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THE EFFECTS OF MORPHINE ON THE RELEASE OF NORADRENALINE FROM THE CAT ISOLATED NICTITATING MEMBRANE AND THE GUINEA-PIG ILEUM MYENTERIC PLEXUS-LONGITUDINAL MUSCLE PREPARATION

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1 Electrical field stimulation of either the cat isolated nictitating membrane or the guinea-pig ileum myenteric plexus-longitudinal muscle preparation caused the release of noradrenaline into the bathing medium.

2 In the cat nictitating membrane, the output per pulse of noradrenaline was constant at frequencies of stimulation from 0.5 to 15 Hz. In the guinea-pig myenteric plexus preparation the output per pulse of noradrenaline increased as the frequency of stimulation was increased from 2 to 16 Hz.

3 Phenoxybenzamine (29.3 μM) caused a marked increase in the noradrenaline output from both the cat nictitating membrane and guinea-pig myenteric plexus preparations.

4 Morphine (0.13-8 μM) inhibited the contractions of the cat nictitating membrane caused by electrical stimulation. This effect was greater at low (1 Hz) than at high (15 Hz) frequencies of stimulation. The site of action is at the nerve-smooth muscle junction.

5 The action of narcotic analgesic drugs on the cat nictitating membrane showed stereospecificity. Naloxone (0.1 μM) reversed the inhibition caused by normorphine (3.2 μM).

6 Morphine (3 μM) reduced the noradrenaline output from the cat nictitating membrane stimulated at 1 Hz but not at 15 Hz. At 1 Hz, the inhibition of noradrenaline output by normorphine (5 μM) was reversed by naloxone (0.25 μM).

7 Morphine (1.5 μM) did not alter the noradrenaline output from the guinea-pig myenteric plexus preparation stimulated at 2 or 16 Hz.

Introduction

Morphine has a very selective action on the peripheral autonomic system and only affects certain neuro-effector junctions. Thus in the adrenergic system, morphine inhibits contractions elicited by nerve stimulation of the cat nictitating membrane both *in vivo* and *in vitro* (Trendelenburg, 1957; Thompson, 1960; Cairnie, Kosterlitz & Taylor, 1961) and of the mouse vas deferens (Henderson, Hughes & Kosterlitz, 1972a). The action of morphine in these tissues is a specific effect and appears to involve an inhibition of noradrenaline release (Cairnie *et al.*, 1961; Henderson *et al.*, 1972a). Except in very high concentrations ($>10\ \mu$M) morphine does not affect the release of noradrenaline from the rabbit isolated heart (Montel & Starke, 1973), vas deferens and portal vein (Hughes, unpublished observations). Why transmission is impaired at only certain synapses is unknown.

The purpose of this investigation was to examine the release of noradrenaline in the cat nictitating membrane at different frequencies of stimulation and the effects of morphine on the relationship between frequency and release. A parallel study has been made on the output of noradrenaline from the guinea-pig ileum myenteric plexus-longitudinal muscle preparation where it is known that morphine inhibits the release of acetylcholine (Paton, 1957; Schaumann, 1957; Cowie, Kosterlitz & Watt, 1968). Some of the results have already been reported to the British Pharmacological Society (Henderson, Hughes & Thompson, 1972b).

Methods

Cat nictitating membrane

Male or female cats of between 2 and 4 kg were anaesthetized by intraperitoneal injection of either

a solution containing urethane (500 mg/kg) and chloralose (50 mg/kg) or a solution of sodium pentobarbitone (40 mg/kg). The medial smooth muscle of the nictitating membrane attached to a portion of cartilage was isolated by the method of Thompson (1958). The tissue was mounted in a 5 ml organ bath containing Krebs solution at 37° C and placed under 1 g tension.

Guinea-pig ileum myenteric plexus-longitudinal muscle

Male guinea-pigs weighing 300-600 g were killed by breaking the neck and exsanguination. A suitable length of ileum was removed and placed in warm Krebs solution; the terminal 10 cm of ileum was not used. The method of dissection of the myenteric plexus-longitudinal muscle preparation was a modification of that reported by Ambache (1954) and is described in full by Kosterlitz, Lydon & Watt (1970). The tissue was mounted in a 3 ml organ bath under 0.5 g tension.

