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Neuronal actions of endorphins and enkephalins among brain regions: A comparative microiontophoretic study

(opiates/naloxone/inhibitory/excitatory)

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ABSTRACT **The brain peptides α- and β-endorphin, leucine- and methionine-enkephalin, as well as the opiate normorphine, have been evaluated by microiontophoresis for their effects on neuronal activity in several regions of the rat brain. In cerebral cortex, brainstem, caudate nucleus, and thalamus, most responsive cells were inhibited by the peptides and by normorphine, while in hippocampus all responsive cells were excited. Both inhibitory and excitatory responses were blocked by the narcotic antagonist naloxone. Occurrence of responsive cells encountered in a particular region was loosely correlated with density of stereospecific opiate binding sites as reported by others. These results are consistent with the hypothesis that the endorphins and enkephalins may represent a new class of central neurotransmitters; among other functions, these peptides may play a role in the regulation of behavior and the expression of psychopharmacological agents such as the opiate alkaloids.**

Recently several peptides that show opiate-like properties have been isolated from brain and pituitary. Methionine (Met^5)- and leucine (Leu^5)-enkephalin were isolated from brain (1, 2), while the larger endorphins were isolated from extracts of hypothalamus and pituitary (3–6). The morphinomimetic activity of the endorphins and enkephalins has been studied extensively in the guinea pig ileum preparation (1, 4, 6), in binding assays with stereospecific opiate-receptors of brain synaptosomal preparations (2, 4, 7–9), and in behavioral studies after injections into cerebrospinal fluid (10–14). In these studies, the potency of β-endorphin exceeded morphine or normorphine, while the enkephalins and α-endorphin were generally less potent than the opiates.

The existence of endogenous peptides with opiate-like actions suggests that these substances may function as neuromodulators or neurotransmitters in the central nervous system. Indeed, enkephalins can modify the excitability of a variety of neurons in the central nervous system when administered by iontophoresis. Most neurons tested were inhibited by these peptides (15–19), although Renshaw cells were excited (20). There have been no reports on the responsiveness of neurons to the endorphins, nor any systematic regional survey of neurons responsive to these peptides.

The present study was undertaken (*i*) to compare the neuronal effects of α- and β-endorphin to the enkephalins and normorphine; (*ii*) to determine the responsiveness of neurons in a variety of regions and compare these results to the density of opiate receptors in the same regions, and (*iii*) to determine the specificity of the responses by administering the selective narcotic antagonist naloxone.

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METHODS

We used 26 rats weighing between 170 and 350 g. In the initial experiments ($n = 8$) the animals were anesthetized with 1.2 g of urethane per kg intraperitoneally. In the remaining experiments ($n = 18$) anesthesia was induced with 350 mg of chloral hydrate per kg intraperitoneally and then maintained for the rest of the experiment by 0.5–1.0% halothane. All animals were tracheotomized and allowed to breath spontaneously. Body temperature was maintained at 35–37°. The use of low halothane levels appeared to facilitate recording of spontaneously discharging neurons, especially in brainstem and caudate nucleus. Individual neurons were recorded through the central barrel (containing 4 M NaCl) of a five-barrel micropipette electrode. The electrodes were prepared immediately before the experiment, as described (21). The drugs placed in the peripheral barrels were: L-glutamate (0.5 M, pH 8), normorphine (0.05 M, pH 4), naloxone hydrochloride (0.1 M, pH 4), Met^5- and Leu^5-enkephalin (0.03 M, in 0.9% NaCl, pH 4), α-endorphin (6 mM in 0.9% NaCl, pH 4) and β-endorphin (3 mM in 0.9% NaCl, pH 4). The peptides were ejected with cationic current by electroosmosis. One of the peripheral barrels always contained 3 M NaCl for neutralizing electrotonic effects of the electrophoretic currents (21, 22). In addition to the standard analysis of drug effects by rate meter display on a polygraph, drug response histograms (23, 24) were generated by a PDP-11 computer (Digital Equipment Co., Maynard, MA; program by K. Liebold) to quantitate peptide effects. Naloxone was also administered subcutaneously, but since it has longlasting effects (4–6 hr), this route of administration was only used once in a given animal at the end of the experiment.

