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A dynorphin-like opioid in the central nervous system of an amphibian

(endorphin/opioid peptide/neuropeptide/toad/brain)

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ABSTRACT **We have provided evidence for the existence of a biologically active opioid in toad (*Bufo marinus*) brain that is immunoreactive with antiserum raised against dynorphin-(1–13). Compared with porcine dynorphin, this opioid is similar in apparent molecular weight on the basis of gel permeation chromatography and is more hydrophobic on the basis of high-performance liquid chromatography. After purification, its opioid biological activity was demonstrated on the guinea pig ileum myenteric plexus–longitudinal muscle preparation. It was found to be less potent, and to have a similar sensitivity to antagonism by naloxone, in comparison with porcine dynorphin. Because it is immunoreactive with antiserum specific for porcine dynorphin, it probably has considerable sequence homology. Generally, the tissue distribution of immunoreactive dynorphin in the toad is similar to that in the rat, with highest concentrations in the neurointermediate lobe of the pituitary. However, the anterior lobe of the toad pituitary contains considerably lower concentrations than are found in the rat anterior lobe. There appear to be three size classes of immunoreactive dynorphin in toad neural tissue, each with apparent molecular weight below 12,000, similar to the size classes of immunoreactive dynorphin found in pig and rat. However, in toad spinal cord (and possibly in brain) there is immunoreactive dynorphin of greater apparent molecular weight, which has not been reported in mammalian tissue. The contribution of each molecular size to the total immunoreactivity varies from tissue to tissue and is different from that observed in the rat.**

Dynorphin is a potent opioid heptadecapeptide, initially purified from porcine pituitary and found to contain the [Leu]-enkephalin sequence at its amino terminus (1, 2). Using antiserum ("Lucia") raised against dynorphin-(1–13) (D13) and specific for the sequence dynorphin-(3–12) (3), immunoreactive dynorphin (ir-Dyn) has been found in pig, beef, and rat pituitary and rat brain and spinal cord (4–7).

This paper describes the presence of ir-Dyn in toad (*Bufo marinus*) pituitary, brain, and spinal cord. We determined the tissue content and molecular size distribution patterns and reported these results in preliminary form (8). Then, using high-performance liquid chromatography (HPLC), we partially purified the toad brain ir-Dyn closest in apparent molecular weight to that of porcine dynorphin and found that it had typical opioid activity in the guinea pig ileum myenteric plexus–longitudinal muscle (GPI) preparation. The substance is recognized by our specific dynorphin antiserum, but it differs somewhat from porcine dynorphin.

MATERIALS AND METHODS

The following opioid peptides were synthesized by Peninsula Laboratories (San Carlos, CA): porcine dynorphin, D13, α-neoendorphin,* and camel β-endorphin (β_c-endorphin). Each peptide was eluted as a single peak on HPLC. Normorphine was obtained from Applied Science (State College, PA), naloxone hydrochloride was from Endo Laboratories (Garden City, NJ). Iodinated peptides were prepared by a modification of the method of Hunter and Greenwood (10), separated from unreacted iodide on a Bio-Gel P-2 column (0.7 × 20 cm; Bio-Rad) with methanol/glacial acetic acid/water, 300:275:1,100 (vol/vol), and purified by HPLC as described (2). Unless otherwise noted, reagents were Baker analyzed reagent grade.

Adult male *Bufo marinus* (200–300 g) were obtained from Carolina Biologicals (Burlington, NC) or Charles Sullivan (Nashville, TN). Tissues from brain, spinal cord, and pituitary were removed immediately after decapitation, dissected on a chilled aluminum plate, and quickly frozen in powdered dry ice. Meningeal tissue and cerebellum were discarded, and the brain was cut posteriorly at the level of the obex. Anterior and neurointermediate lobe (NIL) of pituitary were separated under a dissecting microscope.

Tissues were extracted as described in the legends to Table 1 and Fig. 1, assayed by radioimmunoassay (RIA), and characterized by Sephadex G-50 chromatography.