Contractions of both the isolated nictitating membrane and the myenteric plexus-longitudinal muscle were recorded isometrically and displayed on a pen oscillograph. Electrical field stimulation was used to excite the tissues. Platinum electrodes were fixed vertically on opposite sides of the tissue and supramaximal (1.3 x maximal current) stimuli, 1.0 ms rectangular pulses were used throughout.

Determination of released noradrenaline

Biological and fluorimetric assay methods were used. Superfused spiral preparations of rabbit aortic and iliac arteries were used for the bioassay (Hughes, 1972). After stimulation, the fluid surrounding the nictitating membrane or myenteric plexus (donor tissues) was left for 3 min to permit the released noradrenaline to diffuse from the tissue into the surrounding bathing medium. The bath fluid was then transferred to a cascade system perfusing the assay tissues. The output of transmitter from the donor tissue was determined by bracketing contractions of the assay tissues to the released material with contractions elicited by standard doses of noradrenaline.

In several experiments the noradrenaline which diffused from the tissue into the surrounding bathing fluid after electrical stimulation was estimated fluorimetrically. The fluid was collected in cooled flasks containing disodium edetate (10 mg/ml bath fluid). After adjusting the pH to 8.6, the noradrenaline was adsorbed on alumina columns. After elution with 0.15 M perchloric acid the noradrenaline content was determined fluorimetrically by a modification of the method of O'Hanlon, Campuzano & Horvath (1970) as described by Hughes (1972). Standard amounts of noradrenaline were taken through this procedure in order to determine the total recovery of the amine.

The donor tissues were stimulated at 20 min intervals. Experiments in which the output of noradrenaline from the donor tissues declined by more than 10% per stimulus train were either rejected or further controls were obtained until the outputs were within 10% of each other. In any one experiment the output of noradrenaline after stimulation at a single frequency is the mean of at least two observations.

Electrical stimulation did not produce any destruction of noradrenaline; this was shown after removal of the donor tissues, when 100 pulses at 0.2 or 15 Hz were passed through the bathing fluid containing 0.33 ng/ml of noradrenaline. Bioassay showed that there was no loss of noradrenaline activity.

Endogenous noradrenaline content

The noradrenaline contents of the nictitating membrane and of the myenteric plexus-longitudinal muscle preparation were estimated by fluorimetric assay. After homogenization at 4° C with 4 ml 0.4 M perchloric acid, 0.2 ml 10% (w/v) EDTA solution and 0.2 ml Tris buffer (1 M, pH 8.6), the homogenate was centrifuged for 15 min at 15,000 *g*. The supernatant was concentrated over alumina columns and the noradrenaline content assayed fluorimetrically as described above.

Drugs and solutions

The bathing fluid was a modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.19, $NaHCO_3$ 24, KH_2PO_4 0.93, glucose 11, tyrosine 0.25, ascorbic acid 0.1 and disodium edetate 0.027; it was bubbled with 95% oxygen and 5% carbon dioxide. The drugs used were dextrorphan tartrate (Roche Products), histamine acid phosphate (B.D.H.), 5-hydroxytryptamine-creatine sulphate (B.D.H.), hyoscine hydrobromide (Macfarlan Smith), levorphanol tartrate (Roche Products), morphine hydrochloride (Macfarlan Smith), naloxone hydrochloride (Endo Laboratories), (−)-noradrenaline bitartrate (B.D.H.), normorphine hydrochloride (Dr E.L. May), phenoxybenzamine hydrochloride (Smith, Kline & French), and phentolamine hydrochloride (Ciba).

A time interval of 20 min was used between exposures of the tissues to successive doses of

narcotic analgesics to prevent the occurrence of tachyphylaxis.

Definition of terms

Output of transmitter This term is used to describe the amount of noradrenaline which diffuses from the stimulated tissue into the surrounding bathing fluid. This is not equal to the amount released from nerve endings but is assumed to be proportional to the actual amount released.

Fractional noradrenaline output per pulse This indicates the output of transmitter per pulse as a fraction of the total tissue content of noradrenaline determined after completion of the experiment.

Results

Identification of material released by electrical stimulation

When the assay tissues were superfused with phentolamine (160 nM), contractions to standard doses of noradrenaline and to the material present in the bath fluid after electrical stimulation of the donor tissue were reduced equally whereas contractions elicited by equiactive doses of histamine and 5-hydroxytryptamine were not decreased.