The stereotaxic coordinates (nose 2.4 mm below vertical zero) for recording from the caudate nucleus were: 2.5–3.5 mm lateral to midline, within 0.5 mm posterior to or 1.0 mm anterior to bregma, and 3.3–6.0 mm vertical depth below the cerebral cortical surface. For hippocampus and thalamus, coordinates were 1.5–2.0 mm lateral to midline, 3.0–3.7 mm posterior to bregma, and 2.0 mm vertical depth for hippocampus and 3.5 mm vertical depth for thalamus (nucleus lateralis thalami pars posterior). Units in parietal and somatosensory cortex were tested before the electrode was advanced to deeper structures. To record from cerebellum and brainstem the electrode was angled at 30° with the nose 18 mm below horizontal. The electrode was zeroed on obex and inserted 1.0–2.0 mm lateral to midline and 0.5–2.0 mm anterior to obex. Most units recorded in brainstem were from the bulbar reticular formation and lateral reticular nucleus. At the termination of each experiment the brain was removed, fixed in either glutaraldehyde or paraformaldehyde, sectioned, and examined histologically for electrode placements.

Table 1. Neuronal effects of opioid peptides

Region (cell type)	Met-enkephalin % exc.	% inh.	β-Endorphin % exc.	% inh.	Normorphine % exc.	% inh.
Cerebellum (Purkinje)	18	21	23	23	20	60
	$n = 34$		$n = 13$		$n = 5$	
Cerebral cortex (unidentified)	1	79	25	48	26	52
	$n = 58$		$n = 44$		$n = 27$	
Brain stem (lateral reticular nucleus +)	3	47	23	46	10	75
	$n = 113$		$n = 35$		$n = 20$	
Caudate nucleus (unidentified)	0	83	10	86	9	73
	$n = 18$		$n = 35$		$n = 20$	
Thalamus (unidentified)	0	100	0	100	0	100
	$n = 15$		$n = 5$		$n = 4$	
Hippocampus (pyramidal)	90	5	86	7	92	0
	$n = 19$		$n = 14$		$n = 12$	

Summary of neuronal effects of opioid peptides and normorphine. In each category the total number of cells tested (n) and the percentage of this total that were inhibited or excited is given.

A cell was considered responsive to a drug if it changed its firing rate by more than 20%, the response could be repeated at least twice, there was no net current unbalance, and the response could not be duplicated by an equal amount of current ejected from the balance barrel. A response was considered to be antagonized (by naloxone) if it was reduced by 50%.

In three experiments on caudate nucleus, the animals were given an intracisternal injection of 6-hydroxydopamine (see ref. 25) in order to exclude possible indirect effects by presynaptic actions on dopaminergic nerve terminals.

All peptides were synthesized by one of us (N.L.) and obtained in high purity using methods fully described elsewhere (6, 26).

RESULTS

Characterization of Responses. In cerebral and cerebellar cortex, thalamus, and caudate nucleus, most neurons encountered were inhibited in a dose-dependent and reversible fashion by all the peptides as well as by normorphine (Table 1 and Fig. 1). In contrast to this general response, almost all hippocampal pyramidal cells were excited by these agents (see below). The inhibitions produced by the pentapeptides were typically rapid in onset and neuronal activity recovered quickly (3–10 sec) after the ejection current was terminated (Fig. 1 *A*, *C*, and *D*). With spontaneously firing units, the responses to the endorphins and normorphine generally had a slower time course than the responses to the enkephalins (Fig. 1*D*). The inhibitory action of the peptides could also be demonstrated on glutamate-evoked activity. The inhibition of glutamate responses followed the same time course as the inhibition of spontaneous activity recorded in the same cell. This did not appear to be a specific antiglutamate effect, since the spontaneous firing was always depressed as well as the responses to glutamate.