CM-Sephadex (C-25, Pharmacia, prewashed with 1 M HCl, 1 M NaOH, 0.5 M acetic acid, and 0.1 M acetic acid) was used for concentrating and desalting Sephadex G-50 fractions containing the peak of ir-Dyn nearest the salt volume. Fractions were mixed with 0.2 ml of CM-Sephadex in Bio-Rad disposable columns at 4°C, and the solvent was removed by filtration. The gel was washed twice with 10 ml of distilled water, and ir-Dyn was eluted at room temperature with five 100-μl portions of methanol/0.5 M HCl, 1:1 (vol/vol). Recovery of ir-Dyn was typically 90% or better.

CM-Sephadex concentrates from toad brain were characterized on a reverse-phase HPLC column as described in the legend to Fig. 2. Peak ir-Dyn fractions were lyophilized, then pooled in a small volume of methanol/0.1 M HCl, 1:1 (vol/vol) (referred to hereafter as MeOH/HCl). Recovery of ir-Dyn after HPLC purification was typically 40–60%.

HPLC-purified material was further purified by extraction with immunoaffinity gel [Sepharose-4B coupled to Lucia antibodies as described by Goldstein *et al.* (2)]. Details of the procedure are presented in the legend to Fig. 4.

For estimation of ir-Dyn by RIA, Lucia antiserum and ^{125}I-labeled D13 (^{125}I-D13) were prepared and incubated with the sample as described (3). In order to provide a direct method of assay throughout the purification, the reagent composition of

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Abbreviations: ir-Dyn, immunoreactive dynorphin; D13, dynorphin-(1–13); β_c-endorphin, camel β-endorphin; GPI, guinea pig ileum myenteric plexus–longitudinal muscle; NIL, neurointermediate lobe; RIA, radioimmunoassay.

* The synthesized peptide has the corrected sequence (9), not the originally reported sequence.

the sample in the RIA was changed to 90 μl of 0.1 M acetic acid containing 0.15 M NaCl and 0.1% Triton X-100 (solution A) and 10 μl of MeOH/HCl. Tissue extracts and Sephadex G-50 fractions were in solution A, whereas D13 standards and concentrates were in MeOH/HCl. HPLC fractions were assayed as follows: 10 μl of 6 M HCl was mixed with each fraction to facilitate the transfer of ir-Dyn in the HPLC solvent. A sample from each fraction replaced a similar volume of solution A in the RIA (up to 20 μl), and comparable amounts of HPLC solvent were added to standards.

The assay was validated with criteria described by Ghazarossian *et al.* (3): (*i*) Serial dilutions of toad extracts exhibited parallelism with D13. (*ii*) When combined with toad extract, D13 produced a shift in the dilution profile of the appropriate magnitude and direction. (*iii*) Neither prolonged incubation nor delayed addition of ^{125}I-D13 altered binding in the RIA. (*iv*) ^{125}I-D13 was not degraded during incubation with tissue extracts, as shown by thin-layer chromatography and HPLC.

The GPI preparation was used as described (11) to assess opioid bioactivity after HPLC and immunoaffinity extraction. Inhibition less than 15% or greater than 85% of the initial contraction height was not included in calculations. Dilutions were made in MeOH/HCl. The concentration of ir-Dyn necessary for 50% inhibition of muscle contraction (IC_{50}) was estimated by interpolation of measurements bracketing 50% on a plot of percent inhibition as a function of concentration (on a logarithmic scale). The IC_{50} estimates for determining naloxone K_e were obtained by extrapolation as explained in the legend to Table 2.

RESULTS

Tissue concentrations of ir-Dyn were highest in the NIL of toad pituitary and lowest in the anterior lobe (Table 1). The values in anterior lobe were sufficiently low to suggest that measurable ir-Dyn might have been the result of tissue contamination from NIL. The concentrations of ir-Dyn in toad brain and spinal cord were approximately 1% that in NIL (Table 1). Total ir-Dyn was equally distributed between whole brain and NIL.

