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Isolation and amino acid sequence analysis of a 4,000-dalton dynorphin from porcine pituitary

(opioid/peptide/endorphin/neuropeptide/prohormone)

WALTER FISCHLI*, AVRAM GOLDSTEIN*, MICHAEL W. HUNKAPILLER†, AND LEROY E. HOOD†

*Addiction Research Foundation and Stanford University, Palo Alto, California 94304; and †California Institute of Technology, Pasadena, California 91125

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ABSTRACT **A 4,000-dalton dynorphin was isolated from porcine pituitary. It has 32 amino acids (M_r = 3,986), with the previously described heptadecapeptide (now called dynorphin A) at its amino terminus and a related tridecapeptide, dynorphin B, at its carboxyl terminus. The two peptides are separated by the "processing signal" Lys-Arg.**

Dynorphin was isolated from porcine pituitary (1, 2) and gut (3) and identified as a heptadecapeptide with M_r 2,148. It showed the highest potency of all the known opioid peptides in the guinea pig ileum preparation and a high selectivity for the κ opioid receptor (4, 5). We report here the isolation and characterization of a longer dynorphin-containing peptide from porcine pituitary.

MATERIALS AND METHODS

The starting material for this purification was the same porcine melanotropin concentrate from which the dynorphin heptadecapeptide was isolated previously (1, 2). This powder is a dried oxycellulose adsorbate of an extract of whole porcine pituitaries (generously furnished by J. D. Fisher, Armour, Kankakee, IL). The purification itself was followed by radioimmunoassay using synthetic dynorphin-(1–13) (Peninsula Laboratories, San Carlos, CA) as the standard for measurement of immunoreactive dynorphin (ir-dynorphin); the "Lucia" antiserum, which displays an affinity in the low picomolar range, was used (6).

In the course of isolating the dynorphin heptadecapeptide it had been observed that neutralization of the acetic acid extract from porcine pituitary precipitated 60–70% of ir-dynorphin, which could then be redissolved only partly. Gel permeation analysis of this material on Sephadex G-50 showed that it consisted mainly of ir-dynorphin of higher apparent M_r. For purification of this larger ir-dynorphin, methods and conditions were chosen that did not precipitate any immunoreactive material.

Table 1 shows the scheme of the purification. Details are given in the table legend. ir-Dynorphin from a crude acetic acid extract was adsorbed to CM-Sephadex and eluted with a sodium chloride gradient in acetic acid containing Triton X-100 (step 2a). The immunoreactive material was then extracted with an immunoaffinity resin (step 3a). Antibodies of the same type used in radioimmunoassay were first purified by affinity chromatography on dynorphin-(1–13) coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) and then coupled to Sepharose 4B (2). Adsorption of ir-dynorphin to this resin and subsequent washes with phosphate buffer (pH 6.0) containing 2.5% Triton X-100 could be done without loss of the larger ir-dynorphin, as proved by Sephadex G-50 chromatography. A purification factor of close to 100 and a yield of >50% was achieved at this step.

Concentration and desalting was done on CM-Sephadex (steps 2b and 3b). ir-Dynorphin was adsorbed onto a minimum amount of CM-Sephadex and eluted in a small volume of methanol/0.3 M HCl, 1:1 (vol/vol). Lyophilization, which gave unacceptable losses (2), could thus be avoided during the purification procedure. Purification by HPLC on a C_{18} column (step 4, see Fig. 1) finally yielded five peaks of absorbance and immunoreactivity. A_{228}/A_{254} ratios were 14.3 (peak 1), 20.0 (peak 2), 18.3 (peak 3), 14.8 (peak 4), and 15.9 (peak 5).

RESULTS AND DISCUSSION

The pattern shown in Fig. 1 represents a late (basic) fraction of ir-dynorphin from CM-Sephadex chromatography (step 2a) containing mainly peak 3, whereas earlier fractions contained mostly peak 1 (data not shown). Thus, ir-dynorphin from peak 3 is more basic than that from peak 1 and also is eluted with higher acetonitrile concentrations upon HPLC. The absorbance, at 228 nm, of the total ir-dynorphin from CM-Sephadex was 3 times greater for peak 1 than for peak 3, indicating that peak 1 is the major component in porcine pituitary.

