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Distribution Pattern of Metorphamide Compared with Other Opioid Peptides from Proenkephalin and Prodynorphin in the Bovine Brain

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Abstract: Metorphamide is a [Met]-enkephalin-containing opioid octapeptide with a C-terminal α-amide group. It is derived from proenkephalin and is, so far, the only endogenous opioid peptide with a particularly high affinity for μ opioid (morphine) receptors, a somewhat lesser affinity for κ opioid receptors, and a relatively low affinity for δ opioid receptors. The concentrations of metorphamide in the bovine caudate nucleus, the hypothalamus, the spinal cord, and the neurointermediate pituitary were determined by radioimmunoassay and chromatography separation procedures. Metorphamide concentrations were compared with the concentrations of eight other opioid peptides from proenkephalin and prodynorphin in identical extracts. The other opioid peptides were [Met]-enkephalyl-Arg^6-Phe^7 and [Met]-enkephalyl-Arg^6-Gly^7-Leu^8 from proenkephalin; α-neoendorphin, β-neoendorphin, dynorphin A(1–8), dynorphin A(1–17), and dynorphin B from prodynorphin; and [Leu]-enkephalin, which can be derived from either precursor. All opioid peptides were present in all four bovine neural tissues investigated. Metorphamide concentrations were lower than the concentrations of the other proenkephalin-derived opioid peptides. They were, however, similar to the concentrations of the prodynorphin-derived opioid peptides in the same tissues. Marked differences in the relative ratios of the opioids derived from prodynorphin across brain regions were observed, a finding suggesting differential posttranslational processing. Differences in the ratios of the proenkephalin-derived opioids across brain regions were less pronounced. The results from this study together with previous findings on metorphamide's μ opioid receptor binding and bioactivities suggest that the amounts of metorphamide in the bovine brain are sufficient to make this peptide a candidate for a physiologically significant endogenous μ opioid receptor ligand. **Key Words:** μ opioid receptors—Processing—Precursor. **Sonders M. and Weber E.** Distribution pattern of metorphamide compared with other opioid peptides from proenkephalin and prodynorphin in the bovine brain. *J. Neurochem.* **49,** 671–680 (1987).

More than 20 different opioid peptides have been isolated from mammalian tissues, and all share one of two N-terminal sequences—Tyr^1-Gly^2-Gly^3-Phe^4 and either Met^5 or Leu^5—the canonical [Met]- and [Leu]-enkephalin sequences first determined by Hughes et al. (1975). Three genes encode separate opioid polypeptide precursor molecules: proopiomelanocortin (Nakanishi et al., 1979), proenkephalin (Comb et al., 1982; Noda et al., 1982; Gubler et al., 1983; Howells et al., 1984; Martens and Herbert, 1984), and prodynorphin (Kakidani et al., 1982; Horikawa et al., 1983). The sequences of all known enkephalin-containing opioid peptides can be found within these precursors. Tissue specificity has been observed both in the level of opioid gene expression and in the patterns of posttranslational proteolysis. Not only do different tissues—even different regions of a single tissue—express greater or lesser amounts of an opioid precursor, but a single type of precursor molecule can be cleaved into markedly dissimilar sets of peptides in particular regions. Figure 1 illustrates the precursor structure and major peptide products of proenkephalin and prodynorphin as found in the CNS. Neither the mechanisms controlling the patterns of proteolysis nor the physiological roles of the individual peptides are completely understood, but the striking conservation of bioactive sequences across species suggests that the peptides may have particular functions (Herbert et al., 1983).

One reason for the multiplicity of opioid peptides within the mammalian CNS may be the existence of at least three separate opioid receptor types, namely,

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Abbreviations used: MERF, [Met]-enkephalyl-Arg^6-Phe^7; MERGL, [Met]-enkephalyl-Arg^6-Gly^7-Leu^8; RIA, radioimmunoassay.