Four peaks of ir-Dyn were observed after Sephadex G-50 gel permeation chromatography of toad neural tissue extracts (Fig. 1). The contribution of ir-Dyn in each peak relative to total ir-Dyn varied from region to region: (*i*) In pituitary NIL, most of the ir-Dyn was in peak D (apparent M_r 1,400–1,800). (*ii*) In brain, most of the ir-Dyn was in peaks B (apparent M_r 9,000–11,000) and D. Peak C (apparent M_r 3,800) was also present in a small amount, and peak A (void volume) may have been present as well. (*iii*) In spinal cord, most of the ir-Dyn was in peak A; peaks B and D were also present. These patterns were the same whether the tissue was extracted with 1 M acetic acid at 90°C or 0.1 M acetic acid at 100°C.

Table 1. Dynorphin immunoreactivity in toad pituitary, brain, and spinal cord

Region*	Protein content, mg	Concentration of ir-Dyn, fmol/mg protein	Total ir-Dyn, fmol
Pituitary			
NIL	0.09 ± 0.01	50,500 ± 2,500	4,690 ± 1,220
Anterior lobe†	0.42 ± 0.03	41 ± 3	17 ± 1
Brain	11.0 ± 0.4	496 ± 32	5,490 ± 1,260
Spinal cord	3.17 ± 0.26	341 ± 21	1,060 ± 168

* Tissues were homogenized with a Tissumizer (Tekmar, Cincinnati, OH) in 10 vol (but not less than 1 ml) of 1 M acetic acid at 90°C and incubated 15 min at the same temperature. ir-Dyn was measured in the supernatant solutions after centrifugation (16,000 × *g*, 20 min, 4°C). Protein was estimated according to Lowry *et al.* (12) from the initial homogenate. Values represent means ± SEM for six toads.

† Six lobes were combined for each of six determinations. Values represent means ± SEM per lobe.