Peak 1 coeluted from HPLC as a single symmetrical absorbance peak when mixed with synthetic heptadecapeptide. Peak 1, peak 2, and synthetic heptadecapeptide showed the same elution pattern on Sephadex G-50 and approximately the same potency (per immunoreactive dynorphin equivalent) in the guinea pig ileum bioassay. Relative potencies, expressed as percents relative to that of the synthetic heptadecapeptide tested on the same strips, were (mean ± SEM): Peak 1, 103 ± 4 (n = 8); peak 2, 66 ± 8 (n = 4). Peak 1 was therefore assumed to be the previously isolated heptadecapeptide and peak 2, a derivatized product (possibly oxidized at tryptophan-14). They were not analyzed further.

On gel permeation analysis, HPLC peak 3 proved to be the larger dynorphin with apparent M_r of approximately 3,000. Automatic Edman degradation at Pasadena (8) gave the sequence shown in Fig. 2, corresponding to actual M_r 3,986. The first 31 amino acids were established without ambiguity; there was some uncertainty in regard to threonine-32 because of the small signal. Cycles 33, 34, and 35 yielded no additional amino acid.

To estimate the carboxyl-terminal amino acids of the peptide in peak 3, it was digested with carboxypeptidase A (see legend to Fig. 1). After 10- and 60-min incubations, only the following amino acids were released (as nmol/nmol of peptide): threonine, 0.22 and 0.50; valine, 0.41 and 1.15. The simultaneous release of threonine and valine was seen even after 2-min incubation. Carboxypeptidase Y digestion (10 ng of enzyme and 200 pmol of peptide in 0.025 M pyridinium acetate at pH 5.5) gave, after 60 and 120 min, the following released amino acids

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Abbreviation: ir-dynorphin, immunoreactive dynorphin.

Table 1. Purification scheme for dynorphin peptides in porcine pituitary

Step	Dynorphin: Amount, nmol	Dynorphin: Recovery, %	Specific activity, nmol/mg protein
1. Extract	198	—	0.038
2a. Eluate from CM-Sephadex chromatography	162	82	0.23
2b. Eluate from CM-Sephadex adsorption	128	79	0.48
3a. Immunoextract	67	52	*
3b. Eluate from CM-Sephadex adsorption	57	85	37
4. Eluate from HPLC	32	56	250

Melanotropin concentrate powder (10 g) was extracted in 1 M acetic acid (180 ml, 90°C, 10 min). The ir-dynorphin was then adsorbed to CM-Sephadex (Pharmacia, C-25, 8.5 g, prewashed with 1 M acetic acid) and eluted by a linear gradient of NaCl (0.066–0.6 M; total volume, 1,540 ml) in 1 M acetic acid/1% Triton X-100. The fractions containing ir-dynorphin were pooled (850 ml) and diluted to 0.025 M NaCl with 1 M acetic acid/1% Triton X-100, adsorbed again on CM-Sephadex (2.5 g), and eluted in 30 ml of methanol/0.3 M HCl, 1:1 (vol/vol). After dilution with 150 ml of 0.25 M sodium phosphate, pH 6.0/2.5% Triton X-100, the ir-dynorphin was adsorbed in batches of 25–30 nmol to the immunoaffinity resin (30 min, 4°C). The resin was then washed six times with 50 ml of incubation buffer (30 min per wash, 4°C) and the ir-dynorphin was eluted with four 10-ml portions of 0.5 M acetic acid (30 min per elution, 37°C). ir-Dynorphin was concentrated again on CM-Sephadex (0.5 g), and the methanol/HCl eluate (5–6 ml) was injected, in batches of 1–2 ml, onto the HPLC column. Amounts of dynorphin were determined by radioimmunoassay (see text); protein was measured by the method of Lowry *et al.* (7). Specific activity of pure dynorphin-32 was calculated on the basis of M_r = 3,986.

* Protein was not determined at this step in order to save material.

(nmol/nmol of peptide): threonine, 0.68 and 0.74; valine, 1.14 and 1.18; lysine, 0.16 and 0.23; phenylalanine, 0.24 and 0.28. The nmol of amino acid released per nmol of peptide was always below the theoretical value because the amount of peptide was measured before lyophilization. The estimated amount thus did not account for the usual losses during lyophilization and reconstitution (due to incomplete dissolution in incubation buffer and adsorption to the incubation vial). The results proved that the peptide is not amidated because the carboxypeptidase A used here has no amidase activity (2). Hydrazinolysis (1–100 hr, 100°C), which would give an estimate of the carboxyl-terminal amino acid, showed no free amino acid. The same negative result was obtained with synthetic dynorphin-32-(20–32), in which the carboxyl-terminal amino acid is threonine. Thus, all the results are consistent with the carboxyl-terminal sequence -Phe^{28}-Lys^{29}-Val^{30}-Val^{31}-Thr^{32}(OH). However, tryptophan and proline would not be detectable in our procedure.

