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Opioid activities of human β-casomorphins

Gertrud Koch, Klaus Wiedemann *, and Hansjörg Teschemacher

Rudolf-Buchheim-Institut für Pharmakologie der Justus-Liebig-Universität Gießen, Frankfurter Strasse 107, D-6300 Gießen, Federal Republic of Germany

Summary. Opioid activities of human β-casomorphin-4, -5, -7 and -8 and, for comparison, of the corresponding bovine β-casomorphins were studied in the guinea-pig ileum preparation. Binding parameters, i.e. K_D-values and binding site concentrations, for the interaction of human and bovine β-casomorphins with opioid receptors in rat brain homogenates were determined in inhibition experiments, using [^{3}H]-(D-Ala^2, MePhe^4, Gly-ol^5)enkephalin, [^{3}H]-(D-Ala^2, D-Leu^5)enkephalin and [^{3}H]ethylketazocin as μ-, δ- and κ-opioid receptor ligands. Analysis of binding data was performed using a non-linear curve fitting program. All β-casomorphins examined displayed opioid activity. The affinity was highest for μ-receptors, less so for δ-receptors and lowest for κ-receptors. It is suggested that human β-casomorphins might play a role as "food hormones".

Key words: Human β-casein – Opioid peptides – Opioid receptor ligands – Human β-casomorphins – Computerized binding data analysis

Introduction

Recently, a fragment of the bovine β-casein amino acid sequence, corresponding to amino acid residues 60-66, has been shown to display opioid activity when cleaved from the β-casein molecule. In opioid receptor binding studies and isolated organ preparations this peptide, Tyr-Pro-Phe-Pro-Gly-Pro-Ile, and its fragments shortened at the C-terminus by one, two or three amino acid residues, behave like μ-type opioid agonists (Brantl et al. 1981). In view of origin and pharmacological activity these peptides were named β-casomorphins (Brantl et al. 1979).

Very recently, the primary structure of human β-casein was determined (Greenberg et al. 1984) and sequence comparison with bovine β-casein revealed a 10-residue shifted alignment relationship and 47% identity. Thus, by analogy, human β-casein (51-57) could be supposed to represent a "human β-casomorphin". In fact, except for two amino acids at position 4 and 5 of the sequence, this peptide, Tyr-Pro-Phe-Val-Glu-Pro-Ile, proved to be identical with bovine β-casein (60-66). Thus the opioid activities of human β-casein (51-54), (51-55), (51-57) and (51-58), representing "human β-casomorphin"-4, -5, -7 or -8, were studied in comparison to bovine β-casomorphin-4, -5, -7 and -8 in both the guinea-pig ileum preparation and in opioid receptor binding studies. A non-linear curve fitting computer program was used for binding data analysis.

* *Present address:* Psychiatrische Kliniken der Universität Mainz, Langenbeckstrasse 2, D-6500 Mainz 1, Federal Republic of Germany

Send offprint requests to G. Koch at the above address

Materials and methods

Substances. Synthetic human and bovine β-casomorphins were purchased from Novabiochem, Läufelfingen, Switzerland or Bachem AG, Bubendorf, Switzerland; unlabelled (D-Ala^2, D-Leu^5)enkephalin from Bachem AG, Bubendorf, Switzerland. Unlabelled (D-Ala^2, MePhe^4, Gly-ol^5)-enkephalin was a generous gift from Dr. Römer, Sandoz, Switzerland and unlabelled ethylketazocin a generous gift from Dr. Daniel, Sterling Winthrop, UK. [^{3}H]-(D-Ala^2, MePhe^4, Gly-ol^5)enkephalin (=[^{3}H]DAGO, specific activity: 60 Ci/mmol) was obtained from Amersham Buchler, Braunschweig, FRG and [^{3}H]-(D-Ala^2, D-Leu^5)enkephalin (=[^{3}H]DADL, specific activity: 47 Ci/mmol) and [^{3}H]-ethylketazocin (=[^{3}H]EK, specific activity: 19 Ci/mmol) from New England Nuclear, Dreieich, FRG.