Noradrenaline output from the cat isolated nictitating membrane

After stimulation with trains of 100 pulses, the fractional noradrenaline output per pulse from the untreated nictitating membrane was not significantly different at 0.2, 1 and 15 Hz (Table 1). Incubation of the tissue for 1 h with phenoxybenzamine resulted in a 10 to 25-fold increase in the noradrenaline output at the three frequencies of stimulation. After phenoxybenzamine the noradrenaline output was slightly higher at 16 Hz than at 0.2 Hz but this rise with frequency was much smaller than that found in the rabbit vas deferens or portal vein (Hughes, 1972; Hughes & Roth, 1974).

Noradrenaline output from the guinea-pig myenteric plexus-longitudinal muscle

In this tissue stimulation with trains of 240 pulses at 2 and 16 Hz produced a frequency-output relationship (Table 1) different from that observed in the cat nictitating membrane. In the untreated

Table 1 Effects of phenoxybenzamine on fractional noradrenaline output from the nictitating membrane of the cat and the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Treatment	*Fractional noradrenaline output from nictitating membrane (x 10^5) at*			*Differences (0.2 vs 1 or 15 Hz)*		*Fractional noradrenaline output from myenteric plexus (x 10^5) at*		*Difference between frequencies*
	0.2 Hz	*1 Hz*	*15 Hz*			*2 Hz*	*16 Hz*	
None	0.16 ±0.03	0.34 ±0.11	0.24 ±0.06	+ 0.18 ± 0.11 + 0.08 ± 0.04	(1 Hz) (15 Hz)	1.81 ±0.29	3.52 ±0.70	1.71** ±0.51
Phenoxybenzamine (29.3 μM for 1 h)	4.8 ±1.4	5.3 ±1.2	6.4 ±1.1	+ 0.50 ± 0.61 + 1.57 ± 0.50*	(1 Hz) (15 Hz)	15.3 ±3.5	26.1 ±2.7	10.8*** ±2.1

Each of 3 preparations of the nictitating membrane were stimulated at three frequencies, in the absence or presence of phenoxybenzamine. There were 12 control and 7 treated preparations of the myenteric plexus. The values are the means ± s.e.mean of the fractional output per pulse caused by trains of 100 pulses to the nictitating membrane and trains of 240 pulses to the myenteric plexus. The standard errors of the differences between the means were determined by paired analysis.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

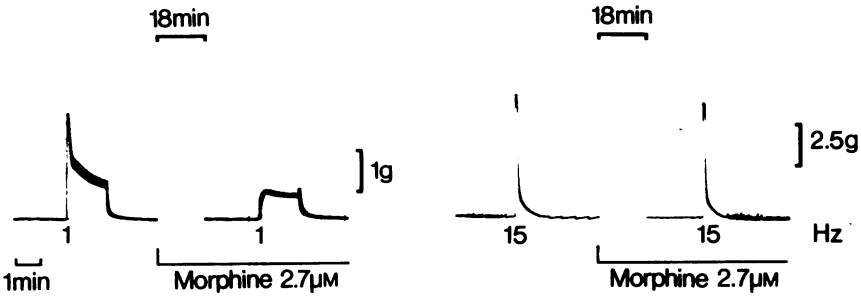

Figure 1 Effect of morphine (2.7 μM) on contractions of the cat isolated nictitating membrane stimulated at 1 and 15 Hz with trains of 100 pulses. These results were repeated in four experiments.

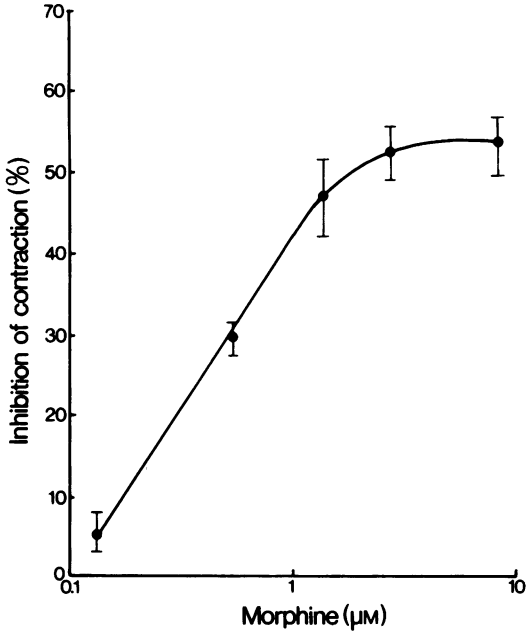

Figure 2 Effect of morphine on contractions of the cat isolated nictitating membrane stimulated at 1 Hz with trains of 30 pulses every 3 minutes. Mean results from 6 preparations. Vertical bars show s.e.mean. Ordinates: inhibition of contraction as a percentage of the initial response. Abscissae: morphine concentration (μM).