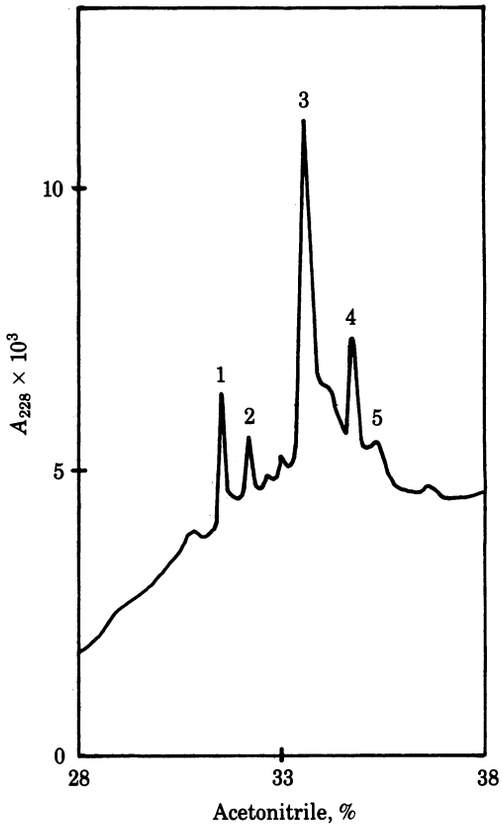

FIG. 1. HPLC of porcine pituitary dynorphins. The figure represents a typical elution pattern of concentrated ir-dynorphin from step 3b. Concentrate (1.5 ml) containing 500 pmol of ir-dynorphin was injected onto a μBondapak C_{18} column (3.9 × 300 mm). Elution was achieved by a linear gradient of acetonitrile (20–50%, 30 min, 1.5 ml/min, 0.6-ml fractions) in 5 mM trifluoroacetic acid. The five peaks emerged at the following acetonitrile concentrations: 31.6% (peak 1), 32.4% (peak 2), 33.6% (peak 3), 34.8% (peak 4), and 35.1% (peak 5). Fractions containing the peaks were acidified with 10 μl of 6 M HCl, pooled, and lyophilized. The residue was taken up in 1.0 ml of methanol/0.1 M HCl, 1:1 (vol/vol) and stored at −70°C.

Immunoreactive peak 4 was eluted from HPLC as a minor component. Sephadex G-50 chromatography showed ir-dynorphin with apparent M_r = 2,500, intermediate between that of the heptadecapeptide and that of dynorphin-32. Sequence analysis gave the first 24 amino acids of dynorphin-32 (actual M_r = 2,970) without ambiguity. Carboxypeptidase A digestion for 60 and 120 min gave the following released amino acids (nmol/nmol of peptide): leucine, 0.53 and 0.54; phenylalanine, 0.60 and 0.58; glycine, 0.02 and 1.44. Hydrazinolysis (9 hr, 100°C) indicated that leucine was the carboxyl-terminal amino acid. We conclude, therefore, that peak 4 is dynorphin-32-(1–24),

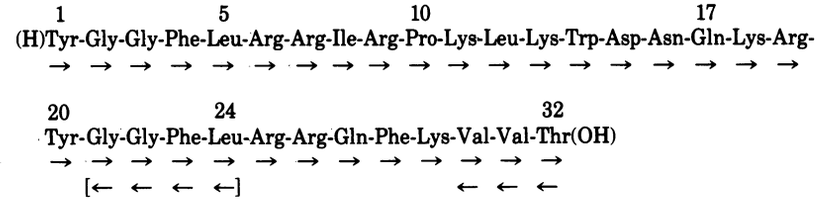

FIG. 2. Sequence of porcine pituitary dynorphin-32. →, Automated Edman degradation on 200–300 pmol of peptide in the gas/liquid/solid phase peptide Sequenator (8) with a sensitivity limit of about 5 pmol of cleaved amino acid. ←, Carboxypeptidase A digestion of peak 3 or [←] peak 4. Peptide (100–200 pmol) was dissolved in 50–100 μl of 0.02 M sodium borate, pH 7.15/0.2 M NaCl/0.1% Triton X-100 and incubated with 100 ng of enzyme at 37°C. Samples (10 μl) were withdrawn, diluted with commercially available sample diluent (30 μl, "Na-S," Dionex), and injected directly into the amino acid analyzer (Dionex, Sunnyvale, CA). Zero-time samples provided correction for free amino acids present initially. The sequence 20–32 is dynorphin B. The original heptadecapeptide 1–17 is now referred to as dynorphin A.

with carboxyl-terminal [Leu]enkephalin, as reported elsewhere (9).