In three experiments, the action of Leu^5-enkephalin was

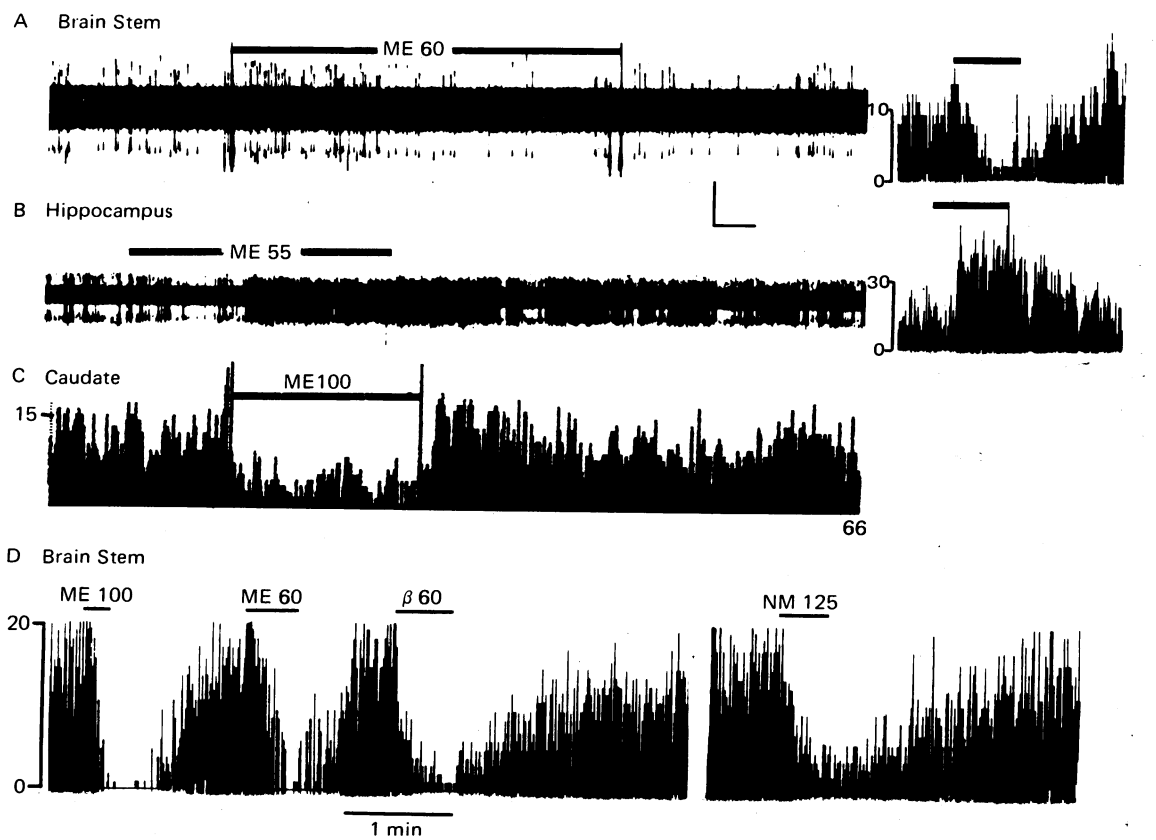

FIG. 1. The effect of opioid substances on the spontaneous activity of central nervous system neurons. (*A*) Oscilloscope record of the discharge of a brainstem neuron that is inhibited by 60 nA Met^5-enkephalin (ME). The rate meter record (1 sec rate integration) of the same response is shown to the right. (*B*) An excitatory response from a hippocampal neuron to Met^5-enkephalin. The calibration in *A* is 150 μV and 4.7 sec and in *B* is 500 μV and 5.7 sec. (*C*) Computer-generated drug histogram of the inhibition of a caudate neuron to Met^5-enkephalin. This histogram sums the spikes of six sweeps each with a duration of 66 sec. The number on the ordinate refers to counts per bin. (*D*) Comparison of the action of Met^5-enkephalin, β-endorphin (β), and normorphine (NM) on the activity of a brainstem neuron.

