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Relative contents and concomitant release of prodynorphin/neoendorphin-derived peptides in rat hippocampus

(opioid peptide/*in vitro* neurotransmitter release/HPLC resolution)

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ABSTRACT **The contents and molecular forms of five different prodynorphin-derived opioid peptides were compared in extracts of rat hippocampus by radioimmunoassay after C_{18}-HPLC resolution. Dynorphin (Dyn) A(1–17) immunoreactivity (ir) and Dyn B-ir were heterogeneous in form; Dyn A(1–8)-ir, α-neoendorphin (αneo)-ir and β-neoendorphin (βneo)-ir each eluted as single homogeneous peaks of immunoreactivity. The fraction of immunoreactivity having the same retention as the appropriate synthetic standard was used to estimate the actual hippocampal content of each peptide. Comparison of these values showed that the concentrations of Dyn B, αneo, and Dyn A(1–8) were nearly equal, whereas both Dyn A(1–17) and βneo were 1/5th to 1/10th the value of the other three. Calcium-dependent K^+-stimulated release of these prodynorphin-derived opioids from hippocampal slices was detected. The stimulated rates of release were highest for Dyn B-ir followed by αneo-ir, then βneo-ir and Dyn A(1–8)-ir with Dyn A(1–17)-ir lowest. The relative rates of stimulated release were in agreement with the relative proportions of peptide present within the tissue. This evidence of the presence and release of these opioid peptides considerably strengthens the hypothesis that this family of endogenous opioids plays a neurotransmitter role in the hippocampus.**

The polypeptide sequence of the dynorphin/neoendorphin precursor (prodynorphin) was recently deduced by Kakidani *et al.* (1) using cDNA cloning techniques. Three [Leu^5]enkephalin segments, each having a unique COOH-terminal extension, are contained in this sequence (Fig. 1). Based on peptide purification and radioimmunoassay (RIA) results, it is thought that the post-translational processing of prodynorphin may yield at least five different opioid peptide products. α-Neoendorphin (αneo) and β-neoendorphin (βneo), originally described by Matsuo and co-workers (2, 3), overlap within the first COOH terminally extended [Leu^5]enkephalin segment. Dynorphin (Dyn) A(1–17), isolated by Goldstein *et al.* (4, 5) and its fragment Dyn A(1–8) (6–8) are present within the second segment. The third opioid segment in prodynorphin (Dyn B) corresponds to the 13-amino acid peptide sequence independently determined by Fischli *et al.* (9) and by Kilpatrick *et al.* (10). This prodynorphin-derived peptide family together with the proenkephalin products and pro-opiomelanocortin opioid products compose the three distinct groups of endogenous opioids (11, 12).

The identified prodynorphin products [Dyn A(1–17), Dyn A(1–8), Dyn B, αneo, and βneo] are widely distributed in brain, spinal cord, and peripheral autonomic ganglia and form a distinct opioid system (13–16). Within the rat hippocampus Dyn A immunoreactivity (ir) is densely concentrated in the granule cell nerve fiber projection (the mossy fibers) innervating the hippocampal pyramidal cells (17, 18). The anatomical organization of the hippocampus makes it accessible to electrophysiologic recording and permits analysis of the function of the endogenous prodynorphin-derived opioids. *In vivo* (19, 20) and *in vitro* (21) studies have shown complex effects of Dyn A(1–17) on the firing rates and excitability of hippocampal pyramidal cells. The high density of dynorphin/neoendorphin-ir fibers in this region and the electrophysiologic effects of administered Dyn A(1–17) suggest that the prodynorphin-derived opioid peptides may function in the control of neuronal excitability within the hippocampus.

An understanding of a possible neurotransmitter role for the prodynorphin-derived opioids in this brain region requires the demonstration that these peptides are present within the neurons in bioactive form and can be released at nerve terminals after appropriate stimulation. In the present study, we sought to determine the relative abundance of the prodynorphin-derived peptides within the hippocampus and to relate these values of stored peptide to the relative distribution of peptide forms that can be released *in vitro* from hippocampal slices.

METHODS

To prepare tissue extracts, hippocampi from male Sprague–Dawley rats (180–220 g) were rapidly dissected, trimmed of subiculum, suspended in 10 vol of hot (90°C) 1 M acetic acid, heated in a boiling water bath for 30 min, sonicated 15 sec (Kontes probe sonicator), and then centrifuged for 60 min at 20,000 × *g* at 4°C. Acid-insoluble protein was assayed by the Lowry procedure (22). Acid-soluble extracts were lyophilized and suspended in a minimal volume of methanol/0.1 M HCl, 1:1 (vol/vol).