preparation the noradrenaline output per pulse at 16 Hz was approximately twice that at 2 Hz. Although, after treatment with phenoxybenzamine (29.3 μM), the noradrenaline outputs at each frequency were increased 8-fold there was still a 2-fold difference in the outputs per pulse at 2 Hz and 16 Hz.

Effect of morphine on contractions of the cat isolated nictitating membrane

Cairnie *et al.* (1961) found that morphine was more effective in reducing the size of the contractions at low frequencies than at high frequencies of stimulation in the cat nictitating membrane *in vivo*. A similar result was obtained in the *in vitro* preparation in which morphine (3 μM) produced a greater inhibition of the contractions due to stimulation at 1 Hz than at 15 Hz (Figure 1).

Morphine produced a dose-dependent inhibition of the contractions of the nictitating membrane stimulated at 1 Hz (Figure 2). The maximal inhibition was 50-60%, the residual contraction was unaffected by hyoscine (1.2 μM) but abolished by either bretylium (12 μM) or phentolamine (310 μM). The concentration of morphine required to produce 50% of the maximal inhibition (ED_{50}) was 0.5 μM. When the contractions due to stimulation at 1 Hz were reduced by normorphine (3.2 μM), which is equiactive with morphine in the guinea-pig ileum (Kosterlitz, Lord & Watt, 1972) and mouse vas deferens (Hughes, Kosterlitz & Leslie, 1974), naloxone (100 nM) completely reversed the inhibition of contraction (Figure 3). Levorphanol (3.2 μM) depressed the responses of the cat nictitating membrane whereas an equal concentration of dextrorphan (3.2 μM) did not. A higher concentration of dextrorphan (32 μM) did depress the response to stimulation, but this was not reversed by naloxone (1.3 μM) whereas the depression due to an equiactive dose

of levorphanol was reversed by naloxone (300 nM).

Effect of morphine on the noradrenaline output from the cat nictitating membrane

The noradrenaline outputs from this tissue stimulated with trains of 100 pulses at 1 and 15 Hz were determined in normal Krebs solution and in Krebs solution containing morphine (3 μM) (Table 2). Since there was considerable variation in the noradrenaline output of different preparations, the results were obtained from paired observations. Morphine produced a 60% decrease in the