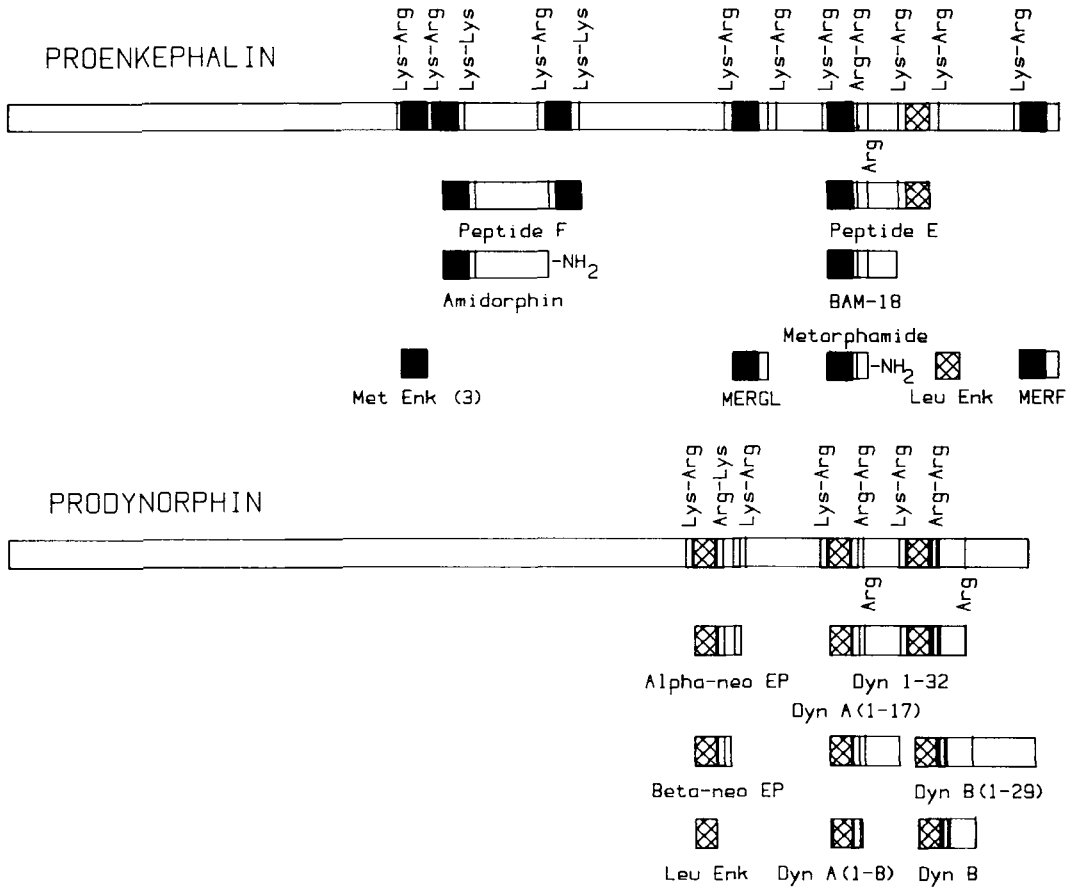

FIG. 1. Structure and proteolytic products of the proenkephalin and prodynorphin precursors in the CNS, including cleavage sites. [Met]-enkephalin and [Leu]-enkephalin could be potentially derived from any parts of their respective precursors that contain these pentapeptide sequences. BAM, bovine adrenal medullary peptide; Dyn, dynorphin; Enk, enkephalin; neo EP, neoendorphin.

the μ, δ, and κ opioid receptors. Certain opioid peptides are known to interact preferentially with one of the receptor types and are, therefore, thought to serve as endogenous ligands for those receptors. The enkephalins, for example, are thought to be the endogenous ligands for the δ opioid receptors, whereas the dynorphins and neoendorphins are thought to be the endogenous ligands for the κ opioid receptors (Chavkin and Goldstein, 1981; Paterson et al., 1983). Which peptide(s) serve as endogenous ligands for the μ opioid receptors is still unclear (Kosterlitz, 1985). Determination of the physiologically relevant endogenous ligands for μ opioid receptors is important because this receptor type is known to mediate many of the pharmacological effects of morphine.

The opioid peptide metorphamide, the first known opioid with a C-terminal α-amide group, was isolated and characterized from bovine caudate nucleus in our laboratory (Weber et al., 1983*a*). Matsuo et al. (1983) independently characterized the same peptide from human pheochromocytoma tissue and called it "adrenorphin." The primary structure of metorphamide is Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH_2 and corresponds to residues 206–213 of the bovine proenkephalin precursor (Noda et al., 1982). Recently, a second amidated opioid peptide, amidorphin, has been identified (Seizinger et al., 1985).

Metorphamide is unique in that it is, so far, the only endogenous opioid peptide with a particularly high affinity for μ opioid receptors, a somewhat lesser affinity for κ opioid receptors, and a much lower affinity for δ opioid receptors. The question of the abundance of metorphamide in brain tissue relative to other opioid peptides is, therefore, of considerable interest. If metorphamide can be demonstrated to be present in significant quantities in various brain regions, it would raise the possibility that the peptide can serve as an endogenous ligand for μ opioid receptors.

To learn more about the generation of metorphamide from its precursor and its abundance in brain tissue relative to other opioid peptides, we undertook a study of its regional distribution in neural tissue and compared its concentration with those of eight other opioid peptides in the same extracts. To do so, we developed highly specific radioimmunoassays (RIAs) for the amidated octapeptide, as well as for [Met]-enkephalyl-Arg^6-Gly^7-Leu^8 (MERGL) and [Met]-en-

kephalyl-Arg^6-Phe^7 (MERF), two other opioid peptides generated from proenkephalin (Stern et al., 1979, 1981; Rossier et al., 1980; Kilpatrick et al., 1981). We used these RIAs in parallel with six previously established assays for other opioid peptides (Weber et al., 1982*a,c,* 1983*b;* Weber and Barchas, 1983) to compare concentrations within identical extracts of the bovine caudate-putamen (henceforth termed caudate), hypothalamus, cervical spinal cord, and neurointermediate lobe of the pituitary. Other RIAs performed on the extracts were for five peptides derived from prodynorphin [α-neoendorphin, β-neoendorphin, dynorphin A(1–17), dynorphin A(1–8), and dynorphin B (rimorphin)] and for [Leu]-enkephalin, which can be generated from either proenkephalin or prodynorphin (Zamir et al., 1984). We chose to assay for these relatively small opioids because these shorter forms are prevalent in brain tissue (Cone et al., 1983; Rossier et al., 1983; Giraud et al., 1984). We found that metorphamide is indeed present in significant quantities in bovine brain tissue, with its abundance being in the same range as that of other opioid peptides, particularly those derived from prodynorphin.

