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Opioid Activities and Structures of α-Casein-Derived Exorphins†

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ABSTRACT: Exorphins, peptides with opioid activity, have previously been isolated from pepsin hydrolysates of α-casein [Zioudrou, C., Streaty, R. A., & Klee, W. A. (1979) *J. Biol. Chem. 254*, 2446–2449]. Analysis of these peptides shows that they correspond to the sequences 90–96, Arg-Tyr-Leu-Gly-Tyr-Leu-Glu, and 90–95, Arg-Tyr-Leu-Gly-Tyr-Leu, of α-casein. These peptides, as well as two of their analogues Tyr-Leu-Gly-Tyr-Leu-Glu (91–96) and Tyr-Leu-Gly-Tyr-Leu (91–95), have now been synthesized and characterized. Their opioid activity was examined by three different bioassays: (a) displacement of D-2-alanyl[*tyrosyl*-3,5-^{3}H]enkephalin-(5-L-methioninamide) and [^{3}H]dihydromorphine from rat brain membranes; (b) naloxone-reversible inhibition of adenylate cyclase in homogenates of neuroblastoma x glioma hybrid cells; (c) naloxonne-reversible inhibition of electrically stimulated contractions of the mouse vas deferens. The synthetic peptide of sequence 90–96 was the most potent opioid in all three bioassays and its potency was similar to that of the isolated α-casein exorphins. The synthetic peptides were totally resistant to hydrolysis by trypsin and homogenates of rat brain membranes, but were partially inactivated by chymotrypsin and subtilisin. The difference in opioid activity of α-casein exorphins may be related to differences in conformational flexibility observed by NMR spectroscopy.

Several families of opioid peptide have been found to occur naturally in the central nervous system. These include the pentapeptide enkephalins (Hughes et al., 1975), the endorphins which are larger (Bradbury et al., 1976; Guillemin et al., 1976; Li & Chung, 1976) and derived from a common precursor along with ACTH and α-MSH (Mains et al., 1977; Nakanishi et al., 1979), and the dynorphins (Goldstein et al., 1979; Kangawa et al., 1979), peptides of intermediate size, derived from yet another precursor (Kakidani et al., 1982). Each of these peptide groups shares the amino-terminal tetrapeptide sequence Tyr-Gly-Gly-Phe followed by Leu or Met. These pentapeptide sequences seem therefore to be the opiate recognition structure for all endogenous opioid peptides, collectively known as endorphins. The remainder of the endorphin sequence may serve to direct the peptide to the appropriate receptor type.

We and others have previously described the existence of peptides, in partial enzymatic digests of proteins derived from foodstuffs, which have opioid activities (Wajda et al., 1976; Klee et al., 1978; Zioudrou et al., 1979; Brantl et al., 1979;

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Henschen et al., 1979). These peptides, because of their exogenous origin, were named exorphins. The structure of one such peptide, derived from β-casein, has been determined (Brantl et al., 1979).

In this paper we describe the structure, synthesis, and opioid activities of the exorphin originally isolated from pepsin digests of α-casein. The peptide is only distantly related in sequence to either the endorphins or β-casein exorphin [also known as casomorphin (Brantl et al., 1979)] and is the only opioid peptide yet studied that requires an amino acid residue prior to the amino-terminal tyrosine for optimal opioid activity.

Materials and Methods

N-t-Boc-L-amino acids[1] were either purchased from Fluka or synthesized from the individual amino acids by reaction with 2-[(*tert*-butoxycarbonyl)oximino]-2-phenylacetonitrile (Itoh et al., 1975). The following amino acid derivatives were prepared by methods cited in the literature: L-leucine benzyl ester hydrochloride (Erlanger & Hall, 1954); *O*-benzyl-L-tyrosine (Wünsch et al., 1958); dibenzyl L-glutamate (Izumiya & Makusumi, 1957); *N-t*-Boc-L-leucine hydroxysuccinimide ester (Anderson et al., 1964); Z_3-L-arginine (Zervas et al., 1957); Z_3-L-arginine *p*-nitrophenyl ester (Wünsch & Wendlberger, 1969); *N-t*-L-(OBzl)tyrosine hydroxysuccinimide ester (Wolman & Klausner, 1971).

The purity of these derivatives was checked (a) by measuring their optical rotation at 589 nm (Perkin-Elmer Polarimeter 140), (b) by their melting points, and (c) by TLC chromatography in the following solvent systems: (A) *n*-BuOH/acetic acid/water (4:1:1), (B) 2-butanol/3% ammonia (70:30), (C) chloroform/methanol/acetic acid (3:1:1), and (D) 2-propanol/sodium pyrophosphate, 50 mM, pH 7.3, in 100 mM sodium chloride (70:30) using silica gel 60 precoated plates (Merck). The peptides were synthesized by conventional solution methods, and removal (a) of the *N-t*-Boc groups was performed with trifluoroacetic acid in CH_2Cl_2 and (b) of the carbobenzoxy and benzyl groups by catalytic hydrogenation with Pd/H_2. The peptides were purified by reverse-phase high-pressure liquid chromatography on a Varian Model 5000 chromatograph and a Varian MCH-10 column.

Amino acid analyses were performed with a Beckman 120-C amino acid analyzer after hydrolysis of the peptides in 6 N HCl (constant boiling point) in the presence of small amounts of purified phenol, at 110 °C for 18 h, in degased, sealed ampoules. The recovery ranged between 87 and 95%.

