Chemical characterization and opioid activity of an exorphin isolated from in vivo digests of casein

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The in vivo formation of an opioid peptide (exorphin) derived from β-casein has been proved for the first time. It was isolated from duodenal chyme of minipigs after feeding with the milk protein casein. The exorphin has been identified as a β-casein fragment by end-group determinations and qualitative amino acid analysis of the purified peptide. This peptide, named β-casomorphin-11, displayed substantial opioid activity in an opiate receptor-binding assay.

Exorphin *Casomorphin* *β-Casein* *(Duodenal digest)* *Opioid activity*

1. INTRODUCTION

The existence of opioid peptides has been described in partial enzymatic digests of proteins derived from foodstuffs [1–3]. These peptides are called exorphins because of their exogenous origin and morphine-like activities.

Such opioid peptides have been discovered recently in enzymatic digests of whole bovine casein and were designated as β-casomorphins because their sequences identified them as fragments of bovine β-casein [4].

The physiological role of casomorphins and other exorphins is not yet understood. Nevertheless, evidence exists that exorphins do affect intestinal motility as well as the release of insulin and glucagon [5–8].

This paper reports on the isolation and characterization of an opioid peptide from duodenal chyme of minipigs after feeding with casein.

2. MATERIALS AND METHODS

2.1. *Collection and extraction of digesta*

Two male adult Göttingen minipigs (35 kg) fitted with T-shaped canulas at the duodenum, 20 cm from the pylorus, were fed with 100 g acid-precipitated casein (Biogen-Casein, Bayr. Milchversorgung, Nürnberg) after overnight fasting. Postprandial duodenum samples were taken for 8 h, frozen immediately in liquid nitrogen and freeze-dried. A control experiment was performed after feeding with a maize starch diet composed as in [9] without casein.

The extraction of 110 g freeze-dried duodenal chyme from 5 collection periods with chloroform/methanol (65:35, v/v), and the following batchwise adsorption/desorption procedure was performed as in [1]. The oily residue from the last desorption step was diluted up to 5 ml with methanol.

2.2. *Thin-layer analyses and preparative separations*

Aliquots (1 μl) of the extraction residue were subjected to the fingerprint technique on silica gel 60 sheets (20 × 20 cm, 0.2 mm, Merck). First, electrophoresis was carried out in pyridine/acetic acid/water (100:4:900, by vol.), pH 6.5, at 10 mA for 1 h. The second dimension was ascending chromatography in chloroform/methanol/20% ammonia (60:30:5, by vol). The peptides were detected by a tyrosine-specific spray reagent according to [10] or by spraying with 0.05% fluores-

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camine (Serva) in acetone. All β-casomorphin standards were obtained from Sigma.

The conditions for preparative electrophoresis were the same as for fingerprinting but the sample was applied as a streak (80 μl/12 cm). Aliquots (12 μl/17 cm) of the material from several electrophoretic separations were applied to silica gel 60 HPLC plates with fluorescent indicator (10×10 cm, Merck) which had been activated at 100°C for 1 h. After development in the solvent system as described above the main peptide band was visualized under UV light (254 nm), eluted with chloroform/methanol (1:1, v/v), evaporated to dryness and redissolved in a small volume of methanol.

The peptide concentration was determined spectrophotometrically with 0.5 ml of an *o*-phthaldialdehyde (OPA, Serva) reagent as in [11] using L-leucine and β-casomorphin-7 as standards (E_{340} = 6000 $M^{-1} \cdot cm^{-1}$ for calculations).

2.3. *Qualitative amino acid composition*

The purified peptide (2–3 nmol, based upon OPA spectrophotometric assay) was first hydrolyzed in 6 M HCl for 18 h at 105°C; the released amino acids were dansylated and identified as described below.

2.4. *N-terminal amino acid analysis*

A solution of the purified peptide (0.5–1 nmol) was dried in vacuo and the dansylation carried out using 3 μl dansyl chloride (Merck) solution in acetonitrile and 6 μl lithium carbonate buffer, pH 9.5 [12]. After reaction for 30 min at 30°C an aliquot (0.5 μl) was withdrawn to check the purity by thin-layer chromatography (TLC). Acid hydrolysis and identification of the N-terminal dansyl amino acid by two-dimensional TLC on 5×5 cm micropolyamide sheets (Schleicher & Schüll) was carried out as in [13].