Radioimmunoassays were carried out on tissue extracts and column fractions using specific antisera as described (8, 23–25). Peptides were iodinated as described (23) and then purified by C_{18}-HPLC. The RIA protocol was as follows: 200 μl of tracer (5,000 cpm) diluted in buffer A (150 mM sodium phosphate, pH 7.4/0.1% bovine serum albumin/0.1% Triton X-100) and 100 μl of antiserum diluted in buffer A were added to a 100-μl sample diluted in 1.0% Triton X-100/0.1 M HCl. This mixture was incubated for 18–24 hr at 4°C; antibody-bound peptide was separated by the dextran-charcoal procedure (23). Each of the antisera used was highly selective; crossreactions with other endogenous opioid peptides were not appreciable (8, 23–25). Opioid peptides were synthesized and purified by N. Ling (Salk Institute).

For release experiments, hippocampal tissue was rapidly dissected and then sliced in two perpendicular planes at 250-μm thickness using a McIlwain tissue chopper. The slices were

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Abbreviations: RIA, radioimmunoassay; ir, immunoreactivity; Dyn, dynorphin; αneo, α-neoendorphin; βneo, β-neoendorphin.

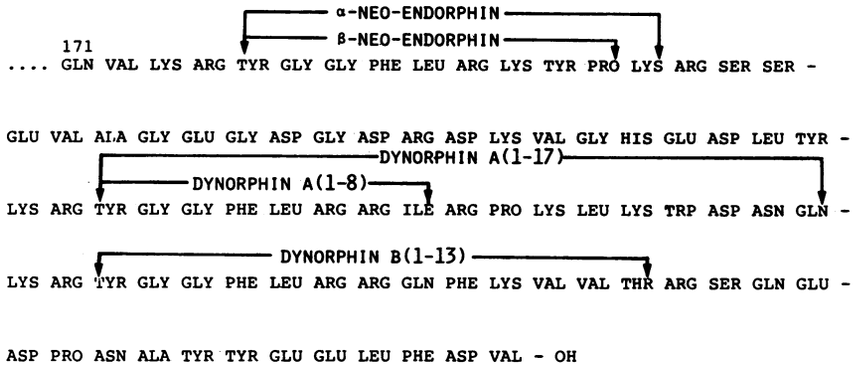

FIG. 1. COOH-terminal portion of porcine prodynorphin sequence (residues 171–256) deduced by Kakidani *et al.* (1). The three [Leu^5]enkephalin-containing segments are bracketed by the putative peptide processing signal "Lys-Arg" (1). αneo and βneo overlap as prodynorphin residues 175–184 and 175–183, respectively. Dyn A(1–17) corresponds to the full sequence of the second segment. Dyn A(1–8) is produced by an unusual cleavage to the left of arginine-217. A similar cleavage to the left of arginine-241 produces Dyn B from the third [Leu^5]-enkephalin-containing segment. The presence of the full sequence of Dyn B(1–29) in brain tissue has not yet been reported.

washed three times with a Krebs-bicarbonate solution modified to contain the following: 127 mM NaCl/3.85 mM KCl/1.8 mM $CaCl_2$/1.8 mM KH_2PO_4/1.18 mM $MgSO_4$/20 mM $NaHCO_3$/11 mM D-glucose/bovine serum albumin (1 mg/ml) (Miles, crystalline)/bacitracin (Sigma) at 30 μg/ml and bubbled with 5% CO_2/95% O_2 (vol/vol) for at least 30 min (pH 7.4). Slices were placed in a 1-inch diameter (1 inch = 2.54×10^{-2} m) superfusion chamber (vol, 0.3 ml) with a 0.45-μm metricel membrane filter (Gelman). Each chamber contained the tissue equivalent of six hippocampi (200–300 mg of tissue per chamber) taken from a pool of hippocampal slices. Slices were superfused with modified Krebs-bicarbonate buffer at 0.4 ml/min at 24°C. To determine K^+-stimulated release of [^{3}H]norepinephrine (New England Nuclear) from hippocampal slices, slices were incubated in 0.5 μM [^{3}H]norepinephrine (10 Ci/mmol; 1 Ci = 37 GBq) for 30 min. Perfusate samples were collected in scintillation vials, mixed with Aquasol II (New England Nuclear), and radioactivity was determined.

To measure endogenous peptidase action, hippocampal slices prepared as described above were incubated at 24°C for 10 min with ^{125}I-labeled Dyn A(1–17) or ^{125}I-labeled [Leu^5]enkephalin in Krebs-bicarbonate buffer. Reactions were terminated by adding an equal volume of hot 2 M acetic acid and boiling for 15 min. Extracts were chilled and centrifuged (5 min, 9,000 × *g*) in a Brinkman Microfuge, and 10-μl portions of supernatant were resolved by TLC on silica plates (LK6DF, Whatman) in butanol/acetic acid/water (4:1:1) as described (26).

