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Characterization of an Endogenous Morphine-like Factor (Enkephalin) in Mammalian Brain

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SUMMARY

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Mammalian brain extracts contain a substance, "enkephalin," which competes for opiate receptor binding in a highly selective fashion. This material behaves in opiate receptor assays as an opiate agonist, with its effects enhanced by manganese and decreased by sodium. The regional distribution of enkephalin parallels that of the opiate receptor itself. Enkephalin activity is degraded by carboxypeptidases A and B and leucinaminopeptidase as well as by chymotrypsin, but is resistant to trypsin and neuraminidase. Thus enkephalin appears to be a peptide. Gel filtration experiments indicate a molecular weight of about 1000.

INTRODUCTION

The striking selectivity of specific opiate binding sites in the brain in terms of their substrate specificity (1-3), regional localization (4, 5), and correlation with pharmacological activity (6) suggests that these sites do not exist fortuitously but might represent receptor sites for a normally occurring opiate-like material. Hughes (7) has elegantly characterized a small peptide in mammalian brain whose regional distribution closely resembles that of the opiate receptor itself and which mimics the capacity of morphine to inhibit electrically induced contractions of smooth muscle preparations. Terenius and Wahlstrom (8, 9) and Pasternak *et al.* (10) described a substance extracted from the brain tissue, which appears to be the same as that reported by Hughes (7), that competes for opiate receptor binding. Cox *et al.* (11) studied a peptide-like material from the pituitary gland which affects smooth muscle preparations in a fashion similar to morphine but whose chemical properties are different from the morphine-like substance in brain tissue. In the present study we describe the properties of a morphine-like factor, which we refer to as "enkephalin,"[4] in mammalian brain tissue.

MATERIALS AND METHODS

Trypsin (205 units/mg), chymotrypsin (45 units/mg), carboxypeptidase A (diisopropyl fluorophosphate, 83.3 units/mg; 61.3 mg/ml), carboxypeptidase B (179 units/mg; 20 mg/ml) leucinaminopeptidase (100 units/mg; 3.54 mg/ml), and neuramin-

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[4] The morphine-like activity of tissue extracts has been named enkephalin (7), morphine-like factors (MLF) (9, 10), and pituitary opiate peptide (POP) (11).

idase (1.13 units/mg) were purchased from Worthington Biochemicals Corporation. [^{3}H]Naloxone (23.6 Ci/mmole) and [^{3}H]dihydromorphine (46 Ci/mmole) were obtained from New England Nuclear Corporation. Levallorphan was the generous gift of Hoffmann-La Roche.

Binding assays. Membranes from male Sprague-Dawley rat brains, minus cerebella, were prepared and assayed as previously described (12). Opiate-specific binding is defined as the difference in binding in the presence of 1 μM levallorphan from that in its absence. All values are opiate-specific binding expressed as the means of triplicate determinations, which varied less than 10% unless otherwise indicated. Total binding was routinely 4–7 times the binding in the presence of 1 μM levallorphan, depending upon the opiate and the concentration used.

Tissue extraction. Brain tissue was homogenized in 10 volumes of 0.32 M sucrose in a glass tube with a Teflon homogenizer and then centrifuged at 100,000 $\times$ *g* for 1 hr. In some later experiments the homogenate was centrifuged at 1000 $\times$ *g* for 10 min, the pellet was discarded, and the supernatant was then centrifuged at 100,000 $\times$ *g* for 1 hr. Similar results were obtained with both types of tissue preparation. The pellet remaining after the 100,000 $\times$ *g* centrifugation was resuspended in 2 volumes of 10 mM Tris-HCl buffer (pH 7.7 at 25°) and immersed in a boiling water bath for 15 min. Enkephalin activity is not altered by boiling for up to 60 min at neutral pH. The boiled homogenate was centrifuged at 100,000 $\times$ *g* for 1 hr, and the clear supernatant was either used immediately or lyophilized. One unit of enkephalin activity was defined as the amount of which yields 50% receptor occupancy in the standard binding assay described above, containing 1 mM $MnCl_2$ and determined according to Colquhoun (13), assuming classical binding interactions. Values determined in this way are a function of assay conditions, including the presence of particular ions.