2.5. *C-terminal amino acid analysis*

The purified peptide (3–5 nmol) was dissolved in 60 μl sodium acetate, pH 5.5, buffer, and after addition of 0.5 μg carboxypeptidase Y (Sigma) incubated at 37°C. Timed aliquots (10 μl) were removed and immediately heated for 3 min in a boiling water bath. Dansylation and identification of the liberated C-terminal amino acid was performed as described above.

2.6. *Opiate receptor-binding assay*

Opiate receptor binding in rat brain membrane preparations was assayed by competition with (−)-D-[^{3}H]naloxone (Amersham, 51 Ci/mmol) according to [14]). The membranes from brains, without cerebellum, of 3 male rats (Wistar) were reconstituted in 30 ml of 0.05 M Tris-HCl buffer, pH 7.4, at 37°C.

Protein concentration was determined by a microprotein assay with the Coomassie G-250 reagent as in [15] using bovine serum albumin as standard. Aliquots (200 μl) of the membrane preparation were incubated for 5 min at 37°C, pH 7.4, with peptides (1–20 nmol), and the incubation continued for 15 min after the addition of [^{3}H]-naloxone (0.55 pmol, 25 000 cpm) in a final volume of 0.5 ml. After rapid filtration through glass fiber circles (Whatman, 2.4 cm) and washing with four 5-ml portions of ice-cold Tris buffer, the filters were transferred into counting vials, immersed in 1 ml TS-1 tissue solubilizer (Zinsser) and kept overnight at room temperature. The filters were extracted by shaking with 15 ml Ready-Solv MP (Beckman) for 1 h. The radioactivity was counted at 40% efficiency after addition of 100 μl acetic acid/water (1:1, v/v) and equilibration at 4°C in a liquid scintillation spectrometer (Packard Tri-Carb 2660). Nonspecific binding was determined in the presence of a large excess (1 μmol/l) of unlabelled naloxone (Sigma).

3. RESULTS

3.1. *Purification*

The peptide map of the duodenal extract displays at least 23 tyrosine-containing peptides (fig.1). No tyrosine-containing peptides were detectable in the duodenal extract after feeding with a protein-free diet.

After preparative electrophoresis the main band including the positions of standard β-casomorphin-7 and -5 (1. dim. in fig.1) was eluted. With HPLC of the electrophoretically prepurified sample on silica gel a more refined preparative separation of the hydrophobic peptides (R_F>0.4) was achieved (fig.2). The detection with fluorescamine (not shown) revealed that the bulk of interfering peptides emerged with R_F values less than 0.35. The application of the tyrosine-specific reagent gave 14 peptides with the main band in the position

