Br. J. Pharmac. (198), **71**, 201–210

ACTION OF ENKEPHALIN ANALOGUES AND MORPHINE ON BRAIN ACETYLCHOLINE RELEASE: DIFFERENTIAL REVERSAL BY NALOXONE AND AN OPIATE PENTAPEPTIDE

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1 Methionine (Met)-enkephalin, leucine (Leu)-enkephalin and their synthetic analogues were tested for effects on the spontaneous release of cortical acetylcholine (ACh) *in vivo*. The ability of naloxone to reverse the action of enkephalins on ACh release was compared with its action against morphine. An enkephalin analogue, structurally related to Met-enkephalin, was tested for opiate antagonistic activity in ACh release experiments.

2 Intraventricular administration of Met-enkephalin, Leu-enkephalin, D-Ala^2-Met^5-enkephalinamide (DALA) and D-Ala^2-D-Leu^5-enkephalin (DALEU) produced a dose-related inhibition of cortical ACh release. Met- and Leu-enkephalin were very similar both in their potency and the time course of their action on ACh release. Both DALA and DALEU were more potent and had a longer duration of action than Leu-enkephalin. Systemic injections of two pentapeptides, D-Met^2-Pro^5-enkephalinamide and D-Ala^2-MePhe^4-Met^5(O)-ol-enkephalin (33,824), produced a sustained inhibition of cortical ACh release.

3 Naloxone, administered systemically following the depression of ACh release induced by either intraventricular injections of enkephalins (DALA or DALEU), or systemic injections of enkephalins (D-Met^2-Pro^5-enkephalinamide or 33,824), reversed this depression and restored the release to baseline levels. The effect of D-Met^2-Pro^5-enkephalinamide on the release of ACh was reversed by naloxone with difficulty. Naloxone also reversed the inhibitory effect of systemic morphine and this reversal was associated with a large overshoot of ACh release. The latter was never observed in the enkephalin experiments.

4 Intraventricular injection of the pentapeptide, D-Ala^2-D-Ala^3-Met^5-enkephalinamide (TAAPM), at doses that did not influence the basal ACh release, blocked or reversed the inhibitory effect of morphine on this release. This peptide did not block the effect of the non-opiate, chlorpromazine, under similar conditions. In two experiments TAAPM failed to reverse the inhibition of ACh release produced by systemically injected enkephalin, D-Met^2-Pro^5- enkephalinamide.

5 Effects of morphine and enkephalin on ACh release are discussed in terms of their action on different opiate receptors.

Introduction

It is widely recognized that the endogenous opiate pentapeptides, methionine (Met) and leucine (Leu)-enkephalin, depress the release of acetylcholine (ACh) and noradrenaline (NA) from the electrically stimulated guinea-pig ileum and the mouse vas deferens respectively (Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris, 1975; Waterfield, Smokcum, Hughes, Kosterlitz & Henderson, 1977). Recent *in vitro* studies have shown that significant differences exist between these two tissues with respect to the rank order of enkephalin potencies, and the ability of the narcotic antagonist, naloxone, to antagonize the effects of enkephalins. These findings, and a number of other observations, have led some investigators to propose that the population of opiate receptors in different tissues is heterogeneous (Lord, Waterfield, Hughes & Kosterlitz, 1977).

Enkephalins have also been shown to modify the release of several neurotransmitter substances from the regions of the central nervous system (CNS). Inhibitory effects of enkephalins have been observed on the *in vitro* release of NA (Taube, Borowski, Endo & Starke, 1976), substance P (Jessel & Iversen, 1977) and dopamine (Subramanian, Mitznegg, Sprügel, Domsche, Domschke, Wünsch & Demling, 1977). Previously we have shown that both Met- and Leu-enkephalin depress the *in vivo* release of cortical ACh following intraventricular injections in rats (Jha-

0007-1188/80/130201-10 $01.00

mandas, Sawynok & Sutak, 1977). These findings have raised the possibility that enkephalins modulate the release of several neurotransmitters in the CNS through their action on opiate receptors. Although a great deal of interest has focussed on comparative actions of enkephalins on the peripheral neurotransmission, to date little information exists concerning the effect of enkephalin analogues on the release of central neurotransmitters. Studies in this regard might be helpful in assessing the relative importance of the endogenous enkephalins as modulators of neurotransmitter release processes in the CNS, and they might also provide insight into the type of opiate receptors activated by enkephalins. As a step in this direction we have investigated the effect of several enkephalins on the central release of ACh. This paper describes the dose and time effect relationships of the naturally occurring and synthetic enkephalins on the spontaneous release of ACh from intact rat cerebral cortex.

