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A comparison of the effects of morphine, enkephalin, kyotorphin and D-phenylalanine on rat central neurones

T.W. Stone

Department of Physiology, St. George's Hospital Medical School, University of London, London SW17 0RE

1 Morphine, Met-enkephalin, kyotorphin and D-phenylalanine have been applied by microiontophoresis to neurones in the globus pallidus and cerebral cortex of rats anaesthetized with urethane.

2 In the pallidum, most cells were inhibited by all the agonists, with a high correspondence between cells inhibited by Met-enkephalin and D-phenylalanine and by Met-enkephalin and kyotorphin. Whereas responses to Met-enkephalin were readily antagonized by naloxone, responses to kyotorphin and D-phenylalanine were not.

3 In the cerebral cortex a high proportion of cells was excited by all four agonists and antagonism by naloxone was less consistent than in pallidum.

4 It is concluded that the naloxone-reversible analgesic effects of kyotorphin and D-phenylalanine may be mediated indirectly, rather than through an activation of opiate receptors.

Introduction

In order to clarify the site of action of opiate compounds in the central nervous system (CNS) a number of groups have applied opiates or opioid peptides to neurones in different regions of the CNS with widely varying results (North, 1979; Klemm, 1981). Whereas several groups obtain largely inhibitory effects of morphine or enkephalin (Dostrovsky & Pomeranz, 1976; Young, Bird & Kuhar, 1977; Carette & Poulain, 1978; Palmer, Morris, Taylor, Stewart & Hoffer, 1978; Zieglgänsberger & Tulloch, 1979; Perkins & Stone, 1980a; Bradley & Brookes, 1981; Hosford & Haigler, 1981; Huffman & Felpel, 1981) others have observed a high proportion of excitatory effects (Davies & Dray, 1976; 1978; Duggan, Davies & Hall, 1976a; Belcher & Ryall, 1978; Deadwyler & Robinson, 1979; Satoh, Akaike & Takagi, 1979; Bradley & Brookes, 1981; Reyes-Vazquez & Dafny, 1982) the direction of responses depending on the region studied and the type of cell.

Attempts to assess the pharmacological relevance of these responses have resulted in reports of naloxone blockade in some cases of inhibitory responses (Satoh, Zieglgänsberger, Fries & Herz, 1974; Frederickson & Norris, 1976; Bradley, Briggs, Gayton & Lambert, 1976; Zieglgänsberger & Bayerl, 1976; Duggan, Hall & Headley, 1976b; Belcher & Ryall, 1978; Palmer *et al.*, 1978; Hosford & Haigler, 1981; Huffman & Felpel, 1981) and in some cases of excitatory responses (Davies & Dray, 1976; 1978; Duggan *et al.*, 1976a; Belcher & Ryall, 1978; Bradley & Brokes, 1981) while in still other examples, none of the opiate agonist actions were readily reversible (Calvillo, Henry & Neuman, 1974; Dostrovsky & Pomeranz, 1976; Gent & Wolstencroft, 1976).

Several authors have argued that part of this complexity arises from nonspecific effects of the opiates and that even the use of naloxone cannot always be regarded as a meaningful test of opiate action as it has nonspecific effects of its own, and can even block the inhibitory actions of γ-aminobutyric acid (GABA) (Dingledine, Iversen & Breuker, 1978; Perkins & Stone, unpublished observations).

This problem was encountered in a previous study of neurones in the rat striatum in which most cells were inhibited by morphine but 18% of cells were excited, and the excitatory responses appeared to be easier to antagonize by naloxone iontophoresis (Perkins & Stone 1980a).

