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THE EFFECT OF NALOXONE ON OPIOID-INDUCED INHIBITION AND FACILITATION OF ACETYLCHOLINE RELEASE IN BRAIN SLICES

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1 The effect of morphine, methionine-enkephalin (Met-enkephalin) and D-Ala^2-D-Leu^5-enkephalin (DADLE) were tested on the spontaneous and electrically-evoked release of acetylcholine (ACh) from superfused slices of guinea-pig thalamus, caudate nucleus and cerebral cortex.

2 At no concentration did morphine, Met-enkephalin or DADLE modify the outflow of ACh at rest but Met-enkephalin in the presence of naloxone, reduced the resting ACh release.

3 Morphine, at a low dose (3 μM) had no effect in slices of cerebral cortex, but it enhanced the evoked release of ACh in thalamic and caudate, slices. At higher doses of morphine (10–30 μM), the ACh release evoked by electrical pulses was significantly inhibited in every area.

4 Met-enkephalin behaved like morphine in thalamic slices, whereas DADLE, a specific δ agonist, produced a slight inhibition of ACh outflow only at 10 μM.

5 Naloxone antagonized the inhibitory effect of morphine in the cerebral cortex and caudate nucleus slices. Naloxone and also spiroperidol blocked the releasing effect of morphine in caudate slices. In contrast naloxone did not affect the increase of ACh release caused by morphine and Met-enkephalin in thalamic slices. The inhibitory effect of both opioids at high doses was reversed by naloxone so that they then enhanced ACh release.

6 A two fold increase of calcium concentration in the Krebs solution prevented the inhibitory effects of morphine 10 μM.

7 It is suggested that two receptors are present in thalamic slices, one of which inhibits and the other facilitates ACh release.

Introduction

Many reports have shown that, *in vivo*, morphine inhibits acetylcholine (ACh) release from the cerebral cortex (Domino, 1979). This effect which is prevented by naloxone (Jhamandas, Phillis & Pinsky, 1971; Jhamandas, Hron & Sutak, 1975; Beani, Siniscalchi & Sarto, 1979; Domino, 1979; Jhamandas & Sutak, 1980), may be due to an action on subcortical sites, since it is abolished by lesions of the medial thalamus or septum (Jhamandas & Sutak, 1976; Pepeu, Garau, Mulas & Marconcini-Pepeu, 1975). Also morphine and the enkephalins have been shown to enhance ACh content and to reduce its turnover in several brain areas (Giarman & Pepeu, 1962; Green, Gliek, Crane & Szilagyi, 1975; Cheney & Costa, 1977; Moroni, Cheney & Costa, 1977; Sethy, 1978).

Recently Wood & Stotland (1980) showed that μ-opiate agonists and enkephalins reduce ACh turnover rate in the hippocampus and parietal cortex, but not in the striatum. On the other hand, some workers have demonstrated increased ACh utilization and release in certain structures (Vasko & Domino, 1976; Jhamandas & Sutak, 1976).

The results of *in vitro* release studies are conflicting. Szerb (1974) showed that morphine (0.3–30 μM) did not modify the ACh release from electrically-stimulated slices of rat cerebral cortex, striatum and hippocampus whilst others found that morphine and enkephalins inhibited ACh release evoked either by KCl or by electrical pulses (Sharkawi & Shulman, 1969; Jhamandas *et al.*, 1975; Subramanian, Mitznegg, Spügel, Domschke, Domschke-Wünsch & Demling, 1977; Iwatsubo & Kondo, 1977).

On the other hand, Vizi, Harsing & Knoll (1977) have shown that β-endorphin and morphine enhanced the ouabain-induced ACh outflow from normal rat striatal slices, while these drugs inhibited the release from striatal slices of animals pretreated with 6-hydroxydopamine. The above discrepancies may be attributed to the presence of multiple opiate receptors in the brain, which could modulate, directly or indirectly and in opposite directions, the activity of the cholinergic neurones in different nuclei or areas.

We have studied the effect of morphine and enkephalins on the spontaneous and stimulus-induced release of ACh from thalamic slices. For comparison

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the analysis was extended to the cerebral cortex and caudate nucleus. Our findings support the view that two kinds of receptors controlling ACh release, one inhibitory and the other excitatory, are present in the guinea-pig thalamus. A preliminary account of these findings has been published (Beani, Bianchi & Siniscalchi, 1981).

