Opioid Peptides Derived from Food Proteins

THE EXORPHINS*

Christine Zioudrou,‡ Richard A. Streaty, and Werner A. Klee

From the Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, Maryland 20014

Peptides with opioid activity are found in pepsin hydrolysates of wheat gluten and α-casein. The opioid activity of these peptides was demonstrated by use of the following bioassays: 1) naloxone-reversible inhibition of adenylate cyclase in homogenates of neuroblastoma X-glioma hybrid cells; 2) naloxone-reversible inhibition of electrically stimulated contractions of the mouse vas deferens; 3) displacement of [^{3}H]dihydromorphine and [^{3}H-Tyr, DAla^2]met-enkephalin amide from rat brain membranes. Substances which stimulate adenylate cyclase and increase the contractions of the mouse vas deferens but do not bind to opiate receptors are also isolated from gluten hydrolysates. It is suggested that peptides derived from some food proteins may be of physiological importance.

Peptides with activity similar to that of morphine and other opioids have been isolated from the brain (1–5) and other sources such as the pituitary (6). These peptides, the endorphins and enkephalins, are synthesized in vivo and may function both as hormones and neurotransmitters.

An alternate source of peptides, some of which may have biological activities, is dietary protein. Because of reports linking wheat gluten (7–9) with mental disorders, we tested pepsin digests of wheat gluten for opioid activity. The present report describes the isolation of some purified peptides with opioid activity from pepsin digests of wheat gluten and α-casein. These peptides are called exorphins (10) because of their exogenous origin and morphine-like activity. Also present in pepsin digests of wheat gluten are stimulatory materials which exhibit activities opposed to those of the exorphins.

MATERIALS AND METHODS

Opiate Assays

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. (11). In routine assays, samples were tested in the presence and absence of the specific morphine antagonist (−)-naloxone (a gift of Endo Laboratories) at 10^{-4} M. Inhibitory activity which is reversed by naloxone is considered to be opiate-like. Naloxone alone has no effect upon the activity of the enzyme. Maximal inhibition of adenylate cyclase by morphine and other opioids varies from 30 to 50% depending upon the enzyme preparation.

Mouse Vas Deferens—Opioid inhibition of the electrically stimu-

lated contraction of the mouse vas deferens was performed as described by Henderson et al. (12). Tests were performed in a 5-ml organ bath filled with Ca^{2+}-free Ringers solution at 37°C. Electrical stimulation was 90 V at 0.1 Hz for 1 ms. Isotonic contraction size was recorded on a Brush recorder through a Statham transducer. (−)-Naloxone (200 nM) reversed opioid inhibition of contractions. (+)-Naloxone (200 nM) had no effect.

Gluten stimulatory fraction was also assayed for its effect on adenylate cyclase activity and the contractions of the mouse vas deferens as described above.

Binding to Opiate Receptors—Binding to opiate receptors in rat brain homogenates was measured at 37°C by competition with [^{3}H]dihydromorphine or [^{3}H-Tyr, DAla^2]met-enkephalin amide (both from New England Nuclear Corp.) as described previously (13).

Determination of Peptide Concentrations

Peptide concentrations were determined from absorption measurements at 210 nm using the value 33 as the extinction coefficient for a 0.1% solution or from amino acid analysis. The extinction coefficient was determined from the spectra of a number of peptides containing from 3 to 20 amino acid residues.

Pepsin twice crystallized and gluten (Lot 7620) were from ICN and α-casein was from Sigma. Gliadin, zein, avenin, secalin, and hordein were a gift from Dr. J. S. Wall, United States Department of Agriculture, Northern Regional Research Laboratory, Peoria, Ill. XAD-2 (Applied Science Labs) was washed exhaustively with iospropyl alcohol and then water prior to use.