In previous work on opiates and central ACh release, we have observed differences between the naloxone-induced reversal of morphine and the two naturally occurring enkephalins (Met- and Leu-enkephalin). The reversal of morphine (Jhamandas & Sutak, 1976), methadone and levorphanol (Jhamandas, Hron & Sutak, 1975) by naloxone is always complete and it is associated with a large overshoot. In contrast, the reversal of Met-enkephalin by naloxone at similar doses is often incomplete and it is not associated with an overshoot phenomenon (Jhamandas *et al.*, 1977). Similar differences between morphine and enkephalin reversal by naloxone have been observed by Duggan, Hall & Headley (1977) in their electrophysiological experiments on the cat spinal cord. It is attractive to consider that differential effects of naloxone against morphine and enkephalins reflect an action of these agonists at different opiate receptors (Lord *et al.*, 1977). However, before this idea can be entertained seriously it is important to consider whether certain factors in morphine and enkephalin experiments have a bearing on the differential action of naloxone against these agonists. The metabolic instability of Met- and Leu-enkephalin *in vivo*, their intraventricular injection, and different ACh collection protocols used in enkephalin and morphine experiments (Jhamandas *et al.*, 1977) are among factors which might have influenced naloxone action and hence contributed to the difference between the two agonists in our previous tests. Recently analogues of enkephalins have been synthesized which unlike Met- or Leu-enkephalin are metabolically stable, and some of which are systemically active in analgesic tests. To minimize the influence of the above factors, in this study we have examined the action of naloxone against several such synthetic analogues of Met- and Leu-enkephalin and compared its effect against morphine. This study also describes the antagonistic action of a pentapeptide against morphine and the selectivity of this antagonism.

Methods

Experiments were performed on Sprague-Dawley rats (250 to 300 g) lightly anaesthetized with a mixture of pentobarbitone and urethane (Jhamandas & Sutak, 1976). The spontaneous release of cortical ACh, in the presence of neostigmine (50 mg/ml) and atropine (0.8 mg/ml), was measured by the cup technique. Experimental details of this procedure, and the method for the biological estimation of ACh, have been described in previous publications (Jhamandas & Sutak, 1976; Jhamandas *et al.*, 1977). In some experiments enkephalins were administered by intraventricular (i.c.v.) injection through a cannula placed in the lateral ventricle, and in these tests the samples of cortical fluid (0.2 ml) were collected bilaterally every 10 min, pooled, and estimated as a single sample. In other experiments enkephalins were injected into the femoral vein, and in these successive samples of cortical fluid from the same side were collected at 10 min intervals, pooled, and assayed as a single sample. Pooling of the collections was necessary to provide adequate sample volume (0.4 ml) for the biological estimation of ACh released from the cortex. Details of individual experiments are provided in the Results Section.

Drugs

Drugs used were atropine sulphate, chlorpromazine hydrochloride, morphine sulphate, naloxone hydrochloride, pentobarbitone sodium and urethane. Weights of morphine and all other drugs refer to the salts. Enkephalins used were: methionine-enkephalin, leucine-enkephalin, D-Ala^2-Met^5-enkephalinamide (DALA), D-Ala^2-D-Leu^5-enkephalin (DALEU), D-Ala^2-MePhe^4-Met^5(0)-ol-enkephalin (33,824), D-Met^2-Pro^5-enkephalinamide, D-Ala^2-D-Ala^3-Met^5-enkephalinamide (TAAPM) and D-Phe^4-Met^5-enkephalinamide (TGG(D-P)M).

Results

Enkephalin effects after intraventricular injections

Effects of four opiate pentapeptides, Met-enkephalin, Leu-enkephalin, D-Ala^2-enkephalinamide (DALA), a synthetic analogue resistant to enzymatic degradation (Pert, Pert, Chang & Fong, 1976) and D-Ala^2-D-Leu^5-enkephalin (DALEU), a peptide with a highly selective action on δ-type of opiate receptors (Miller,

Chang, Cuatrecasas, Wilkinson, Lowe, Beddell & Follenfant, 1978) were evaluated on the cortical release of ACh following intraventricular injections. As indicated by the dose-response relationships represented in Table 1, all four peptides produced a dose-related inhibition of this release. The effects of both Met- and Leu-enkephalin were first evident after an injection of 5 μg, and a maximal effect occurred after a dose of 50 μg. In two experiments a higher dose of Met-enkephalin (100 μg) was also tested but this failed to produce a greater depression of ACh release (data not shown). In the dose range 5 to 50 μg the inhibitory effects of Met- and Leu-enkephalin on ACh release were similar in magnitude, although Met-enkephalin tended to have a slightly greater effect at 50 μg dose. The effects of DALA on the release were observed after injection of only 0.5 μg, and a maximal effect was observed after 5 μg dose of this peptide. The responses elicited by DALA in the dose range 0.5 to 5.0 μg were comparable in size to those elicited by Met- or Leu-enkephalin in the higher dose range (5 to 50 μg), indicating a greater inhibitory potency of this synthetic analogue. DALEU effect on ACh was investigated at three doses. At a dose of 0.5 μg (the lowest dose) this peptide produced a depression of ACh release similar to that produced by DALA at this dose, but at 2.0 and 5.0 μg its depressant action exceeded that of DALA at similar doses. Extensive comparison between the effects of DALEU and other pentapeptides could not be undertaken owing to a limited supply of this peptide, but at the doses examined here, DALEU apparently was the most active of the four enkephalins depressing the release of cortical ACh.