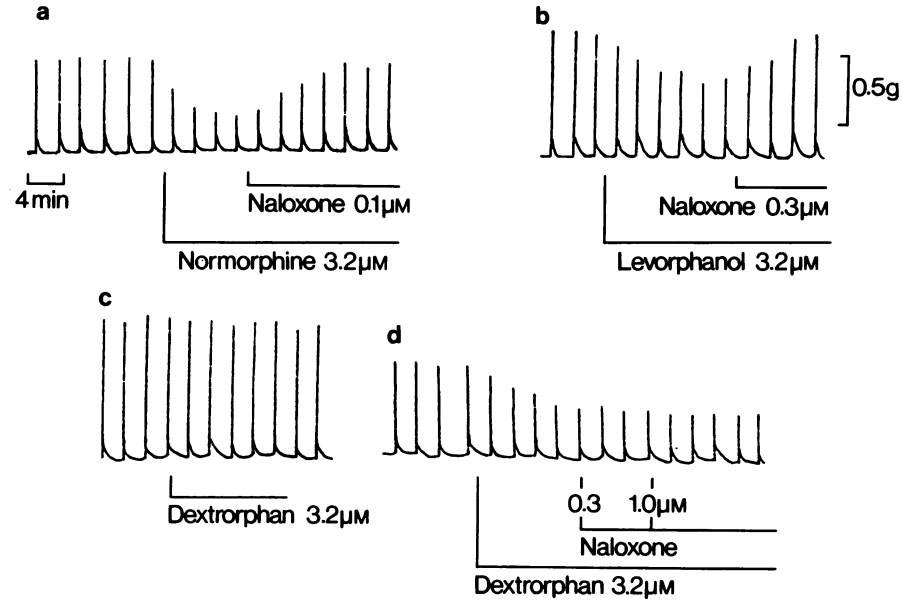

Figure 3 Effect of normorphine, levorphanol and dextrorphan on contractions of the cat isolated nictitating membrane stimulated at 1 Hz with trains of 10 pulses every 3 minutes. (a) Inhibition of contraction by normorphine (3.2 μM) was reversed by naloxone (0.1 μM). (b) Inhibition of contraction by levorphanol (3.2 μM) was reversed by naloxone (0.3 μM). (c) and (d) Dextrorphan (3.2 μM) did not inhibit the contractions; the inhibition of contraction produced by dextrorphan (32 μM) was unaffected by naloxone (1.3 μM). These results were repeated in three experiments.

Table 2 Effects of morphine on fractional noradrenaline output from the nictitating membrane of the cat and the myenteric plexus-longitudinal muscle preparation from the guinea-pig ileum

Treatment	*Fractional noradrenaline output from nictitating membrane (x 10^5) at*		*Difference for frequencies*	*Fractional noradrenaline output from myenteric plexus (x 10^5) at*		*Difference for frequencies*
	1 Hz	*15 Hz*		*2 Hz*	*16 Hz*	
None	0.86 ±0.07	0.92 ±0.29	+0.052 ±0.272	0.94 ±0.23	1.83 ±0.27	+0.89* ±0.35
Morphine	0.28 ±0.06	1.03 ±0.29	+0.74* ±0.24	1.28 ±0.31	1.94 ±0.14	+0.66 ±0.34
Difference for treatment	−0.58** ±0.047	+0.11 ±0.043		+0.34 ±0.38	+0.11 ±0.30	

Each of 4 preparations was stimulated at the two frequencies, in the absence and presence of morphine (nictitating membrane, 3 μM; myenteric plexus, 1.5 μM). The values are the means ± s.e.mean of the fractional output per pulse caused by trains of 100 pulses to the nictitating membrane or trains of 240 pulses to the myenteric plexus. The standard errors of the differences between the means were determined by paired analysis. * $P < 0.05$, ** $P < 0.01$.

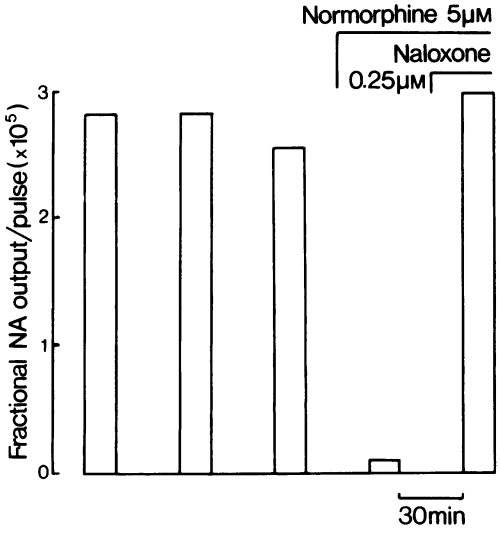

Figure 4 Reversal of normorphine inhibition of noradrenaline output by naloxone. Each column represents the noradrenaline output from the cat isolated nictitating membrane stimulated at 1 Hz with 240 pulses. Tissue pretreated for 1 h with phentolamine (36 μM). Ordinates: noradrenaline output (fractional noradrenaline (NA) output per pulse). Abscissae: time between stimulation periods.

output of noradrenaline at 1 Hz whereas the noradrenaline output at 15 Hz was not significantly altered. This greater effect of morphine on noradrenaline output at the lower frequency of stimulation correlates well with the greater depressant effect of morphine on the contractions (Figure 1).

After treatment of the nictitating membrane with phentolamine (36 μM), the noradrenaline outputs were determined after stimulation with trains of 100 pulses at 1 Hz applied every 30 minutes. Exposure of the tissue to normorphine (5 μM) for 15 min before stimulation resulted in a decrease in the noradrenaline output (Figure 4). When the tissue was subsequently treated with normorphine (5 μM) and naloxone (0.25 μM) for 15 min, the noradrenaline output returned to control levels.