End-Group Analysis. The N-terminal amino acid determination was done by dansylation of 5–8 μg of peptide, hydrolysis, and chromatography of the products on polyamide plates according to the procedure described by Hartley (1970).

The following enzymes were purchased by Sigma: trypsin, bovine pancreas (type III) (EC 3.4.21.1); subtilopeptidase A, bacterial (type VIIII); protease from *Streptomyces griseus* (type VI).

Synthesis of Peptides. (*A*) *N-t-Boc-Leu-Glu Dibenzyl Ester.* A solution of 3.9 g (16 mmol) of *t*-Boc-leucine monohydrate in 20 mL of methylene chloride was mixed with 8 g (16 mmol) of dibenzyl L-glutamate toluenesulfonate in 30 mL of methylene chloride and 2.3 mL of triethylamine. The solution was cooled to 4 °C, and 3.1 g (20 mmol) of HOBT was added followed by a chilled solution of 3.6 g (17 mmol) of DCC in 15 mL of methylene chloride. The solution was stirred overnight at room temperature. The urea was removed by filtration, and the organic phase was washed twice with cold solutions of H_2SO_4 (2%), $NaHCO_3$ (5%), and saturated NaCl. It was then dried over Na_2SO_4 and the solvent evaporated. The oily residue weighed 7.8 g (90% yield) after drying and appeared as a single spot on TLC: R_f 0.73 (solvent B) and R_f 0.25 (solvent C).

(*B*) *N-t-Boc-(OBzl)Tyr-Leu-Glu Dibenzyl Ester.* A solution of 4.45 g (12 mmol) of *t*-Boc-(OBzl)tyrosine in 15 mL of methylene chloride was mixed with 6.65 g (12 mmol) of Leu-Glu dibenzyl ester trifluoroacetate in 30 mL of CH_2Cl_2 and 1.7 mL of triethylamine. To this ice-cold solution were added 2.6 g of HOBT and a solution of 2.7 g of DCC in 15 mL of CH_2Cl_2. The solution was stirred overnight at room temperature. After the removal of the urea, the organic phase was washed twice with cold solutions of 1 N HCl, 1 M $NaHCO_3$, and saturated NaCl. After drying, the solvent was evaporated, and the product was precipitated with petroleum ether. It was recrystallized twice from methanol: yield 4.7 g (50%); mp 138–140 °C; $[\alpha]^{22}$ −11.75° (*c* 2, DMF). The product appeared as a single spot with R_f 0.94 (solvent C). *N-t*-Boc-(OBzl)tyrosine and Leu-Glu dibenzyl ester moved with R_f 0.91 and 0.4, respectively, in the same solvent system.

(*C*) *N-t-Boc-Leu-Gly.* A solution of 3.3 g (10 mmol) of *t*-Boc-Leu hydroxysuccinimide ester in 20 mL of dimethoxyethane was mixed with a solution of 0.75 g of glycine (10 mmol) and 1.68 g of sodium bicarbonate in 20 mL of water. The solution was stirred overnight at room temperature. After filtration the dimethoxyethane was removed in vacuo. The aqueous solution was acidified to pH 2 with saturated citric acid in the cold. The precipitate was extracted with ethyl acetate (3 × 50 mL) and the organic phase washed with water, 1 N $NaHCO_3$, and saturated NaCl. The solvent was evaporated, and the product was precipitated with petroleum ether. It was recrystallized from diisopropyl ether: yield 21 g (73%); mp 117–118 °C; $[\alpha]^{25}$ −22.3° (*c* 2, CH_2Cl_2). The product moved as a single spot with R_f 0.43 (solvent B); *N-t*-Boc-Leu-OSu moves with R_f 0.84.

(*D*) *N-t-Boc-Leu-Gly-(OBzl)Tyr-Leu-Glu Dibenzyl Ester.* A solution of 1.58 g (5.5 mmol) of *N-t*-Boc-Leu-glycine in 10 mL of CH_2Cl_2 was mixed with 4.45 g of (OBzl)Tyr-Leu-Glu dibenzyl ester trifluoroacetate in 15 mL of CH_2Cl_2 and 0.77 mL of triethylamine. To the ice-cold solution were added 0.92 g of HOBT and a solution of 1.24 g of DCC in 10 mL of CH_2Cl_2, and the mixture was stirred overnight at room temperature. After removal of the urea, the solvent was evaporated and the product solidified by addition of iced water. The solid was washed twice with cold solutions of 2% citric acid, 1 N $NaHCO_3$, and water. The dried material was dissolved in methanol and precipitated with diisopropyl ether: yield 3 g (58%); mp 102–106 °C; $[\alpha]^{22}$ −21.55° (*c* 2, methanol). Amino acid analysis was as follows: Gly, 1.06; Glu, 1.0; Leu, 2.01; Tyr, 0.92. The product appeared as a single spot with R_f 0.82 (solvent B). *N-t*-Boc-Leu-Gly and (OBzl)Tyr-Leu-Glu dibenzyl ester trifluoroacetate moved with R_f 0.41 and R_f 0.66, respectively, in solvent B.