Peak 5 was recovered only in trace amounts. Gel permeation analysis showed that the apparent M_r of this material was 5,700 (calibration with the heptadecapeptide and dynorphin-32). It was set apart for further characterization.

The finding of dynorphin-32 is interesting because it seems to be a precursor of two related peptides. Enzymatic attack at Lys^{18}-Arg^{19}, which proves to be a processing signal in proopiomelanocortin (10), preproglucagon (11), preprocalcitonin (12), and preproenkephalin (13–15), would generate an amino-terminal heptadecapeptide (dynorphin A) and a carboxyl-terminal tridecapeptide (dynorphin B). The placement of Lys-Arg shows that dynorphin A is a naturally processed product that terminates at glutamine-17.

Dynorphin B represents the third "big [Leu]enkephalin" besides dynorphin A and α-neo-endorphin-(1–10) (16). Interestingly, it is related to both of them. It shares the sequence 1–7 with dynorphin A but has lysine [which seems to be important for potency and receptor selectivity (17)] shifted from position 11 in dynorphin A to position 10. The homology with α-neo-endorphin-(1–10) (Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys) is also clear; again the sequence 1–7 is the same, except for a conservative change from lysine-7 in α-neo-endorphin to arginine-7 in dynorphin B; and both these peptides have lysine at position 10.

Dynorphin-32-(1–24) could be another naturally processed product from dynorphin-32. It is similar to preproenkephalin peptide E (18), which contains 25 amino acids and carries [Met]enkephalin at the amino terminus and [Leu]enkephalin at the carboxyl terminus (9).

Dynorphin B was synthesized (Peninsula Laboratories, San Carlos, CA) and all the peptides were compared in the guinea pig ileum preparation (19). The concentration of the dynorphin-32 solution used for the bioassay dilutions was estimated by radioimmunoassay and by amino acid analysis of the acid hydrolysates. According to radioimmunoassay referred to dynorphin-(1–13) as standard, concentration was found to be 2.5 μM. The prominent peaks from amino acid analysis of valine, isoleucine, leucine, and phenylalanine yielded estimates of 2.5 ± 0.2 μM. Therefore, the "Lucia" antiserum is equally reactive with dynorphin-(1–13) (against which it was raised) and dynorphin-32. It can be concluded that the intermediate-size peptide dynorphin-32-(1–24) is also fully crossreactive.

In the bioassay, to minimize variability, potencies relative to dynorphin A were estimated by IC_{50} ratios on the same muscle strips. The IC_{50} value for dynorphin A (geometric mean) was 0.40 nM in 58 different preparations, with 95% confidence interval 0.34–0.47 nM. Potencies expressed as percents relative to dynorphin A were (mean ± SEM; n = number of observations): dynorphin-32-(1–24) (peak 4), 42 ± 2 (n = 15); dynorphin-32 (peak 3), 27 ± 3 (n = 8); dynorphin-32-(20–32) (dynorphin B), 7.8 ± 0.5 (n = 16). The naloxone K_e values for all these peptides were found to be the same, 24–28 nM (data not shown). Because naloxone K_e is a measure of receptor selectivity (20) the results indicate that all the peptides act through the same opioid receptor type in guinea pig ileum myenteric plexus, which contains functional μ and κ but not δ, receptors (21). Selective protection experiments (4, 5) and crosstolerance studies (22) have shown that dynorphin interacts with a single highly specific receptor in guinea pig myenteric plexus and brain as well as in mouse vas deferens. That receptor proved to be identical with the previously known κ opioid receptor. That dynorphin B displays the same high κ opioid selectivity as dynorphin A is surprising. Lysine-11 in dynorphin was shown to be essential not only for high potency but also for receptor selectivity. The outcome here suggests that lysine-10 in dynorphin B can fulfill the same requirement for selectivity as does lysine-11 in dynorphin A. We propose that dynorphin B is a dynorphin-like κ opioid agonist.

Interestingly, α-neo-endorphin-(1–10), which is colocalized with dynorphin A (23) and has important structural similarities to dynorphin B, also shows selectivity for κ opioid receptors (unpublished data). It therefore is likely that α-neo-endorphin-(1–10), dynorphin A, and dynorphin B are components of the same precursor and, thus, that the dynorphin gene would encode a set of κ opioid agonists.

Note Added in Proof. At the International Narcotic Research Conference, North Falmouth, MA, June 14–18, 1982, Udenfriend's group (24) reported the isolation of a tridecapeptide from bovine hypothalamus with sequence identical to that of porcine dynorphin B.

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