Peptide Purification

Gluten (100 g) suspended in 2 liters of 0.1 N HCl was treated with pepsin, 2.5 g, with vigorous stirring for 1 h at 37°C. The pH of the hydrolysate was adjusted to 7.5 with 10 N NaOH, the suspension was stirred with 100 g of XAD-2 polystyrene beads for 30 min and filtered, and the resin was washed with 10 liters of water. The materials adsorbed on the resin were eluted with 5 liters of 90% 2-propanol, and the eluate was taken to dryness. The residue was dissolved in water and lyophilized to yield 3.7 g of powder. This material (3.2 g in 50 ml H$_2$O) was applied to an AG50W-X 2 column (H$^+$ form, 2.5 × 21 cm in water) which was then washed with 200 ml H$_2$O and eluted with a linear gradient from 0 to 4 M pyridine/acetate, pH 6.3 (1200 ml). Early fractions, eluting between 0 and 260 ml, were found to stimulate adenylate cyclase and were pooled and lyophilized to yield 7 mg (by A_{210nm}) of material which is the "gluten stimulatory fraction." Fractions eluting between 360 and 660 ml had opioid activity and were lyophilized to yield 0.4 g of material which was further purified in two batches, by preparative thin layer chromatography on 2-mm-thick silica gel plates (Merck) using 1-butanol, methanol, 20% NH$_3$ (4/1/1) as solvent. The silica gel was divided into 10 fractions which were extracted by shaking overnight with 90% methanol. After centrifugation and filtration, the extracts were taken to dryness and assayed. Opioid activity was found in all fractions, but was concentrated in those migrating with an R_F between 0.15 and 0.26, and these were pooled to yield 15 mg of material of which 10 mg, in 1 ml H$_2$O, was applied to a μBondapak C$_{18}$ reversed phase column (0.4 × 30 cm) (Waters Associates) which was eluted with a linear gradient between 0 to 70% acetonitrile at 2.5 ml/min over 15 min. Fractions (2.5 ml) were dried under vacuum, dissolved in H$_2$O, and assayed. The highest specific activity fraction (14) is approximately 10,000 times more

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‡ Visiting Scientist from the Nuclear Research Center "Demokritos," Aghia Paraskevi, Attikis, Athens, Greece.

active than was the unfractionated pepsin digest and contained 28 μg of peptide. This was applied to a silica gel thin layer plate which was developed with isopropyl alcohol, 0.05 M sodium pyrophosphate, pH 7.3, in 0.1 M NaCl (70/30) and the fractions eluted as before. The active fraction (R_F 0.83) was dissolved in 0.5 ml of H_2O and studied as "gluten exorphin."

Similar procedures were used for purifying opioid peptides from pepsin hydrolysates of 10 g of α-casein. After adsorption and elution from XAD-2 resin, the peptides (400 mg) were applied to a DEAE-Sephadex A-25 column (2.5 × 24 cm) which had previously been washed with 2 M NH_4HCO_3 followed by water. The column was eluted with a linear gradient (400 ml) between 0 and 0.5 M NH_4HCO_3. Active fractions, eluting between 200 and 400 ml, were pooled and lyophilized to yield 124 mg of peptides. This material was subjected to preparative thin layer chromatography as described above, and the most active fraction (R_F 0.25, 13 mg) was subjected to reversed phase liquid chromatography, also as described above. Fraction 9 (3 mg) was further purified by thin layer chromatography using the isopropyl alcohol/pyrophosphate solvent, and the most active fraction (R_F 0.75) was dissolved in water and studied as "casein exorphin."

RESULTS

The abilities of varying concentrations of gluten and casein exorphins to inhibit the opiate-sensitive adenylate cyclase of neuroblastoma X-glioma, NG108-15, hybrid cell homogenates (11) are shown in Fig. 1. The exorphins inhibit the enzyme to the same extent as does a saturating amount (2 × 10⁻⁵ M) of morphine, albeit with different potencies. Endogenously derived opioid peptides, such as the enkephalins, exhibit similar behaviors in this assay (14). The inhibitory action of the exorphins in the adenylate cyclase assay is a specific opiate effect since it is blocked by the antagonist, naloxone (Table I). Treatment of gluten exorphin with pronase results in the loss of 70% of its activity, and casein exorphin is completely inactivated by pronase (Table I). Thus, both of these materials are peptides. In similar experiments, other proteinases led to either no inactivation of the exorphins (trypsin) or only partial inactivation (subtilisin, thermolysin, and chymotrypsin).

The exorphins are also able to compete with [³H]dihydromorphine (Fig. 2) and [³H-Tyr, DAla²]met-enkephalin amide (data not shown) for binding to rat brain opiate receptors. Here too, the potency of gluten exorphin is much greater than that of casein exorphin.