(*E*) *N-t-Boc-(OBzl)Tyr-Leu-Gly-(OBzl)Tyr-Leu-Glu Dibenzyl Ester.* A solution of 1.38 g (2.96 mmol) of *N-t*-Boc-(OBzl)Tyr-OSu in 10 mL of dimethylformamide was mixed with 2.90 g (2.96 mmol) of Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester trifluoroacetate in 15 mL of dimethylformamide containing 0.45 mL of triethylamine. After 3 days at room temperature, addition of ice-cold water produced a

[1] Abbreviations: [^{3}H]DALAMID, D-2-alanyl[*tyrosyl*-3,5-^{3}H]enkephalin-(5-L-methioninamide); Me_2SO-d_6, hexadeuteriodimethyl sulfoxide; Me_4Si, tetramethylsilane; *t*-Boc, *tert*-butyloxycarbonyl; Z, carbobenzoxy; DCC, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; [^{3}H]-DMH, [1,7,8(N)-^{3}H]dihydromorphine; OBzl, benzyloxy; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

solid precipitate that was filtered off and washed twice with cold 2 M citric acid and 1 M $NaHCO_3$. It was recrystallized from 2-propanol to give 2.77 g (77%) of peptide: mp 197–198 °C; $[\alpha]^{25}$ −21.2° (*c* 2, CH_2Cl_2). Amino acid analysis was as follows: Glu, 1.03; Gly, 1.0; Leu, 2.05; Tyr, 1.80. The peptide appeared as a single spot with R_f 0.87 (solvent D), and *N-t*-Boc-(OBzl)Tyr-OSu and Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester moved with R_f 0.98 and R_f 0.6, respectively.

(*F*) *N^{α},N^{δ},N^{ω}-Z_3-Arg-(OBzl)Tyr-Leu-Gly-(OBzl)Tyr-Leu-Glu Dibenzyl Ester.* To an ice-cold solution of (OBzl)Tyr-Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester trifluoroacetate, 1.42 g (1.15 mmol), containing 0.16 mL of triethylamine in 15 mL of dimethylformamide was added N^{α},N^{δ},N^{ω}-Z_3-arginine *p*-nitrophenyl ester, 0.930 g (1.1 mmol). The reaction mixture was stirred overnight at 0 °C and 3 days at room temperature. Dry ether (100 mL) was added and the precipitate filtered. The precipitate was washed with ether, methanol, and ethyl acetate: yield 1.5 g (83%); mp 190–191 °C; $[\alpha]^{25}$ −15.5° (*c* 1, DMF). Amino acid analysis was as follows: Arg, 0.926; Glu, 1.05; Gly, 1.02; Leu, 2.02; Tyr, 1.81. The product appeared as a single spot with R_f 0.1 (solvent D). Z_3-arginine *p*-nitrophenyl ester and (OBzl)Tyr-Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester moved with R_f 0.25 and R_f 0.85, respectively.

(*G*) *Arg-Tyr-Leu-Gly-Tyr-Leu-Glu.* N^{α},N^{δ},N^{ω}-Z_3-Arg-(OBzl)Tyr-Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester, 1.4 g (0.83 mmol), in methanol/dimethylformamide and acetic acid was hydrogenated over palladium black for three days. The catalyst was filtered and the solvents were evaporated in vacuo. The material was precipitated by the addition of ether: yield 0.6 g (73%); mp 235–237 °C dec; $[\alpha]^{22}$ −5.3° (*c* 1, DMF); ϵ 2900 M^{-1} cm^{-1} at 275 nm in methanol. Amino acid analysis was as follows: Arg, 0.89; Glu, 1.02; Gly, 1.00; Leu, 2.00; Tyr, 1.8. The peptide appeared as a single spot with R_f 0.73 (solvent D). Anal. Calcd for $C_{43}H_{64}N_{10}O_{12}$ (M_r 913.11): N, 13.55. Found: N, 13.16. Purification was achieved with a Bio-Gel P2 column with 80% 2-propanol/water. On HPLC (column MCH-10, Varian) the peptide elutes at 8.1 min and 38% of the linear gradient 0–70% ammonium acetate buffer, pH 5 (10 mM)/acetonitrile and a flow rate of 3 mL/min. On a C_{18} column (Waters) with a linear gradient 0–70% water/acetonitrile and a flow rate of 3 mL/min, the peptide elutes at 45% acetonitrile.

(*H*) *Tyr-Leu-Gly-Tyr-Leu-Glu.* Amino acid analysis was as follows: Glu, 1.02; Gly, 1.0; Leu, 2.1; Tyr, 1.85. The peptide appeared as a single spot with R_f 0.6 (solvent D): mp 200–205 °C dec; $[\alpha]^{25}$ −15.5° (*c* 1, methanol); ϵ 2850 M^{-1} cm^{-1} at 275 nm in methanol. Anal. Calcd for $C_{37}H_{52}N_6O_{11}$ (M_r 756.9): N, 11.1. Found: N, 10.7. During purification by HPLC the peptide elutes at 7.5 min and 34% of the gradient (column MCH-10, gradient 0–70% ammonium acetate, pH 5/acetonitrile).