RESULTS

To determine the molecular nature of the prodynorphin-derived immunoreactivities detected in hippocampus, acetic acid extracts were resolved by C_{18}-HPLC (Fig. 2). The peaks of Dyn A(1–8)-ir, αneo-ir, and βneo-ir were each nearly homogeneous, and each eluted with a retention time corresponding to its appropriate synthetic standard. These peptides were well resolved by the acetonitrile gradient. The homogeneity of the peaks confirms the specificity of the RIAs and indicates that the immunoreactivities detected are largely due to the presence of the corresponding authentic peptide forms.

Hippocampal Dyn A(1–17)-ir was resolved by C_{18}-HPLC as two peaks of immunoreactivity. The first peak had the same retention time as synthetic Dyn A(1–17) and corresponds to ≈20% of the total eluted immunoreactivity. The majority of Dyn A(1–17)-ir was retained on the C_{18}-HPLC column longer than synthetic standard, signifying greater hydrophobicity. Dyn B-ir (Fig. 2) also eluted as two peaks of immunoreactivity; 40% had the same retention time as synthetic Dyn B. Most of the remaining Dyn B-ir eluted with the same retention as the second peak of Dyn A(1–17)-ir. Molecular sieve column data (Waters Associates, HPLC protein analysis column; data not shown) indicated that the second peak of Dyn A(1–17)-ir had an apparent molecular weight of 3,500–4,000, which is consistent with its longer retention on C_{18}-HPLC and a greater hydrophobicity. The high molecular weight and equal crossreactivities in Dyn A(1–17) and Dyn B RIAs indicate that this material may be Dyn A(1–32), an intermediate precursor of both Dyn A(1–17) and Dyn B described by Fischli *et al.* (9). For each peptide, the fraction of the total immunoreactivity eluting from C_{18}-HPLC having the same retention as synthetic standard is shown in Table 1. Resolution of replicate extracts gave similar results.

The total immunoreactivity of each of the peptides was measured in hippocampal extracts (Table 1). Consistent with

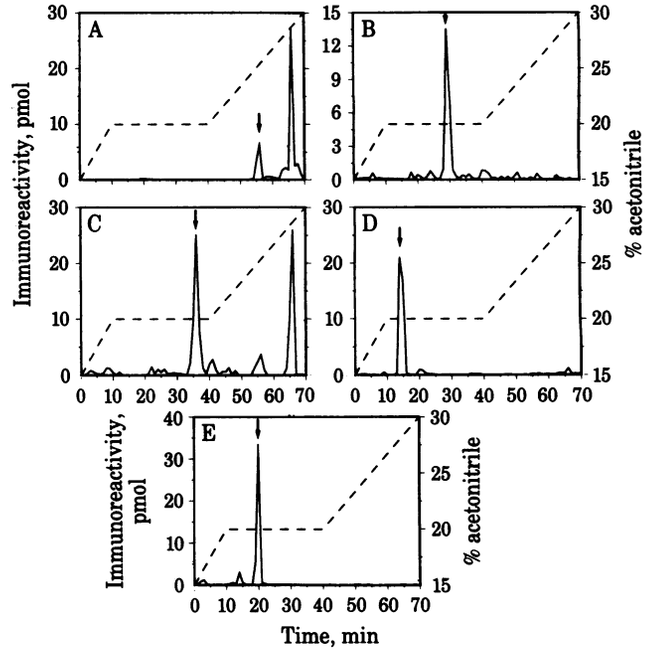

FIG. 2. Hippocampal extracts were resolved by HPLC on a reversed-phase μBondapak C_{18} (Waters) 3.6 × 250 mm column. Tissue extracts were clarified by centrifugation (5 min, 9,000 × *g*, Brinkmann microfuge), and then 50–100 μl (0.6–1.2 mg of protein) was injected. Material was eluted at a flow rate of 1.5 ml/min by a nonlinear gradient of acetonitrile (---) (Burdick and Jackson, Muskegon, MI) in 5 mM trifluoroacetic acid. Absorbance was monitored at 254 nm (Hitachi model 100-10 spectrophotometer) (data not shown). Eluate was collected at 1 min per fraction and then lyophilized. Lyophilized column fractions were dissolved in 1% Triton X-100/0.1 M HCl and then assayed at several dilutions to bring the immunoreactivity content (——) within the linear portion of the appropriate standard curve. Arrows indicate the elution positions of the appropriate synthetic peptide in each panel as determined by both absorbance and RIA. To facilitate comparison, the immunoreactivity values in each graph represent the amount in 100 mg of protein; note that the ordinate scales differ. (*A*) Dyn A(1–17)-ir; (*B*) Dyn A(1–8)-ir; (*C*) Dyn B-ir; (*D*) αneo-ir; (*E*) βneo-ir.