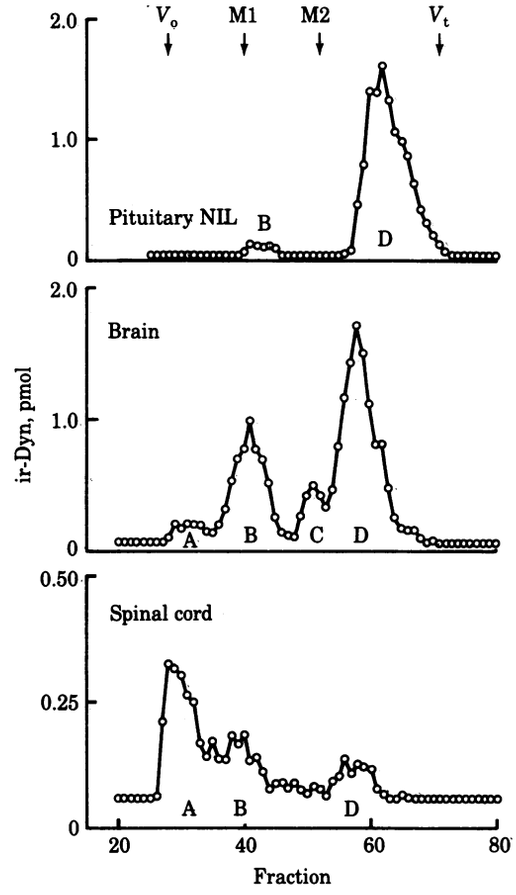

FIG. 1. Gel permeation chromatography of toad pituitary NIL, brain, and spinal cord. Tissues were homogenized in a Tissumizer in 10 vol of 0.1 M acetic acid at 100°C, incubated 30 min at the same temperature, and centrifuged (16,000 × *g*, 20 min, 4°C). Supernatant solutions larger than 6 ml were concentrated by lyophilization and resuspension in 1 M acetic acid containing 0.1% Triton X-100. Routinely, 2.9 ml of the supernatant solution was applied to a Sephadex G-50 fine column (Pharmacia; 90 × 1.5 cm; elution solvent 0.1 M acetic acid in 0.15 M NaCl with 0.1% Triton X-100), then eluted at 5–7 ml/hr at 4°C. Fractions (2.0 ml) were collected, and samples were removed for determination of ir-Dyn. Markers, run in the absence of tissue were: V_o = blue dextran, M1 = cytochrome *c* (Sigma; M_r 12,384, partition coefficient K_{av} = 0.30), M2 = ^{125}I-labeled β_c-endorphin (M_r 3,563, K_{av} = 0.57), V_t = $CoCl_2$. Apparent M_rs for peaks of ir-Dyn were approximated from K_{av} relative to markers: peak A apparent M_r 30,000 or greater (exclusion size of column); peak B apparent M_r 9,000–11,000 (K_{av} = 0.32 and 0.36); peak C apparent M_r 3,800 (K_{av} = 0.55); peak D apparent M_r 1,400–1,800 (K_{av} = 0.72 and 0.77). After immunoextraction of crude brain extract, peak D had K_{av} similar to that of peak D in pituitary NIL. In pituitary NIL, brain, and spinal cord, 9.4, 26.5, and 5.5 pmol of ir-Dyn were applied to the columns, respectively, and 100%, 79%, and 94% of the ir-Dyn applied were recovered.

When peak D from toad brain was subjected to HPLC, a single major peak of ir-Dyn was eluted 27.6 min after the start of the CH_3CN gradient (30.4% CH_3CN, peak D′, Fig. 2). In the same system, porcine dynorphin eluted 6.8 min earlier, at 28.8% CH_3CN. After peak D′ was lyophilized, resuspended in MeOH/HCl, and subjected to HPLC again, the elution position did not change. Moreover, when porcine dynorphin and peak D′ were combined and applied to HPLC, two peaks of ir-Dyn appeared, at 28.8% and 30.4% CH_3CN, respectively. By contrast, α-neo-endorphin and β_c-endorphin were eluted 8 and