MATERIALS AND METHODS

Peptide synthesis

Synthetic metorphamide and four analogs and fragments of metorphamide with C-terminal amide groups were synthesized by the solid-phase method of Merrifield (1963) on *p*-methylbenzylhydrylamine resin (Matsueda and Stewart, 1981). An analog of metorphamide with a free carboxyl group and one with a glycine residue in position 9 were synthesized on carboxymethyl resins substituted with the respective amino acids. After synthesis, resins were treated with HF, and the cleaved peptides were purified by partition chromatography on a Sephadex G-25 column (2 × 30 cm). The column was packed with resin that had previously been swelled in the aqueous phase of a mixture of *n*-butanol/acetic acid/water (4:1:5 by volume). The crude peptides were dissolved and applied to the column in 2–4 ml of this mixture and were eluted with the organic phase. Fractions (4 ml) were collected, and aliquots were spotted onto silica gel TLC plates. The plates were developed in the organic phase of the column eluent, and peptide spots were visualized by spraying the plates with ninhydrin. The desired fractions were pooled, rotary-evaporated, taken up in distilled water, and lyophilized. The peptides thus obtained were homogeneous by TLC and reverse-phase HPLC. Amino acid analysis confirmed the correct composition of the synthetic peptides. All eight other synthetic peptides used in this study were obtained from Peninsula Laboratories (San Carlos, CA, U.S.A.).

RIA procedures

Antisera to synthetic peptides were raised in male or female New Zealand white rabbits by injecting peptide conjugated to bovine thyroglobulin with water-soluble carbodiimide, as previously described (Weber et al., 1982*b*). The resulting antisera were used in RIAs with synthetic peptides as standards and ^{125}I-labeled peptides (specific activity, ~100 Ci/mmol) as tracers. The iodination and RIA procedures have been described in earlier articles (Weber et al., 1982*a,b*). Antisera and RIAs for [Leu]-enkephalin (Weber et al., 1983*b*), α- and β-neoendorphin (Weber et al., 1982*a,c*), dynorphin B (Weber and Barchas, 1983), and dynorphin A(1–8) and A(1–17) (Weber et al., 1982*a*) have been characterized and described.

Tissue extraction

Bovine hypothalami, caudate nuclei, cervical spinal cords, and neurointermediate lobes of pituitaries were dissected at a local slaughterhouse and immediately frozen on dry ice. The frozen tissues were stored at −70°C until they were used, at which time they were weighed and then extracted in 10 volumes of acidic acetone (acetone/water/12 *M* HCl, 40:6:1 by volume) in a ground glass homogenizer or Osterizer blender. The supernatant was filtered through cheesecloth and defatted with ~10 volumes of petroleum ether (b.p. 35–65°C), and the residual organic solvent was removed from the aqueous phase by a constant nitrogen stream (5–10 h). The extract was centrifuged again at 30,000 *g* for 15 min, desalted, and concentrated with a C-18 Sep-Pak cartridge (Waters Associates). Studies of recovery efficiency of the extraction process were performed for metorphamide, MERF, MERGL, [Leu]-enkephalin, and dynorphin A(1–8) and A(1–17) by adding 100,000 cpm of each of these six radioiodinated tracer peptides to six individual caudate nuclei before homogenization. Each caudate plus tracer was homogenized, extracted, defatted, and exposed to a nitrogen stream exactly as described above. Separate aliquots of each extract were recentrifuged, desalted, and concentrated in an identical manner to the other tissue extracts. Eluates and effluents were counted for radioactivity, and the percentage of recovered tracer was calculated. Our experimental protocol gave an average recovery of 30.0 ± 2.0% (mean ± SEM).

For comparing the extraction efficiency of metorphamide in acidic acetone and acetic acid, bovine caudate nuclei were also extracted by boiling in 10 volumes of 0.1 *M* acetic acid for 15 min and then homogenized in an Osterizer blender. After centrifugation of the extract at 30,000 *g* for 15 min, 50-μl aliquots of the supernatant were directly assayed in the metorphamide RIA. In this case, the RIA was modified to accommodate up to 50 μl of 0.1 *M* acetic acid by diluting the synthetic metorphamide standards in 0.1 *M* acetic acid containing 0.1% bovine serum albumin. We found that acetic acid extraction yielded 12 pmol of immunoreactive metorphamide/g of tissue, whereas acidic acetone extraction yielded 22 pmol/g.

Chromatography procedures

Four caudate nuclei (40 g of tissue) were extracted, defatted, desalted, and concentrated as described above. The extract was diluted with an equal volume of acetic acid, to which 20,000 cpm of metorphamide tracer was added, and was chromatographed on a Sephadex G-50 column (2.5 × 120 cm) using 50% acetic acid as the eluent. Aliquots of the 5-ml fractions were assayed for all peptides except α-neoendorphin.

Aliquots of 1.5 ml from sequential fractions were pooled [fractions 47–50 for dynorphin B, 51–55 for α-neoendorphin, and 54–58 for both metorphamide and dynorphin A(1–8)], evaporated under a steady stream of nitrogen, and dissolved in 1 ml of 5% acetic acid. In each of the three sample HPLC runs, 250 μl of the extract and 50 μl of 5% acetic

acid containing (Gly^9)-^{125}I-metorphamide tracer (20,000 cpm; ~113 fmol) were combined and injected onto the octadecylsilica column (Altex Ultrasphere ODS; particle size, 5 μm; 4.9 × 250 mm). Blank runs preceded and standard runs followed each of the three sample runs. Injections for the standard runs contained 1.0 μg of the synthetic peptide plus tracer in 500 μl of 5% acetic acid. Metorphamide sulfoxide was formed by dissolving 5 μg of the peptide in 0.054% hydrogen peroxide and letting the mixture stand for 10 min before dilution with acetic acid. HPLC buffer A consisted of 50 m*M* dibasic sodium phosphate/phosphoric acid/5% methanol (pH 2.7), and buffer B was 100% acetonitrile. The flow rate was 1.5 ml/min, and fractions were collected over 1-min intervals. The gradient is described in the legend of Fig. 4 along with results of four RIAs performed on 100-μl aliquots of fractions that were evaporated under reduced pressure and reconstituted in 50 μl of RIA buffer before assay.

