-casomorphin, and the tetrapeptide acid (Tyr-Pro-Phe-Pro-Ile) (Fig. 1A). Met- and Leu-enkephalins show virtually no cross-reactivity (data not shown). β-Casomorphin and β-casomorphin-5 show 0.01 to 0.02% cross-reactivity. This may account for the activity detected in those fractions on HPLC which do not correspond to morphiceptin.

Anti-β-casomorphin antibody has a high affinity to β-casomorphin. An IC₅₀ value of 2 nM was obtained in the standard RIA (Fig. 1B). Its cross-reactivity to morphiceptin, β-casomorphin-5, and β-casomorphin-4 was always less than 0.01%. Des-Tyr-β-casomorphin (Pro-Phe-Pro-Gly-Pro-Ile) shows about 3% cross-reactivity (Fig. 1B).

Isolation of Morphiceptin—The procedure for the isolation of morphiceptin is summarized in Scheme 1. An acetic acid extract of casein hydrolysate was chromatographed on Bio-Gel P-2 (Fig. 2). The peak of β-casomorphin activity precedes the morphiceptin activity. Fractions 33–55 were pooled for the purification of β-casomorphin and morphiceptin. The pooled fractions (33–55) were absorbed on Amberlite XAD-2 and eluted with methanol. This methanol extract was dried under nitrogen, lyophilized, and subjected to preparative HPLC. Four major peaks of morphiceptin-like material were detected. The largest peak, which corresponds to morphiceptin, was found in fractions 23–28 (Peak II). The minor peaks, fractions 16–18 (Peak I), 33–36 (Peak III), and 39–42 (Peak IV) were identified later (Fig. 3). Fractions from Peak II were rechromatographed on Magnum 9 and then on an analytical reverse-phase and ion-exchange column (Fig. 4). Both morphiceptin radioimmunoactivity and µ-receptor binding activity were determined for each fraction from the analytical reverse-phase column (Fig. 5). Activities coincided exactly at the same fractions and eluted with the same retention time.
Isolation of Morphiceptin from Casein Digest

Fig. 2. Gel filtration of morphiceptin and β-casomorphin immunoreactivity from a casein digest. The digest (35 g) was applied to Bio-Gel P-2 (2.8 x 53 cm) and eluted with acetic acid. Five-ml fractions were collected. An aliquot of each fraction was assayed for morphiceptin (●) and β-casomorphin (○) immunoreactivities using the standard RIA described under "Materials and Methods." Absorbance of the eluate was monitored at 280 nm (△).

Fig. 3. Separation of morphiceptin-like and casomorphin-like material from pooled fractions 33–55 of Fig. 2 by semipreparative reverse-phase HPLC. A Magnum 9 column (Whatman, Partisil PX-10/50 ODS) was used with a 0 to 50% gradient of acetonitrile in 20 mM ammonium acetate buffer (pH 4.2) for 2 h at 2 ml/min to elute the peptides. Four-ml fractions were collected and an aliquot of the diluted sample was assayed for morphiceptin (●) and β-casomorphin (○) activity by RIA. Absorbance of the eluate was monitored at 280 nm (△).

as synthetic morphiceptin (Fig. 5). The μ-receptor selectivity of purified P-II was confirmed by comparing the displacement binding curves of [125I]-FK 33824 (μ-agonist) and [125I]-DADLE (δ-agonist). Like synthetic morphiceptin, natural morphiceptin potently inhibits [125I]-FK binding but only partially inhibits [125I]-DADLE binding to a rat brain membrane preparation (Fig. 6).

It should be pointed out that because of the small quantity of morphiceptin in our preparation we have been extremely careful to avoid any contamination by synthetic morphiceptin at any step. In all chromatography and HPLC, columns were extensively washed and samples injected only after we had not detected any morphiceptin-like activity in a prior control run. Thus, it is unlikely that the purified material is contaminated by synthetic morphiceptin.

The total morphiceptin-like activity in 10 g of the casein digest was about 40 nmol. The final purified material, containing about 5 nmol (12% yield) of morphiceptin equivalent, was subjected to further characterization by mass spectrometry.

Isolation of β-Casomorphin—A similar procedure was employed to obtain β-casomorphin, and the morphiceptin-like β-casomorphin-like materials from the other peaks seen on semipreparative HPLC (Fig. 3). Two peaks of immunoreactivity eluted from Magnum 9 with retention times corresponding to the two minor morphiceptin-like activities (Peaks III and IV of Fig. 3) after morphiceptin. Both were purified to homogeneity with Magnum 9 and an analytical reverse-phase HPLC.
Isolation of Morphiceptin from Casein Digest

The total β-casomorphin-like activity in 10 g of casein digest is about 18 μmol, 400 times more than that of the morphiceptin in Peak II. Peak III material, when eluted from an analytical reverse-phase column, has a retention time corresponding to synthetic β-casomorphin, while Peak IV material is eluted behind β-casomorphin. The specific activity of the β-casomorphin of Peak III is consistent with that of β-casomorphin; Peak IV has 0.3% cross-activity based on absorbance at 280 nm.