Methods

Acetylcholine release

Guinea-pigs, weighing 400–500 g, were killed by decapitation. The following areas were removed: right and left parietal cortex, caudate nucleus (head and body) and thalamus. Both thalami were dissected from the rest of the brain with a transverse cut to separate the thalamic complex from the corpora quadrigemina and with another semicircular cut along the capsula interna reaching the anterior commissura. The hypothalamus was excluded with a horizontal cut at the sulcus hypothalamicus level. The tissue was placed in cold oxygenated Krebs solution and was sliced (0.400 mm thick) with a fresh tissue microtome. The slices from one animal were allowed to equilibrate for 30 min at room temperature. Then, they were transferred into superfusion chambers (volume 0.9 ml), and superfused (0.5 ml/min) with Krebs solution, bubbled with 95% O_2 plus 5% CO_2, at 37°C.

As a general rule, these experiments consisted of two cycles. Each cycle was divided into two 5 min stimulation periods, at different frequencies, each preceded by 5 min of rest. Electrical stimulation of the slices was performed at different frequencies (1, 2 or 5 Hz) with rectangular pulses of alternating polarity, current strength 30 mA/cm^2, duration 5 ms. The details of this technique and calculation of the electrically-evoked ACh extra-release are described by Beani, Bianchi, Giacomelli & Tamberi (1978).

The drugs were added 15 min before starting the 2nd cycle of stimulation. The effect of drug treatment in cortical and thalamic slices was evaluated by comparing the 2nd cycle (treated) release with that of the 1st cycle (internal controls).

In the caudate nucleus slices the effect was assessed by comparing the release during drug-treatment with that of 2nd cycle of non-treated external controls (see results).

Acetylcholine assay

ACh released from brain slices was bioassayed on guinea-pig ileum pretreated (60 min) with tetrodotoxin (0.03 mM) and maintained in a Tyrode solution containing morphine (3 μM) and cyproheptadine (3 nM) (see Beani *et al.*, 1978).

The samples were assayed for their ACh content with suitable standards. The ACh standards were added to Krebs solutions, both with and without drugs, in order to avoid possible interference in the bioassay. The usual controls, i.e. atropine treatment or alkaline hydrolysis, were made to ensure biological response specificity.

Materials

The following drugs were used: D-Ala^2-D-Leu^5-enkephalin (from Bachem Feinchemikalien, Switzerland), methionine-enkephalin (Met-enkephalin), physostigmine sulphate and acetylcholine chloride (from Sigma Chemical Company Ltd, U.S.A.), morphine HCl and naloxone (from Solars, Italy), tetrodotoxin (from Sankyo, Japan).

Krebs solution was of the following composition (mM): NaCl 118.5, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 10, $NaHCO_3$ 25, choline 0.02 and physostigmine 0.03.

Statistical analysis of results

The data were analysed by the paired and non-paired Student's *t* test. Probability levels of less than 0.05 were taken to indicate statistical significance.

Results

Morphine and naloxone effects on acetylcholine release from cerebral cortex

As previously reported, the ACh release in this brain area remained constant in subsequent stimulation cycles, thus results of the first cycle could be used as controls (Beani *et al.*, 1978). Morphine at all concentrations tested did not change the resting release. The drug (30 μM), at 5 Hz, caused a small but significant inhibition of the evoked release but had no effect at 1 Hz. Naloxone (10 μM), itself was ineffective, but counteracted the inhibitory effect of morphine (Figure 1).