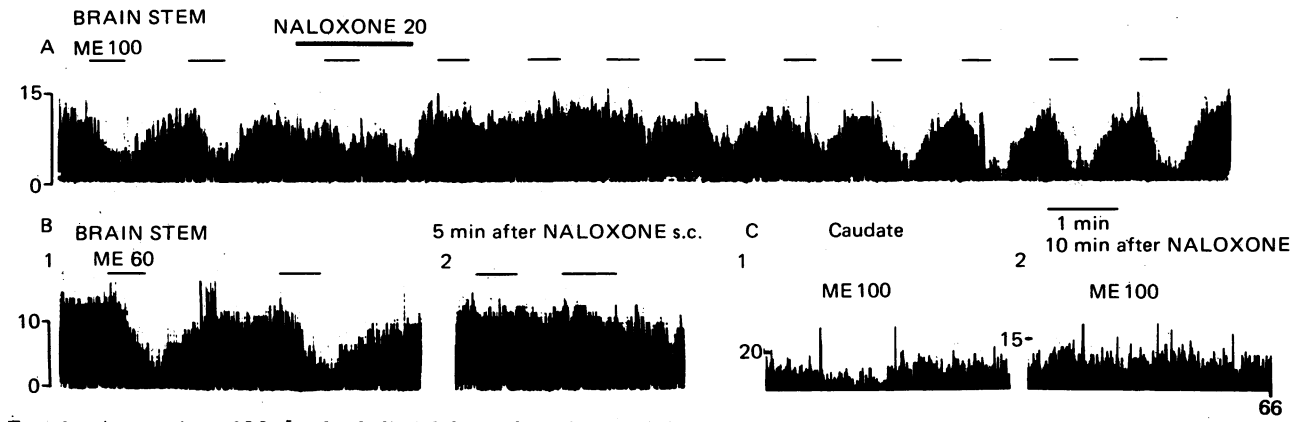

FIG. 2. Antagonism of Met[5]-enkephalin inhibition by naloxone. (*A*) A short iontophoretic application of naloxone reversibly blocks the inhibitory response of a brainstem neuron. (*B*) A subcutaneous injection of naloxone (8 mg/kg) blocks the inhibitory response of another brainstem neuron. (*C*) A similar action of naloxone (8 mg/kg subcutaneously) in a caudate neuron, as documented by computer-generated drug histograms immediately before and 3 min after naloxone. Each histogram is composed of six sweeps of 66-sec duration.

compared to that of Met[5]-enkephalin on 25 brainstem neurons; both peptides required similar ejection currents and a similar proportion of neurons were inhibited by both. The action of α-endorphin was examined on 32 brainstem neurons and was found to inhibit only three of these cells, but a number of technical problems, including "noisy" drug barrels often indicative of poor release (27), may have limited the amount of drug ejected. Thus, further experiments with α-endorphin are required. The somewhat larger proportions of neurons inhibited by Met[5]-enkephalin compared to β-endorphin is probably of little significance since the molarity of the β-endorphin solution in the pipette was approximately one-tenth that of the Met[5]-enkephalin solution.

The major effect produced by normorphine was also inhibition of neuronal activity. However, with normorphine, a reduction in spike height occurred with ejection currents in excess of 60 nA, and particular attention was required to ensure that all spikes were counted during the ejection of normorphine. In spite of these precautions this "local anesthetic action" may have contributed to the somewhat higher percentage of cells inhibited by normorphine compared to the peptides. The peptides caused little change in spike size (see Fig. 1*A*).