RESULTS

Regional localization of enkephalin and opiate receptor binding. In preliminary experiments (10) the regional variation in enkephalin content in the rat and calf brain tissue resembled variations in opiate receptor binding. Definitive evidence that enkephalin levels differ regionally requires that in all brain areas enkephalin activity vary linearly with the amount of tissue extract. In nine discrete regions of calf brain, we measured enkephalin content at three concentrations of tissue extract (Table 1). In the same brain regions opiate receptor binding was determined by the stereospecific binding of [^{3}H]naloxone.

The ability of enkephalin to inhibit opiate receptor binding is dose-related and not restricted to one opiate. In all brain regions examined, enkephalin activity increases progressively with amount of tissue extract (Fig. 1 and Table 1). Approximately half-maximal inhibition of both [^{3}H]dihydromorphine and [^{3}H]naloxone binding occurs at enkephalin concentrations corresponding to 37 mg of original tissue weight. Similar inhibition of ^{3}H-opiate receptor binding was obtained using [^{3}H]levorphanol, [^{3}H]levallorphan, [^{3}H]-diprenorphine, and [^{3}H]etorphine. Thus enkephalin activity relates to selective competition for opiate receptor sites regardless of the ^{3}H-ligand.

Regional differences in enkephalin activity closely resemble those of opiate receptor binding. The caudate nucleus has the highest levels of both, with somewhat lower contents in the hypothalamus. The enkephalin concentration in spinal cord and pons is about one-third the level in the caudate nucleus, while opiate receptor binding in these two regions is about two-thirds of the caudate nucleus values. In the parietal cerebral cortex and thalamus, enkephalin concentration is about one-sixth of the caudate nucleus values, while levels of enkephalin in the cerebellum and medulla oblongata are only about $^1/_{10}$ of those in the caudate nucleus. The lowest levels of both opiate receptor binding and enkephalin occur in the corpus callosum, whose enkephalin content is only about $^1/_{50}$ of that of the caudate nucleus. Although the rank orders for enkephalin and opiate receptor binding are the same in the brain regions examined, regional variations are more marked for enkephalin than for opiate receptor binding. These re-

TABLE 1

Regional Localization of enkephalin and opiate receptor binding

Bovine brain regions were dissected and homogenized in 0.32 M sucrose, and a small aliquot was assayed for [^{3}H]naloxone binding. The remainder was extracted for enkephalin as described in MATERIALS AND METHODS. Quantities of extract equivalent to the wet weight of tissue extracted were then added in a standard binding assay with [^{3}H]naloxone and 1 mM $MnCl_2$, and units of inhibition were determined as described in the text. [^{3}H]Naloxone binding was determined by assaying the aliquots from the various regions with [^{3}H]naloxone and 100 mM NaCl. Regional distribution experiments were repeated three times. "Protein" values of enkephalin extracts were determined by the method of Lowry *et al.* (14).

Tissue extracted	Weight	Enkephalin distribution			Opiate receptor [^{3}H]naloxone binding
		[^{3}H]Naloxone binding	Calculated amount of enkephalin	Enkephalin activity	
	mg	*cpm*	*units*	*units/mg protein*	*cpm/mg protein*
Control		2865	0	0	
Caudate nucleus	25	1250	5.4	480	3195
	50	820	11.0		
	100	400	28		
Hypothalamus	25	1655	2.8	250	2820
	50	1265	5.2		
	100	700	13.6		
Spinal cord	25	1990	1.5	140	2050
	50	1490	3.8		
	100	1125	6.6		
Pons	25	2107	1.2	135	2310
	50	1650	2.8		
	100	1250	2.7		
Parietal cerebral cortex	25	2110	1.2	80	1735
	50	1780	2.4		
	100	1340	4.6		
Thalamus	25	2315	0.6	75	1795
	50	1990	1.5		
	100	1430	4.0		
Cerebellum	25	2330	0.6	50	860
	50	2070	1.2		
	100	1670	2.8		
Medulla oblongata	25	2170	1.0	50	885
	50	1900	1.8		
	100	1920	1.8		
Corpus callosum	25	2340	0.5	10	600
	50	2621	<0.1		
	100	2500	0.1		

gional variations are generally, but not entirely, similar to those reported by Hughes in rabbit brain (7), by Pasternak *et al.* (10) in rat brain, and by Simantov *et al.* (15) in monkey brain.