RESULTS

Specificity of antisera

The metorphamide antiserum (R1-3) that we developed and used in the RIA was sensitive (Fig. 2) and highly specific for the amidated C-terminal of the octapeptide. Table 1 shows the results of a cross-reactivity study in which five synthetic analogs and fragments of metorphamide as well as 13 related peptides were used for testing the antiserum's specificity. The RIA required the C-terminal amide group as part of the recognition site, because des-amide-metorphamide had virtually no cross-reactivity. Furthermore, it is clear that the RIA required the five C-terminal amino acids of metorphamide for full recognition, because shorter fragments had low or negligible cross-reactivities. It is interesting that the antibodies did not differentiate between leucine and methionine residues at position 5 of metorphamide, as both peptides showed full cross-reactivity.

Antisera to MERGL (R3-2) and MERF (R1-2) were also characterized for cross-reactivity to several synthetic peptides. The only detectable cross-reactivity our MERF antiserum displayed was to MERF-NH_2, whereas the MERGL antiserum displayed very slight binding to some C-terminally extended enkephalin sequences. It did not bind detectably to [Met]-enkephalin.

Affinity of metorphamide antiserum

The lower curve of Fig. 2 is a displacement curve in which synthetic metorphamide was used to displace ^{125}I-metorphamide tracer from the antigen binding sites of metorphamide R1-3 antiserum. The seven concentrations of synthetic standard displaced the tracer in a normal fashion, and the assay was highly sensitive, as indicated by the half-maximal displacement (IC_{50}) value of 450 p*M* (135 fmol/tube). Offset from the synthetic standard curve are RIA data of serial dilutions of hypothalamus extract 4. The parallel between the curves suggests that the antiserum is binding in an identical fashion to the synthetic and tissue-derived immunoreactive substances.

Chromatography

To confirm that the antigens from neural tissues recognized by our antibodies were similar or identical to those synthetic peptides against which the antibodies were raised, we compared the behavior of the RIA-immunoreactive substances from bovine caudate with that of metorphamide tracer in Sephadex G-50 gel permeation chromatography and followed this by reverse-phase HPLC of G-50 fractions. Immunoreactivity profiles of metorphamide, the dynorphins, and α-neoendorphin plus the peak locations of MERGL, MERF, and [Leu]-enkephalin in G-50 chromatography are displayed in Fig. 3. None of the RIAs showed immunoreactivity peaks preceding the ones indicated in Fig. 3. This suggests that extended forms of the eight opioid peptides were present at undetectably low levels in G-50 fractions, or else that our antisera do not cross-react with them.

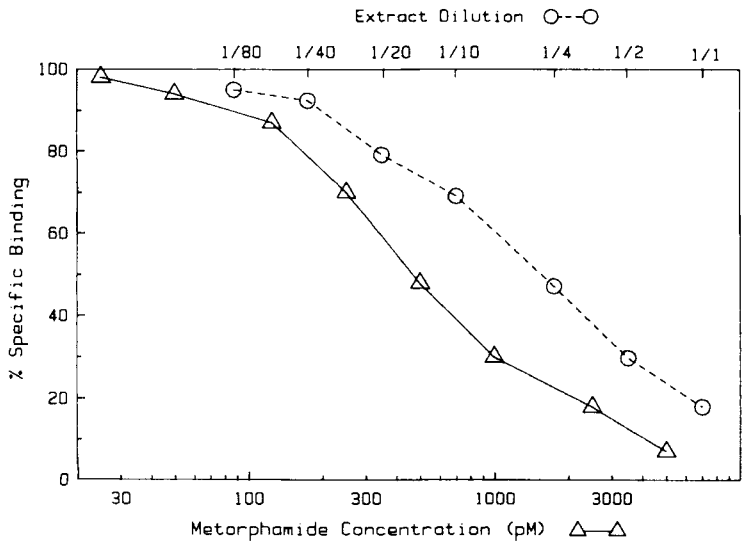

FIG. 2. Semilogarithmic plot of tracer displacement curves for metorphamide antiserum R1-3 using synthetic peptides (△) and hypothalamus extract 4 (○). The ordinate displays the percentage of radioactive tracer specifically bound by antiserum in the RIA. The abscissa for lower curve is the concentration of synthetic metorphamide in each assay tube, and that for the upper curve represents what fraction of the 50-μl RIA sample volume was extract (assay volume, 300 μl). The data for the two curves come from separate RIAs. The synthetic standard RIA was performed in quadruplicate. The hypothalamic extract RIA was performed in duplicate. The final dilution of antibody in both assays was 1:64,000. The upper curve is arbitrarily offset from the lower curve.