The time course of the inhibitory effect of Leu-enkephalin, DALA and DALEU, following a single intraventricular injection at different doses of these peptides, is illustrated by curves shown in Figure 1. Regardless of the peptide or the dose employed, the peak effect always occurred during the first 10 min collection period following the injection. The rate of recovery of release to baseline levels depended on the peptide and the dose producing inhibition of ACh release. A visual inspection of curves shown in Figure 1 indicated that the rate of recovery was fastest from the actions of Leu-enkephalin and was slowest from the effects of DALEU. After doses of the peptide producing a comparable inhibition of release, the recovery from DALA was significantly more prolonged than from Leu-enkephalin. The time course of Leu-enkephalin and Met-enkephalin action was compared at only one dose (20 μg). At this dose there was no significant difference between the duration of their inhibitory effects (not shown). None of the three enkephalins, Met-, Leu-enkephalin or DALA, influenced cortical ACh release following a single intravenous injection at a dose of 2.5 mg/kg. The systemic effect of DALEU was not tested.

Enkephalin effect after systemic injection

In previous experiments two opioid pentapetides, D-Met^2-Pro^5-enkephalinamide (Bajusz, Rónai, Székely, Gráf, Dunai-Kovács & Berzétei, 1977) and D-Ala^2-MePhe^4-Met^5(0)-ol enkephalin (33,824) (Roemer, Buescher, Hill, Pless, Bauer, Cardinaux, Closse, Hauser & Huguenin, 1977), have been shown to induce analgesia following their peripheral administration. In this study the ability of these peptides to influence cortical ACh release following their intraventricular injection was investigated. As illustrated in Figure 2, after a single injection of these peptides at a

Table 1 Comparative effects of four enkephalins of the spontaneous release of cortical acetylcholine (ACh)

Dose (μg/rat, i.c.v.)	% control release of ACh[1]			
	Met-enkephalin	*Leu-enkephalin*	*DALA*[2]	*DALEU*[3]
0.5	—	—	85.0 ± 3.1 (4)	78.6 ± 4.8 (3)
1	—	—	77.2 ± 5.0 (3)	—
2	—	—	76.3 ± 4.7 (3)	57.9 ± 3.3 (3)
5	83.9 ± 3.6 (3)	78.1 ± 5.2 (3)	52.1 ± 2.4 (4)	40.0 (2)
10	59.6 ± 4.5 (3)	56.9 ± 4.1 (4)	52.7 ± 2.9 (3)	—
20	57.8 ± 3.0 (3)	51.6 ± 8.0 (3)	—	—
50	42.9 ± 1.0 (4)	49.8 ± 1.9 (4)	—	—

Results are expressed as mean ± s.e. mean. Number of experiments shown in parentheses.

[1]Control release represents average release during two 10 min collections immediately preceding the enkephalin injection, and each value shown represents release during the first 10 min collection period following this injection.

[2][D-Ala^2-Met^5-enkephalinamide].

[3][D-Ala^2-D-Leu^5-enkephalin].

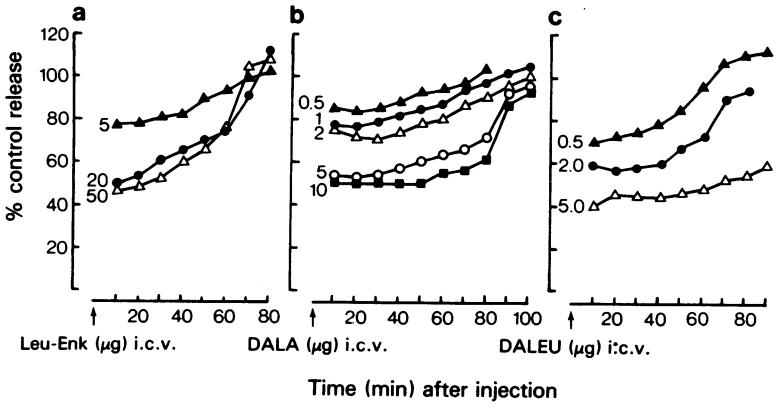

Figure 1 Time course of inhibitory effects of leucine-enkephalin (Leu-Enk), D-Ala^2-Met^5-enkephalinamide (DALA) and D-Ala^2-D-Leu^5-enkephalin (DALEU) on the cortical release of acetylcholine (ACh) following a single intraventricular (i.c.v.) injection into the lateral ventricle. The numbers shown on the figure represent the dose (μg/rat) of enkephalin injected into the ventricle. Each curve represents an average of at least three experiments except that curves shown for DALEU which represent single experiments. Control release (100%) in each case is the average of two 10 min collection periods immediately preceding the injection of the enkephalin.