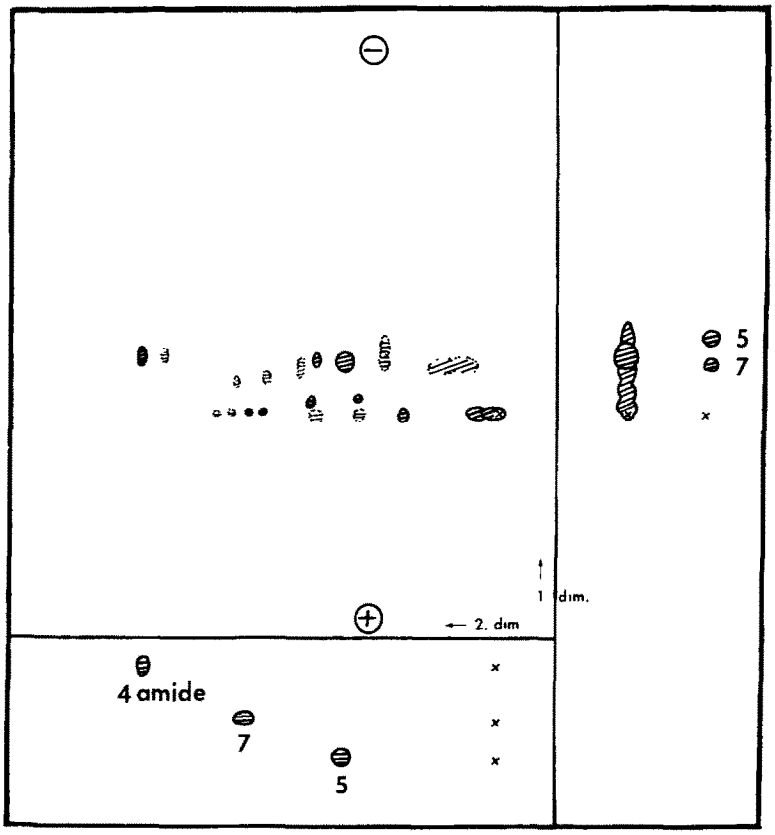

Fig.1. Fingerprint of the tyrosine-containing peptides from duodenal chyme extract on silica gel. Synthetic casomorphins were applied as standards. 1. dim., electrophoresis; 2. dim., ascending chromatography. For details see section 2.2.

of β-casomorphin-5 (R_F 0.42) and two minor bands corresponding to β-casomorphin-7 (R_F 0.63) and β-casomorphin-4 amide (R_F 0.88). The main peptide band as indicated by an arrow in fig.2 was eluted from several HPLC plates for further characterization. After rechromatography, the eluted peptide (77 nmol) was homogeneous as proved after dansylation and by two-dimensional TLC on micropolyamide layer.

3.2. *Characterization and opioid activity*

Because of the small quantities of the purified peptide the hydrolysed sample was dansylated and analysed for qualitative amino acid composition by TLC. The constituents found were Pro, Tyr, Phe, Gly, Ile, Leu, Asx, Ser. When subjected to N-terminal group analysis by the dansyl chloride method it revealed tyrosine as the N-terminus.

In preliminary experiments it was found that the experimental conditions for C-terminal analysis enabled the slow sequential release of the first 3 amino acids from synthetic β-casomorphin-5 and -7. By treatment of the purified peptide with carboxypeptidase Y for 30 min only leucine was released and consequently recognized as the C-terminus.

As the purified peptide had been isolated from a duodenal digest of bovine casein, the sequences of α_s-, β- and κ-casein were examined for regions containing the determined end-groups and amino acid residues. It was indeed discovered that the composition found is only compatible with a fragment of β-casein (genetic variant A2 or A3) [16] originating at Tyr 60 and extending to Leu 70: Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu.

Our results allow the conclusion that the purified peptide contains the whole β-casomorphin-7 sequence (N-terminal) plus the next 4 amino acids in

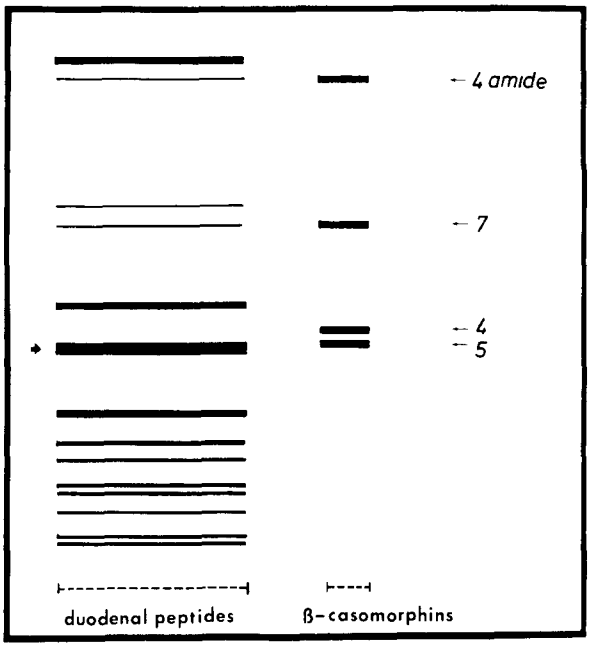

Fig.2. Thin-layer chromatogramm of the electrophoretically prepurified sample from duodenal chyme extract. Synthetic casomorphins were applied for comparison. For details see section 2.2.