(*I*) *Arg-Tyr-Leu-Gly-Tyr-Leu.* Amino acid analysis was as follows: Arg, 0.96, Gly, 1.0; Leu, 1.97; Tyr, 1.90. The peptide appeared as a single spot with R_f 0.21 (solvent B) and R_f 0.75 (solvent D): mp 185–190 °C dec; $[\alpha]^{25}$ −2.6° (*c* 1, methanol); ϵ 2850 M^{-1} cm^{-1} at 275 nm in methanol. Anal. Calcd for $C_{38}H_{57}N_9O_9$ (M_r 784): N, 16.07. Found: N, 15.75. During purification by HPLC the peptide elutes at 9.4 min and 41% of the gradient (column MCH-10, gradient 0–70% ammonium acetate, pH 5/acetonitrile).

(*J*) *Tyr-Leu-Gly-Tyr-Leu.* Amino acid analysis was as follows: Gly, 1.02; Leu, 2.0; Tyr, 1.92. The peptide appeared as a single spot with R_f 0.55 (solvent D): $[\alpha]^{25}$ −12.8° (*c* 1.1, methanol); ϵ 2890 M^{-1} cm^{-1} at 275 nm in methanol. Anal. Calcd for $C_{32}H_{45}N_5O_8$ (M_r 627.7): N, 11.15. Found: N, 10.9.

Preparation of Rat Brain Homogenates (Fraction P_2). The brains without the cerebellum from three to four male rats were homogenized in 10 volumes of 0.32 M sucrose by using 12 strokes of a loosely fitted Teflon–glass homogenizer. The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant fraction was centrifuged at 10000*g* for 10 min. The pellet P_2 was suspended in the original volume of 0.32 M sucrose and stored at −70 °C (Whittaker, 1959).

Binding to Opiate Receptors. Binding of the peptides to the P_2 fraction was measured at 0 °C for 120 min, by competition with [[^{3}H]Tyr]-D-Ala^2-Met-enkephalinamide, 16 Ci/mmol (3 mM), or with [^{3}H]dihydromorphine, 65 Ci/mmol (1.5 mM) (Amersham), as described by Klee & Streaty (1974). Three experiments were performed with duplicate points for each concentration curve.

Adenylate Cyclase Activity. The opiate-sensitive adenylate cyclase activity of homogenates of neuroblastoma x glioma NG108-15 hybrid cells was measured as described by Sharma et al. (1975).

Mouse Vas Deferens Assay. Opioid inhibition of the electrically stimulated contractions of the mouse vas deferens was performed as described by Henderson et al. (1972). Electrical stimulation was 40 V at 0.1 Hz for 0.6 ms. Naloxone, 10^{-7} M, was used to reverse the inhibition of contractions.

Hydrolysis of the α-Casein Exophins by Proteases. The hydrolysis of the peptides by proteolytic enzymes was followed by HPLC on a Varian MCH-10 column and with a gradient of potassium phosphate buffer, 10 mM (pH 4.8)/acetonitrile (0–70%). The run was 15 min at 3 mL/min. Under these conditions the peptides are eluted at 40, 45, and 52% acetonitrile for the sequences 91–96, 90–96, and 90–95, respectively. The incubation mixtures contained 1–2 mM peptide in 50 mM Tris-HCl, pH 7.8, and 50 μg/mL enzyme. The rat brain membrane (fraction P_2) contained 0.5 mg of protein/mL. Samples, 50 μL, were withdrawn at different times of incubation at 27 °C, and the hydrolysis was followed by measuring the disappearance of the peptide peak monitored at 275 nm.

NMR Measurements. NMR spectra were recorded on a Varian XL-100 Fourier transform instrument connected with a Varian 620/L-100 computer. Spectra were routinely recorded at 30 °C. The proton spin–lattice relaxation times (T_1) were measured by using the inversion recovery method 180°–τ–90° pulse sequence (Freeman & Hill, 1971) or the 90°–τ–90° pulse sequence (McDonald & Leigh, 1973). A linear regression analysis was used for the estimation of T_1's with a correlation coefficient ≥0.980.

Results

In our earlier studies we showed that exorphins, peptides with opioid activity, are present in pepsin digests of several proteins including wheat gluten and α-casein (Klee et al., 1978; Zioudrou et al., 1979; Zioudrou & Klee, 1979). Although α-casein exorphin was appreciably less potent than that derived from wheat gluten, its purification to essential homogeneity was facilitated by the larger amounts present. The α-casein exorphins were purified by several chromatographic procedures described previously. Final purification was achieved by HPLC chromatography on a C_{18} column and a gradient of water/acetonitrile (0–70%). The fraction with opioid activity which was eluted between 45 and 50% of the gradient was further purified by TLC chromatography on silica gel plates (solvent D) and showed R_f 0.75. This active fraction when

Table I: Amino Acid Analysis of an Opioid Peptide Isolated from Pepsin Hydrolysates of α-Casein[a]

amino acid	molar ratio	theoretical α-casein peptide 90–95[b]	90–96[c]
Lys	0.07		
His	0.04		
Arg	*1.11*	1	1
Asp	0.16		
Thr	0.07		
Ser	0.19		
Glu	*0.52*		1
Pro	0		
Gly	*1.00*	1	1
Ala	0.10		
Val	0.10		
Met	0		
Ileu	0.19		
Leu	*1.76*	2	2
Tyr	*1.94*	2	2
Phe	0.15		

[a] A total of 10–15 μg of peptides was subjected to hydrolysis. [b] Sequence 90–95: Arg-Tyr-Leu-Gly-Tyr-Leu. [c] Sequence 90–96: Arg-Tyr-Leu-Gly-Tyr-Leu-Glu.

subjected to N-terminal group analysis as described by Hartley (1970) showed only amino-terminal arginine.