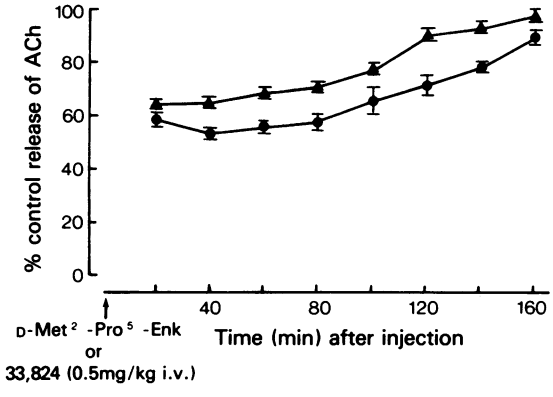

Figure 2 The inhibitory effect of D-Met^2-Pro^5-enkephalinamide (●) and D-Ala^2-MePhe^4-Met^5(O)-ol-enkephalin (33,824) (▲) on the spontaneous release of acetylcholine (ACh) after a single intravenous injection of these peptides. Control release values, representing average release of ACh during two 20 min collection periods preceding the injection, were 2.4 ± 0.1 (●, $n = 4$), and 2.3 ± 0.1 (▲, $n = 4$) ng, 20 min^{-1} 0.25 cm^{-2}.

dose of 0.5 mg/kg there was a depression of cortical ACh release. As in earlier tests (Figure 1) the peak effect was apparent in the first collection period which followed the injection. It should be noted that in these tests the release of ACh was measured at 20 min intervals. Both peptides reduced the release of ACh by about 40%, and the recovery from their effect occurred very slowly. The duration of inhibitory effect of these two peptides on ACh release exceeded that of the highest doses of Leu-enkephalin or DALA (Figure 1).

Reversal of enkephalin and morphine effects by naloxone

As the cortical release of ACh was inhibited by intraventricular injection of metabolically stable enkephalins (DALA and DALEU), and by two systemically active enkephalins (D-Met^2-Pro^5-enkephalinamide and 33,824), experiments were performed to examine the action of naloxone against these pentapeptides. In the following tests, the reversal of inhibitory action of these enkephalins and morphine by systemically administered naloxone was investigated. The results are illustrated in Figures 3 and 4. Figure 3 shows naloxone action on the depression of ACh release evoked by intraventricular DALA and DALEU. Naloxone (0.2 mg/kg) promptly reversed their effect and restored the release to the baseline level in the collection period immediately following its injection, a result similar to that observed previously in experiments with Met- and Leu-enkephalin (Jhamandas *et al.*, 1977). Figure 4 shows naloxone action against two systemically injected enkephalins and morphine. In

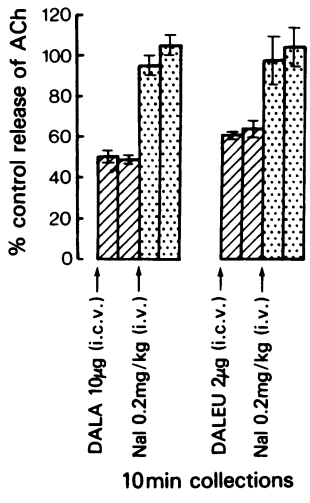

Figure 3 The action of intraventricularly (i.c.v.) administered D-Ala^2-Met^5-enkephalinamide (DALA) and D-Ala^2-D-Leu^5-enkephalin (DALEU) on the spontaneous release of cortical acetylcholine (ACh) and their reversal by intravenous (i.v.) naloxone (Nal). Control release values, representing average release during the two pre-injection collections were 2.4 ± 0.3 (DALA) and 2.1 ± 0.1 ng 10 min^{-1} 0.25 cm^{-2} (DALEU). Values shown are mean of four experiments; vertical lines show s.e. mean.

contrast to the preceding tests (shown in Figure 3), naloxone (0.2 mg/kg) induced a very sluggish reversal of the inhibitory effect of D-Met^2-Pro^5-enkephalinamide (Figure 4a). When the dose of naloxone in subsequent tests was increased five fold (1.0 mg/kg) it still induced a slow reversal of the inhibition produced by this peptide, although in this case the rate of reversal appeared to be a little more rapid than in earlier tests with a lower dose of the antagonist (Figure 4b). Naloxone (0.2 mg/kg) administered after a systemic injection of 33,824, producing an inhibition of ACh release, promptly reversed its depressant action and restored the release to baseline levels (Figure 4c). In marked contrast to enkephalin experiments (Figures 3 and 4a–c), naloxone (0.2 mg/kg) given after an injection of morphine, which depressed ACh release to a comparable level, not only reversed the depressant action of the alkaloid agonist but also produced a large overshoot of the release. The post-naloxone overshoot, exceeding the baseline level by about 150%, was never observed in enkephalin experiments regardless of the dose, route of administration or the type of enkephalin employed in these experiments. At doses (0.2 to 1.0 mg/kg) used in the present study, naloxone has been repeatedly shown to have no effect of its own on the spontaneous release of ACh from the cerebral cortex, although it does enhance the cortical release evoked by electrical stimulation of subcortical areas (Jhamandas & Sutak, 1976).