Distribution of Responsive Cells. The highest proportion of tested cells inhibited by the peptides and normorphine were found in caudate nucleus and thalamus, while cerebellum had the lowest percentage of inhibitions. Since the same electrodes were used to record Purkinje cells in cerebellum and neurons in brainstem, the greater sensitivity of brainstem neurons is particularly striking. Furthermore, in cerebellum about as many cells were excited as were depressed. In brainstem, sensitive cells often occurred in clusters and a higher percentage of cells responded in lateral reticular nucleus than in medial reticular formation. In cerebral cortex and brainstem a somewhat higher proportion of cells were excited by β-endorphin than by Met[5]-enkephalin. Destruction of the dopaminergic nigral-striatal pathway with 6-hydroxydopamine did not grossly alter the predominantly inhibitory action of the peptides on caudate neurons.

A surprising finding in the present study was the potent excitatory effects of the peptides and normorphine on hippocampal pyramidal cells (Figs. 1*B* and 3). The regional specificity of this excitatory action could be clearly demonstrated with the same electrode by recording from cells in the overlying cerebral cortex and the underlying thalamus during a single penetration. Thus, while cells in the cortex responded with inhibition to the peptides, as soon as the electrode entered the hippocampus, only marked excitatory responses were observed. Further advancement of the electrode into the thalamus again revealed exclusively inhibitory responses.

No tachyphylaxis was observed either to the excitatory or inhibitory action of the peptides in any of the regions examined, even though the peptides were often applied repeatedly to the same cell for periods in excess of 1 hr.

Naloxone Antagonism of Peptide Effects. To determine whether the responses observed with the peptides were related to the activation of opiate receptors, the specific opiate antagonist, naloxone, was administered both by iontophoresis from an adjacent barrel of the microelectrode and by subcutaneous injections. By either route, naloxone antagonized both the excitations and the inhibitions (Figs. 2 and 3) in all regions encountered, including cerebellum. Iontophoretic administration of naloxone alone, in every region, depressed neuronal activity directly when ejected with currents of 40 nA or more. This depression was due in large part to a local anesthetic action and could easily be separated from its opiate antagonist properties, since the cell recovered very quickly from the former action (see Fig. 2*A*). On 17 cells that were inhibited by Met[5]-enkephalin, iontophoresis of naloxone antagonized the response of 14 cells. β-Endorphin inhibitions were antagonized by naloxone in all four cells tested. Subcutaneous injection of naloxone also antagonized the inhibitory effect of Met[5]-enkephalin on two brainstem neurons and one caudate neuron (Fig. 2 *B* and *C*) as well as the inhibition of two caudate neurons by β-endorphin. The parenteral dose of naloxone required to demonstrate an antagonism, usually in excess of 2 mg/kg, was considerably higher than the doses reported to block the behavioral effects of opiates. In addition, the antagonism observed with parenteral naloxone could be partially overcome by increasing the ejection current. Even after doses of 20 mg of naloxone per kg, it was possible to find cells that were still inhibited by each of the peptides.

The excitatory effects of the peptides in all the regions examined were also sensitive to naloxone by either route. Five of seven excitatory responses to Met[5]-enkephalin were blocked by iontophoresis of naloxone, three of the blocked cells being in hippocampus (Fig. 3*A*). All five excitatory responses to β-endorphin, including cells in cerebral cortex, brainstem, and caudate nucleus, were also reduced by naloxone (Fig. 3*C*). The antagonism could not be explained by a local anesthetic action since peptide excitatory responses were reduced with doses of naloxone that had little effect on glutamate excitation (Fig. 3*A*) or on the spontaneous activity (Fig. 3*C*). Subcutaneous injec-