In the present study therefore it was decided to compare the actions of morphine and Met-enkephalin with those of the dipeptide kyotorphin (L-Tyr-L-Arg) in the rat cerebral cortex and globus pallidus. Kyotorphin was originally described by Takagi, Shionii, Ueda & Amano (1979a,b) and is thought to provoke the release of endogenous enkephalins from nerve terminals. As there are now

several lines of evidence to suggest the existence of an enkephalinergic pathway to the pallidum (Cuello & Paxinos 1978; Bayon, Shoemaker, Lugo, Azad, Ling, Drucker-Colin & Bloom 1981) the primary rationale of the present study was that local application of kyotorphin might release enkephalin from nerve terminals onto physiologically relevant sites, and any resulting change of neuronal firing could be compared with that produced by exogenous enkephalin and morphine. It has been shown by Satoh, Kawajiri, Yamamoto, Akaike, Ukai & Takagi (1980) that kyotorphin and Met-enkephalin have similar effects on cell firing in the dorsal horn of the spinal cord and the hind brain.

In addition, the effects of D-phenylalanine have been examined in the present study as this compound has been reported to produce naloxone reversible analgesia (Cheng & Pomeranz, 1979; Allava, Castellano & Oliverio, 1980) and to show cross-tolerance with morphine in behavioural studies (Filibeck, Castellano & Oliverio, 1981).

Methods

Male rats were anaesthetized with urethane, 1.5 g/kg i.p. and held in a stereotaxic frame. The cerebral cortex was exposed and the dura mater removed. The exposed surface of the cortex was covered with warmed saline throughout the experiment and the rectal temperature was maintained at 37°–38°C by an automatically controlled heating blanket.

Neurones in the globus pallidus were studied at co-ordinates of 0.8 mm anterior to bregma; Lateral 3.0; Vertical 5.5–8.0 (Pellegrino, Pellegrino & Cushman, 1979). The regular firing pattern of cells, which we have reported previously to be characteristic of the pallidum (Perkins & Stone, 1980b; 1981) was taken as confirmation of electrode position during the experiments.

All drugs were applied by microiontophoresis from seven-barrelled micropipettes containing glass fibres to permit rapid filling of the barrels immediately before use. The tip of the micropipettes was broken back to an overall size of 4–8 μm under microscopic observation. A separate single glass microelectrode containing 1 M potassium acetate was glued alongside the multibarrel assembly for recording unit activity (Stone, 1973). Spikes were amplified, gated by a window discriminator, counted and displayed on a Grass polygraph either by a resetting integrator (reset time 1 s) or by a continuous ratemeter with a time constant of 2 s. When testing the effects of naloxone as an antagonist, agonists were applied in an automatically controlled time cycle so as to reduce any variations of response size due to retaining and ejecting currents (Bradshaw, Szabadi & Roberts, 1973).

The following compounds were used: morphine sulphate, 50 mM, pH 5 (McFarlan Smith); Met-enkephalin, 10 mM, pH 4 (Dr S. Wilkinson); D-phenylalanine, 20 mM, pH 4 (Sigma); kyotorphin, 10 mM, pH 4 (Sigma); naloxone HCl, 20 mM, pH 4 (Endo Labs). The pH of solutions was adjusted with 1 M HCl and all compounds were ejected as cations. One barrel of the multibarrel pipette was filled with

Table 1 The direction of responses of neurones in the globus pallidus to morphine (M), Met-enkephalin (Enk), kyotorphin (K) and D-phenylalanine (D-Phe) applied by microiontophoresis

Cell	*M*	*Enk*	*K*	*D-Phe*
1	I	I	I	I
2	I	I	I	I
3	I	I	I	I
4	I	I	I	I
5	I	I	O	I
6	I	O	I	I
7	I	O	O	O
8	I	O	O	I
9	O	O	O	I
10	O	O	E	O
11	O	I	O	I
12	O	I	O	I
13	O	I	I	O
14	O	I	I	O
15	O	I	I	I
16	O	I	I	I
17	E	I	I	O
18	E	I	O	I

I = inhibited; E = excited; O = no response.

1M NaCl at pH 4 to test for current and pH effects. Results from any cell which exhibited changes of firing rate in response to control currents were discarded.

It proved impracticable to test for antagonism by naloxone against all four agonists at once, because of the continuously increasing naloxone concentration produced over the entire cycle length of several minutes. Naloxone was therefore tested against pairs of agonists.