If enkephalin is a normally occurring

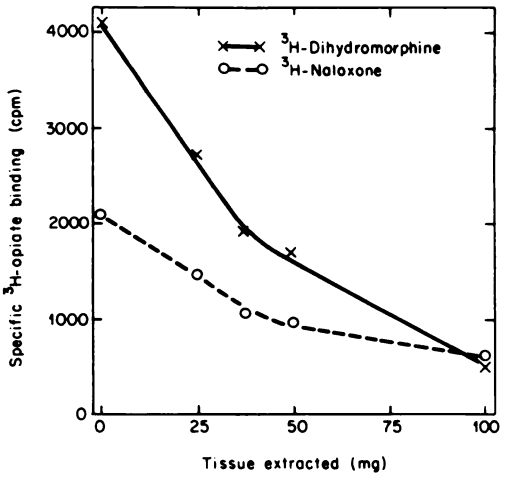

FIG. 1. *Concentration dependence for enkephalin activity of calf caudate nucleus extracts*

Various amounts of extract from calf caudate nucleus (expressed as milligrams of tissue extracted) were included in a standard assay with [^{3}H]- naloxone (40,000 cpm/tube) or [^{3}H]dihydromorphine (50,000 cpm/tube) and 1 mM $MnCl_2$. Specific opiate binding was determined from triplicate samples as described in MATERIALS AND METHODS. Binding values varied less than 10%, and the experiment was repeated three times.

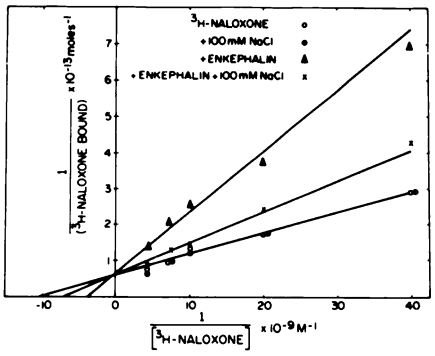

FIG. 2. *Effect of varying [^{3}H]naloxone concentration on enkephalin inhibition of opiate receptor binding*

Calf caudate nucleus extract equivalent to 50 mg of tissue, wet weight, was added to a standard binding assay with [^{3}H]naloxone (0.25–2.3 nM) with and without NaCl (100 mM). Opiate-specific binding was determined from triplicate samples as previously described (12). Binding of [^{3}H]naloxone in the absence of enkephalin was identical in the presence and absence of NaCl, consistent with the finding that preliminary incubation abolishes the sodium-induced increase in [^{3}H]naloxone binding (12). Thus a single line has been drawn for control binding in the presence and absence of NaCl (● and ○).

substrate for the opiate receptor, one might expect it to compete directly with opiates for receptor binding. Examination of the influence of enkephalin on the binding of various concentrations of [^{3}H]- naloxone indicates that enkephalin does inhibit opiate receptor binding in a competitive fashion (Fig. 2).