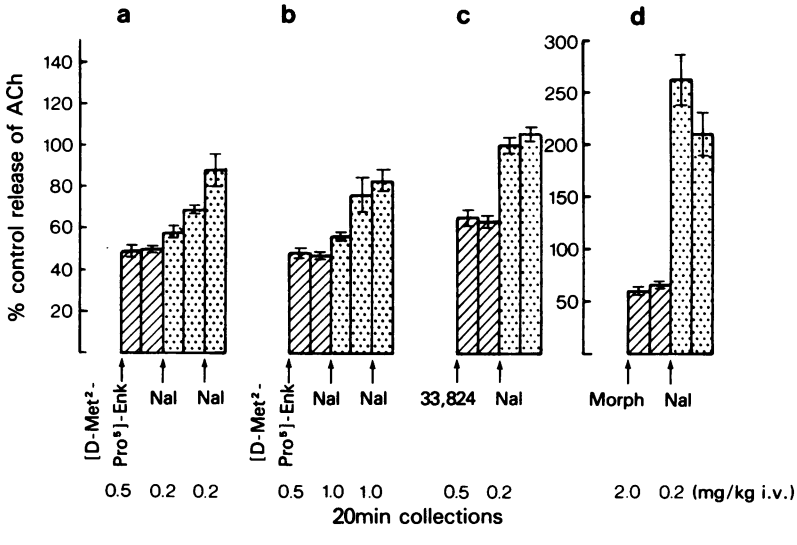

Figure 4 The inhibitory effects of systemically active enkephalins, D-Met^2-Pro^5-enkephalinamide, D-Ala^2-MePhe^4-Met^5 (O)-ol-enkephalin (33,824) and morphine on the spontaneous release of acetylcholine (ACh), and their reversal by systemic naloxone (Nal). All agents shown were administered intravenously. Control release values, representing release during two collections immediately preceding the first injection were: (a) 2.4 ± 0.2 (n = 4); (b) 2.3 ± 0.1 (n = 4); (c) 2.3 ± 0.2 (n = 8); (d) 2.3 ± 0.4 ng 20 min^{-1} 0.25 cm^{-2} (n = 6). Each column represents mean value; vertical lines show s.e. mean. Note the change of scale in (d).

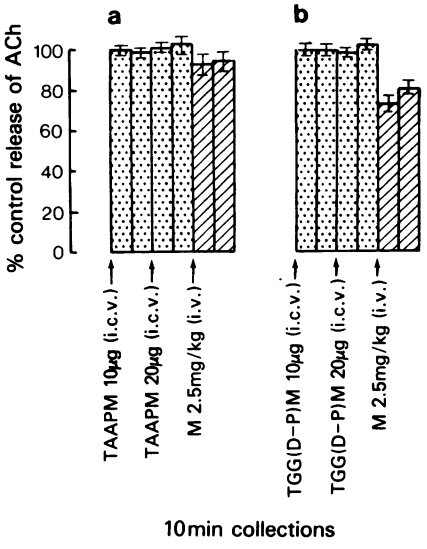

Figure 5 The effect of two pentapeptides, D-Ala^2-D-Ala^3-Met^5-enkephalinamide (TAAPM) and D-Phe^4-Met^5-enkephalinamide (TGG(D-P)M) on the spontaneous release of acetylcholine (ACh) and their influence on the action of morphine (M) on this release. The effect of morphine in the absence of the pretreatment with these peptides (control morphine response) is shown in Figure 6. Control release values, representing the release during two collections immediately preceding the first injection, were: (a) 2.5 ± 0.2; (b) 2.3 ± 0.1 ng 10 min^{-1} 0.25 cm^{-2}. Values shown represent mean of 3 experiments; vertical lines show s.e. mean.

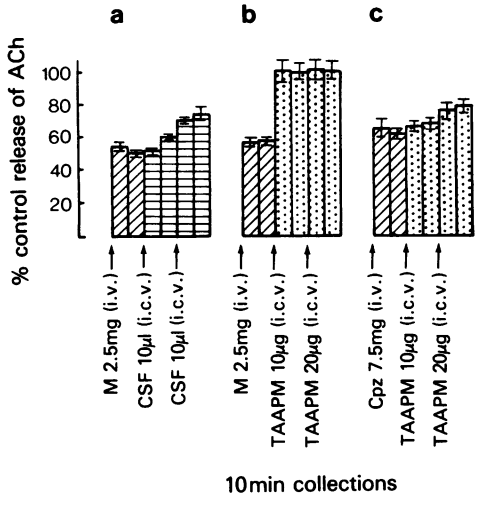

Figure 6 The reversal of inhibitory action of morphine (M) on the release of cortical acetylcholine (ACh) by the pentapeptide D-Ala^2-D-Ala^3-Met^5-enkephalinamide (TAAPM) (b), and the failure to reverse the inhibitory action of chlorpromazine (Cpz) by this peptide (c). The doses of TAAPM injected were contained in the volumes of CSF shown in control experiments in (a). Control release values, representing the average release during the two collection periods preceding the first injection, were (left to right), 2.3 ± 0.2, 2.3 ± 0.1, 2.6 ± 0.1 ng 10 min^{-1} 0.25 cm^{-2}. Values shown are mean of 3 experiments; vertical lines show s.e. mean.