Table 1. Immunoreactivity in 1 M acetic acid extracts of rat hippocampus

	Total immunoreactivity, pmol per g of protein	Immunoreactive fraction	Actual tissue content, pmol per g of protein
Dyn A(1–17)	413 ± 110 (10)	0.18	74
Dyn A(1–8)	427 ± 51 (13)	0.77	329
Dyn B	1,140 ± 260 (8)	0.40	456
αneo	496 ± 27 (7)	0.89	441
βneo	47 ± 14 (4)	0.80	38

Immunoreactivity is expressed as immunoequivalent pmol per g of original tissue protein. Immunoreactive fraction corresponds to the percentage of the total ir eluting from C_{18}-HPLC columns at the same retention time as the appropriate synthetic peptide standard (see Fig. 2). Numbers in parentheses represent number of extracts tested. The actual tissue contents are the fractions of total immunoreactivity equivalent to the nominal peptide.

previous reports (27), Dyn B-ir was the most abundant. Dyn A(1–17)-ir, Dyn A(1–8)-ir, and αneo-ir contents were nearly equal, and βneo-ir was least abundant. The relatively high proportion of Dyn A(1–17)-ir in hippocampus is in contrast to other rat brain regions in which Dyn A(1–17)-ir was reported to be only 10–20% the level of Dyn A(1–8) (8). The percentage of the total immunoreactivity of each peptide corresponding to its appropriate synthetic peptide standard as determined by C_{18}-HPLC was used to estimate the actual tissue content of each opioid. These corrected contents show that Dyn B and αneo are nearly equally abundant, followed by Dyn A(1–8), Dyn A(1–17), and then βneo. This correction adjusts the estimates of Dyn A(1–17) and Dyn B contents as only these two RIAs detect a significant amount of other peptide forms in acid extracts.

Prodynorphin can be cleaved to yield either Dyn A(1–17) or Dyn A(1–8); in hippocampus the latter two peptides are present in a 1:5 ratio. The sum of the corrected Dyn A(1–17) and Dyn A(1–8) contents equals one-third of the total prodynorphin-derived opioid content. Similarly, the precursor can yield either αneo or βneo, and these are present in a 10:1 ratio. Together, αneo and βneo contents compose nearly one-third of the total prodynorphin-derived peptide content.

Fig. 3 shows the hippocampal opioid release rates. The peak rate of K^+-stimulated release was 2- to 10-fold more than basal release. K^+ superfusion in the absence of calcium did not stimulate peptide release. It is evident that not only are each of these prodynorphin-derived opioids present in hippocampus, but each can be released by K^+-induced depolarization through a Ca^{2+}-dependent mechanism.

Rates of K^+-stimulated release are shown in Table 2. These values are the mean release rates in the presence of 50 mM KCl minus the basal rates. The stimulated rate of Dyn B-ir release was highest and was followed by those of αneo-ir, Dyn A(1–8)-ir, βneo-ir, and Dyn A(1–17)-ir. Stimulated release of each of the peptides was 1–2% of the total content except for βneo-ir, which was 5–8% of the total βneo-ir present in the chambers. The rank order of release rates is not in agreement with the rank order of corrected peptide contents. βneo was present at lower tissue concentrations than either Dyn A(1–8) or Dyn A(1–17), yet it was released at a higher rate. However, because the measured rates of release were not corrected for possible post-release degradation or for possible differences in recovery, underestimation of the actual release rates is likely, because of their sensitivity to tissue peptidases (26, 27) and because of nonspecific adsorption to surfaces (26–28).

To estimate catabolism rate and recovery, trace amounts of ^{125}I-labeled Dyn A(1–17) were added to the Krebs-bicarbonate