The exorphins also behave as opiates when tested for their ability to inhibit electrically stimulated contractions of the mouse vas deferens (Fig. 3). *Part A* of the figure shows the inhibitions of contraction size elicited by three different concentrations of gluten exorphin. In the experiments shown, the inhibitions are 61, 73, and 86% at gluten exorphin concentrations of 3, 6, and 15 ng/ml, respectively. The inhibition is reversed by the (−)-isomer of naloxone, but as is also shown in the figure, is not affected by the enantiomeric (+)-isomer (15). *Part B* of the figure shows that casein exorphin also inhibits contractions of the mouse vas deferens, and that (−)-naloxone, but not (+)-naloxone, reverses the inhibition. Casein exorphin is much less potent than is gluten exorphin in this assay.

A comparison of the opioid properties of the exorphins with those of met-enkephalin and morphine is shown in Table II. Remarkably, the potency of gluten exorphin in each of the assays is comparable to that of met-enkephalin. Casein exorphin, on the other hand, is much less potent in all assay systems used. The relative opioid potencies of gluten and casein exorphins vary with the assay. Thus, gluten exorphin is least active in the adenylate cyclase assay where casein exorphin is most active. Amino acid analysis of gluten and casein exorphins at their present stage of purification showed that both are mixtures of a major and minor peptidic component. Further purification is in progress. Because of these
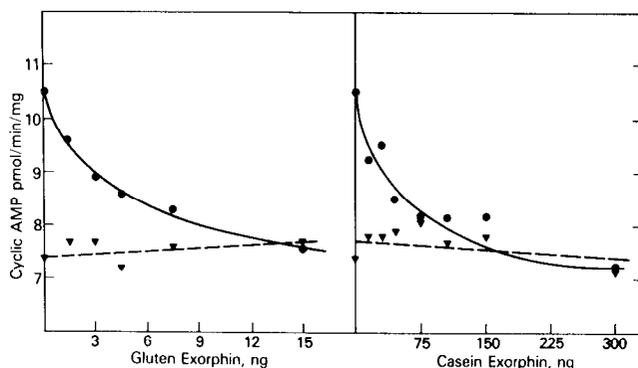

FIG. 1. Effect of varying concentrations of exorphins on the activity of NG108-15 adenylate cyclase in the presence (▲) and absence (●) of 2 × 10⁻⁵ M morphine.

TABLE I

Effects of exorphins on adenylate cyclase activity in the presence and absence of naloxone before and after pronase treatment

Pronase hydrolysis was carried out at pH 7.5 for 6 h at 37°C with gluten exorphin at 10 μg/ml and pronase at 20 μg/ml, or with casein exorphin at 75 μg/ml and pronase at 10 μg/ml. Pronase was inactivated by boiling and 1- and 2-μl aliquots of gluten exorphin and casein exorphin, respectively, were assayed. The data are mean values of duplicate determinations which differ from the mean by 5% or less. The experiment has been replicated four times for gluten exorphin and two times for casein exorphin.

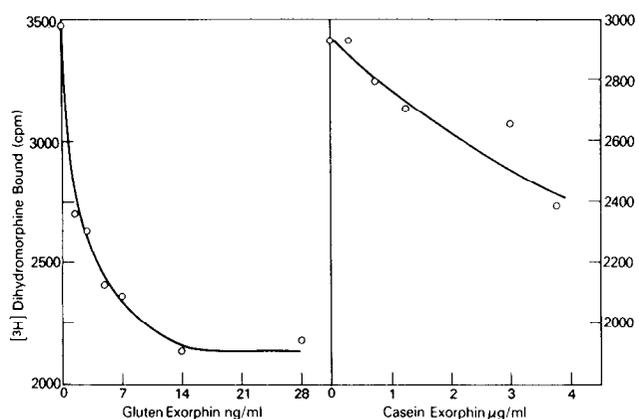| Additions	Adenylate cyclase activity			
	Untreated		Pronase-treated	
		Naloxone		Naloxone
	pmol cyclic AMP/min/mg			
None	16.5	16.0	17.0	18.1
Gluten exorphin	12.5	16.5	15.2	16.4
Casein exorphin	14.5	18.5	17.8	17.7

FIG. 2. Displacement of [³H]dihydromorphine from rat brain membranes by gluten and casein exorphins.

uncertainties as to the purity and molecular weight, we expressed the activities of the exorphins on a weight rather than on a molar basis.