Antagonism of morphine effect by an enkephalin analogue

As a control experiment for possible artifacts resulting from either intraventricular injections or the osmotic effects of peptide solutions in ACh release experiments, it was decided to administer enkephalin analogues which have poor biological activity. Preliminary tests in this laboratory showed that the two peptides, D-Phe^4-Met^5 (TGG(D-P)M) and D-Ala^2-D-Ala^3-Met^5-enkephalinamide (TAAPM) had a very weak inhibitory action on the contractions of the guinea-pig isolated ileum (unpublished), and therefore these relatively 'inactive' enkephalins were administered to rats in our ACh release experiments. As expected, at doses of 10 to 20 μg, both peptides failed to influence the spontaneous release of ACh over the time period during which other enkephalins reduced this release. This lack of enkephalin effect on the spontaneous release observed in the present study was reminiscent of the action of naloxone in our earlier tests (Jhamandas & Sutak, 1976). The latter showed that naloxone by itself did not modify the spontaneous release of ACh but it effectively antagonized the morphine effect. Therefore it was of interest to test whether the two 'inactive' enkephalins, TAAPM and TGG(D-P)-M, would antagonize the action of morphine on the release of cortical ACh. This was tested in subsequent experiments and the results are shown in Figure 5. As noted above, both peptides failed to alter the release of ACh on their own (Figure 5). However, an injection of morphine 2.5 mg/kg (a dose slightly higher than that which depressed ACh release by about 40% in experiments shown in Figure 4) administered after TAAPM pretreatment did not produce the expected inhibition of ACh release (Figure 5). The significant reduction of morphine by TAAPM indicated that this peptide had blocked the inhibitory action of morphine. In contrast, morphine (2.5 mg/kg) administered after pretreatment with TGG(D-P)M still produced a significant inhibition of the release of ACh (Figure 5), although in this case morphine effect was reduced by about 10% when compared with control response (see later experi-

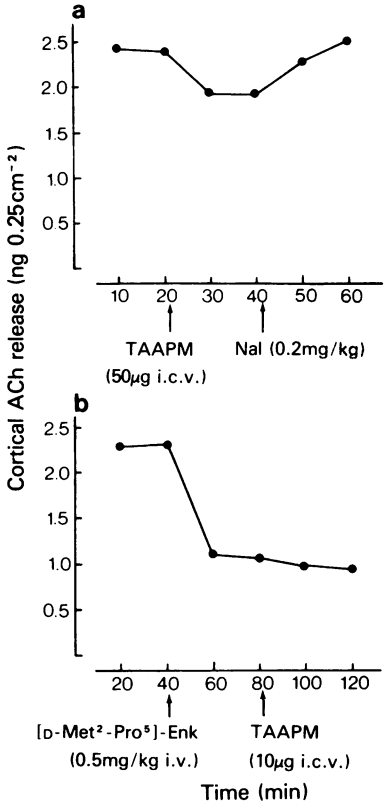

Figure 7 (a) Inhibitory effect of a large dose of the pentapeptide D-Ala^2-D-Ala^3-Met^5-enkephalinamide (TAAPM) on the cortical release of acetylcholine (ACh) and the reversal of this effect by systemic injection of naloxone. (b) The failure of TAAPM to reverse the inhibitory action of D-Met^2-Pro^5-enkephalinamide on the cortical release of ACh. Values shown on each curve are an average of 2 experiments.

ments, Figure 6). Thus TGG(D-P)M, unlike the peptide TAAPM, did not block the morphine effect on ACh release.

Blockade of the morphine effect by TAAPM stimulated interest in the possibility that this peptide might also reverse the inhibitory effect of morphine on ACh release. Experiments were subsequently performed to test this possibility and the results are depicted by histograms shown in Figure 6. Figure 6a shows the effects of injection vehicle (CSF) alone on the inhibitory action of systemic morphine. The vehicle had no significant effect on morphine action and the post-morphine release shown in the histogram simply represents a gradual recovery from the inhibitory action of this agonist. In separate experiments (Figure 6b), injection of TAAPM (10 μg), contained in the same volume of the vehicle (10 μl), promptly reversed the depressant action of morphine on ACh release. This reversal was not associated with an overshoot phenomenon. Administration of a second injection of TAAPM produced no additional change in ACh release. To test the selectivity of TAAPM against morphine, this peptide was administered after a comparable depression of ACh release induced by the non-opiate, chlorpromazine. The results (Figure 6c) showed that TAAPM did not reverse chlorpromazine-induced depression, indicating that the reversal of morphine effect by this peptide was indeed selective. To test whether TAAPM would act as an antagonist of enkephalins, it was injected after the systemically active pentapeptide, D-Met^2-Pro^5-enkephalinamide, in two experiments similar to those described above. In both experiments TAAPM, at a dose which had previously reversed morphine action, completely failed to reverse the inhibitory action of D-Met^2-Pro^5-enkephalinamide on ACh release, and in fact it slightly augmented this action (Figure 7b). These findings, and the results of preliminary tests showing a weak inhibitory activity of TAAPM on the guinea-pig ileum, suggested that at a certain dose level this peptide might have the ability to alter ACh release by itself. The effect of a higher dose of TAAPM (50 μg) was therefore tested on the release of cortical ACh. As shown in Figure 7a this dose of the peptide depressed ACh release by about 20% (2 experiments) and its action, like that of other enkephalin analogues, was reversed by systemic naloxone. The ability of TAAPM to antagonize morphine at lower doses, and to inhibit ACh release by itself at a higher dose in these tests suggested that this peptide was acting as a partial opiate agonist. Its antagonistic activity against other opiates and enkephalins was not examined in this study.