Opiate agonist properties of enkephalin. Several properties of opiate receptor binding distinguish agonists from antagonists. Low concentrations of sodium selectively depress agonist binding (16, 17), as do low concentrations of protein-modifying reagents (12) and certain enzymes (18). By contrast, manganese and certain other divalent cations selectively enhance the binding of opiate agonists (19). Accordingly, we sought to determine how treatment of opiate receptor-containing brain membranes with ions and reagents might influence the ability of enkephalin to compete for opiate binding sites. Sodium chloride in concentrations of 5–100 mM markedly depressed the ability of enkephalin to inhibit [^{3}H]naloxone binding (10). This represents a selective effect of sodium, because similar concentrations of potassium chloride have no effect on enkephalin inhibition of naloxone binding. This accords with our previous observations that sodium, but not potassium, selectively decreases opiate agonist binding (16, 17). Manganese, which enhances agonist binding (19), facilitates the inhibition by enkephalin of [^{3}H]- naloxone binding (10) (Fig. 3). The influence of these ions on enkephalin inhibition of naloxone binding suggests that enkephalin functions as an opiate agonist. These observations are consistent with the direct demonstration by Hughes (7) that enkephalin has opiate agonist biological activity, and resemble earlier observations with these ions (9, 10).

Previously we reported that opiate agonists and antagonists are differentiated by the extent to which sodium alters their ability to bind to the opiate receptor. Different variations have been found for the influence of manganese upon receptor binding of opiates. The extent to which sodium or manganese alters the potency of

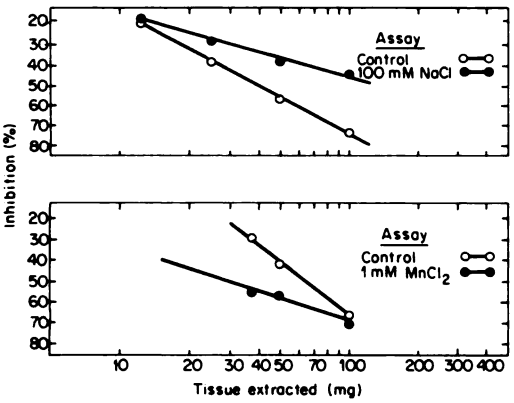

FIG. 3. *Log probit analysis of sodium and manganese effects on enkephalin inhibition of [^{3}H]- naloxone binding*

Various amounts of calf caudate nucleus extract, equivalent to the amount of tissue extracted, were added to a standard assay using [^{3}H]naloxone (50,000 cpm/tube) and the appropriate ion (NaCl, 100 mM; $MnCl_2$, 1 mM). Opiate-specific binding was determined from triplicate samples, which varied less than 10%, and inhibition was determined from the appropriate control. The two ions were tested in different experiments with different batches of enkephalin. Each experiment was repeated three times.

opiates in competing for [^{3}H]naloxone binding sites predicts the character of drugs as pure antagonists, agonists, or mixed agonist-antagonists (16, 17). The extent of the shifts in potency produced by various ions depends on the condition of the assay procedure, especially temperature (20). Under the present experimental conditions morphine and dihydromorphine become 2–3 times less potent in the presence of 100 mM NaCl than in sodium-free medium, while they become 1.3–1.5 times more potent when assayed with 1 mM manganese chloride. The potency of enkephalin as an inhibitor of [^{3}H]naloxone binding is reduced about 3-fold in the presence of 100 mM NaCl and increased about 2-fold in the presence of 1 mM manganese chloride, resembling the behavior of morphine or dihydromorphine (Fig. 3). By contrast, under the same experimental conditions, antagonists such as naloxone are unaffected by both NaCl or manganese chloride, and mixed agonist-antagonists would display intermediate changes (19).

Low concentrations of protein-modifying reagents and enzymes such as trypsin and chymotrypsin alter the opiate receptor to decrease agonist binding with no influence on antagonist binding (12, 18). These reagents and enzymes act by increasing the sensitivity of agonist binding to sodium-induced decreases, and their effects are only minimally apparent in the absence of sodium. Accordingly, we compared the influence of three protein-modifying reagents, trypsin and chymotrypsin treatment of brain membranes upon the ability of enkephalin to compete for [^{3}H]naloxone and [^{3}H]dihydromorphine binding in the presence and absence of sodium, (Table 2). In the absence of sodium enkephalin inhibition of [^{3}H]naloxone binding is affected only minimally and to a variable

TABLE 2

Enkephalin inhibition of opiate binding to receptors treated with reagents and enzymes