Results

In these experiments neurones sensitive to an agonist usually responded within 30 s of starting the agonist ejection. Neurones failing to respond to an agonist ejection of 100 nA lasting 1 min were therefore considered to be unresponsive to that agonist.

Most responsive cells responded to ejection of agonists using currents in the range 40–75 nA. Although there was some overlap, the latency and duration of a response tended to correlate with its direction, excitatory responses (Figure 4) being generally slower in onset and longer in duration than inhibitory responses (Figures 2 and 3).

Globus pallidus neurones

A total of 18 cells were fully characterized in terms of their responses to all 4 agonists: morphine, Met-enkephalin, kyotorphin and D-phenylalanine (D-Phe). Results from a number of cells which showed variable responses, either in direction or size, or which were not held long enough to test with all 4 agents were discarded.

The patterns of responses observed on these 18 cells are summarized in Table 1, from which it can be seen that morphine inhibited 8 and excited 2 neurones, enkephalin and D-Phe inhibited 13 while kyotorphin inhibited 10 and excited 1 cell. Examples of firing rate records from pallidal neurones are shown in Figures 1 and 2.

Table 2 represents an attempt to collate this data by comparing neuronal responses to each pair of agonists. It is interesting to note the relatively high correlation of responses to the Enk/K and Enk/D-Phe pairs compared with other groupings, and the low correlation of responses to M/Enk. This distinction is made particularly striking by an examination of Table 3 which summarizes the effects of naloxone, applied with a current of up to 72 nA for 8 minutes, on the agonist responses. The results using naloxone from all cells on which naloxone itself produced noticeable effects, either as a change of spike height or shape, or as a change of firing rate, were discarded. It may be seen that naloxone consistently antagonized inhibitory responses to Met-enkephalin and morphine while showing poor antagonism towards kyotorphin and D-Phe. Figure 3 illustrates the antagonism by naloxone of responses to Met-enkephalin while responses to D-Phe are not affected.

Cerebral cortex

A total of 18 cortical neurones were also fully characterized for their responses to the four agonists, and the pattern of response are summarized in Table 4. Morphine here inhibited 5 cells and excited 5, Met-enkephalin inhibited only 2 but excited 9, kyotorphin inhibited 4 and excited 2, while D-Phe inhibited only 1 cell and excited 11. Perhaps as a result of the prolonged time course of many of the excitatory responses (Figure 4) repeated excitatory responses could be elicited on a given cell with no evidence of tachyphylaxis.

Table 5 presents a comparison of data for the

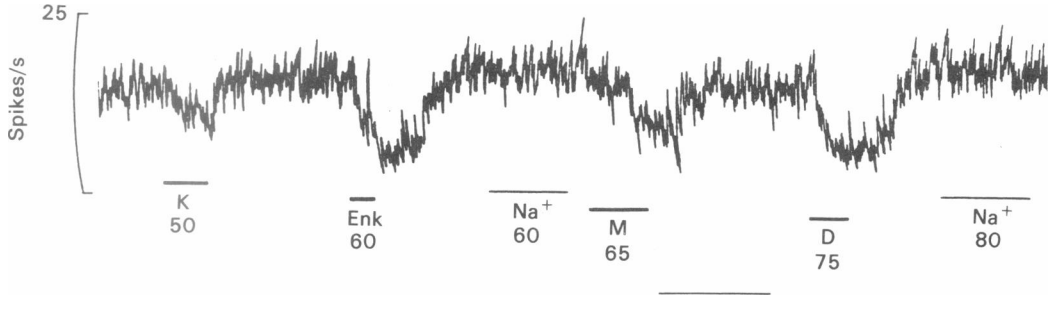

Figure 1 Ratemeter record of the firing rate of pallidal cell number 2 (see Table 1) in response to the iontophoretic application of kyotorphin (K), Met-enkephalin (Enk), sodium ions as a current control (Na^+), morphine (M) and D-phenylalanine (D). On this cell all four agonists were inhibitory. In this and all subsequent figures the bars indicate the duration of drug application and the numbers below the drug abbreviations indicate the ejecting currents in nanoamperes. The ordinate scale is firing rate in spikes per s; the time bar is 1 min.