Morphine and naloxone effects on acetylcholine release from caudate nucleus

In previous experiments it was shown that the evoked ACh release in this brain area increased by 20–30% with time (Bianchi, Tanganelli & Beani, 1979); therefore the effect of drug treatment on caudate nucleus slices was assessed by comparing the release of treated slices with that of untreated ones (external controls, 2nd cycle). Morphine had no effect on the resting release of ACh. At 3 μM, the drug significant-

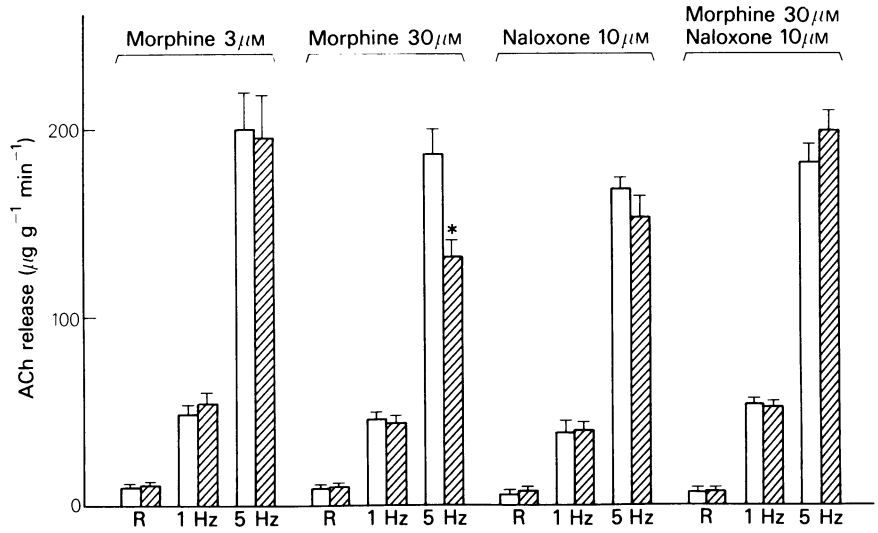

Figure 1 Effect of morphine and naloxone on acetylcholine (ACh) release from slices of guinea-pig cerebral cortex kept at rest (R) and stimulated for 5 min at different frequencies. Morphine and naloxone were added to the Krebs solution between the first (control) and second cycle. Columns show mean values; vertical lines show s.e.mean. Open columns = control; hatched columns = treated. $*P < 0.05$ significantly different from controls (first cycle), *t* test for paired data.

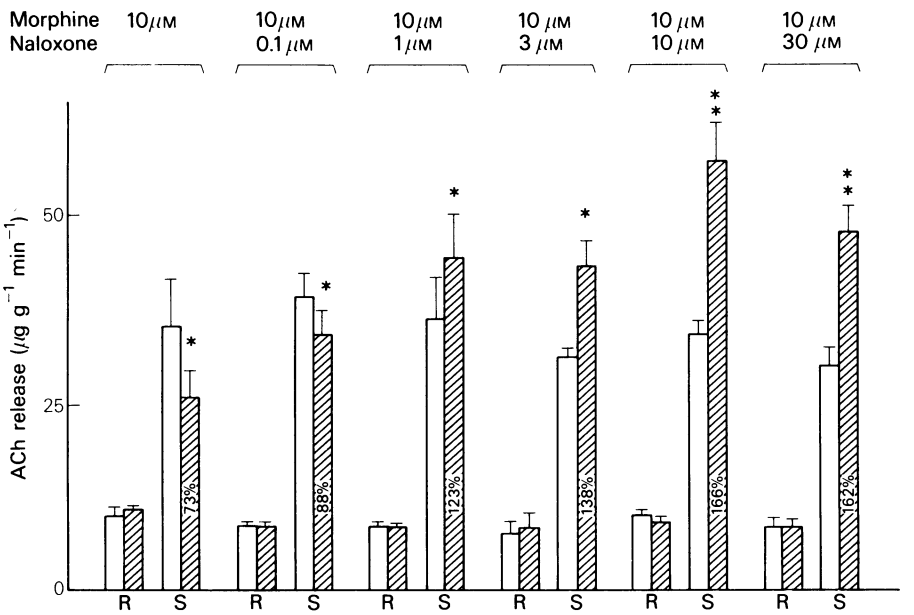

Figure 2 Effect of morphine, in the presence of different naloxone concentrations, on acetylcholine (ACh) release from slices of guinea-pig thalamus kept at rest (R) and stimulated from 5 min at 2 Hz (S). Morphine and naloxone were added to Krebs solution between the first cycle (control, open columns) and second cycle (treated, hatched columns). The percentage changes with respect to the control are also shown. Vertical lines indicate s.e.mean. $*P < 0.05$ significantly different from controls (first cycle), *t* test for paired data; $**P < 0.01$ significantly different from controls (first cycle), *t* test for paired data.