Discussion

Since the discovery of two morphinomimetic pentapeptides, Met- and Leu-enkephalin in the CNS (Hughes *et al.*, 1975), there has been considerable interest in the comparative pharmacology of these peptides and their synthetic analogues. A number of different studies have pointed to the existence of quantitative or qualitative differences between the two naturally occurring pentapeptides. In tests on isolated peripheral tissues (Waterfield *et al.*, 1977; Shaw & Turnbull, 1978), and opiate receptor binding assays (Simantov & Snyder, 1976), Met-enkephalin was found to have a greater potency than Leu-enkephalin. In some behavioural studies, qualitative differences have been observed between these two peptides with respect to low intensity pain (Leybin, Pinsky, LaBella, Havelicek & Rezek, 1976), motility (Chang, Fong,

Pert & Pert, 1976) and memory (Rigter, 1978). Recently Vaught & Takemori (1979) have reported that in mice, Leu-enkephalin, but not Met-enkephalin, modified analgesia, tolerance and dependence. These investigators suggested that Leu-enkephalin may be the important physiological modulator of narcotic action.

These reports raise the possibility that Met- and Leu-enkephalin might differ significantly in their effects on the release of neurotransmitters in the CNS sensitive to the opiate peptides. This study examined the comparative effects of these two peptides, and their synthetic analogues (DALA and DALEU), on the release of cortical ACh *in vivo*. All of these enkephalins reduced the spontaneous release of ACh in a dose-related fashion, although they differed in the potency and the time course of their action on this release. Synthetic analogues, DALA and DALEU, clearly exceeded the naturally occurring peptides both in potency and the duration of their inhibitory effect, but no significant difference was apparent between Met- and Leu-enkephalin in this respect. The latter finding is in contrast to previous reports of significant differences between these peptides in the isolated tissue or behavioural tests, but it is in accord with the results of Malick & Goldstein (1977) who showed that both Met- and Leu-enkephalin produced analgesia in rats and were equipotent. The similarity between Met- and Leu-enkephalin actions on cortical ACh release suggests that, in considering the endogenous opiate peptides as neuromodulators of transmitter (ACh) release in the CNS, both peptides should be regarded as important physiological modulators of this phenomenon. However, the present study cannot completely exclude the possibility of a significant difference existing between these enkephalins, since their effects have been studied without inactivating their metabolism, and hence the estimates of potency only reflect apparent potency of these peptides. Estimates of true potency, obtained in the absence of enkephalin metabolism *in vivo*, might reveal an important difference between the effects of Met- and Leu-enkephalin and provide insight into the relative importance of these peptides in the CNS. However, at present it is difficult to prevent enkephalin metabolism completely in the intact brain, although certain non-specific inhibitors such as bacitracin can reduce this to some extent (Craves, Law, Hunt & Loh, 1978). Estimation of true potencies of enkephalins and firm conclusions regarding their relative importance as neuromodulators, must await development of more specific and effective inhibitors of enkephalin metabolism.

Notwithstanding the fact that enkephalins (Met and Leu) are degraded very rapidly in the brain (Craves *et al.*, 1978), it has been possible to demonstrate clearly their inhibitory actions on the release of central ACh in the present study. Although the effects of these peptides were not as long-lasting as those of their metabolitically stable analogues, DALA or DALEU, they were still of considerable duration when compared with their analgesic actions. In the past, analgesic effects of Met- and Leu-enkephalin have been difficult to demonstrate (Chang *et al.*, 1977), and when observed these effects were found to be of a very short duration (2 to 3 min) (Malick & Goldstein, 1977). The transient analgesic actions of Met- and Leu-enkephalin have been attributed to their rapid degradation by the brain tissue enzymes (Pert *et al.*, 1976). However, on the basis of a study of enkephalins of varying metabolic stability, some investigators have found that there is no simple correlation between the metabolic stability and the analgesic activity of enkephalins (Bajusz, Patthy, Kenessey, Gráf, Székely & Rónai, 1978). These investigators suggest that the latter should be closely related to factors such as improved receptor binding and transport properties. It is possible that the anti-release effects of Met- and Leu-enkephalin are more profound than their analgesic effects because of certain differences between the receptors, or between the post-receptor coupling mechanisms, that are involved in the expression of these two effects of enkephalins. Alternatively, the effect of enkephalin on ACh release might fall into a category of drug-induced responses where the effect persists after the removal of drug from the target site. This possibility is diminished by the fact that naloxone can terminate the enkephalin effect, indicating that the peptide must be present at the opiate receptors during the response.