Bioassay. Opioid activities of β-casomorphins were determined in the guinea-pig ileum longitudinal muscle/myenteric plexus preparation. Preparation, mounting and electrical stimulation (frequency: 0.1 Hz; pulse: 60 V; duration: 0.5 ms) were carried out as described by Kosterlitz et al. (1970) and Schulz and Goldstein (1972).

Binding studies. Affinities of β-casomorphins for μ-, δ- and κ-binding sites and binding site concentrations were determined in inhibition experiments making use of [^{3}H]DAGO as μ-ligand, [^{3}H]DADL as δ-ligand and [^{3}H]EK as κ-ligand. The assays were performed according to the method of Pert and Snyder (1973) and Magnan et al. (1982) with modifications. In brief, brains (without cerebellum) of 5-months-old female Wistar rats were homogenized in 50 mmol/l Tris/HCl buffer, pH 7.4 at 0° C. The homogenate was centrifuged at 20,000 × *g* for 5 min and the pellet was then resuspended in Tris buffer. Centrifugation and resuspension were repeated three times; a 2% (w/v) final homogenate was prepared. One ml of homogenate was incubated at 25° C for 1 h with unlabelled DAGO, DADL, EK or β-casomorphins in concentrations between 10^{-11} to 10^{-3} mol/l together with [^{3}H]DAGO (0.73 nmol/l), [^{3}H]DADL (0.43 nmol/l) or [^{3}H]EK (0.78 nmol/l), respectively. Incubation with [^{3}H]EK (0.78 nmol/l) was performed in the presence of unlabelled DAGO and DADL (concentrations were 100 times greater

than [^{3}H]EK concentrations), in order to suppress μ- and δ-binding of [^{3}H]EK without affecting κ-binding. Samples were filtered through prewetted Whatman GF-B glass fibre filters; radioactivity retained on the filters was measured by liquid scintillation spectrometry.

Data analysis. Estimation of IC_{50}-values from the guinea-pig ileum preparation was performed using a non-linear least squares curve fitting program (A. Rücker, Hochschulrechenzentrum, University of Gießen) which is based on the MODFIT program as described by McIntosh and McIntosh (1980).

Binding parameters, i.e. dissociation constants (K_D-values) and binding site concentrations for the interactions of β-casomorphins with μ-, δ- and κ-binding sites were determined with the non-linear curve fitting program LIGAND as described by Munson and Rodbard (1980). It is based on a mathematical theory developed by Feldman: the equilibrium composition of a ligand-binding system, including any number of ligands reacting with any number of classes of binding sites, is characterized by solving the mass action equations (Feldman 1972; Feldman et al. 1972).

Data analysis was performed by simultaneously fitting the data from a homologous (labelled ligand/corresponding unlabelled ligand) and a heterologous (labelled ligand/β-casomorphin) inhibition experiment including data from all three repetitions of experiments. One, two or three binding site models were assumed and fitted to the data. By means of an F ratio test, which compared the mean square errors in each fit, a decision on the best model was made. For three binding site models, the fit did not improve significantly as compared to the two binding site models or convergence could not be obtained. In order to reduce the complexity of the fit for multiple inhibition curves, within the fit for two or three binding sites, the ligands of the homologous experiments were constrained to share the same affinities for all classes of binding sites. Affinities for DAGO, DADL and EK for opioid receptors were determined in a separate fit.

All computer programs ran on a Control Data Corporation (CDC) Cyber 174 computer using the Network Operating System (NOS) version 2.