Rat brain membranes were prepared and treated with reagents as previously described (12). Specific binding of [^{3}H]naloxone in untreated tissue was 1536 cpm and 1636 cpm in the presence and absence of 100 mM NaCl. Enkephalin extracts (from 50 mg of calf caudate nucleus) lowered binding 48% in the presence and 65% in the absence of NaCl. Trypsin (0.5 μg/ml), chymotrypsin (50 μg/ml), *N*-ethylmaleimide (10 μM), iodoacetamide (5 mM), and *p*-chloromercuribenzoate (10 μM) inhibited the binding of [^{3}H]naloxone 20%, 45%, 0%, 0%, and 8% in the presence of NaCl and 68%, 74%, 28%, 13%, and 52% in the absence of NaCl, and inhibited the binding of [^{3}H]dihydromorphine 74%, 82%, 63%, 68%, and 81% in the presence of NaCl and 59%, 76%, 46%, 54%, and 60% in the absence of NaCl, respectively. All values presented are specific opiate binding determined from triplicate samples, which varied by less than 8%. The experiment was replicated three times.

Receptor treatment	Percent of inhibition in untreated tissue			
	[^{3}H]Naloxone		[^{3}H]Dihydromorphine	
	+NaCl	−NaCl	+NaCl	−NaCl
	%	%	%	%
Trypsin	46	79	110	118
Chymotrypsin	55	118	110	124
N-Ethylmaleimide	55	94	110	107
Iodoacetamide	51	113	115	134
p-Chloromercuribenzoate	64	75	105	110

extent by treating membranes with trypsin, chymotrypsin, *N*-ethylmaleimide, iodoacetamide, or *p*-chloromercuribenzoate. However, in the presence of 100 mM NaCl, which facilitates the depression of agonist binding, all these treatments reduce by 40–50% the ability of enkephalin to compete for [^{3}H]naloxone binding sites. Thus, in terms of responses to protein-modifying reagents and enzymes, enkephalin again behaves as an opiate agonist.

These treatments should affect receptor binding of dihydromorphine, an agonist, in the same way as they affect enkephalin itself, so that one would not predict any changes in competition by enkephalin for [^{3}H]dihydromorphine binding sites. In accordance with this prediction, none of the enzymatic or protein-modifying reagents alters the extent to which enkephalin competes for [^{3}H]dihydromorphine binding sites, whether assays are conducted in the presence or absence of sodium. It should be noted that all these treatments produce a marked depression of [^{3}H]dihydromorphine binding itself, which is greater when assays are conducted in the presence of sodium.

Cross-reactivity of enkephalin and opiate receptor binding in different species. The drug specificity of the opiate receptor is closely similar in numerous vertebrate species (21). Differences in some properties of partially purified enkephalin from various species (7–10) suggest that there may be chemical distinctions in the structure of enkephalin from different animal sources. If such differences were physiologically important, enkephalin-receptor interactions might be species-specific, so that enkephalin from one species might have greater affinity for the opiate receptor of the same than of other species. To evaluate this possibility, we compared the abilities of enkephalin preparations from rat, calf, and guinea pig brain to compete for receptor binding in membrane preparations from these three species (Table 3). In all species, inhibition of [^{3}H]dihydro- morphine binding is dependent on the enkephalin concentration. Enkephalin from each species produces the same degree of

TABLE 3

Cross-reactivity of enkephalin from rat, calf, and guinea pig

Enkephalin from rat, calf, and guinea pig was prepared as described in MATERIALS AND METHODS and lyophilized. Appropriate amounts of enkephalin were then added to a standard binding assay containing [^{3}H]dihydromorphine and tissue from whole rat brain, guinea pig brain, or calf caudate nucleus, all prepared with a preliminary incubation as previously described (12).

