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THE ROLE OF SUPERSENSITIVITY TO ACETYLCHOLINE IN THE PRODUCTION OF TOLERANCE TO MORPHINE IN STIMULATED GUINEA-PIG ILEUM

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1 Morphine caused a dose-dependent reduction in both the height of contraction and acetylcholine release from coaxially stimulated strips of guinea-pig ileum.

2 Exposure of the tissue to morphine for 90 min produced acute tolerance to the effect of subsequent doses of morphine on contraction height.

3 There was no change in the ability of morphine to suppress acetylcholine release.

4 The responses of morphine-tolerant ileum to exogenous acetylcholine were enhanced 3 to 10-fold.

5 If the ileum did not show tolerance to morphine it did not become more sensitive to acetylcholine.

6 The results presented suggest that tolerance to morphine could result from a form of disuse supersensitivity.

Introduction

In a number of independent studies, morphine has been shown to inhibit the release of acetylcholine in the brain (Beleslin & Polak, 1965; Jhamandas, Pinsky & Phillis, 1970; Domino & Wilson, 1973; Matthews, Labreque & Domino, 1973). It is significant that this effect occurred with the relatively low concentrations of morphine needed to produce euphoria, analgesia and respiratory depression. In the same low concentrations morphine prevents the contractions of the coaxially stimulated guinea-pig ileum, by inhibiting acetylcholine release (Paton, 1957; Schaumann, 1957; Cox & Weinstock, 1966).

Other similarities exist between the actions of narcotic analgesic drugs on guinea-pig ileum, and those on the central nervous system. In both tissues, the effects of morphine can be antagonized by naloxone or nalorphine (Cox & Weinstock, 1966; Matthews *et al.*, 1973). Guinea-pig ileum has also been shown to exhibit tolerance to the effects of morphine on repeated administration, a property characteristic of morphine's effects on the central nervous system (Paton, 1957; Fennessy, Heimans & Rand, 1969). This tissue has therefore been considered a good experimental model for studying both acute and chronic effects of morphine (Paton, 1957).

One of the current theories attempting to explain the phenomenon of tolerance to morphine is that proposed by Collier, (1965, 1968) but which so far has lacked experimental substantiation. He suggested that morphine produces its acute effects by reducing the release of neurotransmitter(s). The continued inhibition of transmitter release brings about an increase in the number of active receptors for that transmitter. Thus, morphine apparently loses its effect (tolerance) because the reduced amount of transmitter becomes hyperactive. Removal of morphine allows the normal flow of transmitter to interact with the activated effector area, resulting in an exaggerated response or 'withdrawal symptoms'.

The failure of morphine to reduce the contractions of the stimulated ileum on repeated administration, could result from a loss of its ability to inhibit acetylcholine release. Alternatively, consistent with Collier's hypothesis, an increase could occur in the sensitivity of the muscle to acetylcholine, without any change in the quantity of transmitter released.

The purpose of the present study was to use the stimulated guinea-pig ileum preparation to examine experimentally Collier's hypothesis.

Methods

Male guinea-pigs weighing between 300-500 g were used. The animals were killed; a 4 cm strip of

ileum was cut about 20 cm from the ileo-caecal junction and suspended in Krebs-Hensleit solution in a 5 ml organ bath. The ileum was stimulated coaxially according to the method described by Paton (1957). The stimulus parameters were: frequency, 0.05 Hz; duration, 0.5 ms; voltage, supramaximal, 40 V. Contractions were recorded isotonically by means of a Statham strain gauge on a multichannel polygraph.

Establishment of tachyphylaxis to morphine

Dose-response curves were obtained of the effect of morphine in reducing the height of contraction of the stimulated ileum. A concentration of morphine, which produced approximately 70% inhibition of the twitch, was introduced into the reservoir of fluid supplying the bath containing the preparation. This enabled us to wash the preparation as required, while maintaining a constant concentration of morphine in contact with the tissue. A contact period of 90 min was chosen because it was found that tachyphylaxis to morphine could thus be most reliably reproduced. After 90 min the morphine solution was replaced by normal Krebs-Hensleit solution. The tissue was thoroughly washed several times before dose-response curves to morphine were re-established.