TABLE 1. *Specificity of antisera for Met-enkephalin-containing peptides*

Peptide	Antiserum (% cross-reactivity) Metorphamide R1-3	MERF R1-2	MERGL R3-2
Metorphamide (Y G G F M R R V-NH_2)	100		
Y G G F L R R V-NH_2	100		
Y G G F M R R V	0.03		
Y G G F M R R V G	0.02		
F M R R V-NH_2	100		
M R R V-NH_2	0.18		
R R V-NH_2	0.06		
BAM-22	0.005		
BAM-12	0.006		ND
Dynorphin A (1–8)	ND		0.005
Dynorphin A (1–17)		ND	
α-Neoendorphin		ND	0.004
[Leu]-Enkephalin		ND	ND
[Met]-Enkephalin	ND		ND
Y G G F M R	ND	ND	0.004
Y G G F M R R	ND	ND	0.003
MERF	ND	100	ND
MERF-NH_2		2	
MERGL	ND	ND	100
β-Endorphin			0.002

The percentage cross-reactivity was derived from RIAs in which a single antiserum and tracer were used with dilutions of the respective synthetic peptide standards, ranging from 10 p*M* to 1 μ*M*. Values of the concentrations of metorphamide, MERF, or MERGL that gave half-maximal inhibition of tracer binding to antiserum (IC_{50}) were divided by similar values for the peptides with which they were compared, yielding a percentage cross-reactivity. All RIAs were done in duplicate. Metorphamide antiserum R1-3 was used at a final dilution of 1:64,000, and the IC_{50} was 450 p*M* (135 fmol/tube); MERF antiserum R1-2 was used at 1:3,000, and the IC_{50} was 1,000 p*M* (300 fmol/tube); and MERGL antiserum R3-2 was used at 1:30,000, and the IC_{50} was 500 p*M* (150 fmol/tube). Single letter amino acid abbreviations are as follows: Y for tyrosine, G for glycine, F for phenylalanine, L for leucine, M for methionine, R for arginine, and V for valine. ND, no cross-reactivity detectable at a peptide concentration of 1 μ*M*; BAM, bovine adrenal medullary peptide.

Results of four RIAs performed on fractions of three HPLC runs are displayed in Fig. 4. Locations of immunoreactivity peaks in HPLC fractions for caudate-derived immunoreactive metorphamide, metorphamide sulfoxide, α-neoendorphin, and dynorphin A(1–8) never varied more than one fraction from those of the synthetic standards, whereas the extracted immunoreactive dynorphin B peak preceded the synthetic standard by two fractions. The plot of metorphamide immunoreactivity exhibits a major peak at precisely the fraction in which synthetic metorphamide sulfoxide elutes and a smaller peak preceding by

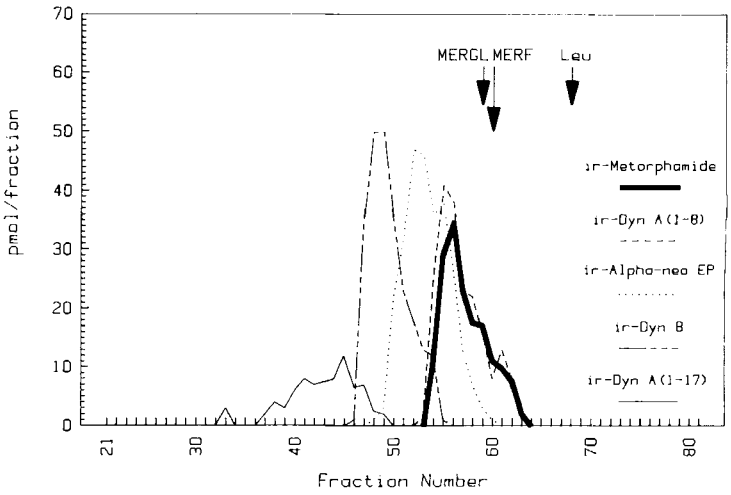

FIG. 3. Opioid peptide immunoreactivities of four extracted caudate nuclei in Sephadex G-50 gel permeation chromatography. Tissue extracts were prepared by the protocol described in Materials and Methods and diluted with an equal volume of acetic acid containing ~50,000 cpm of ^{125}I-metorphamide (~225 fmol). This mixture was loaded onto the 2.5- × 120-cm column and eluted with 50% acetic acid. Five-milliliter fractions were collected and counted for radioactivity. The peak of ^{125}I-metorphamide tracer coeluted with the immunoreactive (ir) metorphamide peak. Duplicate 5- or 20-μl aliquots of each fraction were dried under reduced pressure and RIAs for the eight peptides (not dynorphin B) were performed. The peak profiles of five peptide immunoreactivities are shown; the locations of the peak fraction of the remaining three peptide immunoreactivities are indicated by arrows. Dyn, dynorphin; Leu, [Leu]-enkephalin; neo EP, neoendorphin.