Species	Enkephalin	Receptor species					
		[^{3}H]Dihydromorphine binding			Enkephalin inhibition		
		Rat	Calf	Guinea pig	Rat	Calf	Guinea pig
	mg	*cpm*	*cpm*	*cpm*	*%*	*%*	*%*
	None	4006	5266	2385			
Rat	1	3557	4520	2127	12	15	11
	2	3190	3893	1765	20	27	26
	5	2398	2806	1203	40	47	50
	10	1421	1651	596	65	69	75
Calf	1	3023	3737	1731	25	29	28
	2	2229	2822	1164	44	46	51
	5	1227	1222	458	69	77	81
	10	401	411	0	90	92	100
Guinea pig	1	3298	4311	1961	18	18	18
	2	2808	3610	1577	30	32	34
	5	1886	2359	872	53	55	63
	10	846	1139	326	79	78	86

inhibition of opiate receptor binding in membranes from all three species. Moreover, the pattern of increasing opiate receptor competition with increasing enkephalin concentration is the same for all species. This suggests that enkephalin-receptor interactions do not vary markedly among these species.

Sensitivity of enkephalin extracts to enzymatic degradation. Hughes (7) suggested that brain enkephalin is a peptide, because it is readily degraded by carboxypeptidase A and leucinaminopeptidase. We assessed the sensitivity of enkephalin from calf brain to treatment with a variety of enzymes (Table 4). Carboxypeptidase A, in a dose-dependent fashion, inhibits the ability of calf enkephalin to compete for [^{3}H]naloxone binding. Maximal effects are produced to a similar extent by carboxypeptidases A and B and leucinaminopeptidase (Table 4), similar to results with rat enkephalin (10). By contrast, trypsin and neuraminidase have negligible effects upon brain enkephalin from either the calf or, as previously observed (10), from the rat. Although chymotrypsin markedly reduces the activity of enkephalin preparations from rat (10), it has considerably less effect on calf enkephalin.

The relative influences of these enzymes upon calf brain enkephalin are very similar to those reported by Hughes for enkephalin preparations from the pig brain (7, 22), but differ from pituitary enkephalin, which is sensitive to trypsin but resistant to carboxypeptidases A and B and leucinaminopeptidase.

Gel chromatography of enkephalin. To estimate the molecular weight of brain enkephalin, to assess whether the enkephalin activity of crude extracts is attributable to one or more substances, and to partially purify the substance, we chromatographed calf caudate nucleus extracts on Bio-Gel P_2 columns (Fig. 4). All fractions were assayed in the absence and presence of sodium chloride and manganese chloride. The principal peak of eluted enkephalin activity, like enkephalin activity of crude brain extracts, becomes more potent in inhibiting [^{3}H]- naloxone binding when assays are conducted

TABLE 4

Sensitivity of calf enkephalin to enzymatic degradations

Lyophilized calf caudate nucleus extract (6.7 mg) was dissolved in 0.06 ml of distilled water and allowed to react with the indicated enzyme at the appropriate concentration for 30 min at 37°. The enzymes were then inactivated by immersing the samples in a boiling water bath for 15 min; the samples were then centrifuged, and 0.015 ml of the clear supernatant was tested in a standard binding assay with [^{3}H]dihydromorphine and 1 mM $MnCl_2$ with no added sodium. Leucinaminopeptidase had 2.5 mM $MgCl_2$ present as a cofactor. The resuspended extract contained Tris-HCl buffer from the extraction, and therefore all enzymatic reactions were conducted at pH 7.4. Similar results were obtained with rat extracts. This experiment was replicated three times. Values are specific binding and were determined from triplicate samples, which varied less than 10%.