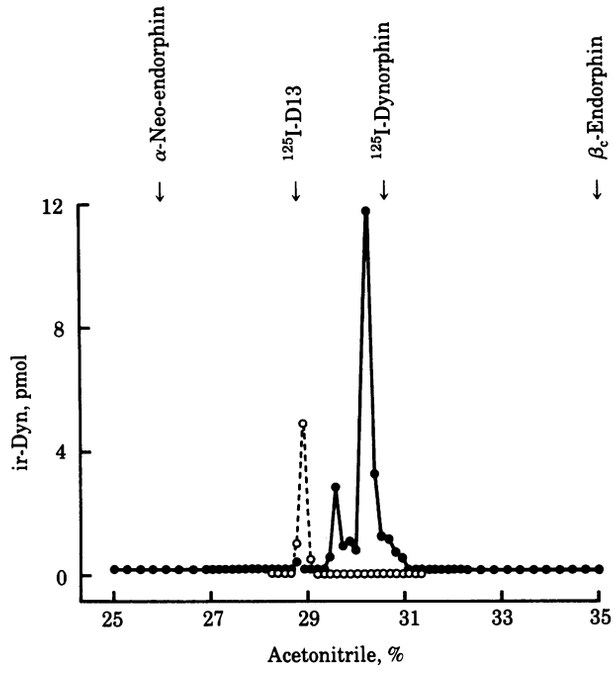

FIG. 2. Reverse-phase HPLC of toad brain peak D (——) and of porcine dynorphin (---). A Waters Associates system was used, with a μBondapak C_{18} column (3.9 × 300 mm). One reservoir contained 880 μl of trifluoroacetic acid in 1 liter of triple-distilled Millipore-filtered water (12 mM) and the other reservoir contained 880 μl of trifluoroacetic acid in 1 liter of CH_3CN (HPLC grade). Reservoirs were mixed with a Waters 660 solvent programmer. Columns were equilibrated in 25% CH_3CN (vol/vol) containing 12 mM trifluoroacetic acid. Starting with sample injection, reservoir pumps were programmed to produce a linear gradient to 35% CH_3CN over 45 min at 1 ml/min. Fractions (0.6 ml) were collected and assayed for ir-Dyn. Markers run in the presence of samples were: ^{125}I-D13, which eluted 20.4 min after the start of the gradient (28.9% CH_3CN), and ^{125}I-labeled dynorphin (^{125}I-dynorphin), which eluted 28.8 min after the start of the gradient (30.6% CH_3CN). The CM-Sephadex concentrate of toad brain peak D (54.2 pmol of ir-Dyn) was applied to the column and eluted as one major peak (D') 27.6 min after the start of the gradient (30.4% CH_3CN). Recovery of ir-Dyn from the column was 52.8%. The minor peak of ir-Dyn that eluted 24 min after the start of the gradient (29.6% CH_3CN) represented 8% of the ir-Dyn applied to the column. A second and steeper gradient from 35% to 90% CH_3CN in 30 min (not shown), yielded no more ir-Dyn. ir-Dyn was less than 0.3 pmol per fraction after HPLC of blank preparations extracted in the same manner as tissue and carried through the purification. The retention times of peak D' and iodinated markers did not vary by more than 2.4 min in six additional HPLC characterizations in which 300 toad brains were purified (in batches of 50) as described in *Materials and Methods*. Once this purification was completed, the HPLC column was calibrated further. Porcine dynorphin (12.1 pmol, based on RIA estimate) was applied to the column, and eluted, as shown, in a single peak 20.8 min after the start of the gradient (28.8% CH_3CN). Recovery of ir-Dyn from the column was 54.6%. α-Neo-endorphin and β_c-endorphin were applied to the column separately, and were eluted as single peaks 8 and 45 min after the start of the CH_3CN gradient (26% and 35% CH_3CN), respectively, based on absorbance at 228 nm.

45 min after the start of the CH_3CN gradient (26% and 35% CH_3CN; Fig. 2), respectively.

Peak D' inhibited the electrically stimulated twitch in the GPI preparation, and this inhibition was completely reversed at a high concentration of the specific opioid antagonist naloxone (Fig. 3 *Upper*). Opioid activity relative to immunoreactivity was estimated for peak D' and porcine dynorphin. IC_{50} for peak D' was 1.3 ± 0.1 nM compared with an IC_{50} for porcine dynorphin in the same preparations of 0.42 ± 0.07 nM (mean ± SEM; n = 5).† Thus the potency of peak D' was about one-third that of porcine dynorphin. However, because it is not known if peak D' is fully immunoreactive with our antiserum, the molar IC_{50} of peak D' may be greater, and hence the potency less, than that indicated here.