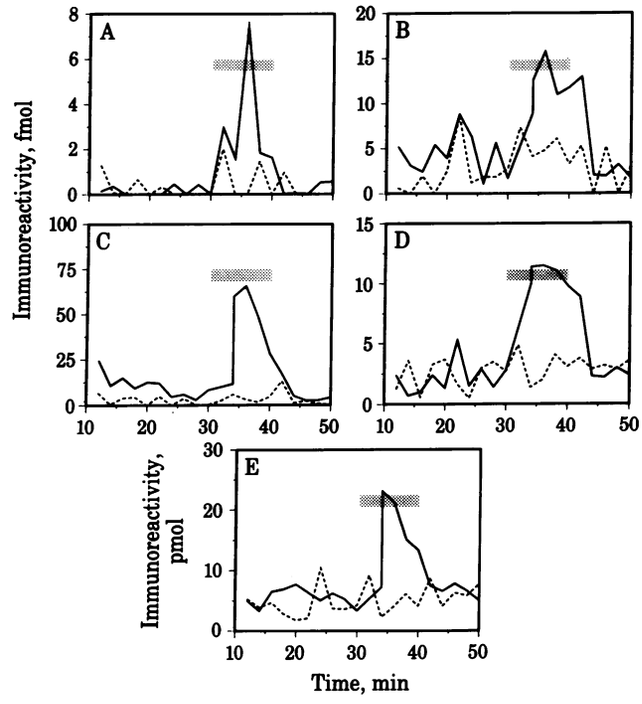

FIG. 3. After an initial 10-min wash, superfusate samples were collected at 2-min intervals, immediately boiled for 15 min, and then frozen and lyophilized. After 30 min of superfusion with normal Krebs-bicarbonate, the superfusion buffer was changed to 50 mM KCl Krebs-bicarbonate for 10 min; later the slices were again superfused with normal Krebs-bicarbonate. Three to five chambers were run simultaneously with the eluates for each assayed in different RIAs. To correct for the high salt concentration in the lyophilized superfusate samples, 0.8-ml aliquots of each superfusion buffer were boiled and lyophilized as described above and then included in the RIA as blanks that were subtracted from the release fraction values. Appropriate peptides were also diluted in buffer containing ion concentrations equivalent to the release fractions and included in the RIAs as standards. For K^+ stimulation, the Krebs-bicarbonate buffer ion concentrations were adjusted to 50 mM KCl and 87 mM NaCl. K^+ was superfused for 10 min (hatched bar). For experiments in the absence of Ca^{2+} (---), $CaCl_2$ was replaced by 1 mM cobalt chloride in both the normal and 50 mM KCl Krebs-bicarbonate solutions. Data points shown are means of three independent chambers. (*A*) Dyn A(1–17)-ir; (*B*) Dyn A(1–8)-ir; (*C*) Dyn B-ir; (*D*) αneo-ir; (*E*) βneo-ir.

before superfusing hippocampal tissue slices. Recovery of the added ^{125}I-labeled Dyn A(1–17) in the collected fractions was >90%, and the peptide was intact as determined by resolving the eluted radioactivity by TLC. Catabolism was also measured in slices prepared in normal Krebs-bicarbonate (100 mg of tissue per 500 μl), incubated with 1 nM ^{125}I-labeled Dyn A(1–17) at 24°C for 10 min, and then extracted with hot 1 M acetic acid. TLC analysis of the resulting acid extract showed that only 35% of the radioactivity migrated with intact ^{125}I-labeled Dyn A(1–17) extracted at zero incubation time. Similarly, after incubation of hippocampal slices with ^{125}I-labeled [Leu^5]enkephalin as described above, only 20% of the radioactivity migrated the same as intact ^{125}I-labeled [Leu^5]enkephalin extracted at zero incubation time. These results were confirmed by analysis of the extracts on C_{18}-HPLC. The actual extent of endogenous peptide degradation after release is unknown and obviously depends on the actual tissue exposure.

A peptidase inhibitor was then prepared to protect endogenous prodynorphin-derived peptides from post-release degradation. [Leu^5]Enkephalin degradation has been reported to be inhibited by a mixture of 0.3 μM thiorphan/20 μM bes-

Table 2. Rate of K^+-stimulated release

	Unprotected release		Release in presence of peptidase inhibitor cocktail	
	Basal rate	Stimulated rate above basal	Basal rate	Stimulated rate above basal
Dyn A(1–17)-ir	1.3 ± 0.36 (3)	1.6 ± 0.63 (3)	1.8 ± 0.53 (6)	1.00 ± 0.76 (6)
Dyn A(1–8)-ir	2.1 ± 1.3 (3)	5.8 ± 1.1 (3)	1.1 ± 0.20 (3)	4.9 ± 0.7 (3)
Dyn B-ir	2.8 ± 2.1 (3)	23.0 ± 9.6 (3)	3.6 ± 0.82 (3)	7.8 ± 2.2 (3)
αneo-ir	2.7 ± 0.54 (7)	11.0 ± 2.6 (7)	3.5 ± 0.83 (7)	3.9 ± 0.62 (7)
βneo-ir	3.0 ± 0.25 (3)	6.1 ± 1.3 (3)	3.3 ± 1.10 (6)	1.7 ± 0.55 (6)