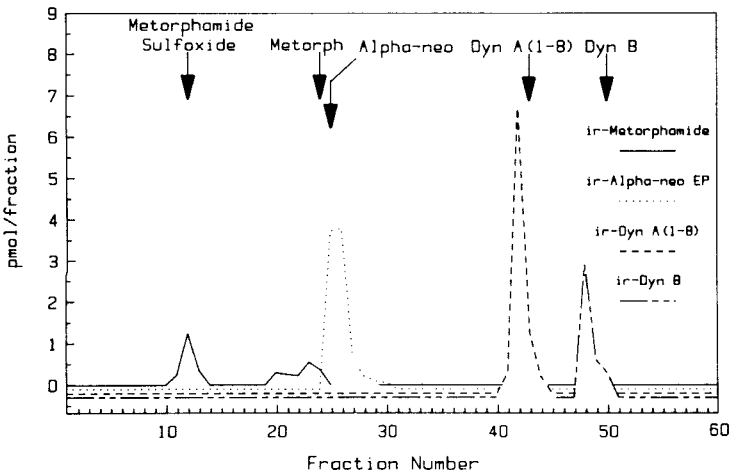

FIG. 4. RIAs for opioid immunoreactivity following reverse-phase HPLC of G-50 extracts. Arrows indicate peak fractions of standard runs (see text for preparation details). Four RIAs as indicated in the graph were performed on aliquots of three HPLC sample runs. The metorphamide (Metorph) and dynorphin (Dyn) A(1–8) RIAs were performed on aliquots of HPLC fractions collected after pooled, reconstituted aliquots from G-50 fractions 54–58 were run. The α-neoendorphin (Alpha-neo EP) RIA was done on the HPLC fractions following injection of pooled G-50 fraction 51–55, and the dynorphin B RIA on the HPLC fractions was done after injection of pooled G-50 fraction 47–50. The flow rate was 1.5 ml/min. Fractions were collected over 1-min intervals. The acetonitrile gradient was in 50 m*M* dibasic sodium phosphate/0.1% phosphoric acid/5% methanol (pH 2.7): 0–10% acetonitrile in 5 min; 10–20% in 50 min; and 20–60% in 10 min. We used an Altex Ultrasphere ODS (particle size, 5 μm) 4.9- × 250-mm column. Duplicate 100-μl aliquots of HPLC fractions were dried under reduced pressure before reconstitution with RIA buffer during the assay. ir, immunoreactive.

one fraction the position of synthetic metorphamide. It is likely that the sulfoxide derivative of metorphamide forms during the gel permeation chromatography step. We have previously reported that immunoreactive metorphamide from extracts of whole guinea pig brain elutes in reverse-phase HPLC at the position of synthetic metorphamide sulfoxide if the extracts have first been run through a G-50 gel permeation chromatography column in 50% acetic acid (Sonders et al., 1984). If the extracts are assayed directly by HPLC, immunoreactive metorphamide coelutes with synthetic metorphamide. In accordance with the cross-reactivity data (Table 1) that Met^5 is not a strict requirement for antibody recognition, the sulfoxide form of metorphamide is readily detected by the antiserum.

RIA results on brain regions

Concentrations of immunoreactive metorphamide and eight other opioid peptides from identical extracts of three different regions of the bovine nervous system and of pituitary are listed in Table 2 and graphed in Fig. 5. The neurointermediate lobe of the pituitary held the highest and the cervical spinal cord the lowest concentrations of all nine peptides. All of the proenkephalin-derived peptides, [Leu]-enkephalin included, were more concentrated in the caudate than in the hypothalamus, whereas the prodynorphin-derived peptides, with the notable exception of dynorphin A(1–8), were more abundant in the hypothalamus. The immunoreactive metorphamide concentration was found to range between 6 and 18% of the concentration of MERF or MERGL and between 10 and 26% of the [Leu]-enkephalin concentration. In all tissues, the concentration of immunoreactive metorphamide was in the same range as the concentrations of the dynorphin A or neoendorphin peptides (Table 2 and Figs. 3 and 4). The values for the peptides given in Table 2 and Fig. 4 have not been corrected for recovery. The average extraction efficiency for the peptides (not determined for α-neoendorphin) was determined to be 30 ± 2.0% (mean ± SEM); thus, the true concentrations of the opioid peptides in regions of the bovine nervous system are likely to be three (or more) times as high as the values indicated.

DISCUSSION

In this article, we report the development and use of a highly specific RIA for the amidated opioid octapeptide metorphamide. From our cross-reactivity data, it is clear that the recognition site of the antibody used in the RIA requires at least the five C-terminal amino acids and a terminal α-amide group for binding. Several lines of evidence indicate that the peptide recognized by our antiserum is authentic metorphamide: (a) Metorphamide is present in the caudate, because it was previously isolated and sequenced from that tissue (Weber et al., 1983*a*). (b) Immunoreactive metorphamide in serial dilutions of hypothalamic extract behaves like the synthetic peptide in RIAs. (c) The immunoreactivity peak of caudate extract in Sephadex G-50 gel permeation chromatography coincides precisely with the radioactivity peak of synthetic ^{125}I-metorphamide. (d) The metorphamide immunoreactivity peaks of the current caudate extract cochromatograph in separate HPLC assays with peaks of synthetic metorphamide and its sulfoxide form. Although the specificity of our antiserum unequivocally detects the C-terminal of metorphamide, the isolation and chromatographic data indicate that the metorphamide in our extracts is not N-terminally extended.

Immunoreactive metorphamide levels in the areas surveyed are, on average, 12% of those of MERGL and 10% of MERF. We have previously demonstrated metorphamide immunoreactivity in eight brain re-

TABLE 2. *Concentrations of immunoreactive opioid peptides in bovine brain regions determined by RIA*