Relative affinities (Table 2) of β-casomorphins for [^{3}H]DAGO- (K_{D11}, K_{D12}), [^{3}H]DADL- (K_{D2}) and [^{3}H]EK-binding sites (K_{D3}) were derived according to the equation (Paterson et al. 1983):

$$\text{Rel. aff. (DAGO, DADL or EK binding sites)} = \frac{\frac{1}{K_{D(11,12,2 \text{ or } 3)}}}{\frac{1}{K_{D11}} + \frac{1}{K_{D12}} + \frac{1}{K_{D2}} + \frac{1}{K_{D3}}}.$$

Results

In the guinea-pig ileum longitudinal muscle/myenteric plexus preparation human β-casomorphins displayed opioid activity. The respective IC_{50}-values in comparison with IC_{50}-values for bovine β-casomorphins are given in Table 1.

Human β-casomorphins were 3 to 30 times less potent than bovine β-casomorphins and 300 – 600 times less potent than normorphin. The rank order of potencies for human β-casomorphins was the same as for the bovine β-casomorphins.

Table 1. Opioid activities of β-casomorphins. IC_{50}-values indicate substance concentrations (μmol/l) causing a 50% inhibition of electrically induced contractions of the guinea-pig ileum myenteric plexus/longitudinal muscle preparation: values are means from 12 determinations, standard deviations were less than 16% of mean values. Naloxone (1.4 μmol/l), a specific opioid antagonist, reversed or blocked substance-induced inhibition of contractions

Human	IC_{50} (μmol/l)	Bovine	IC_{50} (μmol/l)
β-Casomorphin-8	14.55	β-Casomorphin-8	3.33
β-Casomorphin-7	29.00	β-Casomorphin-7	5.14
β-Casomorphin-5	13.50	β-Casomorphin-5	0.53
β-Casomorphin-4	27.60	β-Casomorphin-4	3.60
Normorphin	0.05		

Table 2. Dissociation constants and relative affinities (in parentheses) of human and bovine β-casomorphins for [^{3}H]DAGO, [^{3}H]DADL and [^{3}H]EK binding sites in rat brain homogenates. Binding data were obtained from inhibition experiments (duplicate determination in three independent experiments) for determination of binding parameters by the computer program LIGAND. Two site models were chosen, when the *F*-test, included in the program, indicated a significantly better fit ($P < 0.05$) compared to the respective on site model

	K_D-values (μmol/l) (relative affinities)			
	[^{3}H]DAGO Binding sites		[^{3}H]DADL Binding site	[^{3}H]EK Binding site
	K_{D11}	K_{D12}	K_{D2}	K_{D3}
Human β-casomorphin-8	3 (0.92)	65 (0.043)	77 (0.036)	1,700 (0.0016)
Human β-casomorphin-7	6 (0.88)	100 (0.053)	78 (0.067)	960 (0.0055)
Human β-casomorphin-5	20 (0.51)		21 (0.485)	2,000 (0.0051)
Human β-casomorphin-4	4 (0.63)		7 (0.361)	330 (0.0077)
Bovine β-casomorphin-8	0.8 (0.88)	10 (0.070)	17 (0.041)	60 (0.0117)
Bovine β-casomorphin-7	2 (0.85)		12 (0.142)	340 (0.0050)
Bovine β-casomorphin-5	0.4 (0.79)	1.6 (0.198)	43 (0.007)	91 (0.0035)
Bovine β-casomorphin-4	1.4 (0.97)		51 (0.027)	1,800 (0.0008)

Binding of β-casomorphins to [^{3}H]DAGO, [^{3}H]DADL and [^{3}H]EK binding sites was tested in rat brain homogenates; the inhibition curves may be seen from Fig. 1 and the dissociation constants and relative affinities are listed in Table 2.