Peak I material was also purified to homogeneity by a similar procedure. This material showed very poor receptor binding activity and was not further characterized.

Comparison of the Materials from Peaks II, III, and IV—Elution profiles for morphiceptin and β-casomorphin were compared on analytical reverse-phase HPLC. Retention times of 12, 14.5, and 18.5 min (Fig. 7) were observed for Peaks II, III, and IV, respectively. Peak II corresponds to synthetic morphiceptin, Peak III to β-casomorphin (Fig. 7A). The mo-

Fig. 4. Purification of morphiceptin on a reverse-phase (A) and ion-exchange (B) HPLC column. Peak II from the Magnum 9 column was applied to an analytical HPLC column (A) and eluted with a gradient of 0 to 50% acetonitrile in 20 mM ammonium acetate buffer for 30 min with a flow rate of 2 ml/min. The active fractions from the analytical column were then lyophilized and dissolved in water and applied to an ion-exchange HPLC column (Partisil 10 SAX/25) (B). Morphiceptin activity was present in the void volume. Absorbance of the eluate was monitored at 280 nm (— — ).

Fig. 5. Elution profiles of RIA and radioreceptor activity (RRA) of purified morphiceptin in analytical reverse-phase HPLC. Peak II from Fig. 4 was applied to an analytical HPLC column and eluted with a gradient of 10 to 40% of acetonitrile in 10 mM ammonium acetate buffer (pH 4.2) for 30 min with a flow rate of 1 ml/min. Fractions (0.5 ml) were collected and assayed in RIA and RRA (125I-FK 33824 binding). HPLC profiles of synthetic morphiceptin (TPFPNH₂), β-casomorphin, and various fragments are shown at the bottom: TPFA, Tyr-Pro-Phe-Pro; TPFPG, Tyr-Pro-Phe-Pro-Gly; TPF, Tyr-Pro-Phe.

Fig. 6. Comparison of the μ- (○, ■, □, △) and δ-receptor (△) binding activities of purified morphiceptin, β-casomorphin, and δ-prolyl-β-casomorphin with synthetic morphiceptin. The binding of 125I-labeled μ-agonist, FK 33824 (○, ■, □, △) and δ-agonist [D-Ala²,D-Leu⁵]enkephalin (△) to rat brain membrane preparation were performed at 24 °C for 60 min in the absence and presence of various dilutions of synthetic (○), purified morphiceptin (△, ■), purified casomorphin (□) or δ-prolyl-δ-casomorphin (△). The results are expressed as per cent of the control value of the total specific binding. The molarity of purified morphiceptin was estimated by its activity in RIA. The molarities of purified β-casomorphin and δ-prolyl-δ-casomorphin were based on their absorbance at 280 nm.
**Isolation of Morphiceptin from Casein Digest**

**Fig. 7.** HPLC profiles of Peaks II, III, and IV and synthetic morphiceptin and β-casomorphin. A, elution profiles of synthetic morphiceptin and β-casomorphin. B, profile of Peak II material. Activity is expressed as nmol/fraction of morphiceptin RIA activity. C, elution profile of Peak III material. The results are expressed as β-casomorphin RIA activity in each fraction. D, elution profile of Peak IV material. The results are expressed as β-casomorphin RIA activity in each 1-ml fraction (---). A Partisil PXL 10/25 ODS (Whatman) column was used. The flow rate was 2 ml/min, a 10 to 50% acetonitrile gradient was employed for 30 min, and 1-ml fractions were collected.

| Amino acid composition of purified peak III and IV materials derived from casein | Amino acid ratio | Nearest integer |
|---|---|---|
| Peak III peptide | Phenylalanine | 1.00 | 1 |
| | Proline | 2.84 | 3 |
| | Glycine | 1.19 | 1 |
| | Isoleucine | 1.08 | 1 |
| | Tyrosine | 0.88 | 1 |
| Peak IV peptide | Phenylalanine | 0.82 | 1 |
| | Proline | 3.66 | 4 |
| | Glycine | 1.02 | 1 |
| | Isoleucine | 0.91 | 1 |
| | Tyrosine | 0.59 | 1 |

Peaks III and IV were found to be 50 to 100 times less potent than morphiceptin in displacing [³⁵S]FK 33824 from µ-receptors (Fig. 6). The potency of Peak III is similar to that of synthetic β-casomorphin. Peak IV seems slightly more potent than Peak III.