Region	Immunoreactivity (pmol/g of tissue)								
	Neoendorphin		Dynorphin A		Dynorphin B	Metorph-amide	[Leu]-Enkephalin	MERGL	MERF
	β	α	(1–8)	(1–17)					
Neurointermediate lobe (n = 6)	4,825 ± 829	355 ± 59	784 ± 127	180 ± 41	791 ± 125	147 ± 27	1,441 ± 317	1,390 ± 228	3,049 ± 505
Caudate (n = 4)	9.0 ± 0.2	15.0 ± 0.9	34.8 ± 1.1	5.3 ± 0.4	43.7 ± 3.1	22.0 ± 3.0	83.8 ± 3.9	120.2 ± 9.0	171.4 ± 6.1
Hypothalamus (n = 6)	22.3 ± 2.1	85.3 ± 11.7	22.4 ± 2.4	12.5 ± 1.5	56.5 ± 5.3	6.5 ± 1.1	39.6 ± 9.7	58.3 ± 8.6	112.0 ± 18.4
Spinal cord (n = 6)	7.17 ± 1.55	4.59 ± 0.86	1.21 ± 0.28	3.39 ± 0.74	3.58 ± 0.74	1.69 ± 0.22	12.32 ± 0.69	21.08 ± 2.22	12.27 ± 1.25

Data are mean ± SEM values from n individually extracted issues. Eight dilutions of each extract were assayed in duplicate. Those closest to the IC_{50} of the individual assay were chosen for averaging.

gions of male Sprague-Dawley rats and guinea pigs, and like its pattern of distribution in the cow, rodent brain metorphamide concentrations vary regionally between 10 and 20% those of MERGL or MERF (Sonders et al., 1984; Zamir et al., 1985). Levels of metorphamide immunoreactivity in cow caudate, hypothalamus, and spinal cord are comparable to those in rat brain regions, although guinea pig levels are two to seven times higher. A notable distinction between the cow and rodent patterns is that in the neurointermediate lobes of neither rodent did we detect metorphamide, whereas among bovine tissues assayed, it was most concentrated in the pituitary. In contrast to the relatively constant ratios of MERF and MERGL to metorphamide in brain regions and pituitary, the ratios of the opioid products derived from the prodynorphin precursor vary much more widely, a finding suggesting regional differences in the specificities of synthetic or degradative proteolysis. It seems likely that the observed differences in peptide ratios result from region-specific proteolysis; however, we cannot rule out the possibility that differential axonal transport of opioids may be the mechanism responsible for the apparent concentration differences.

Although neoendorphin, dynorphin A, and dynorphin B sequences exist in stoichiometric ratios within the prodynorphin precursor molecule, we find about five times as much of the neoendorphin peptides as dynorphin A peptides or dynorphin B in the neurointermediate lobe. In the caudate, however, both dynorphin B and the sum of dynorphin A(1–8) and A(1–17) peptides were twice as abundant as the neoendorphins. MERF and MERGL are also present in proenkephalin in a stoichiometric ratio, and yet MERF is

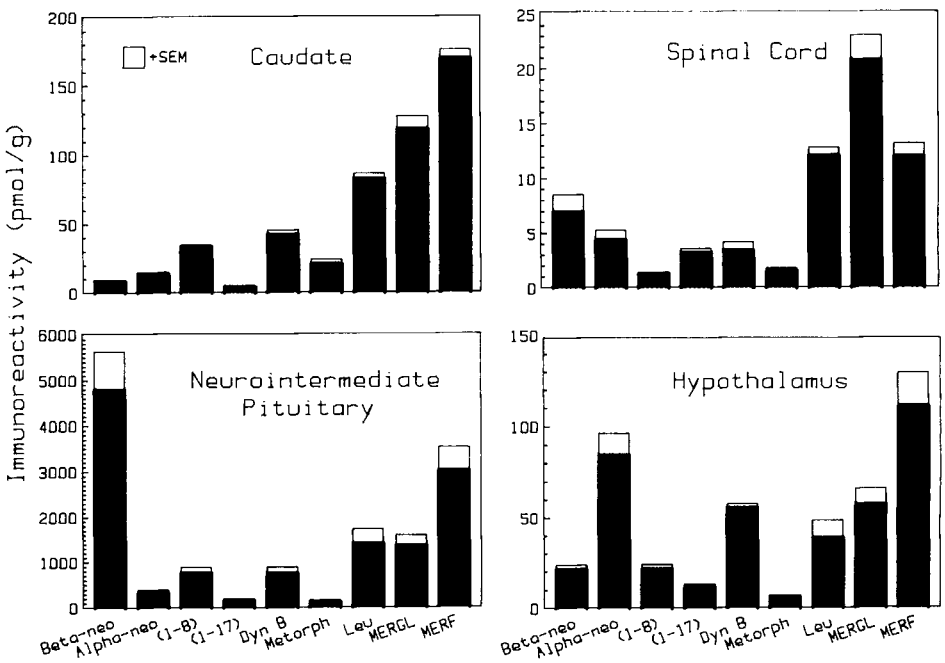

FIG. 5. Graphic representation of opioid peptide concentrations determined from RIAs (see Table 2). Beta-neo and Alpha-neo, β- and α-neoendorphin, respectively; (1–8) and (1–17), dynorphin A(1–8) and A(1–17), respectively; Dyn B, dynorphin B; Metorph, metorphamide; Leu, [Leu]-enkephalin.

1.4 to three times as abundant as MERGL in brain and pituitary, although only about half as abundant in spinal cord.