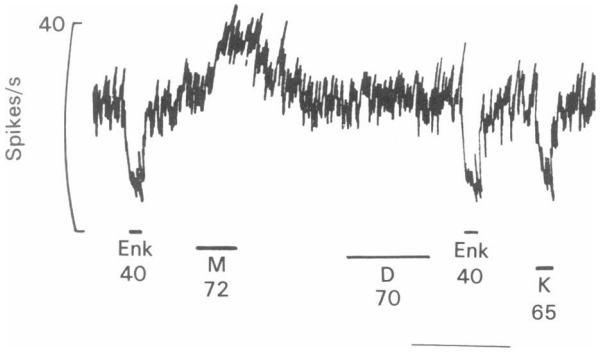

Figure 2 Ratemeter record of the firing rate of pallidal cell 17 (see Table 1) in response to the iontophoretic application of Met-enkephalin (Enk), morphine (M) and D-phenylalanine (D) and kyotorphin (K). Note the clear, rapid inhibitory responses to Met-enkephalin and kyotorphin whereas morphine is excitatory and D-phenylalanine ineffective. Details as for Figure 1.

various pairs of agonists, and it will be noted that there is far less correlation between agonist responses in cortex than was observed in the pallidum (Table 2). The best correlation however was again observed for the Enk/D-Phe pairing. Table 6 summarizes the effects of naloxone, applied with a current of up to 85 nA for 12 min, on the agonist responses. The results using naloxone were discarded for all cells showing changes of spike shape or firing rate in response to naloxone itself. While some antagonism was shown towards morphine and enkephalin, naloxone consistently failed to modify responses to D-Phe as seen in the pallidum.

Discussion

In the cerebral cortex there is in general less correspondence between neuronal responses to the various agonists (Table 5) than was apparent in pallidum (Table 2) but it is particularly striking that many of the cell responses, including those responding simi-

Table 2 Direction of pallidal neuronal responses of those cells responding similarly to agonist pairs

Agonist pair	*Number of cells responding similarly*			*Cells responding differently*
	I	*O*	*E*	
M/Enk	5	2	0	11
M/K	5	3	0	10
M/D-Phe	7	3	0	8
Enk/K	9	3	0	6
Enk/D-Phe	10	2	0	6
K/D-Phe	7	1	0	10

Abbreviations as in Table 1.

Table 3 Effect of naloxone on pallidal neurone responses to opioid agonists

Agonist	*Effects of naloxone*	
	No. of cells inhibition antagonized/total tested	*No. of cells excitation antagonized/total tested*
M	3/4	0/1
Enk	7/8	—
K	1/6	—
D-Phe	1/9	—

Abbreviations as in Table 1.

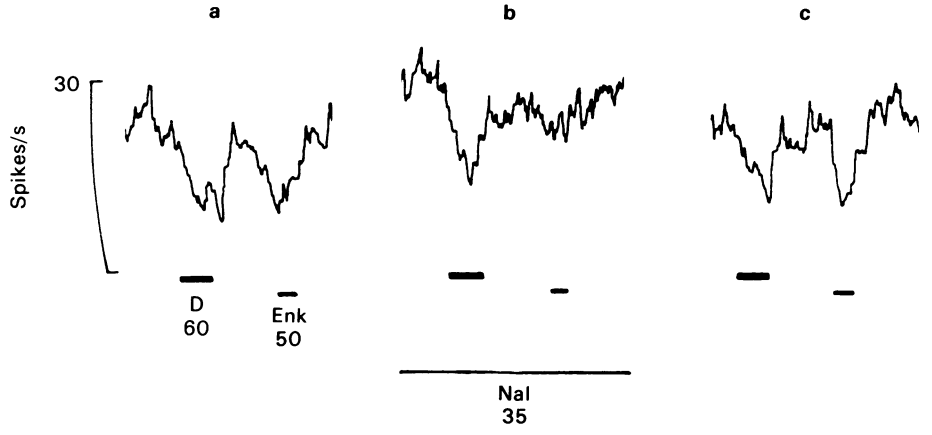

Figure 3 Ratemeter records of the firing rate of pallidal cell 12 (see Table 1) in response to the iontophoresis of D-phenylalanine (D) and Met-enkephalin (Enk) (a). The application of naloxone (Nal) for 6 min caused a block of responses to enkephalin but not D-phenylalanine (record b) with recovery occurring 5 min later (c).