Table 1 Effect of morphine and naloxone on acetylcholine release from slices of guinea-pig caudate nucleus kept at rest and stimulated for 5 min at different frequencies

Treatment	No of Expts	Acetylcholine release ($ng\,g^{-1}\,min^{-1}$) No stimulation	1 Hz	5 Hz
Controls	5	166±26	253±37	770±71
Morphine 1 μM		152±16	229±41	795±118
Controls	9	130±20	298±34	768±167
Morphine 3 μM		120±10	380±26* (127%)	815±73
Controls	7	127±11	282±40	791±79
Morphine 30 μM		131±20	212±26* (75%)	663±66* (84%)
Controls	5	116±21	249±39	714±106
Naloxone 10 μM		114±13	235±27	725±108
Controls	5	138±8	287±4	846±11
Morphine 3 μM + naloxone 1 μM		136±7	268±26	776±30
Controls	8	111±3	310±29	814±60
Morphine 30 μM + naloxone 10 μM		124±18	340±29	785±61
Controls	4	112±7	273±20	—
Morphine 3 μM + spiroperidol 0.25 μM		127±13	295±40	—

Morphine and naloxone were added to the Krebs solution between the first and second cycle. In parentheses the percentage changes with respect to the control. (Second cycle of untreated slices, see methods). Values are mean ± s.e.mean.

*$P < 0.05$ significantly different from controls, t test for non-paired data.

ly increased ACh release evoked at 1 Hz. Inhibition, both at 1 and 5 Hz, was observed when the morphine concentration was raised to 30 μM (Table 1). Naloxone 10 μM abolished both morphine facilitation and inhibition. In order to check whether the increase in ACh release induced by morphine depended on dopamine inhibition of the cholinergic structures, spiroperidol was tested. In aggreement with Vizi *et al.* (1977) the dopamine antagonist abolished morphine facilitation (Table 1).

Morphine and naloxone effects on acetylcholine release from thalamic slices

In this tissue the average ACh release at rest was $9.57\,ng\,g^{-1}\,min^{-1} \pm 0.55$ (60 expts). When field stimulation was applied to the tissue, the increase in ACh outflow at 1 Hz was negligible (data not given) while at 2 Hz it was $29.7\,ng\,g^{-1}\,min^{-1} \pm 1.82$ and at 5 Hz $102.9\,ng\,g^{-1}\,min^{-1} \pm 6.43$ (60 expts).

In this preparation the spontaneous and evoked ACh outflow remained constant for 2–3 h so that the replication of up to three stimulation cycles at 2 and 5 Hz was possible. Thus the first cycle was used as control. Morphine did not modify the ACh release at rest (Table 2) but the opiate, at a relatively low concentration of 3 μM increased the stimulus-evoked release at 2 Hz. At higher concentrations (10–30 μM) morphine inhibited ACh outflow both at low and high stimulation rates (Table 2).

The pretreatment of the thalamic slices with naloxone (agonist/antagonist ratio 3:1) had no effect on the facilitation of ACh release induced by morphine (3 μM) at 2 Hz. Conversely naloxone reversed morphine (10–30 μM) inhibition into a dose-dependent facilitation ($r = 0.68$, $P < 0.01$) (Table 2). Unmasking of the naloxone excitatory effect is further shown in Figure 2. In these experiments the progressive reversal of morphine (10 μM) inhibition was obtained by increasing concentrations of naloxone.

The reversal was evident (i.e. + 23%) when the agonist/antagonist ratio was 10:1 and it was maximal (i.e. + 66%) when the agonist/antagonist ratio was 1:1 (Figure 2). When 5 Hz stimulation was used, naloxone abolished morphine inhibition without unmasking any facilitation (Table 2).