Enzyme	Concentration	[^{3}H]Dihydromorphine binding	
		Control	Enkephalin
	ml^{-1}	*cpm*	*cpm*
Chymotrypsin	0	4655	1060
	0.01 mg	4890	1251
	0.03 mg	4882	1482
	0.1 mg	4591	1350
	1.0 mg	4545	1456
Trypsin	0	4655	1060
	0.01 mg	4689	849
	0.03 mg	5016	897
	0.1 mg	4278	932
	1.0 mg	4783	1225
Carboxypeptidase A	0	4729	2406
	73 units	4581	3447
	146 units	4409	3545
	583 units	4423	3660
Carboxypeptidase B	0	4729	2406
	82 units	4880	3768
	153 units	4713	4006
Leucinaminopeptidase	0	4729	2406
	5.9 units	4585	3544
	11.8 units	4840	3603
	23.6 units	4982	3905
	59 units	5105	4165
Neuraminidase	0	4805	2406
	50 μg	4750	2380

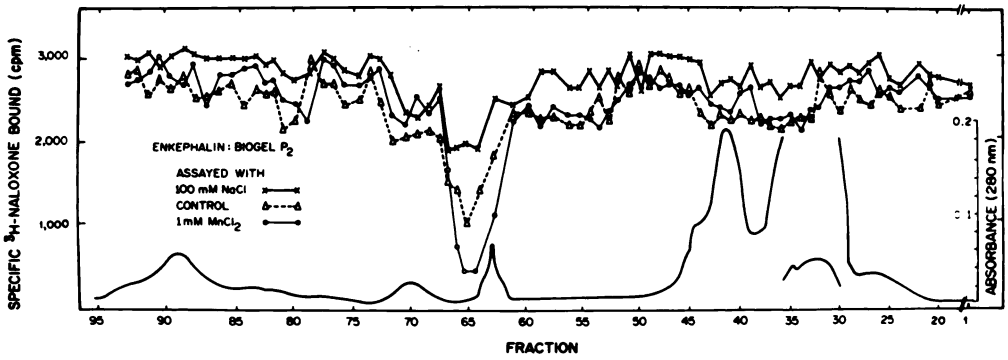

FIG. 4. *Characterization of enkephalin by gel chromatography*

Calf caudate nuclei were extracted as described in MATERIALS AND METHODS and lyophilized. Then 3 ml (750 mg of extract, pH 7.7, at 25°) were layered on a Bio-gel P_2 (100–200 mesh) column, 2.5 × 90 cm, and developed with distilled H_2O overnight at 4°. The volume placed on the column was 0.7% of the total column volume, V_t. Ninety-five 6-ml fractions were collected, and 0.3 ml of each was tested in a standard [³H]naloxone binding assay in the presence and absence of 100 mM NaCl or 1 mM $MnCl_2$. Identical elution patterns and ion sensitivity were seen in six identical columns, five columns of Bio-Gel P_2, 1.5 × 90 cm with 200 mg of extract in 1 ml, and four columns of Bio-Gel P_2 (200–400 mesh), 0.9 × 30 cm with 30 mg of extract on 0.2 ml.

in the presence of manganese and less potent in the presence of sodium. The peak of enkephalin activity does not correspond to any major peak of ultraviolet-absorbing material, indicating a substantial purification of the enkephalin activity applied to the column. The lyophilized material initially applied to the column represents 750 mg of tissue extract, whereas the lyophilized material from the peak of enkephalin activity eluted from the column weighs only about 30 mg, suggesting a purification of at least 25-fold.

To obtain an estimated molecular weight, crude enkephalin extracts were applied to a 200–400 mesh Bio-Gel P_2 column. The elution characteristics of enkephalin activity were compared with those of compounds of known molecular weight (Fig. 5). In these experiments enkephalin activity appears to possess a molecular weight of approximately 1000. In other experiments rat brain enkephalin activity also showed a molecular weight of about 1000.[5] Although, in an original communication, Hughes (7) reported a molecular weight for pig brain enkephalin activity of about 700, he more recently revised this to 1000, similar to our findings (10, 22). Terenius and Wahlstrom (8) examined an enkephalin-like substance from rat brain and obtained a molecular weight of 1000–1200. By contrast, pituitary enkephalin appears to possess a molecular weight of 1750 (11). Because absorption delays can occur with small peptides, one must be cautious in interpreting molecular weight determinations obtained by gel chromatography.

[5] G. W. Pasternak, R. Simantov, and S. H. Snyder, unpublished observations.