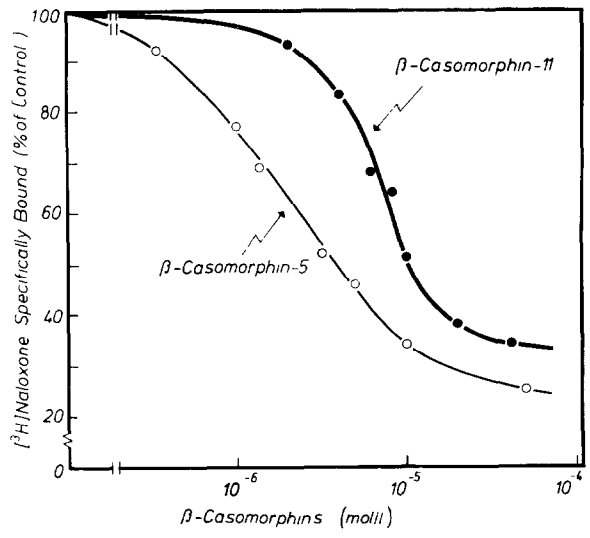

Fig.3. Displacement of specifically bound [^{3}H]naloxone from particulate fractions of rat brain homogenates by β-casomorphin-11 in comparison to synthetic β-casomorphin-5. The incubation volume (0.5 ml) contained 0.7 mg protein, 1.1 nmol/l [^{3}H]naloxone and the indicated concentrations of casomorphins. Control specific binding was 1806 cpm (77% of the total bound). Values are means of 3 separate determinations.

the sequence of β-casein. Moreover, it displayed opioid activity in an opiate receptor-binding assay (fig.3) and hence was referred to as β-casomorphin-11. As shown in fig.3, the isolated β-casomorphin-11 exhibited a weaker but substantial (micromolar) affinity for opiate receptors as compared with synthetic β-casomorphin-5 which is known to be a very potent opioid peptide in several bioassays [17,18].

4. DISCUSSION

The present results show that a peptide with opioid activity, termed β-casomorphin-11, can be isolated in vivo from duodenal chyme of minipigs after feeding with casein whereas no casomorphin-like peptides could be detected after a casein meal in the gastric chyme (H. Meisel, unpublished).

β-Casomorphins are hidden in an inactive state inside the casein structure. To exert biological effects in vivo, they must be produced in the intestinal tract but resist complete degradation by intestinal proteases. Because of the known specificity of pepsin and pancreatic peptidases, cleavage of peptide bonds releasing significant amounts of β-casomorphins from the corresponding β-casein sequence is unexpected. Furthermore, the proline-rich β-casomorphin structure itself was described as being highly resistant towards proteolysis by gastric and pancreatic enzymes [4,17]. However, the intestinal brush border contains high levels of peptidase activity [19], so that the action of several peptidases from the intestinal epithelium could contribute to the rise of free casomorphins. Authors in [20] and [21] have shown that the in vitro digestion of buffalo β-casein released neither β-casomorphin-7 nor a fragment of it but a putative precursor (decapeptide) which was further digested by incubation with brush border peptidases, especially with dipeptidyl peptides IV from human intestine; the peptides were not checked for their opioid activities.

The question arises as to whether the isolated β-casomorphin-11 may be considered as a precursor for smaller casomorphin fragments, because two further peptides from duodenal chyme have indeed shown a chromatographic behaviour corresponding to standard β-casomorphin-7 and -4 amide, respectively (fig.2).

It seems likely that β-casomorphin-11 itself can

interact with receptors in the intestinal tract, or may be degraded to further opioid fragments in the brush border. Such highly hydrophobic fragments are expected to be absorbed into the bloodstream and to bind to brain opiate receptors as well as to those of peripheral organs [2,3]. Both rapid brush border degradation and high absorption rate could be possible explanatations for the relatively low amounts of β-casomorphin-11 found in duodenal chyme (77 nmol isolated vs about 1.0 mmol ingested).

In the present study the formation of an opioid peptide has been proved for the first time in vivo, and under physiological conditions. however, it is not yet clear whether the opioid β-casomorphins could reach concentrations of physiological significance. Thus, their possible physiological significance has to be elucidated by further in vivo studies.

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