Changes in response of ileum to exogenous acetylcholine

Dose-response curves to applied acetylcholine were established by injection of several doses repeatedly before any morphine was given, during the morphine perfusion, and again after the original Krebs-Hensleit solution had been replaced. In a few experiments responses to potassium chloride or histamine were also obtained before and after perfusion of the ileum with morphine solution.

Effect of morphine on acetylcholine release

Two pieces of ileum were taken from the same guinea-pig and set up under identical conditions in adjacent organ baths, and stimulated as described above. Dose-response curves to morphine were obtained on both pieces of ileum, simultaneously. Care was taken to ensure that responses to morphine closely resembled each other for the two tissues before continuing with the experiment.

Physostigmine was then introduced into the fluid, perfusing one piece of ileum, to give a concentration of 1 μg/ml. Since it was impossible to record the responses to morphine on the stimulated ileum in the presence of physostigmine, the second strip served as a control to indicate the development of tachyphylaxis to morphine.

After the introduction of physostigmine to the bathing fluid, the tissue was allowed to equilibrate for 40 minutes. The fluid was changed and exactly 5 min later, withdrawn from the organ bath with a 5 ml syringe. This procedure was repeated several times and served to establish the control release of acetylcholine from the stimulated ileum. Similar collections of bathing fluid were then made in the presence of at least two doses of morphine, given twice.

Both physostigmine-treated and control preparations were perfused for 90 min with morphine, as described above. In some experiments, additional collections of bath fluid were made during the morphine perfusion. In all experiments the whole procedure was repeated, after replacing the morphine-containing bathing fluid with the original Krebs-Hensleit solution containing physostigmine.

Assay of acetylcholine

All samples of bath fluid were frozen immediately and stored at -20°C, together with known amounts of acetylcholine made up in the same Krebs-Hensleit solution plus physostigmine. They were assayed the following day on guinea-pig ileum treated with neostigmine and morphine, as described by Paton (1957).

Student's t test was used to determine the statistical significance of the difference between the means. All values are the mean $\pm$ standard error of the means.

Drugs

The drugs used were, acetylcholine bromide, physostigmine salicylate, morphine sulphate, neostigmine bromide, potassium chloride, histamine chloride. Concentrations are expressed in g of the salt per ml of bath fluid.

Results

Changes in response to morphine after chronic application

In occasional experiments, the effect of morphine in reducing the contractions of the stimulated ileum diminished with each successive application. The development of tachyphylaxis in this way was exceedingly variable and could be abolished by increasing the time interval of dosing to 20 minutes.

A much more consistent tachyphylaxis to morphine was established by perfusing the ileum for 90 min, with a concentration of morphine that

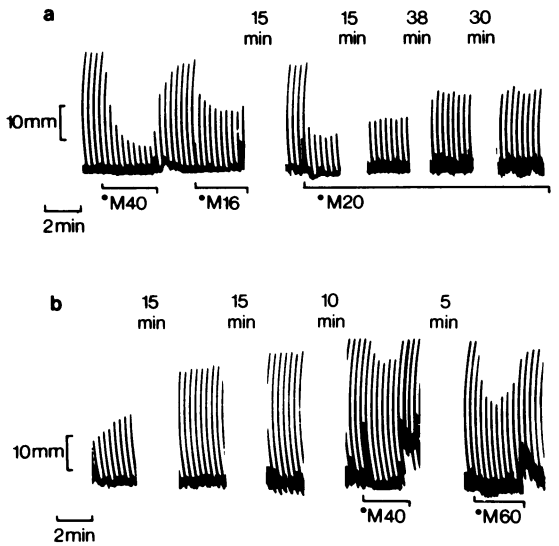

Fig. 1 Reduction in effect of morphine on coaxially-stimulated guinea-pig ileum after chronic morphine perfusion. Isotonic contractions of guinea-pig ileum. Stimulus parameters: frequency, 0.05 Hz, duration of single shocks, 0.5 ms, supramaximal voltage, 40 V. Horizontal bars indicate presence of morphine (M) in bath (ng/ml). Figures denote time interval in min between successive sections of record. Between record (a) and (b) tissue was washed repeatedly with Krebs-Hensleit solution.