Another pattern of differential proteolysis our data highlight is that in which a single bioactive sequence can be cleaved into a shorter one that has perhaps variant bioactivity (Corbett et al., 1982; James et al., 1984). This sort of proteolysis presumably takes place in the cleavage of Lys^{10} from α-neoendorphin to yield β-neoendorphin or in the cleavage of Ile^8-Arg^9 within the dynorphin A(1–17) sequence to yield dynorphin A(1–8). The neurointermediate pituitary and hypothalamus display the most divergent ratios of α- to β-neoendorphin: roughly 13 versus 0.25. The spinal cord is also unusual in this regard, because it is the only region assayed with a higher concentration of dynorphin A(1–17) than A(1–8). Also, in the spinal cord, the concentration of metorphamide is most similar to that of dynorphin A(1–8), whereas it is closer to that of dynorphin A(1–17) in the neurointermediate lobe and hypothalamus. The concentration of metorphamide in the caudate is intermediate between those of dynorphin A(1–8) and A(1–17).

Besides the concentration values, there are other similarities between metorphamide and dynorphin A(1–8). Most obvious is that the two octapeptides are identical in six of their amino acids: Dynorphin A(1–8) has a leucine in position 5 rather than a methionine and an isoleucine rather than an α-amidated valine at the C-terminal. Both peptides contain intact double arginine residues within their extracted forms, even though such double basic sequences can serve as cleavage sites. The Arg^7 has been demonstrated to be important for conferring κ receptor activity in the guinea pig ileum assay (Chavkin and Goldstein, 1981; Corbett et al., 1982), although the neoendorphins act at κ sites yet have a Lys^7.

A second similarity is their location within the precursor molecules, with metorphamide starting at position 206 of the bovine precursor and dynorphin A at 207 of the human precursor. Both are followed by [Leu]-enkephalin sequences starting at position 226, and C-terminally extended forms of both metorphamide and dynorphin A are known that end with [Leu]-enkephalin and dynorphin B sequences: peptide E and dynorphin-32, respectively.

A third similarity between metorphamide and dynorphin A(1–8) is that the cleavage sites for generation of the two peptides from their respective precursors are identical: a conventional double basic amino acid pair, Lys-Arg, at the N-terminal and an atypical single basic Arg-Pro at the C-terminal. A glycine residue follows metorphamide's Val^8 and precedes the Arg-Pro sequence, which is the likely source of metorphamide's C-terminal α-amide group, given the near universality of the X-Gly-basic-basic (Mains et al., 1983; Richter, 1983) precursor sequence to amidated neural and hormonal peptides. It should be noted that several other bioactive peptides, including dynorphin B, are generated from precursors by cleavage at single arginine residues, although only somatocrinin (growth hormone-releasing factor) is also known to undergo a similar C-terminal amidation adjacent to the single arginine cleavage site (-Leu-Gly/Arg/Gln-Val-) (Gubler et al., 1983).

Metorphamide's C-terminal α-amide group is critical in conferring on the opioid peptide its notably high affinity to the μ opioid receptors and, to a lesser extent, to the κ opioid receptor (Weber et al., 1983*a*). The C-terminal α-amidation also renders metorphamide stable to carboxypeptidase A degradation (Weber et al., 1983*a*). Such resistance may be suggestive of a neuroendocrine role for metorphamide. Supporting evidence for this role comes from the finding that the highest concentration of metorphamide we have found in any tissue is from 300 to 500 pmol/g in the human adrenal medulla (authors' unpublished data). Miyata et al. (1984) also reported that the metorphamide content in that tissue is nearly equal to that of MERF. C-terminal amidation is widespread among peptide neurohormones and transmitters such as substance P, Arg-vasopressin, vasoactive intestinal polypeptide, gastrin, gastrin-releasing peptide, neuropeptide Y, α-melanotropin, calcitonin, calcitonin-gene-related peptide, corticotropin-releasing factor, thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, and others. Some of these peptides have well-defined functions, but little characterization of metorphamide's physiological effects has yet been done. Preliminary experiments have shown that intracerebroventricular and intracisternal application of metorphamide in mice and rabbits produces naloxone-reversible analgesia, respiratory depression, and bradycardia (Xu et al., 1985). As analgesia is thought to be mediated by μ opioid receptors, this finding may suggest an interaction of metorphamide with μ opioid receptors under certain in vivo experimental conditions. This is an interesting observation, because metorphamide has particularly high affinity for μ opioid receptors in vitro (Weber et al., 1983*a*). These findings also raise the possibility that metorphamide may be a physiologically important endogenous μ opioid receptor ligand.

A functional role of metorphamide as an endogenous μ opioid receptor ligand requires that the peptide be present in neural tissue in sufficient quantities to allow activation of μ opioid receptors in response to physiological stimuli. The results of the present study demonstrate that metorphamide indeed occurs in significant quantities relative to other opioid peptides in the CNS. It has been noted that metorphamide is present in significantly lower amounts in the brain than either MERF and MERGL (Miyata et al., 1984; Sonders et al., 1984; present study), two major proenkephalin processing products. However, metorphamide is considerably more potent than MERF, MERGL, or [Met]-enkephalin in several opioid receptor binding assays and bioassays (Weber et al., 1983*a,b*). Indeed,

the affinity of metorphamide to the μ opioid receptors is similar to the affinities of α-neoendorphin and dynorphin A(1–8) to κ opioid receptors (Corbett et al., 1982; Höllt et al., 1983). As the concentration of metorphamide is in the same range as that of dynorphin A(1–8) and α-neoendorphin, it is likely that the amounts of metorphamide in CNS tissue are sufficient to allow for a physiological interaction of metorphamide with the μ opioid receptor, just as the amounts of endogenous dynorphin A(1–8) and α-neoendorphin are thought to be sufficient to allow for a physiological interaction of those peptides with κ opioid receptors. Physiological studies are required to confirm the possible role of metorphamide as an endogenous μ opioid receptor ligand.

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