DISCUSSION

The major finding of the present study is the demonstration in mammalian brain extract of an endogenous morphine-like substance, enkephalin. The enkephalin activity identified by competing for opiate receptor binding sites in studies by ourselves (10) and by Terenius and Wahlstrom (8, 9) appears to be the same or closely similar to the material identified by Hughes (7, 22, 23) from its ability to inhibit electrically induced contractions of smooth muscle. Cox *et al.* (11) and Teschemacher *et al.* (24) reported a peptide-like material extracted from the pituitary which inhibits electrically induced contractions of guinea pig intestine and whose activity is antagonized by naloxone. In contrast to the material studied by ourselves, Hughes (7, 22, 23), and Terenius and Wahlstrom (8, 9), the pituitary substance has a molecular weight in excess of

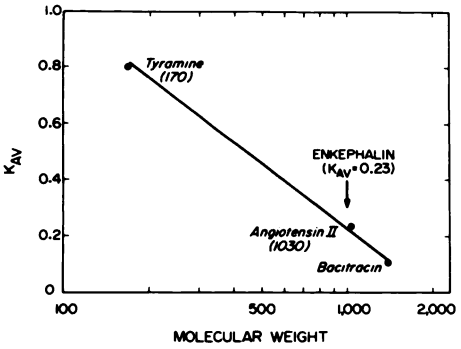

FIG. 5. *Molecular weight determination of enkephalin by gel chromatography*

Thirty milligrams of lyophilized calf caudate nucleus extract were dissolved in 0.2 ml of water and run on a Bio-Gel P_2 (200-400 mesh) column (0.9 × 30 cm) at 4°. Sixty fractions of 0.45 ml each were collected and assayed for their ability to inhibit [^{3}H]naloxone binding. The elution profile and peak of inhibition are almost identical with those in Fig. 4. The inhibition was found primarily in tubes 25 and 26. The column was calibrated with insulin (mol wt 6000), bacitracin (1400), angiotensin II (1030), and tyramine (170). Insulin marked the void volume (tube 15), while the other markers were included in the gel: bacitracin, tube 21; angiotensin II, tube 26; and tyramine, tube 46. K_{av} was determined for each standard [$K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume for the compound, V_0 is the void volume, and V_t is the total volume of the column] and plotted as the logarithm of molecular weight vs. elution volume. The peak of inhibition has K_{av} = 0.23, which corresponds to a molecular weight of approximately 1000.

1700 and is sensitive to trypsin digestion and insensitive to carboxypeptidase A and leucinaminopeptidase (11).

In our studies competition by crude brain extracts for opiate receptor binding appears attributable to a "specific" enkephalin effect rather than a combination of specific and nonspecific actions. The activity is eluted homogeneously from Bio-Gel P_2 columns. Moreover, the marked regional variations in enkephalin activity measured in crude extracts parallel variations in opiate receptor binding, which would not be anticipated if a major portion of the enkephalin-like activity were attributable to nonspecific influences.

In accordance with the demonstration of Hughes that enkephalin is a mixture of two peptides whose respective amino acid sequences are H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH (23), the enkephalin material studied here appears to be a peptide, because of its sensitivity to carboxypeptidase A and B and leucinaminopeptidase. The lack of effect of trypsin upon enkephalin activity is consistent with the supposition that lysine or arginine in the interior region of the peptide is not crucial for activity. However, biological activity would also be resistant to trypsin if the COOH-terminal residue were lysine or arginine.

It is striking that the characteristics of enkephalin appear to be essentially the same whether the material is assayed by competition for opiate receptor binding or by its inhibitory action on neurotransmission in the mouse vas deferens. Thus, when assayed by both techniques, the molecular weight appears to be about 1000 and the regional distributions in brain tissue and enzyme sensitivities are very similar. Moreover, in both assay systems, enkephalin behaves as an opiate agonist. Purified enkephalin isolated in our laboratory elicits naloxone-reversible analgesia similar to that produced by morphine after injection through a cannula into the central gray matter of rat brain.[6]

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