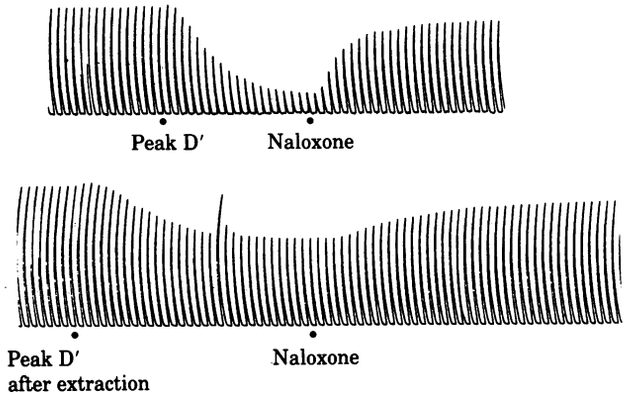

FIG. 3. Opioid activity of HPLC-purified toad brain peak D on the GPI. Electrically stimulated twitch occurs every 10 sec. (*Upper*) Peak D' from HPLC, prepared from 300 toad brains as described in *Materials and Methods* and legend to Fig. 2, was lyophilized and reconstituted in MeOH/HCl. Five microliters (5.8 pmol of ir-Dyn) was added at the dot. The second dot shows addition of naloxone hydrochloride, 5 μM final concentration (bath volume = 5 ml). For this preparation, IC_{50} for porcine dynorphin was 0.23 nM (corresponding to 1.2 pmol in bath) on the basis of RIA estimate. (*Lower*) Peak D' was incubated with immunoaffinity gel as described in the legend to Fig. 4. Immunoaffinity gel extract (15 μl, 1.4 pmol of ir-Dyn) was added at the dot. The second dot shows addition of naloxone hydrochloride, 100 nM final concentration. For this preparation, IC_{50} for porcine dynorphin was 0.36 nM (corresponding to 1.8 pmol in bath) on the basis of RIA estimate. The isolated high trace is an artifact of a power surge in the amplifier.

After immunoaffinity extraction, peak D' inhibited the electrically stimulated twitch in the GPI preparation, and this inhibition was reversed by naloxone (Fig. 3 *Lower*). If all opioid activity present in peak D' were associated with ir-Dyn, little or no opioid activity should remain in the incubation mixture after immunoextraction; and recoveries of opioid activity and ir-Dyn in the gel extract should be comparable and similar to the recoveries in a known dynorphin extract. These conditions were met for peak D' (Fig. 4), and therefore it appears that all (or nearly all) opioid activity in peak D' is associated with ir-Dyn. By contrast, with α-neo-endorphin (43 pmol), 125% of the initial bioactivity was recovered in the incubation mixture after extraction with immunoaffinity gel (not shown).

Like porcine dynorphin, the opioid bioactivity of peak D' was less sensitive to naloxone antagonism than that of normorphine, as determined by comparison of the apparent dissociation constants (K_e) for naloxone on the same GPI preparations: 22, 17, and 4 nM for porcine dynorphin, peak D', and normorphine, respectively (Table 2).

DISCUSSION

The distribution of ir-Dyn across tissues in the toad is generally similar to that reported for the rat (4–7). For example, the high content of ir-Dyn in toad pituitary NIL is similar to that seen

† For each agonist, data were obtained on two GPI preparations and IC_{50} values were estimated as described in *Materials and Methods*. The results for both preparations were combined, because neither slopes nor IC_{50} values differed significantly from preparation to preparation. Slopes of the dose–response relationship between immunoreactivity and opioid bioactivity were consistent with the theoretical slope based on the mass law equation.