Amino acid analysis of the puurified exorphin gave results as shown in Table I. When the known amino acid sequence of α-casein is examined for regions containing these amino acids (Table I), it is clear that the composition found is only compatible with peptides of sequence originating at Arg residue 90 and extending to Leu-95 or Glu-96 (Mercier et al., 1970; Dayhoff, 1972). The presence of a nonintegral number of Glu residues suggests that α-casein exorphin is a mixture of approximately equal amounts of α-casein 90–95 and α-casein 90–96. In order to confirm this assignment we synthesized these peptides as well as several related ones.

The synthetic α-casein peptides Arg-Tyr-Leu-Gly-Tyr-Leu-Glu (90–96) and Arg-Tyr-Leu-Glu-Tyr-Leu (90–95) have identical chromatographic properties as the isolated α-casein exophine; i.e., in solvent D they show R_f values of 0.74 and 0.75, respectively, and migrate on HPLC columns similarly to that of the natural product.

Both α-casein 90–95 and α-casein 90–96 have opioid activity as measured by several standard assay procedures. Thus, α-casein 90–96 competes with [^{3}H]DALAMID for binding to rat brain opiate receptors with better than micromolar affinity (Figure 1A), whereas α-casein 90–95 exhibits a somewhat weaker but still substantial affinity (Figure 1B). Interestingly, α-casein 91–96, with the amino-terminal Tyr characteristic of all endogenous opioid peptides has only about one-tenth of the opiate receptor affinity of α-casein 90–96 with its amino-terminal Arg residue. Also of interest is the observation that α-casein 90–96 displaces only about half the amount of [^{3}H]DALAMID displaceable by α-casein 90–95 or by morphine at saturating concentrations (Figure 1). Similar results were obtained for the displacement of [^{3}H]-DHM from the receptors. Again α-casein 90–95 displaces 54% of the [^{3}H]DHM whereas α-casein 90–96 displaces 25% (data not shown).

The synthetic casein exorphins also exhibit opioid activity in several bioassays. These include inhibition of adenylate cyclase in neuroblastoma x glioma hybrid cell membranes (Figure 2) and inhibition of electrically stimulated contractions of the mouse vas deferens (Figure 3). Importantly, in both cases the inhibition is blocked or reversed by the opiate antagonist, naloxone.

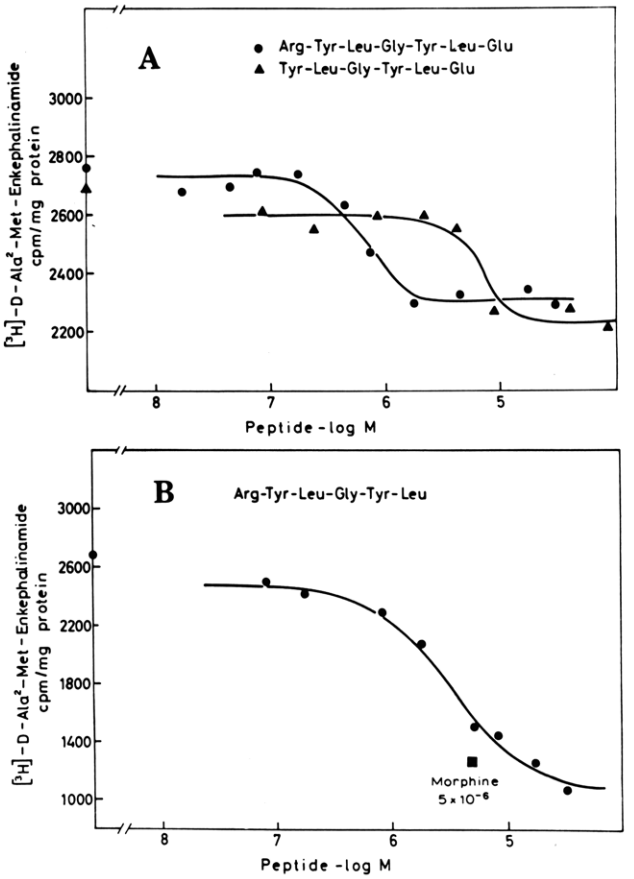

FIGURE 1: Displacement of [^{3}H]DALAMID from rat brain membranes by α-casein exorphins. Assays were performed as described under Materials and Methods. Each point is the mean of duplicate determinations from three different experiments.

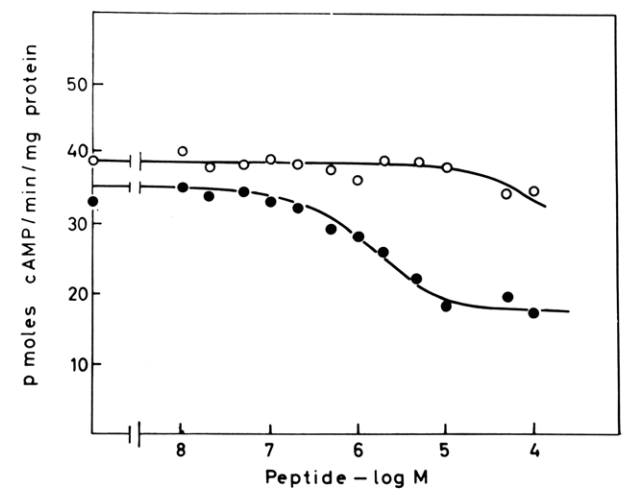

FIGURE 2: Inhibition of adenylate cyclase of NG108-15 hybrid cell homogenates by α-casein 90–95 in the presence (open circles) and absence (filled circles) of 0.1 μM naloxone. Each point is the mean of duplicate experiments.