In the course of our studies on exorphins, we found that pepsin hydrolysates of gluten also contain a substance which stimulates both NG108-15 adenylate cyclase activity and electrically induced contractions of the mouse vas deferens. A partially purified gluten stimulatory fraction, GSF[1] (25 μg/ml), stimulates the contractions of the mouse vas deferens by 150%, as shown in Fig. 3C. Also seen in the figure, after adjustment of the recorder sensitivity to keep the contractions

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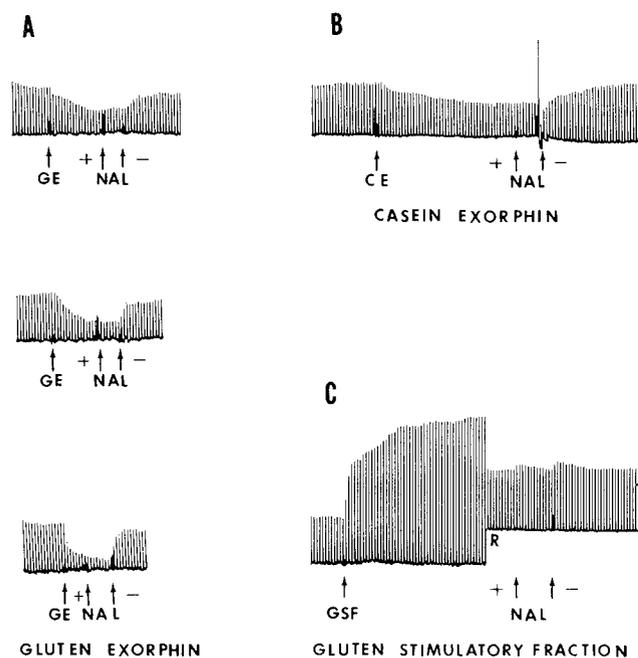[1] The abbreviation used is: GSF, gluten stimulatory fraction.

FIG. 3. The effects of exorphins and gluten stimulatory fraction (*GSF*) on the electrically stimulated mouse vas deferens. At the *arrows*, the following additions were made. *A*: *GE*, gluten exorphin (3, 6, 15 ng/ml) from *top* to *bottom*; *B*: *CE*, casein exorphin (7 μg/ml); and *C*: *GSF*, gluten stimulatory fraction (26 μg/ml). *R* indicates that the recorder scale has been reduced by a factor of 2.5. In each case, + *NAL* indicates 200 nM (+)-naloxone and − *NAL* indicates 200 nM (−)-naloxone.

TABLE II
Concentrations of exorphins (nanograms/ml) for half-maximal inhibitions in several opiate assays

	Brain receptor binding	Adenylate cyclase	Vas deferens
Gluten exorphin	2	30	2
Casein exorphin	3500	320	8000
Met-enkephalin	20[a]	7	7[b]
Morphine sulfate	1	570	190[b]

[a] Determined at 25°C.
[b] Calculated from the data of Lord *et al.* (16).

on scale, is the fact that neither isomer of naloxone affects the activity of GSF. The adenylate cyclase activity of NG108-15 cell homogenates is also stimulated by GSF, to the extent of 50% at the optimal concentration of 300 μg/ml.[2]

Pepsin hydrolysates of gluten are very complex mixtures that contain a number of different fragments with stimulatory or with opiate activities. Similarly, pepsin hydrolysates of casein contain many opioid peptides. Our work to date has concentrated upon one representative of each class of substance. Other exorphins and stimulatory materials behave in qualitatively similar ways but may differ significantly in potencies and structure.

We have also tested pepsin hydrolysates of a number of other proteins for their effects upon NG108-15 adenylate cyclase activity. Based upon the results, summarized in Table III, we can divide these proteins into five groups, as indicated. Grain proteins generally are found to show stimulatory activity, which is sometimes mixed with naloxone-reversible, inhibitory activity. Although the gliadin listed here, derived from

[2] *Note Added in Proof*—*The activities of this fraction have recently been shown to be due to adenosine (Zioudrou, C., Londos, C., and Klee, W. A., unpublished data).*

TABLE III
Effects of pepsin hydrolysates of proteins on the adenylate cyclase activity of NG108-15 hybrid cell homogenates

Pepsin hydrolysis was performed in 0.1 N HCl at 37°C with 50 mg/ml of protein and 1 mg/ml of pepsin for 1 h; the hydrolysates were neutralized with NaOH and 20-μl aliquots were assayed.