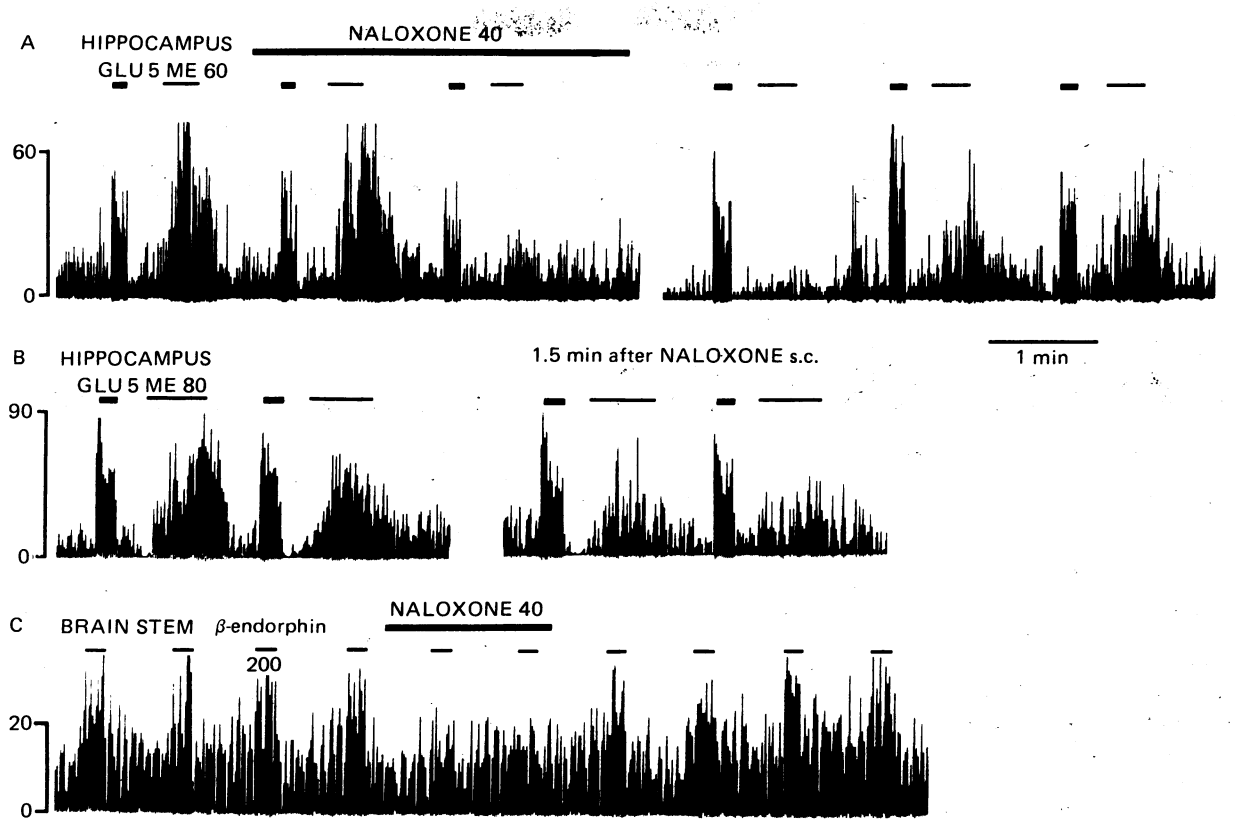

FIG. 3. Naloxone antagonism of excitatory responses to the opioid peptides. (*A*) Iontophoresis of naloxone reversibly and selectively blocks the excitatory action of Met^5-enkephalin on a hippocampal neuron. The break in the record is 8.5 min. (*B*) A subcutaneous injection of naloxone (10 mg/kg) also selectively antagonizes the Met^5-enkephalin excitation of another hippocampal neuron. (*C*) The excitation of a brainstem neuron by β-endorphin is also reversibly blocked by iontophoretically applied naloxone.

tions of naloxone antagonized the excitation of two hippocampal neurons by Met^5-enkephalin (Fig. 3*B*).