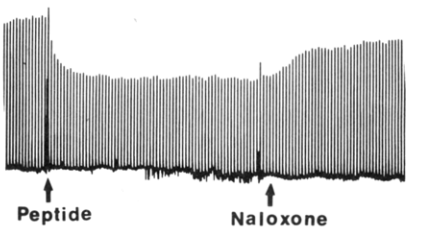

FIGURE 3: Inhibition of the contractions of electrically stimulated mouse vas deferens by α-casein 90–96 (20 μM) and its reversal by naloxone, 0.2 μM.

Table II: Opioid Activities of α-Casein Exorphins

opiate assay	IC_{50} (μM) isolated α-casein exorphin[a]	synthetic α-casein peptides[b] 90–96	90–95	91–96	91–95
displacement of [^{3}H]DALAMID from rat brain membranes		0.7[c]	3.6[c]	5.2[c]	16.0[c]
displacement of [^{3}H]DHM from rat brain membranes	3.8	1.2	12.0	45.0	
inhibition of adenylate cyclase in homogenates of NG108-15 cells	0.3	0.8	2.0	4.0	
inhibition of electrically stimulated mouse vas deferens	11.0	30.0	70.0	>100.0	

[a] Opioid peptides from pepsin hydrolysates of α-casein. The IC_{50} values (μM) were calculated from the peptide concentration as determined from the amino acid analysis or from the absorption measurements at 210 nm (Zioudrou et al., 1979). [b] Sequence 90–96: Arg-Tyr-Leu-Gly-Tyr-Leu-Glu. [c] The apparent dissociation constants (K_D) of the peptides 90–96, 90–95, 91–96, and 91–95 are 0.16, 0.80, 1.15, and 3.60 μM. They were calculated by the method of Cheng & Prusoff (1973) from the corresponding IC_{50} values and the K_D value 0.87 nM for the binding of D-Ala^2-L-Met^5-enkephalinamide to rat brain membranes obtained by Scatchard analysis.

Table III: Amide Proton Chemical Shifts and Temperature Dependencies of α-Casein Opioid Peptide Sequences[a]

amino acid	90–96 δ[b]	90–96 $\Delta\delta/\Delta T$[c]	90–95 δ	90–95 $\Delta\delta/\delta T$	91–96 δ	91–96 $\Delta\delta/\Delta T$
Tyr	8.85	2.8	8.61	5.0		
Leu	8.28	4.0	8.20	5.8	8.64	3.3
Gly	7.90	3.9	7.93	4.2	8.12	5
Tyr	8.12	4.7	7.97	6.7	8.12	5
Leu	8.61	<0.6	8.33	6.5	7.93	3.65
Glu	7.95	4.0			8.12	5

[a] Sequence 90–96: Arg-Tyr-Leu-Gly-Tyr-Leu-Glu; concentration of peptide/trifluoroacetates 45–50 mM in Me_2SO-d_6. [b] δ from Me_4Si. [c] $\Delta\delta/\Delta T \times 10^{-3}$ per degree.

The potencies of the synthetic α-casein exorphins and those of the exorphin isolated from pepsin digests of α-casein in several opiate assays are summarized in Table II. The natural exorphin exhibits a range of potencies in the several assays which are very similar to those of α-casein 90–96. Because the assays of the various peptides were performed at different times with different tissue preparations, the agreement seen in activity is not perfect. Furthermore, the natural material is apparantly a mixture of α-casein 90–96 and 90–95.

We have previously reported (Zioudrou et al., 1979) that the isolated α-casein exorphin is resistant to trypsin. Incubation of the peptide with trypsin for 6 h at 25 °C at pH 7.5 did not affect the activity of the peptide in inhibiting the adenylate cyclase of neuroblastoma x glioma hybrid cell membranes. Chymotrypsin and subtilisin under the same conditions inactivated α-casein exorphin by 20% and 25%, respectively. However, treatment with Pronase abolished the activity. The synthetic α-casein peptides 90–96, 90–95, and 91–96 are also relatively resistant to most proteolytic enzymes. The peptides are totally resistant to the action of trypsin and to the proteolytic enzymes present in rat brain membranes (fraction P_2) after 3-h incubation at 27 °C but are degraded by α-chymotrypsin and subtilisin with half-lives of 30 and 60 min, respectively. Under the same conditions the peptides are degraded totally by Pronase.

In an effort to relate the opioid activities of the synthetic α-casein exorphins to their conformations, we examined their NMR spectra. To minimize intermolecular interactions, we used solutions of the peptides (45–50 mM) in their cationic form as trifluoroacetates in Me_2SO-d_6. Chemical shift assignments, in ppm from trimethylsilane, were made by selective irradiation (Figures 4 and 5). The assignments of the aromatic protons of the tyrosines-2 and -5 in α-casein 90–96 and 90–95 and of the tyrosines-1 and -5 in α-casein 91–96 were made from the corresponding peptides where tyrosines-4 or -5 were substituted with 3,5-dideuterio-L-tyrosine. The chemical shift/temperature coefficients ($\Delta\delta/\Delta T$) of the amide protons are shown in Table III, and the spin–lattice relaxation times (T_1) of the aromatic protons of the tyrosines are listed in Table IV.