The present study also examined the effects of two enkephalins which are of interest because of their special pharmacological properties. The pentapeptide, DALEU, is interesting because its potency is nearly 1200 times higher than that of morphine in the mouse vas deferens assay while in the guinea-pig ileum it is only twice that of morphine (Miller *et al.*, 1978). DALEU therefore might be regarded as a ligand which is much more selective for the δ-type of opiate receptors which predominate in the mouse vas deferens, and less selective for the μ-type of receptors which predominate in the ileum and are primarily morphine-sensitive (Lord *et al.*, 1977). In this study, DALEU produced a very strong and sustained inhibition of ACh release from the cortex, its effects in this respect exceeding those of DALA. This observation indicates that δ-type of opiate receptors, as well as the μ-type, are linked to the neuronal mechanisms releasing cerebral ACh. The pentapeptide, 33,824, is of pharmacological interest because in behavioural tests its profile resembles that of β-endorphin (Roemer *et al.*, 1977). We have observed that this pentapeptide is very active in depressing the contractions of electrically stimulated rat vas deferens (unpublished observations), a preparation which is sensitive to low doses

of β-endorphin but not morphine (Lemaire, Magnan & Regoli, 1978). In these release experiments, 33,824 produced a long-lasting inhibition of ACh release after a relatively low dose (0.5 mg/kg). This finding suggests that β-endorphin, which has not been tested in these release experiments, might be a powerful inhibitor of cortical ACh. The fact that enkephalins, and possibly endorphins, can modify the release of cerebral ACh provides a model for studying neurochemical effects of these peptides *in vivo*. This model could be useful in the study of comparative actions of the short and long opiate peptides, as well as effects of agents affecting the release or metabolism of these peptides.

The results of this study sustain our earlier impression that naloxone produces a differential reversal of the enkephalin and the morphine effect on cortical ACh release. Several differences between enkephalin and morphine became apparent in the present reversal experiments. First, the inhibition of certain peptides, notably D-Met^2-Pro^5-enkephalinamide, was resistant to naloxone, as indicated by its slow reversal by a dose of the antagonist five times that used to reverse the morphine effect. Secondly, the post-naloxone overshoot of ACh release, consistently observed in morphine tests, was never observed in enkephalin experiments, regardless of the type of enkephalin used or the route of its administration. Thirdly, the inhibitory action of morphine on ACh release was reversed by the pentapeptide TAAPM, while that of an enkephalin (D-Met^2-Pro^5-enkephalinamide) was not affected by this peptide. The fact that a difference between the reversal of morphine and enkephalins was observed in experiments involving metabolically stable or systemically active enkephalin analogues clearly indicates that it is not related to factors such as the agonist stability or the route of administration, as might have appeared initially on the basis of an earlier investigation on met-enkephalin (see Introduction). Differences between morphine and enkephalin, as regards their reversal by the antagonists, must therefore be based on other factors. Duggan *et al.* (1977), who observed similar differences between the action of naloxone against morphine and enkephalins in electrophysiological tests on cat spinal neurones, have suggested that these might be related to different levels of opiate receptor activation by these agonists. This explanation is unlikely to apply in the release experiments since morphine and enkephalins produced a comparable inhibition of ACh release (indicating a similar level of receptor activation).

The difference between the profiles of morphine and enkephalin reversal noted in this investigation could be viewed in the context of the suggestion by Lord *et al.* (1977) that there are multiple opiate receptors, and that these agonists might be acting on separate opiate receptors. At certain peripheral junctions (e.g. the mouse vas deferens) enkephalins are relatively resistant to naloxone, and in binding assays on brain homogenates enkephalins displace [^{3}H]-naloxone less readily than morphine (Lord *et al.*, 1977), suggesting that the population of receptor influenced by morphine and enkephalins might be different. In the ACh release experiments performed here, the relative ease of morphine reversal by naloxone and the overshoot associated with it may reflect an action of the alkaloid mainly on one type of opiate receptor, while the difficulty associated with enkephalin reversal by naloxone, or the lack of overshoot phenomenon, may reflect enkephalin action on a second type of opiate receptor. The results of experiments with TAAPM, in which a difference between the reversal of morphine and an enkephalin was observed, may also be interpreted in terms of their action on different opiate receptors, recognizing the fact that results of these did not strictly parallel those of naloxone experiments. The overshoot in ACh release induced by naloxone was not seen after TAAPM in morphine experiments, and while naloxone reversed enkephalins, TAAPM failed to do so. Unlike naloxone, TAAPM has some intrinsic activity and this fact may have contributed to the differences between the two antagonists. However, the fact remains that in tests with both antagonists the profiles of morphine and enkephalin reversal were different, and this suggests that the agonists might be interacting with distinct opiate receptors. It is unlikely that morphine and the enkephalins interact with one type of opiate receptor exclusively, but more probably there is a certain degree of overlap between their actions. This is supported by the observation that the inhibitory effects of Met- and Leu-enkephalin on ACh release are reduced in morphine-tolerant animals (Jhamandas & Sutak, 1978).

The action of enkephalins on the release of cortical ACh release suggests that neuronal mechanisms which release these peptides in the CNS might interact with those releasing ACh at the cerebral cortex or other regions of the CNS. The characteristics of such 'enkephalinergic-cholinergic' interactions remain to be elucidated in future work.

The authors gratefully acknowledge the generous gifts of enkephalins from Dr Robert Fredrickson (The Lilly Research Laboratories, Indianapolis, Indiana, U.S.A.), Dr S. Wilkinson (The Wellcome Research Laboratories, Langely Court, Beckenham, Kent), and Dr D. Roemer (Sandoz, A. G., Basel, Switzerland); they also thank Endo Laboratories for the gift of naloxone hydrochloride. This work was supported by the Medical Research Council of Canada. We thank Dr W. J. Racz for helpful criticism of the manuscript.

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(Received December 13, 1979.)