Effect of morphine on the noradrenaline output from the myenteric plexus-longitudinal muscle preparation

After exposure of the tissue to morphine (1.5 μM) for 5 min the output of noradrenaline at 2 and 16 Hz was not significantly altered from control values (Table 2). Therefore, unlike the cholinergic innervation of the myenteric plexus, the adrenergic innervation is not sensitive to morphine.

Discussion

The inhibition of noradrenaline output from the cat nictitating membrane by morphine is due to a stereospecific effect at a morphine receptor site because the inhibitory effects are reversed by naloxone, and levorphanol has a much greater depressant effect than its dextro-isomer, dextrorphan. Since the concentrations of morphine required for this effect do not affect noradrenaline uptake into peripheral (Montel & Starke, 1973) or central neurones (Ciofalo, 1972), morphine would appear to act on the noradrenergic nerve terminals to decrease transmitter release.

The nictitating membrane may be added to the relatively few tissues with autonomic innervation in which a specific morphine receptor has been demonstrated. In the myenteric plexus of the guinea-pig ileum, the rabbit vagus-sinoauricular node junction (Kosterlitz & Taylor, 1959; Kennedy & West, 1967) and the mouse vas deferens, the possibility has not been excluded that morphine receptors may be present at sites other than the nerve-smooth muscle junction. On the other hand, the smooth muscle cells of the nictitating membrane are innervated by fibres which have their cell bodies in the superior cervical ganglion and no ganglion cells have been found within the smooth muscle (Gardiner, Hellmann & Thompson, 1962). Therefore, the inhibitory effect of morphine on noradrenaline release would appear to be due to an action on the nerve terminals innervating the smooth muscle.

The mechanism of noradrenaline release from the morphine-sensitive nictitating membrane differs in certain aspects from that in morphine-insensitive tissues such as the rabbit vas deferens and portal vein and the guinea-pig myenteric plexus. In the latter tissues, the output of noradrenaline per pulse increases at least 10-fold with a rise in frequency of stimulation from 0.5 to 16 Hz (Hughes, 1972; Hughes & Roth, 1974) while in the nictitating membrane there is only a 1.3-fold increase over a frequency range of 0.2 to 15 Hz. Although phenoxybenzamine increases the output of noradrenaline, it has little effect on the relationship of frequency to noradrenaline output in the tissues mentioned above. This observation would appear to exclude the possibility that neuronal and extraneuronal uptake (Hughes, 1972) or post-junctional or pre-junctional (Starke, 1972) α-adrenoceptors play any major role in determining this relationship. In this connection it should be emphasized that the selection of

experimental conditions is of great importance, since the fractional output increases with increasing train length until a rapid decline sets in when the train length exceeds 400-500 pulses (Hughes & Roth, 1974); moreover, long periods of stimulation deplete the noradrenaline in the tissue stores irreversibly.

The greatest difference in noradrenaline output per pulse between the nictitating membrane and the other tissues is found at frequencies below 0.5 Hz. In the presence of phenoxybenzamine, the fractional output from the nictitating membrane is at these low frequencies similar to that at high frequencies ($5\text{-}6 \times 10^{-5}$) whereas in the rabbit vas deferens the fractional output falls from 1×10^{-4} at 16 Hz to less than 1×10^{-5} at 0.5 Hz (Hughes & Roth, 1974). It may be significant that in the nictitating membrane morphine has its most prominent effect at low frequencies of stimulation (0.1-0.4 Hz) at which the noradrenaline output is high in comparison to morphine-insensitive tissues. A similarly high noradrenaline output at low frequencies has also been observed in the morphine-sensitive mouse vas deferens (Henderson *et al.*, 1972a; Henderson & Hughes, unpublished observations).

In the cholinergic system, Greenberg, Kosterlitz & Waterfield (1970) have shown that at low stimulus frequencies acetylcholine release is higher from the guinea-pig myenteric plexus than from that of the rabbit; morphine inhibits acetylcholine release in the guinea-pig but not in the rabbit.

Therefore, in both the adrenergic and the cholinergic systems, the relationship between frequency and output at synapses at which morphine inhibits transmitter release is different from that at synapses at which morphine has no effect. The mechanisms underlying these observations are as yet unknown.

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