In the [^{3}H]DAGO inhibition experiment affinities of bovine β-casomorphins for the respective binding sites were found to be higher than affinities of human β-casomorphins. In the [^{3}H]DADL inhibition experiment, however, human

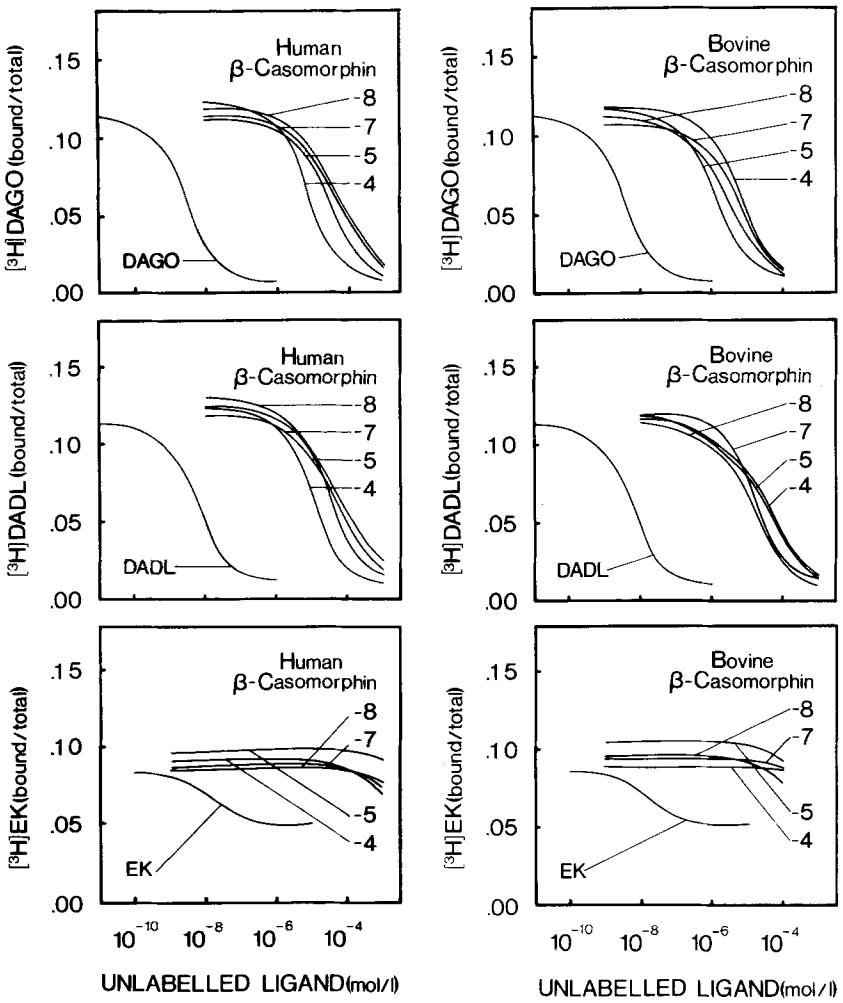

Fig. 1
Inhibition curves: Inhibition of binding of [^{3}H]DAGO, [^{3}H]DADL and [^{3}H]EK to opioid receptors in rat brain membranes by unlabelled β-casomorphin, DAGO, DADL or EK. The bound fraction of the total concentration of labelled ligand in the assay tube is plotted versus the added dose of unlabelled ligand. The curves were obtained by fitting one or, in case of a significantly better fit, two site models to data from three repeats of the respective homologous and heterologous experiments by the LIGAND computer program

β-casomorphin-4 and -5 showed higher affinities than the corresponding bovine β-casomorphins, but the converse was true for β-casomorphin-7 and -8. Affinities of β-casomorphins for the first [^{3}H]DADL-binding site (K_D-values not shown in Table 2) were found to be the same as for the first [^{3}H]DAGO-binding site.

K_D-Values of DAGO for the [^{3}H]DAGO-binding sites were 0.8 nmol/l and 36 nmol/l, of DADL for the [^{3}H]DADL-binding site 3 nmol/l and of EK for the [^{3}H]EK-binding site 8 nmol/l. Binding capacities for the [^{3}H]DAGO-binding sites were 17 pmol/g tissue (one site model) or 7 and 10 pmol/g tissue (two site model), for the [^{3}H]DADL-binding sites 29 pmol/g tissue (one site model) or 7 and 21 pmol/g tissue (two site model) and for the [^{3}H]EK-binding site 25 pmol/g tissue.