larly to pairs of agonists are excited in the cortex rather than depressed (Table 5). It has been reported on several previous occasions that excitatory responses to opiates are a reflection of non-specific actions of cell firing, that is, actions unrelated to their antinociceptive properties, or are mediated indirectly. For example the excitation seen in response to morphine and enkephalins of pyramidal cells in the hippocampus has been attributed to an inhibition of inhibitory interneurones (Zieglgänsberger, French, Siggins & Bloom, 1979; Haas & Ryall, 1980; Nicoll, Alger & Jahr, 1980). The relatively slow onset and prolonged time course of excitatory responses compared to inhibitory responses seen in the present study as well as a previous one (Perkins & Stone 1980a; Stone & Perkins, 1979) would be consistent with such an indirect action, resulting from the diffusion of the opiates to nearby interneurones. However, it must be emphasized that there is no evidence from the present results which would exclude a direct action on neurones being responsible for these slow excitations.

In the pallidum the best correlations between neuronal responses occurred for Met-enkephalin and

Table 4 The direction of responses of neurones in the cerebral cortex to morphine (M), Met-enkephalin (Enk) kyotorphin (K) and D-phenylalanine (D-Phe) applied by microiontophoresis

Cell	*M*	*Enk*	*K*	*D-Phe*
1	I	I	O	O
2	I	O	E	E
3	I	O	O	E
4	I	E	I	O
5	I	E	O	E
6	E	E	I	E
7	E	E	O	E
8	E	E	O	O
9	E	O	O	O
10	E	O	I	E
11	O	I	O	I
12	O	E	E	E
13	O	E	O	E
14	O	E	O	E
15	O	E	O	E
16	O	O	I	O
17	O	O	O	E
18	O	O	O	O

Abbreviations as in Table 1.

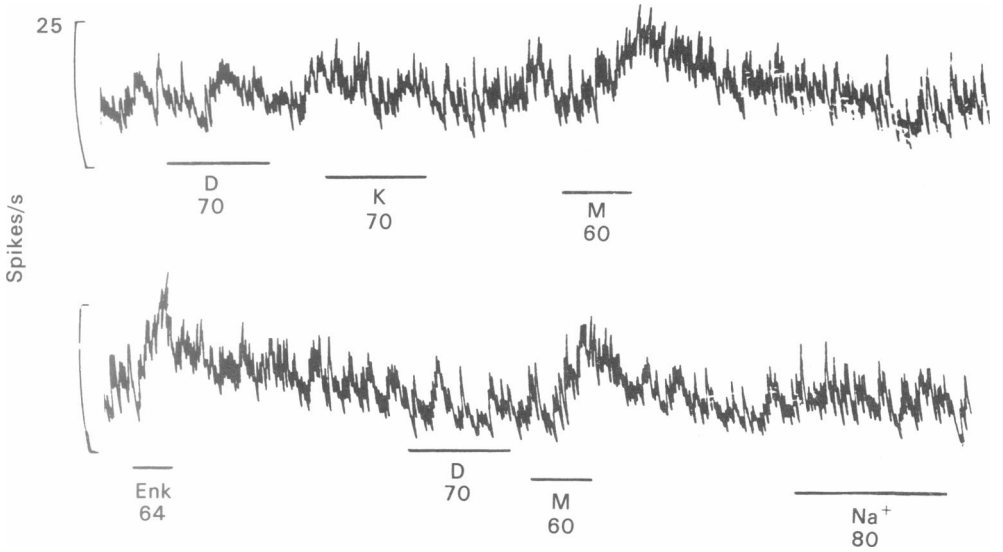

Figure 4 Record of the firing rate of cortical cell 8 (see Table 5) in response to the iontophoresis of D-phenylalanine (D), kyotorphin (K), morphine (M), Met-enkephalin (Enk) and sodium ions (Na^+). The two sections of this record are continuous. Details as for Figure 1.