Basal release rates (fmol/min) are the means and SEM for the 15 min before and 10 min after 50 mM potassium superfusion. Numbers in parentheses represent number of chambers. Stimulated release rates (fmol/min) are the means of the differences between the release rate during the 10-min superfusion period and the basal release rate. For release experiments in the presence of peptidase inhibitors, bacitracin was replaced with 0.3 μM thiorphan [gifts of R. Chipkin (Schering Pharmaceuticals) and B. Roques (Université René Descartes)], 10 μM captopril (Squibb), 20 μM bestatin (Sigma), and 20 μM polylysine (M_r, 4,000; Sigma).

tatin/10 μM captopril (29). These inhibitors did protect ^{125}I-labeled [Leu^5]enkephalin from degradation (70% intact after 10 min), but did not decrease ^{125}I-labeled Dyn A(1–17) degradation by hippocampal tissue slices. Addition of 20 μM poly(Lys), reported to decrease degradation of ^{125}I-labeled Dyn A(1–13) and [^{3}H]Dyn A(1–17) in washed brain membranes (26, 30), did decrease ^{125}I-labeled Dyn A(1–17) degradation by hippocampal slices from 35% intact in the absence of polylysine to 68% intact [lower poly(Lys) concentrations provided less protection]. Therefore, 20 μM polylysine/20 μM bestatin/10 μM captopril/0.3 μM thiorphan was used to protect endogenously released opioids from degradation. The effect of this mixture on the stability of the other opioids was not evaluated. In separate experiments, we observed that this peptidase-inhibitor mixture decreased the K^+-stimulated release of [^{3}H]norepinephrine by about 25% without affecting spontaneous release. Nevertheless, because of the significant amount of protection provided by the mixture, opioid release in its presence was measured.

As shown in Table 2, the K^+-stimulated release rates of the five prodynorphin-derived opioids were also decreased by the mixture but to an unequal extent. The release rate of βneo-ir was most affected. Whether the differences among the peptides in release rates in the presence and absence of peptidase inhibitors was due to differential effect on nonspecific adsorption or post-release catabolism is not known. The relative amounts of each prodynorphin-derived opioid released in the presence of the peptidase-inhibition mixture are generally concordant with their relative tissue contents. Again, αneo-ir and βneo-ir release account for about one-third of the total stimulated release of the prodynorphin-derived opioids, Dyn A(1–17)-ir and Dyn A(1–8)-ir release rates account for another one-third of the total, and the rest is Dyn B-ir.

DISCUSSION

We have examined the processing and release of the prodynorphin-derived opioids in rat hippocampus. The major findings were that the prodynorphin peptides Dyn A(1–17), Dyn A(1–8), Dyn B, αneo, and βneo are present in hippocampal extracts and can be released *in vitro* after K^+ stimulation by a Ca^{2+}-dependent mechanism. These data complement earlier reports of Ca^{2+}-dependent K^+-stimulated release of Dyn A-ir (31, 32) and αneo-ir (31) from rat posterior pituitary *in vitro*. Our chromatographic analysis of opioid peptides in hippocampal extracts allowed the resolution of the molecular forms of opioid immunoreactivity and permitted the accurate estimation of the peptide contents. Regional differences in processing have been suggested (8, 25); however, the demonstrated heterogeneity of molecular form indicates that the apparent regional differences of Dyn A(1–17) and Dyn B may be due to differing concentrations of intermediate precursors.

The full details of the processing of prodynorphin are not yet known; other opioid peptide products of prodynorphin [e.g., [Leu^5]enkephalin and Dyn B(1–29)] may potentially be derived from the precursor. We have measured [Leu^5]enkephalin in these hippocampal preparations by C_{18}-HPLC and RIA (data not shown). Its content was roughly equal to Dyn B, and its stimulated release was about 2-fold greater than Dyn B-ir. Immunocytochemical studies indicate that Dyn A(1–17)-ir, Dyn B-ir, and αneo-ir are confined to the mossy fiber system (17, 18), whereas the proenkephalin-derived peptides are more widely distributed in the hippocampus (33–35). [Met^5]Enkephalin-ir content in rodent hippocampus has been reported to be 2- to 10-fold greater than [Leu^5]enkephalin-ir content (36, 37). This ratio is consistent with the 3:1 ratio in the proenkephalin sequence and suggests that proenkephalin is the major source of [Leu^5]enkephalin. Nevertheless, the relative contributions of proenkephalin and prodynorphin to the [Leu^5]enkephalin content remain to be directly established.