Discussion

Opioid activities of bovine β-casomorphins were examined by Brantl et al. (1981; 1982). Bovine β-casomorphins displayed a much higher activity in the guinea-pig ileum than in the mouse vas deferens. Further, in inhibition experiments using [^{3}H]dihydromorphine as a μ- and [^{3}H]DADL as a δ-ligand, Brantl et al. (1982) found IC_{50}-values for β-casomorphins to be 5 to 40 times lower for the inhibition of binding of the δ-ligand than for the μ-ligand. Affinities for the κ-receptor were found to be very low. Thus, β-casomorphins were suggested to be μ-ligands.

Opioid activities of human β-casomorphin-4 and -5 were examined by Brantl (1984) in the guinea-pig ileum preparation and human β-casomorphin-4, -5, -6 and -8 were examined by Yoshikawa et al. (1984) in opioid receptor binding assays using [^{3}H]naloxone as a labelled μ-ligand, and, for some experiments, [^{3}H]DADL giving IC_{50}-values as binding parameters.

The present studies were undertaken to examine opioid activities of human β-casomorphins and, for comparison, bovine β-casomorphins (however, bovine β-casomorphin-8 had not been studied at all, so far) using the guinea-pig ileum preparation as a test system. In particular, binding parameters should be determined, i.e. μ-, δ- and κ-receptor affinities (K_D-values) and binding capacities, which, until now, had not been investigated, neither for human nor for bovine β-casomorphins.

In the binding studies performed DAGO, DADL and EK were used as labelled or unlabelled opioid receptor ligands. DAGO was chosen as a selective μ-ligand, which is known to be 220 times more active at μ- than at δ-receptors. DADL was taken as δ-ligand displaying, however, a partial μ-affinity. EK is reported to be a κ- as well as a μ- and δ-ligand; thus, unlabelled DAGO and DADL were added

when [^{3}H]EK inhibition experiments were performed (Paterson et al. 1983).

Binding data analysis indicated β-casomorphin binding to two binding sites in the [^{3}H]DAGO as well as in the [^{3}H]DADL experiments (Table 2), i.e. the computer fit was significantly better for the two binding site model than for the one binding site model for some of the β-casomorphins. The two binding sites found in the [^{3}H]DAGO inhibition experiment may represent two μ-receptor subtypes which might be identical with those characterized by Toll et al. (1984) and by Lutz et al. (1984). Eventually β-Casomorphins might be useful in further studies on μ-receptor subtypes.

As found for some β-casomorphins, binding capacities and K_D-values for the first [^{3}H]DAGO and the first [^{3}H]DADL binding site proved nearly identical. Thus, in the [^{3}H]DADL experiment, the first [^{3}H]DADL-binding site might represent a μ-binding site and the second one a δ-binding site; this assumption is compatible with reports on partial μ-affinity of DADL (Paterson et al. 1983).

Relative affinities were calculated assuming two μ-, one δ- and one κ-binding site (Table 2). Both, human and bovine β-casomorphins, were found to bind particularly to μ-receptors. However, for human β-casomorphin-4 and -5, considerably higher relative affinities to δ-receptors were found than for their corresponding bovine β-casomorphins. Thus, substitution of the amino acid residues Pro and Gly in the bovine β-casomorphin amino acid sequence by the residues Val and Glu obviously caused an affinity shift in favour of δ-receptor binding.

In the gastrointestinal tract the presence of opioid receptors has been demonstrated (Pert and Snyder 1973) as well as opioid effects on fluid secretion (Beubler and Lembeck 1979) or on smooth muscle tone (Chapman et al. 1950; Daniel et al. 1959). It is tempting to speculate that after milk intake β-casomorphins might be released from β-casein and might act on the gastrointestinal tract as exogenous "food hormones" (Morley 1982).

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