kyotorphin, and for Met-enkephalin and D-Phe. In view of the evidence for an enkephalinergic projection to the pallidum (Cuello & Paxinos, 1978; Bayon *et al.*, 1981) this would be consistent with the suggestions that kyotorphin can release endogenous enkephalin from nerve terminals, and since D-Phe can induce a naloxone-sensitive analgesia in rats (Filibeck *et al.*, 1981), it would be consistent with the

Table 5 Direction of cortical neurone responses of those cells responding similarly to agonist pairs

Agonist pair	*Number of cells responding similarly*			*Cells responding differently*
	I	*O*	*E*	
M/Enk	1	3	3	11
M/K	1	6	0	11
M/D-Phe	0	2	3	13
Enk/K	0	4	1	13
Enk/D-Phe	1	3	7	7
K/D-Phe	0	4	2	12

Abbreviations as in Table 1.

Table 6 Effects of naloxone on cortical neurone responses to opioid agonists

	Effects of naloxone	
Agonist	*No. of cells inhibition antagonized/total tested*	*No. of cells excitation antagonized/total tested*
M	3/3	0/3
Enk	2/2	1/4
K	0/2	—
D-Phe	0/1	0/6

Abbreviations as in Table 1.

idea that D-Phe can somehow activate opioid receptors. It was of some surprise then to find that responses to neither kyotorphin nor D-Phe could be prevented by naloxone on cells on which responses to Met-enkephalin could be readily antagonized. Several explanations for this present themselves. For example in experiments *in vivo* or in brain slices either of these compounds could be acting indirectly, perhaps activating the distant cell bodies of enkephalinergic projection neurones rather than acting directly on the nerve terminals or opiate receptors. One would not, in that case, see naloxone-sensitive responses to localized application.

A second possibility might be that kyotorphin and D-Phe are interacting with a different type of opioid receptor from that responsible for the effects of Met-enkephalin. Bradley & Brookes (1981) have indeed reported that μ, δ and κ agonists cause depression of neuronal activity in the rat caudate nucleus, but as all such responses were blocked by naloxone, this cannot account for the present results.

Alternatively it is possible that the iontophoretic technique is inappropriate for detecting local effects of these compounds. Thus, Takagi *et al.*, (1979a,b) used 1–10 μM of kyotorphin applied to striatal slices for 15 min in order to produce a modest release of enkephalin. A slowly developing response over 15 min would be difficult to detect iontophoretically.

It is interesting to note that in the experiments of Satoh *et al.* (1980) the effects of kyotorphin on single neurone activity were said to be antagonized by naloxone. However, only 2 of 4 neurones depressed by kyotorphin exhibited this antagonism, and as no other control depressants were employed in this study, the specificity of naloxone cannot be assessed. It is quite possible that, as in the present study, a dose of naloxone which would antagonize responses to Met-enkephalin would *not* affect responses to kyotorphin.

Whatever the explanation for the present failure to reverse D-Phe and kyotorphin responses by naloxone, the fact remains that both are quite effective depressants of pallidal neurone firing. The possibility should therefore be considered that such a direct inhibition of upper motor neurones could for example, contribute to the behavioural effects of these substances (Takagi *et al.*, 1979b; Filibeck *et al.*, 1981).

Thus the original objective of this study has been only partly realized. While the predicted correlation of responses to Met-enkephalin and kyotorphin was observed in the pallidum, with the rapid time course and naloxone-reversibility of the enkephalin response supporting the view that a local opioid receptor can cause neuronal inhibition, the resistance to naloxone of responses to kyotorphin and D-Phe suggests that their analgesic properties may involve opioid systems only indirectly.

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