A major concern in the quantitative interpretation of the release data is the assumption that the recoveries of these peptides are equal. Differences among the peptides in their adsorptive losses or catabolic rates after release could have a large effect on the estimated relative release rates. We tried to maximize recovery in these experiments by adding specific peptidase inhibitors, boiling the chamber eluate promptly after collection, incubating the release chambers at 24°C instead of 37°C, and having an incubation chamber of minimal volume. These modifications provided protection of exogenous ^{125}I-labeled Dyn A(1–17) and ^{125}I-labeled [Leu^5]enkephalin from degradation; however, significant differences between the degradation of exogenous and endogenous peptide may exist. Quantitation of the endogenous peptide recoveries was not possible. The decrease of release rate in the presence of the peptidase-inhibition mixture indicates that any increase in recovery due to protection of peptide was offset by the depressive effects of the mixture on release. Clearly, selective and specific peptidase inhibitors are required.

The homogeneity of the Dyn A(1–8)-ir, αneo-ir, and βneo-ir on C_{18}-HPLC indicates that the immunoreactivities released during K^+ superfusion are likely to be the authentic molecular forms. In a separate study (38), we collected the peptides released, resolved the Dyn A(1–17)-ir on C_{18}-HPLC, and determined that 80% of the immunoreactivity eluted with the same retention as authentic Dyn A(1–17). The nature of the released

Dyn B-ir has not yet been established.

In addition, an important consideration is that superfusion for 10 min with 50 mM KCl may not mimic the normal mode of peptide release. The relative amounts of the prodynorphin-derived opioids released could conceivably be affected by the frequency and intensity of stimulation. However, K^+ stimulation may at least indicate all the forms that have the potential of being released (for review, see ref. 39). Release of prodynorphin-derived opioids can be stimulated by other means. In a previous report (38), we found that Dyn A(1–17)-ir release from hippocampal slices can also be induced by veratrine or kainic acid to the same extent as by 50 mM KCl.

Comparison of peptide content and release rates may provide information about prodynorphin processing. If Dyn A(1–17) were an intermediate in the synthesis of Dyn A(1–8), one might expect to measure preferential release of Dyn A(1–8). This was not observed. Although Dyn A(1–17) content was 1/10th that of Dyn A(1–8), Dyn A(1–17)-ir release rate was only 1/5th that of Dyn A(1–8)-ir. Thus Dyn A(1–17) is probably not an intermediate in the biosynthesis of Dyn A(1–8). In contrast, similar reasoning suggests that αneo is an intermediate in the βneo biosynthesis.

Within the hippocampus, the Dyn B, αneo, and Dyn A(1–8) forms predominate both in relative content and release. The potential concomitant release of the five prodynorphin-derived opioid peptides from mossy fiber terminals indicates that they may have a coordinate action. Their pharmacological characteristics are known to be quite similar. They are selective κ agonists differing only in potency (40). The potency differences in hippocampus have not yet been determined and depend on the nature of the opioid receptor types present.

In hippocampus, opiate alkaloids and opioid peptides are thought to increase the excitability of hippocampal pyramidal cells by inhibiting an inhibitory interneuron. We have shown that Dyn A(1–17) also has this effect in the CA1 region of the hippocampus (21). Masukawa and Prince (41) reported that, in the CA3 pyramidal cell region that receives the mossy fiber input, opioids excite these cells, presumably by a disinhibitory mechanism similar to that described by Zieglgansberger *et al.* (42) in the CA1 region. The effects of opioids in the CA3 region may be complex (19, 20) and require further study, and the actions of the other prodynorphin-derived opioids need to be defined. Ultimately, an understanding of the effects of these peptides at the cellular level will provide insight into their role in the functioning of the hippocampus in the intact animal.

Our results suggest that multiple forms of the prodynorphin molecule exist separately within the defined mossy fiber pathway of the rodent hippocampus. The data suggest that all forms present are candidates for neuronal release. Electrophysiologic data suggest that several of these releasable forms are also potent agonists. Therefore, in contrast with other peptide systems in endocrine and neuronal systems, the prodynorphin-derived peptides appear to offer a profusion of potential agonists. The next critical step is to determine how many of these forms are physiologically released, how their actions are exploited, and what functional advantages such multiple agonist systems could offer.