Effect of enkephalins on acetylcholine release from thalamic slices

The specificity of the morphine effect in this tissue was checked by testing the effects of Met-enkephalin

Table 2 Effect of morphine and naloxone on acetylcholine release from slices of guinea-pig thalamus kept at rest and stimulated for 5 min at different frequencies

Treatment	No of Expts	Acetylcholine release ($ng\,g^{-1}\,min^{-1}$) No stimulation	2 Hz	5 Hz
Controls	5	10.3 ± 0.9	32.6 ± 5	113 ± 19
Morphine 1 μM		10.8 ± 0.9	31.6 ± 5	116 ± 12
Controls	13	10.5 ± 0.6	30.4 ± 2.1	103 ± 5
Morphine 3 μM		11.2 ± 0.6	35.4 ± 1.8* (120%)	103 ± 6
Controls	7	10.0 ± 1.2	35.2 ± 6	117 ± 13
Morphine 10 μM		10.7 ± 0.7	25.7 ± 4* (73%)	104 ± 10
Controls	7	9.4 ± 0.9	34.1 ± 4.7	117 ± 14
Morphine 30 μM		11.0 ± 1	20.6 ± 2.4** (62%)	98 ± 9* (86%)
Controls	7	10.0 ± 0.5	26.1 ± 3.8	93 ± 10
Naloxone 10 μM		9.8 ± 0.5	27.8 ± 4	87 ± 10
Controls	7	9.8 ± 0.6	32.2 ± 4	102 ± 11
Morphine 3 μM + naloxone 1 μM		11.2 ± 0.7	38.7 ± 5* (119%)	110 ± 9
Controls	5	7.5 ± 0.5	31.1 ± 0.9	115 ± 8
Morphine 10 μM + naloxone 3 μM		8.3 ± 1.5	43.1 ± 3.8* (138%)	114 ± 11
Controls	5	9.2 ± 0.9	28.0 ± 4	92 ± 12
Morphine 30 μM + naloxone 10 μM		10.1 ± 1.4	43.6 ± 6** (157%)	87 ± 7

Morphine and naloxone were added to the Krebs solution between the first (control) and second cycle. In parentheses the percent changes with respect to the control (first cycle). Values are mean ± s.e.mean.
*$P < 0.05$ significantly different from controls (first cycle), *t* test for paired data; **$P < 0.01$ significantly different from controls (first cycle), *t* test for paired data.

and D-Ala^2-D-Leu^5-enkephalin (DADLE), a peptide with highly selective action on δ-type opiate receptors (Miller, Chang, Cuatrecasas, Wilkinson, Lowe, Beddel & Follenfant, 1978). As shown in Table 3, the enkephalins did not modify the spontaneous ACh release. Up to 3 μM DADLE did not affect the evoked ACh release, but at 10 μM significantly reduced it and the effect was prevented by naloxone at 10 μM. Conversely, Met-enkephalin at 10 μM, significantly increased ACh output evoked by 2 Hz stimulation (Table 3).

When the Met-enkephalin concentration was raised to 30 μM, the drug inhibited ACh release elicited by low frequency stimulation. Naloxone (10 μM) did not affect the facilitatory effect of Met-enkephalin but the antagonist increased the Met-enkephalin (10 μM) induced facilitation and reversed the inhibition caused by higher doses of the peptide into a facilitation (Table 3).

It is worth noting that, when naloxone (10 μM) was present, Met-enkephalin (10–30 μM) inhibited ACh release at rest. This effect was seen in slices pretreated for 30 min with tetrodotoxin at 0.5 μM (controls: $9.5 \pm 0.9\,ng\,g^{-1}\,min^{-1}$; Met-enkephalin plus naloxone-treated: $6.1 \pm 0.6\,ng\,g^{-1}\,min^{-1}$; tetrodotoxin: $9.1 \pm 0.2\,ng\,g^{-1}\,min^{-1}$; tetrodotoxin plus Met-enkephalin plus naloxone-treated: $6.2 \pm 0.8\,ng\,g^{-1}\,min^{-1}$, mean of 5 expts).

Dependence of morphine effects on calcium

Since it is known that calcium antagonizes many of the actions of morphine (Ross, 1978), we tried to check if the increased Ca^{2+} concentration in the medium modified the morphine effects on release of ACh. A doubling of the $[Ca^{2+}]$ increased the stimulus-evoked ACh release but did not change the resting outflow (Figure 3). The increase of $[Ca^{2+}]$ prevented the inhibition by morphine (10 μM) and seemed also to prevent facilitation of ACh release by morphine in the presence of naloxone.