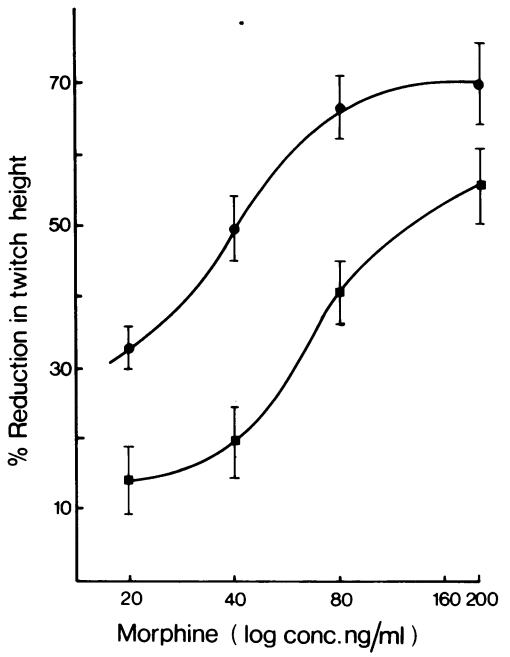

Fig. 2 Responses to morphine before (•) and after (▪) exposure of guinea-pig ileum to morphine (40 ng/ml) for 90 minutes. Each point represents mean ± s.e. mean from 17 experiments.

gave an initial reduction in contraction height of 65-75%. After such prolonged contact with morphine, the original active doses were much less effective in causing a reduction in the twitch (Figure 1). The duration of diminished responsiveness to morphine was not determined accurately in our experiments but it was found to exceed 40 minutes. Dose-response curves for the effect of morphine before and after chronic administration are shown in Figure 2. The extent of the shift varied in individual experiments from 2 to 20 times.

In 5 out of 21 experiments, when the morphine perfusion fluid was replaced by Krebs-Hensleit solution, the contractions of the stimulated ileum not only regained their control height, but were significantly larger than those prior to the morphine administration. Such an increase in the twitch height is shown in Figure 1.

Effect of morphine on twitch height and acetylcholine release after chronic application

In 8 experiments the effect of morphine was measured simultaneously on the height of contraction of the stimulated ileum, and on the amount of acetylcholine released per stimulus. Morphine, administered at a dose of either 40 or 80 ng/ml, reduced both the contraction height and acetylcholine output in all experiments. After the ileum had been perfused with solution containing morphine for 90 min, these same doses no longer caused reduction in contraction. However, acetylcholine release was still inhibited to the same extent as it had been before the morphine perfusion. These results are shown in Figure 3. Since the control output of acetylcholine varied between 0.2 to 3.5 ng ml^{-1} $stimulus^{-1}$, for different preparations, the effect of morphine was expressed as the percentage reduction of the initial control release.

In 6 control experiments in which morphine was omitted from the perfusion fluid, acetylcholine release remained unchanged after 90 min in 4 cases, and rose by 12 and 18% in the last two.

Changes in response to acetylcholine after chronic morphine application

In each experiment where chronic morphine administration resulted in a diminution or complete loss of the effect of the original doses of

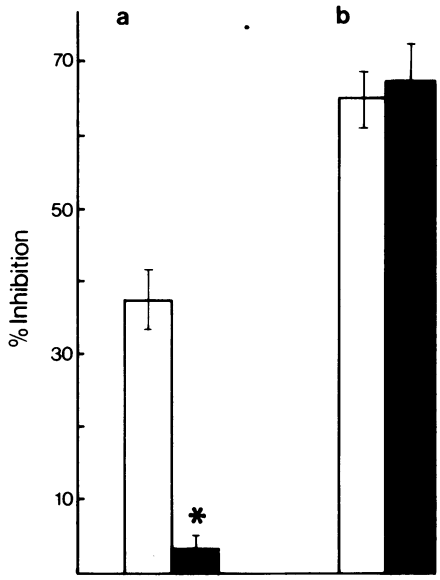

Fig. 3 Effect of chronic morphine perfusion on reduction in twitch height and acetylcholine release from guinea-pig ileum. (a) Inhibition of contraction. (b) Inhibition of acetylcholine release. Open columns, response in presence of morphine (40 ng/ml) before exposure of ileum to morphine (40 ng/ml) for 90 minutes. Closed columns, response in presence of morphine (40 ng/ml) after chronic exposure to morphine. Each column represents mean from eight experiments. Vertical bars indicate s.e. mean. * $P < 0.001$ for two values.