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1. Kakidani, H., Furutani, Y., Takahashi, H., Noda, H., Morimoto, Y., Hirose, T., Asai, M., Inayama, S., Nakanishi, S. & Numa, S. (1982) *Nature (London)* **298**, 245–249.
2. Minamino, N., Kangawa, K., Chino, N., Sakakibara, S. & Matsuo, H. (1981) *Biochem. Biophys. Res. Commun.* **99**, 864–870.
3. Kangawa, K., Minamino, H., Chino, N., Sakakibara, S. & Matsuo, H. (1981) *Biochem. Biophys. Res. Commun.* **99**, 871–877.
4. Goldstein, A., Tachibana, S., Lowney, L. I., Hunkapiller, M. & Hood, L. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6666–6670.
5. Goldstein, A., Fischli, W., Lowney, L., Hunkapiller, M. & Hood, L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7219–7223.
6. Minamino, N., Kangawa, K., Fukoda, A. & Matsuo, H. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1475–1481.
7. Seizinger, B. R., Hollt, V. & Herz, A. (1981) *Biochem. Biophys. Res. Commun.* **102**, 197–205.
8. Weber, E., Evans, C. J. & Barchas, J. D. (1982) *Nature (London)* **299**, 77–79.
9. Fischli, W., Goldstein, A., Hunkapiller, M. W. & Hood, L. E. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5435–5437.
10. Kilpatrick, D. L., Wahlstrom, A., Lahm, H. W., Blacher, R. & Udenfriend, S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6480–6483.
11. Cox, B. M. (1982) *Life Sci.* **31**, 1645–1658.
12. Bloom, F. E. (1983) *Annu. Rev. Pharmacol. Toxicol.* **23**, 151–170.
13. Vincent, S. R., Hokfelt, T., Christensson, I. & Terenius, L. (1982) *Neurosci. Lett.* **33**, 185–190.
14. Watson, S. J., Khachaturian, H., Akil, H., Coy, D. H. & Goldstein, A. (1982) *Science* **218**, 1134–1136.
15. Watson, S. J., Khachaturian, H., Taylor, L., Fischli, W., Goldstein, A. & Akil, H. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 891–894.
16. Weber, E. & Barchas, J. D. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 1125–1129.
17. Weber, E., Roth, K. A. & Barchas, J. D. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3062–3066.
18. McGinty, J. F., Henriksen, S. J., Goldstein, A., Terenius, L. & Bloom, F. E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 589–593.
19. Henriksen, S. J., Chouvet, G. & Bloom, F. E. (1982) *Life Sci.* **31**, 1785–1788.
20. Walker, J. M., Moises, H. C., Coy, D. H., Baldrighi, G. & Akil, H. (1982) *Science* **218**, 1136–1138.
21. Gruol, D. L., Chavkin, C., Valentino, R. J. & Siggins, G. R. (1983) *Life Sci.* **33** Suppl. 1, 533–536.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
23. Ghazarossian, V. E., Chavkin, C. & Goldstein, A. (1980) *Life Sci.* **27**, 75–86.
24. Cone, R. I. & Goldstein, A. (1982) *Neuropeptides* **3**, 97–106.
25. Weber, E., Evans, C. J., Chang, J. K. & Barchas, J. D. (1982) *Biochem. Biophys. Res. Commun.* **108**, 81–88.
26. Leslie, F. M. & Goldstein, A. (1982) *Neuropeptides* **2**, 185–196.
27. Cone, R. I., Weber, E., Barchas, J. D. & Goldstein, A. (1983) *J. Neurosci.*, in press.
28. Ho, W. K. K., Cox, B. M., Chavkin, C. & Goldstein, A. (1980) *Neuropeptides* **1**, 143–152.
29. Corbett, A. D., Paterson, S. J., McKnight, A. T., Magnan, J. & Kosterlitz, H. W. (1982) *Nature (London)* **299**, 79–81.
30. Houghten, R., Bartlett, J. & Ostresh, M. (1983) *Life Sci.*, in press.
31. Seizinger, B. R., Maysinger, D., Hollt, V., Grimm, C. & Herz, A. (1982) *Life Sci.* **31**, 1757–1760.
32. Anhut, H. & Knepel, W. (1982) *Neurosci. Lett.* **31**, 159–164.
33. Gall, C., Brecha, N., Karten, H. J. & Chang, K.-J. (1981) *J. Comp. Neurol.* **198**, 335–350.
34. Bloch, B., Baird, A., Ling, N., Benoit, R. & Guillemin, R. (1983) *Brain Res.* **263**, 251–257.
35. Williams, R. G. & Dockray, G. J. (1983) *Neuroscience* **9**, 563–586.
36. Hong, J. S. & Schmid, R. (1981) *Brain Res.* **205**, 415–418.
37. Hoffman, D. W., Altschuler, R. A. & Gutierrez, J. (1983) *J. Neurochem.*, in press.
38. Chavkin, C., Bakhit, C. & Bloom, F. E. (1983) *Life Sci.*, **33** Suppl. 1, 13–16.
39. Orrego, F. (1979) *Neuroscience* **4**, 1037–1057.
40. James, I. F., Fischli, W. & Goldstein, A. (1984) *J. Pharmacol. Exp. Ther.*, in press.
41. Masukawa, L. M. & Prince, D. A. (1982) *Brain Res.* **249**, 271–280.
42. Zieglgansberger, W., French, E. D., Siggins, G. R. & Bloom, F. E. (1979) *Science* **205**, 415–417.