Table 3 Effect of Met-enkephalin (ME) and D-Ala^2-D-Leu^5-enkephalin (DADLE) on acetylcholine release from slices of guinea-pig thalamus at rest and stimulated for 5 min at different frequencies

Treatment	No of Expts	Acetylcholine release ($ng\,g^{-1}\,min^{-1}$) No stimulation	2 Hz	5 Hz
Controls	5	8.3±0.7	34.3±3.5	116±11
ME 10 μM		10±1.1	41±2.8* (123%)	118±20
Controls	3	7.9±1.5	31 ±8	95.5±0.2
ME 30 μM		8 ±1.6	22 ±3.2 (79%)	98±6
Controls	9	7.9±0.5	22.9±1.8	—
DADLE 3 μM		8.1±0.5	25.9±5	—
Controls	3	8.9±2.5	29.5±5.3	—
DADLE 10 μM		9.6±3.1	17.1±4.2* (57%)	—
Controls	3	9.9±0.9	25.6±2.8	—
ME 10 μM + naloxone 10 μM		5.2±0.3* (53%)	43.1±6.7* (168%)	—
Controls	3	8.4±1.3	24.5±1.7	—
ME 30 μM + naloxone 10 μM		4.5±0.7* (54%)	51.1±10.8* (204%)	—
Controls	3	8.9±2.5	29.5±5.3	—
DADLE 10 μM + naloxone 10 μM		8.8±2.3	26.9± 1.7	—

The drugs were added to the Krebs solution between the first (control) and second cycle. In parentheses the percent changes with respect to the control (first cycle). Values are mean ± s.e.mean.
*$P < 0.05$ significantly different from controls (first cycle), t test for paired data.

Discussion

In many studies *in vitro* it has been found that high concentrations of morphine are required to inhibit ACh release from central cholinergic structures. The concentrations used in our study (1–30 μM) were high, but reasonably similar to those achieved *in vivo* in various animal species after analgesic doses (Szerb & McCurdy, 1955; Johannesson & Schon, 1963).

Our experiments clearly show that the effects of morphine and the enkephalins on electrically-evoked ACh release vary in different brain areas. In the cerebral cortex, in agreement with Szerb (1974), a high dose (30 μM) of morphine produced a slight, naloxone-sensitive, inhibition of ACh release at 5 Hz. In the caudate nucleus, the opiate was active at lower doses when it enhanced ACh release elicited at 1 Hz; at higher doses it inhibited release of ACh at 5 Hz. The thalamic slices behaved like caudate slices, but naloxone did not block the morphine-induced facilitation of ACh release. However the antagonist converted the morphine and Met-enkephalin inhibition at high doses, into a facilitation of ACh release.

It is evident that morphine and enkephalins were able to inhibit ACh release in all the areas studied, provided high concentrations of the opioids were used. On the other hand, at low doses of the opioids, an increase in ACh outflow was observed in caudate and thalamic slices. The ACh facilitation mechanism appears to be different in these two cerebral areas. The enhanced ACh output in the caudate nucleus could represent opioid inhibition of dopaminergic inhibitory input to cholinergic cells since not only naloxone but also spiroperidol blocks this effect. However, the increased release seen in thalamic slices appears to involve an interaction with a different receptor since it is not blocked by naloxone.

The pharmacological evidence for multiple opioid receptors in the CNS stems from behavioural and neurophysiological observations (Martin, Eades, Thompson, Huppler & Gilbert, 1976), as well as from binding studies (Pert, Aposhian & Snyder, 1974; Lord, Waterfield, Hughes & Kosterlitz, 1976; 1977; Chang, Cooper, Hazum & Cuatrecasas, 1979; Gillan, Kosterlitz & Paterson, 1980). According to Terenius (1980), at least four opioid receptor sub-