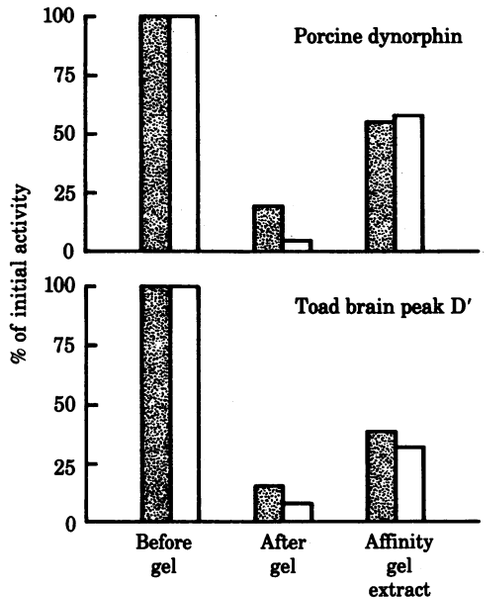

FIG. 4. Recovery of opioid bioactivity on GPI (shaded bars) and ir-Dyn (open bars) before and after immunoaffinity extraction. Porcine dynorphin (12 pmol), toad brain peak D' (35 pmol), or α-neo-endorphin (75 pmol) in 25 μl of MeOH/HCl (or MeOH/HCl alone) was combined with 325 μl of Krebs–Ringer tissue bath solution (11 mM NaCl/4.75 mM KCl/2.54 mM $CaCl_2$/1.19 mM KH_2PO_4/1.20 mM $MgSO_4$/25.0 mM $NaHCO_3$/11 mM glucose containing 20 μM choline chloride and 0.125 μM mepyramine maleate bubbled with 95% O_2/5% CO_2). Opioid activity and ir-Dyn were determined from 100-μl and 10-μl samples, respectively (Before gel). Then 120 μl of bath solution containing immunoaffinity resin (60-μl bed volume) was added. After a 4-hr incubation at 4°C and centrifugation (Eppendorf microcentrifuge), the supernatant solution was removed and opioid activity and ir-Dyn were determined (After gel). The gel was then washed with two 400-μl vol of distilled water, then incubated with 30 μl of MeOH/0.5 M HCl, 1:1 (vol/vol) at 37°C for 15 min to extract adsorbed peptides. Opioid activity and ir-Dyn were determined in the supernatant solution after centrifugation (Affinity gel extract). For each measurement, estimates of bioactivity were corrected for the small effect of MeOH/HCl alone. For porcine dynorphin before gel, 6.2 pmol of opioid bioactivity (estimation relative to porcine dynorphin standard tested on the same tissue strips) and 5.0 pmol of ir-Dyn (estimated by RIA) are represented by 100%. For toad brain peak D' before gel, 8.6 pmol of opioid bioactivity and 15.5 pmol of ir-Dyn are represented by 100%. Results for α-neo-endorphin are presented in the text. The loss of about half of each added peptide prior to the before-gel sampling was presumably due to adsorption to the walls of the reaction vessel (13).

in rat, beef, and pig. However, the negligible amounts of ir-Dyn in toad anterior lobe are a contrast to the considerably higher amounts in mammalian anterior lobe. In general, tissue concentrations of ir-Dyn in pituitary NIL, brain, and spinal cord are higher than those reported for mammalian tissue.

After gel permeation chromatography, three sizes of ir-Dyn with apparent M_r smaller than 12,000 are eluted in positions similar to those reported for ir-Dyn in porcine and rat neural tissues (4, 5, 7, 14). In toad neural tissue, the relative contribution of each molecular size ir-Dyn to the total immunoreactivity varies from tissue to tissue in a pattern different from that seen in the rat. In toad pituitary NIL, peak D (the predominant ir-Dyn) has apparent M_r 1,400–1,800 and is eluted in a position similar to that of porcine pituitary dynorphin (14). However, the intermediate sized ir-Dyn in porcine and rat pituitary NIL is not present in toad NIL. In toad brain, there are two major molecular size forms of ir-Dyn: peak B (apparent M_r 11,000) is eluted in a position similar to that of the largest form of ir-Dyn reported in rat brain (14), and peak D is eluted in a position similar to that of porcine pituitary dynorphin. In toad spinal cord, the predominant ir-Dyn, which is eluted in the void volume (M_r 30,000 or greater), is considerably larger than any ir-Dyn found in rat or pig.

Peak D from toad brain, partially purified by HPLC, is eluted as a single peak (peak D'), with a longer retention time than porcine dynorphin. We will refer to this HPLC-purified preparation as "toad dynorphin" for purposes of discussion.

Toad dynorphin inhibits the electrically stimulated twitch of the GPI, and this effect is reversed by the specific opioid antagonist naloxone. Its opioid potency in the bioassay, relative to its dynorphin immunoreactivity, is one-third that of porcine dynorphin. This difference in potency could be even greater, because toad dynorphin may not be fully immunoreactive with the antiserum.