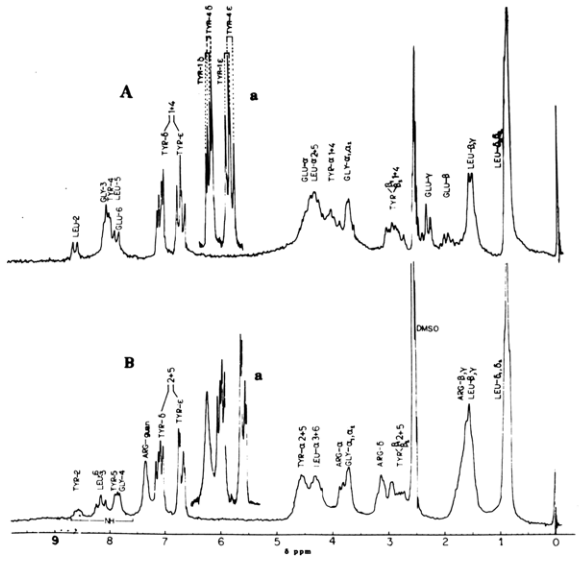

FIGURE 4: Proton NMR spectra of α-casein peptides. (A) Tyr-Leu-Gly-Tyr-Leu-Glu (91–96); (insert a) enlarged aromatic region. The assignments of δ and ε protons of tyrosines-1 and -4 were done by analyzing the proton NMR spectrum of the peptide Tyr-Leu-Gly-(3,5-dideuterio)Tyr-Leu-Glu. (B) Arg-Tyr-Leu-Gly-Tyr-Leu (90–95); (insert a) enlarged aromatic region.

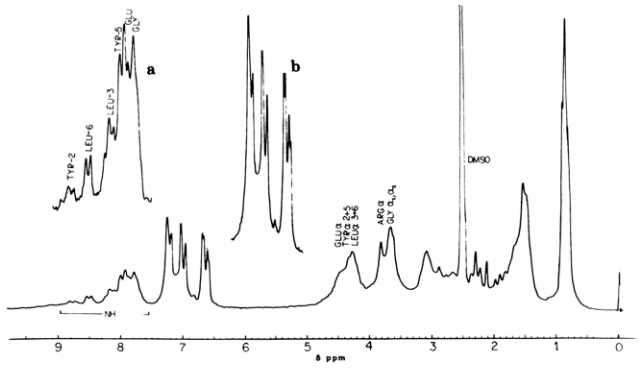

FIGURE 5: Proton NMR spectrum of α-casein peptide sequence 90–96 (Arg-Tyr-Leu-Gly-Tyr-Leu-Glu). (Insert a) Enlarged amide proton region; (insert b) enlarged aromatic region.

Table IV: Spin-Lattice Relaxation Times (T_1) of the Aromatic Protons of α-Casein Peptide Sequences at 30 °C[a]

	90-95		90-96		91-96	
	δ[b]	T_1[c]	δ	T_1	δ	T_1
Tyr-A						
δ	7.13	0.359	7.05	0.240	7.11	0.210
δ	7.05	0.324	6.96	0.217	7.03	0.210
ε	6.72	0.550	6.71	0.393	6.75	0.300
ε	6.64	0.534	6.62	0.390	6.67	0.323
Tyr-B						
δ	7.10	0.320	7.05	0.240	7.08	0.195
δ	7.02	0.264	6.96	0.217	7.00	0.295
ε	6.70	0.500	6.67		6.68	0.321
ε	6.62	0.476	6.57		6.60	0.407
leucine methyl protons	0.87	0.190	0.86	0.160	0.86	0.183

[a] Sequence 90-96: Arg-Tyr-Leu-Gly-Tyr-Leu-Glu. [b] δ from Me_4Si. [c] The T_1's were measured by the 90°-τ-90° pulse sequence method (McDonald & Leigh, 1973) and calculated by using a linear regression analysis with a correlation coefficient ≥0.98.

The amide protons of Tyr-2, Leu-5, and Tyr-6 of α-casein 90–96 show a smaller chemical shift variation with temperature than do the corresponding protons of α-casein 90–95. Smaller temperature dependencies of amide protons could be due to hydrogen bonding (Stern et al., 1968) or shielding from solvent (Lewis et al., 1973). Furthermore, the aromatic protons of the two tyrosines of α-casein 90–95 are well resolved whereas those of 90–96 are superimposed, and the relaxation times of these protons are larger in α-casein 90–95 than in 90–96. Each of these findings suggests that the aromatic side chains have appreciably more conformational freedom in the hexapeptide 90–95.