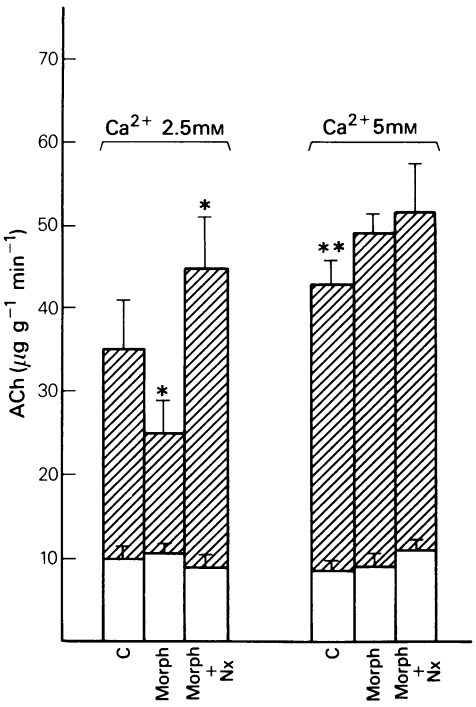

Figure 3 Effect of morphine and naloxone on spontaneous and evoked acetylcholine (ACh) release from guinea-pig thalamus slices kept in normal and double CA^{2+} concentration. (C = Control; Morph = morphine 1×10^{-5} M; Nx = naloxone 3×10^{-5} M). Each value is the mean of 6–7 expts; open column = testing ACh release; hatched section of column = release evoked by stimulation at 2 Hz. Vertical lines show s.e.mean. $^{*}P < 0.05$ significantly different from control; $^{**}P < 0.01$ significantly different from slices treated with calcium 2.5 mM.

types μ, δ, κ, σ (naloxone-sensitive) and an excitatory one (naloxone-insensitive) are present in the CNS. The facilitatory effect of morphine and Met-enkephalin in thalamic slices are unlikely to be due to interaction with μ or κ receptors since the effect is naloxone-insensitive (Kosterlitz, Paterson & Robson, 1981). Also δ receptors are not likely to be involved since the selective δ-agonist, DADLE, only inhibits ACh release.

Therefore, it is possible that the facilitation of ACh output depends on the interaction of morphine and Met-enkephalin with an excitatory, naloxone-insensitive receptor, similar to that postulated by Terenius (1980). The increase of ACh release could represent some ill-defined, unspecific excitatory effects of the opioids, such as those described by Bradley & Brookes (1981). However, this appears unlikely because the facilitation of ACh release induced by morphine was evident at lower doses than those required for the naloxone-sensitive inhibition of release.

Our observations fit well with the results obtained by others in experiments performed on whole animals. In fact, Vasko & Domino (1976) showed that morphine at a low dose (1 mg/kg s.c.) increased locomotor activity and, concomitantly, increased ACh utilization in the thalamus. Moreover, Jhamandas & Sutak (1976) found that naloxone, either alone or after morphine, facilitated the increase of cortical ACh release caused by medial thalamus stimulation. Morphine and enkephalins did not modify resting ACh release in any area tested (Szerb, 1974; Vizi *et al.*, 1977). However, in thalamic slices Met-enkephalin, in the presence of naloxone, significantly reduced the spontaneous ACh output. Since this effect was observed in tetrodotoxin-treated slices the reduction could not be due to opioid inhibition of cholinergic nerve activity.

The morphine-induced inhibition of ACh release was prevented by a doubling of the $[Ca^{2+}]$ in the perfusion medium; this is in accordance with other results (Ross, 1978; Bennet & Lavidis, 1980). The failure of morphine to increase ACh release in the presence of naloxone and raised $[Ca^{2+}]$ could be a ceiling effect. However, the maximal neurosecretory capacity was not reached in the presence of 5 mM Ca^{2+}. In fact, as shown in Table 3, the naloxone reversal of Met-enkephalin doubled the ACh release at 2 Hz. Therefore, the lack of a further increase in ACh outflow induced by morphine in the presence of raised $[Ca^{2+}]$ cannot be ascribed to a ceiling effect.

In conclusion, these experiments suggest that in the guinea-pig thalamus different receptors may control ACh release. One receptor is inhibitory and naloxone-sensitive, the other enhances ACh release and is naloxone-insensitive. At present, further work is needed to decide whether or not these receptors are located on the cholinergic structures, i.e. if they modulate ACh release directly or indirectly

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