Protein	Adenylate cyclase		
	Control	Naloxone	Type of effect
	pmol cyclic AMP/min/ mg		
A Gliadin (wheat)	9.4	11.8	Naloxone-reversible inhibition
α-Casein	8.8	15.2	
Bovine serum albumin[a]	8.2	10.5	
B Gluten (wheat)	22.2	23.8	Stimulation and naloxone-reversible inhibition
Zein (amize)	23.5	25.6	
Hordein (barley)	24.1	27.7	
C Avenin (oats)	19.9	19.4	Stimulation
Secalin (rye)	14.9	15.6	
Ribonuclease A[a]	14.4	14.7	
D Soy α-protein	6.3	6.6	Inhibition not reversed by naloxone
Cytochrome[a]	7.5	6.4	
α-Lactalbumin[a]	3.3	4.6	
E Edestin	12.1	12.5	None
Ovalbumin	11.1	12.6	
γ-Globulin (bovine)	12.0	13.1	
Control	11.9	11.2	

[a] Duration of the pepsin hydrolysis was 2 h.

Ponca wheat, did not show stimulatory activity, some gliadins from commercial sources did so (10). The pepsin hydrolysates of proteins in Class D inhibit adenylate cyclase activity but the inhibition is not reversed by naloxone. This suggests the existence of biologically active peptides in these digests which are not opiate in nature. Finally, pepsin digests of proteins in Category E are inert in this assay. The presence of opiate activity in commercial hydrolysates of casein has previously been observed by Wajda *et al.* (17).

DISCUSSION

The results presented here have shown that peptides with morphine-like activities, which we call exorphins, can be isolated from some food proteins after treatment with the stomach proteinase, pepsin. Since the opiate assays which we have used so far are performed with cell homogenates or isolated organ preparations, it seems pertinent to ask whether these findings have physiological significance. Although an unequivocal answer to this question must await the results of whole animal experiments, a number of less direct lines of evidence is presently available. In order for exorphins to function as opioid peptides in the central nervous system *in vivo* they must: (*a*) be produced in the gastrointestinal tract, (*b*) survive degradation by intestinal proteases, (*c*) be absorbed, without degradation, into the bloodstream, (*d*) cross the blood-brain barrier and thereby reach central opiate receptors, (*e*) interact as opiates with these receptors.

The conditions of the digestion of gluten or casein with pepsin used in this work, namely 0.1 M HCl at 37°C for 1 h, are similar to conditions to which such proteins may be subjected in the stomach after a meal containing wheat or

milk products. Thus, in all likelihood, exorphins will be produced normally in the stomach. The exorphins which we have studied are resistant to the intestinal proteinases trypsin and chymotrypsin. Thus, the exorphins may be expected to survive extensive degradation in the intestine. Some peptides are now known to be absorbed, without prior degradation, from the gastrointestinal tract into the bloodstream. Perhaps, most pertinent to the present studies is the work of Hemmings *et al.* (18, 19) which showed that, after feeding of ^{131}I-labeled gliadin to rats, labeled peptides, which retain the ability to react with anti-gliadin antibodies, are found in appreciable amounts both in the blood and the tissues of the animals. Significantly, such peptide material was found in the brain as well. Thus, some peptide fragments of gluten do indeed reach the brain. Direct evidence that the exorphins will do so is not yet available, although some peptides have been shown to cross the blood-brain barrier (20). Finally, we have presented evidence that the exorphins will bind to brain opiate receptors as well as to those of peripheral organs. In summary, exorphins may normally reach opiate receptors in the central nervous system and trigger their function.

We have also found substances in pepsin hydrolysates of wheat gluten which stimulate NG108-15 adenylated cyclase and the mouse vas deferens. These stimulatory materials do not interact directly with opiate receptors but may nevertheless be considered to be functional antagonists of the opiates since their stimulatory actions oppose the inhibitory actions of the exorphins. Stimulation of the adenylate cyclase of intestinal mucosa cells would lead to diarrhea and could therefore play a role in the gluten enteropathy, celiac disease. Wheat gluten has been implicated by Dohan (7, 8) and his colleagues in the etiology of schizophrenia and supporting evidence has been provided by others (9). Our experiments provide a plausible biochemical mechanism for such a role, in the demonstration of the conversion of gluten into peptides with potential central nervous system actions.

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Opioid peptides derived from food proteins. The exorphins.

C Zioudrou, R A Streaty and W A Klee

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