Discussion

The opioid activities of the synthetic α-casein peptides summarized in Table II show that Arg-Tyr-Leu-Gly-Tyr-Leu-Glu (α-casein 90–96) is more potent than the other related analogues and almost equipotent with the exorphin isolated from pepsin hydrolysates of α-casein. The synthetic α-caseins 91–96 and 91–95, both with an amino-terminal tyrosine, are relatively inactive. Thus, in this particular group of opioid peptides, extension at the amino-terminal end by the addition of an arginine residue increases potency by a factor of 5–35, depending upon the assay used. It has been reported that an amino-terminal extension of Met-enkephalin (Tyr-Gly-Gly-Phe-Met) by an arginine residue (Chang et al., 1976; Day et al., 1976; Law et al., 1977; Ling et al., 1978; Dutta et al., 1977) to yield the β-lipotropin sequence 60–65 or by a Lys-Arg dipeptide to yield the β-lipotropin sequence 59–65 (Ling et al., 1978) reduces opiate activity slightly. In fact, β-lipotropin 60–65 is almost equipotent with Met-enkephalin binding to rat brain membranes and in the guinea pig ileum bioassay, but it has 80 times less activity than Met-enkephalin in inhibiting the adenylate cyclase of homogenates of NG108-15 cell membranes (Zioudrou & Klee, 1979). Thus, addition of an amino-terminal arginine residue can increase or decrease opioid activity depending upon the subsequent sequence.

At pH 7.5, where most biological assays are performed, α-casein 90–96 bears no net charge, whereas α-casein 90–95 has a single net positive charge which could facilitate its interaction with a postulated anionic site on the receptor (Beckett & Casy, 1965). On the other hand, the net negative charge of α-casein 91–96 might reduce its affinity for the receptor. An uncharged analogue, α-casein (91–96) in which the carboxyl-terminal glutamic acid was substituted by glutamine (Tyr-Leu-Gly-Tyr-Leu-Gln), inhibits the electrically stimulated vas deferens with an IC_{50} of 35 μM as compared to over 100 μM for α-casein (91–96) (C. Zioudrou, unpublished results). Thus, the negative charge may account for most of the decreased potency of the Tyr amino-terminal exorphin when compared to their Arg-extended analogues.

The α-casein peptides studied here differ both in their potencies and in the extents to which they displace [^{3}H]DALAMID and [^{3}H]DHM from receptors. Although α-casein 90–95 is not the most potent of the peptides tested in this study, it can displace approximately twice as many molecules of [^{3}H]DALAMID and [^{3}H]DHM from brain membranes as can the other α-casein exorphins (Figure 1). These results may be explained by the greater conformational flexibility of α-casein 90–95 suggested by the NMR studies. DALAMID is believed to interact equally well with both μ and δ opiate receptors (Kosterlitz et al., 1980), and it is conceivable that α-casein 90–96, because of conformational restrictions, can only displace DALAMID from one class of receptor whereas α-casein 90–95 is able to interact with both classes of receptor. Further work will be needed to establish the receptor type specificity of the α-casein exorphins. In addition, a study of several proton and carbon-13 resonance parameters of these peptides at physiological pH values, currently under way, may give more information on their structure–activity relationships.

The opioid activities of isolated and synthetic α-casein exorphins were measured with cell homogenates or isolated organ preparations. In order for such peptides to exert biological effects in vivo and in particular in the brain, they must be produced in the gastrointestinal tract, resist hydrolysis by peptidases and lysosomal enzymes, and cross the blood–brain barrier. The results show that the α-casein exorphins, which are produced by pepsin digestion, are relatively resistant to the action of other proteases. In addition, as shown by their amino acid composition, and their chromatographic and solubility properties, the peptides are highly hydrophobic and could easily cross the blood–brain barrier as it has been reported for metabolically stable analogues of enkephalin (Rapoport et al., 1979; Pardridge et al., 1981). Preparation of radioactively labeled casein exorphins will allow testing of the physiological significance of such peptides.

Registry No. Arg-Tyr-Leu-Gly-Tyr-Leu-Glu, 83471-49-2; Arg-Tyr-Leu-Gly-Tyr-Leu, 83471-50-5; Tyr-Leu-Gly-Tyr-Leu-Glu, 83471-51-6; Tyr-Leu-Gly-Tyr-Leu, 86374-13-2; *N*-*t*-Boc-Leu-Glu dibenzyl ester, 71667-87-3; *t*-Boc-leucine, 13139-15-6; dibenzyl L-glutamate toluenesulfonate, 2791-84-6; *N*-*t*-Boc-(OBzl)Tyr-Leu-Glu dibenzyl ester, 86374-14-3; *t*-Boc-(OBzl)tyrosine, 2130-96-3; Leu-Glu dibenzyl ester, 86374-21-2; *N*-*t*-Boc-Leu-Gly, 32991-17-6; *t*-Boc-Leu hydroxysuccinimide ester, 3392-09-4; glycine, 56-40-6; *N*-*t*-Boc-Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester, 86374-15-4; (OBzl)-Tyr-Leu-Glu dibenzyl ester, 86374-16-5; *N*-*t*-Boc-(OBzl)Tyr-Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester, 86374-18-7; *N*-*t*-Boc-(OBzl)Tyr-OSu, 27601-29-2; Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester, 86374-17-6; N^{α},N^{δ},N^{ω}-Z_3-Arg-(OBzl)Tyr-Leu-Gly-(OBzl)-Tyr-Leu-Glu dibenzyl ester, 86374-20-1; (OBzl)Tyr-Leu-Gly-(OBzl)Tyr-Leu-Glu dibenzyl ester, 86374-19-8; N^{α},N^{δ},N^{ω}-Z_3-arginine *p*-nitrophenyl ester, 14611-35-9; trypsin, 9002-07-7; chymotrypsin, 9004-07-3; subtilisin, 9014-01-1; Pronase, 9036-06-0.

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