We have shown that the potency of toad dynorphin remains the same after immunoaffinity extraction, and furthermore, that recovery is comparable to that of porcine dynorphin. Therefore, opioid activity in this preparation is associated with its immunoreactivity to the dynorphin-specific antiserum. The antiserum, as described previously (3), recognizes the sequence dynorphin-(3–12), and does not crossreact with [Met]enkephalin, [Leu]enkephalin, α-endorphin, or human β-endorphin. In addition, it does not crossreact with α-neo-endorphin (unpublished observation).

In the GPI bioassay, toad dynorphin, like porcine dynorphin, is less sensitive to naloxone antagonism than is normorphine, as determined by estimations of the apparent dissociation constants for naloxone. Thus the opioid receptor specificity of toad dynorphin appears to be similar to that of porcine dynorphin.

The retention time of toad dynorphin on reverse-phase HPLC is longer than that of porcine dynorphin, suggesting that it is more hydrophobic. The longer retention time could not be explained by fragmentation to a biologically active dynorphin peptide shorter than dynorphin-(1–14), because all such fragments have shorter retention times than porcine dynorphin, as judged by their characterization in a similar HPLC system (15).

Although the retention times on HPLC for dynorphin fragments larger than D13 have not been determined, it is unlikely that any such fragment would account for the difference in opioid potency reported here. D13 and its COOH-terminally extended derivatives have the same potency as porcine dynorphin itself in the GPI (2, 16). Therefore potency would not be expected to differ for dynorphin fragments larger than D13.

It is likely, therefore, that toad dynorphin has a sequence that differs slightly from that of porcine dynorphin, while retaining enough homology to have similar biological and immunoreactive properties. An analogous example would be the substitution of isoleucine for phenylalanine at position 3 of arginine vasopressin to form vasotocin, the predominant form of antidiuretic hormone in amphibia (17).

We have provided evidence for the existence of a biologically active opioid in toad brain that is immunoreactive with dynorphin-specific antiserum. Its tissue distribution, apparent molecular size, and opioid receptor selectivity are similar to those of porcine dynorphin. It probably differs somewhat in its amino acid sequence from porcine dynorphin, and this difference may justify isolation and sequence analysis. The lower oxygen demand of amphibian tissue and the somewhat simpler nervous system suggest that the toad may provide a good model for *in vitro* studies of dynorphin processing and release. Differences in the molecular size forms of ir-Dyn in toad and rat suggest that there may also be species differences in dynorphin processing.

We thank Dr. Walter Fischli and Keiko Otsu for kindly providing immunoaffinity gel, Louise Lowney and Drs. Charles Chavkin, Brian

Table 2. Estimation of naloxone K_e for HPLC-purified toad brain peak D'

Agonist (L)	Conc. of L, nM	GPI prepa-ration	IC_{50},* nM Before naloxone	IC_{50},* nM After 50 nM naloxone	Naloxone K_e,† nM
Normorphine	50	A	22	380	3
(initial determination)	100	B	42	645	4
Porcine dynorphin	0.27	A	0.36	1.2	21
	0.41	B	0.38	1.3	22
Toad brain‡	0.70	A	0.70	2.8	17
	0.93	B	1.2	4.8	17
Normorphine	50	A	22	316	4
(final determination)	100	B	35	612	3

* IC_{50} was estimated by extrapolation of a single-point determination on the theoretical curve for mass action, $\log[IC_{50}] = ([50 - Y]/57.6) + \log[L]$, in which Y = percent inhibition of muscle contraction and [L] = agonist concentration. After addition of agonist, Y was determined after inhibition reached a plateau. Then 50 nM naloxone was added and Y was determined again, correcting for the small agonist effect of naloxone alone (19% for strip A, 9% for strip B). These preparations were unusually sensitive to normorphine.

† Naloxone K_e is the apparent dissociation constant of the antagonist, computed from the equation $K_e = C/(DR - 1)$, derived from the mass law for competitive antagonism, in which C is the concentration of naloxone (here, 50 nM) and DR is dose ratio of agonist—i.e., the ratio of IC_{50} doses in the presence and absence of the antagonist.

‡ Molar values are based upon estimated ir-Dyn.

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