**Amino Acid Compositions**—The purified materials from Peaks III and IV were subjected to amino acid analysis. The results are shown in Table I. Peak III contains Tyr:Phe:Pro:Gly:Ile (1:1:3:1:1) and Peak IV Tyr:Phe:Pro:Gly:Ile (1:1:4:1:1). Peak III was β-casomorphin and Peak IV 8-prolyl-β-casomorphin. These assignments were confirmed by mass spectrometry.

**Mass Spectrometry**—Fast atom bombardment was used to ionize underivatized peptides. The resulting [M + H]⁺ ions were fragmented by collision-activated dissociation. These experiments as well as analysis of the daughter ions produced by collision-activated dissociation were carried out on a triple quadrupole mass spectrometer (see Ref. 11). The dominant fragmentation involved the cleavage of an N-protonated amide bond. In one case, the bond ruptured, leaving an acylium ion containing the N-terminal amino acid and n total amino acids. The fragment is designated [An]⁺. A [Zₙ]⁺ fragment series resulted from cleavages involving the loss of a substituted ketene and a proton rearranged to the positive nitrogen-containing fragment. This positively charged fragment bears the carboxyl-terminal amino acid.

The fast atom bombardment spectrum of synthetic morphiceptin gave a base peak at m/z 522, [M + H]⁺. The collision-activated dissociation spectrum of m/z 522 generated from fast atom bombardment of standard morphiceptin is shown in Fig. 8. The [Aₙ]⁺ and [Zₙ]⁺ ions are indicated. The isolated morphiceptin sample (Peak II) exhibited an [M + H]⁺ at m/z 522 whose collision-activated dissociation spectrum compares well with that in Fig. 9. The differences were due to concentration and matrix effects and because the mass window in the first quadrupole was wider for the isolate than for the standard.

Peak III was identified as β-casomorphin. It exhibited [M + H]⁺ at m/z 790 and a peak for [M + Na]⁺ of equal height at m/z 812 in the fast atom bombardment mass spectrum. The collision-activated dissociation spectrum of the [M + H]⁺ ion of Peak III is given in Table II and is consistent with the structure of β-casomorphin.
Isolation of Morphiceptin from Casein Digest

FIG. 8. Collision-activated dissociation mass spectrum of synthetic morphiceptin.

FIG. 9. Collision-activated dissociation mass spectrum of morphiceptin derived from casein.

Peak IV exhibitions at m/z 887 [M + H]+ and m/z 909 [M + Na]+. The collision-activated dissociation spectrum of m/z 887 supports the assignment of the structure of 8-prolyl-β-casomorphin (Table III).

DISCUSSION

Large numbers of biologically active neural or endocrine peptides terminate with an α-amide group at the COOH terminus, for example, corticotropin-releasing factor, gastrin, secretin, vasoactive intestinal peptide, cholecystokinin, growth hormone-releasing factor, substance P, bombesin, neuropeptides, melanocyte stimulating hormones, and thryrotropin-releasing factors (12–17). For most of these peptides, the COOH-terminal amide is required for optimal biological activity (12, 13). Two COOH-terminally amidated opioid peptides (metorphamide and amidorphin) have also recently been isolated from adrenal medulla and brain (18–20). The pancreas also produces a family of α-amidated peptides, the pancreatic polypeptides (i.e. NPY and PPY) (21). An enzyme
with the ability to convert peptides to α-amidated peptides has recently been identified from pituitary glands (22, 23) and tumor cells (24). This enzyme is selective for peptides in which a neutral amino acid is followed by glycine (22-24). This enzyme is present in the secretory granules of pituitary cells and can be released upon stimulation (24). Since α-amidated peptides are widely distributed in the gastrointestinal tract, including the pancreas, a similar enzyme may be present in the pancreatic enzyme mixture which was used to digest casein. Moreover, residues 60-64 of bovine β-casein (Tyr-Pro-Phe-Pro-Gly) match the specificity for this enzyme, and it is conceivable that morphiceptin is produced from β-casein or its fragments (i.e., β-casomorphin) by a similar α-amidating enzyme. With the large quantity of casein or its fragments, it is most likely that they originate from β-casein in contrast to enkephalins which have been shown to prefer β-receptors (9, 25). Morphiceptin and its analogs have been shown to produce analgesia, catalepsy (2, 3), and physical dependence in vivo (26), and to inhibit intestinal smooth muscle contraction (1). It is tempting to speculate that these exorphins may play a role in the infant-mother bonding relationship that is essential for the survival of infants. Recently, other exogenous opioids from food sources have been described (27-29). The demonstration that dietary sources of exorphins may produce effects in vivo (28, 30) also suggests that exorphins such as morphiceptin and β-casomorphin could have physiological roles.

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