DISCUSSION

In the present study, both the percentage of cells affected by the opiate-like peptides and the type of response encountered (excitation and inhibition) depended upon the region of the central nervous system examined. Of all the regions examined, the cerebellum had the least number of responsive cells, while both excitations and inhibitions were observed. This finding is consistent with the low enkephalin levels (28) and the low density of stereospecific opiate binding sites reported for the cerebellum (29). However, the fact that naloxone antagonized the responses indicates that opiate receptor mechanisms can be detected in cerebellum with the iontophoretic technique. In cerebral cortex, brainstem, caudate nucleus, and thalamus, areas reported to have a high density of stereospecific binding sites (29), a higher proportion of cells responded to the peptides with naloxone-sensitive inhibition. These results agree with previous results on the enkephalins (15–19), but some investigators have had difficulty in blocking the peptide responses with naloxone (16, 17). In the present study, excitatory responses were also blocked by naloxone. The higher parenteral dose of naloxone required to block neuronal responses compared to behavioral effects of these peptides may be related to the high local concentration of agonist that can be expected in the immediate vicinity of the iontophoretic electrode.

Hippocampal pyramidal cells, unlike all other neurons examined in this study, were strongly excited by the peptides, and these excitations were selectively blocked by naloxone. A similar excitatory action, although of faster time course (20), has been found with Met^5-enkephalin on Renshaw cells. Interestingly, both types of cells are known to be strongly excited by acetylcholine (20, 30), and the opiate and peptide excitations of Renshaw cells have been closely linked to nicotinic receptors (20, 31). The acetylcholine responses in the hippocampus have both nicotinic and muscarinic properties (30). Further studies will be required to determine whether the excitation of hippocampal pyramidal cells by opiate-like peptides is related to endogenous cholinergic mechanisms in these regions.

It is unlikely that the inhibitory responses observed in this study are due to a presynaptic action (see refs. 32 and 33), since the postsynaptic excitatory action of glutamate is depressed (see also ref. 18) by the peptides. The mechanism underlying this postsynaptic effect is unclear.

There are limitations in establishing the relative potency of compounds released from microelectrodes, especially when electroosmosis is used (see refs. 26 and 34). Nevertheless, it was our impression that the responses to the endorphins and normorphine were of longer duration than the responses to the enkephalins. This might result from the more rapid enzymatic breakdown of the enkephalins, and it would be interesting to see whether enkephalin analogs that are resistant to degradation (35) have more prolonged actions. Leu^5- and Met^5-enkephalin appeared to be equipotent. α-Endorphin appeared to be considerably less potent than β-endorphin, but, as noted above, further experiments with α-endorphin are needed to verify this impression. The somewhat higher percentage of excitations seen with β-endorphin compared to Met^5-enkephalin in the cerebral cortex and brainstem suggests that differences in the pharmacological action of these two peptides may exist.

The effects of opiate-like peptides on neuronal activity

demonstrated in the present study, taken with biochemical (1, 2, 3–7) and histochemical (ref. 36, also see ref. 6) evidence for their existence in brain, are consistent with the hypothesis that these peptides are neurotransmitters in the central nervous system. When the cells of origin of peptide-containing fibers have been determined, it may then be possible to proceed with studies to satisfy more completely the criteria for a neurotransmitter. Crucial points in such future analyses will be the questions of whether the endorphin- and enkephalin-containing fibers are mutually inclusive systems, whether the length of the peptide released by neuronal activity is subject to modulation, and whether intermediate length peptides (such as the α-, γ-, and δ-endorphins; see ref. 37) may participate in such modulatory changes. Such questions are predicated on the assumptions that the endorphins of brain origin are indeed cleavage products of a much larger progenitor molecule such as β-lipotropin, as seems to be the case for the endorphins of pituitary origin (7, 5). The results presented here indicate to us that the cellular roles of endorphins and enkephalins cannot be generalized across all brain regions, and that no single cellular action of any peptide will yield an integrative picture of the way in which opiate alkaloids produce complex analgesic, euphoric, and addictive responses.

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