morphine on twitch height, there appeared a concomitant enhancement of the response to acetylcholine on the ileum. This enhanced responsiveness lasted for at least 40 min, as long as the effect of morphine on the stimulated ileum was depressed. In 9 of these experiments, the maximum response to acetylcholine was unchanged after morphine treatment, but the whole dose-response curve was shifted significantly to the left, so that the ED_{50} was reduced. In two other experiments, there was little reduction in ED_{50}, but the maximum contraction obtainable with acetylcholine was increased by at least 20%. In the remaining four experiments, the maximum effect of acetylcholine was increased and the ED_{50} reduced. The mean value for the maximum contraction to acetylcholine for all 15 experiments did not differ significantly after morphine perfusion from the mean control value (Table 1). The mean ED_{50} value for acetylcholine on the morphine-tolerant ileum was however significantly lower than the mean control figure (Table 1). Figure 4 shows the mean responses of all the tissues to various doses of acetylcholine before and after tolerance to morphine had been induced, expressed as a percentage of the maximum response. These dose-response curves for acetylcholine were obtained at the same time as the curves for morphine antagonism of the twitch height, shown in Figure 2.

In 9 other experiments, no significant tachyphylaxis to morphine developed. In most of these, the maximum response to acetylcholine was considerably reduced after morphine perfusion. This caused a reduction in the calculated ED_{50} even though these preparations were not more sensitive to acetylcholine (Table 1).

In 7 experiments in which morphine was omitted from the perfusion fluid, the maximum response to acetylcholine and the ED_{50} remained unchanged in 4, the maximum was reduced by 15% and 30% in 2 and the ED_{50} reduced by 50% in one.

Changes in response to other antagonists after chronic morphine perfusion

In some experiments, dose-response curves were established to KCl as well as to acetylcholine before and after chronic morphine treatment. Although in one or two experiments there

Table 1 Responses of guinea-pig ileum to acetylcholine and histamine before and after chronic morphine perfusion.

Preparation	*Agonist*	*Mean* ED_{50} *(ng/ml ± s.e.)*	P	*Mean maximum height (mm ± s.e.)*	P	n
Control	Acetylcholine	10.9 ± 2.8	< 0.05	45 ± 2.8	> 0.2	15
Morphine-tolerant	Acetylcholine	5.0 ± 0.9		49 ± 3.5		
Control	Histamine	11.2 ± 1.4	< 0.02	54 ± 2.2	> 0.4	4
Morphine-tolerant	Histamine	5.8 ± 0.6		57 ± 2.3		
Control	Acetylcholine	8.4 ± 1.6	> 0.5	57 ± 4.8	< 0.02	9
Morphine-non-tolerant	Acetylcholine	7.4 ± 1.5		38 ± 5.3		

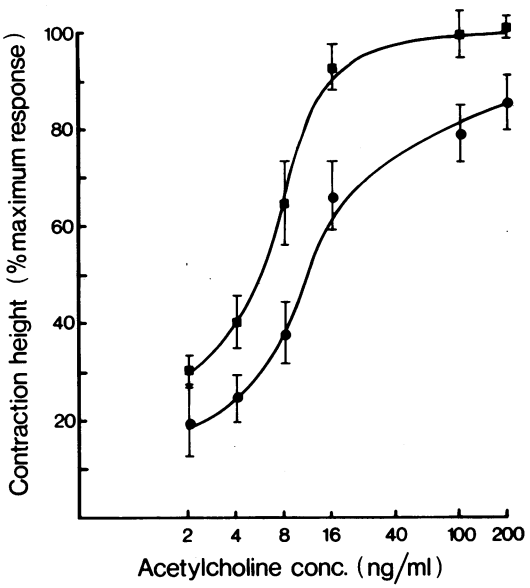

Fig. 4 Responses of guinea-pig ileum to acetylcholine before (•) and after (▪) exposure of ileum to morphine (40 ng/ml) for 90 minutes. Each point represents mean ± s.e. mean from 5 to 15 experiments.

appeared to be an increase in the response to KCl after morphine perfusion, it was less marked than the increase in response to acetylcholine. In other experiments the responses to KCl were generally too variable both before and after morphine treatment to enable us to draw any conclusions about this agent.

In another series of experiments, histamine was substituted for KCl. This time we found a clear-cut increase in the responsiveness of the morphine-tolerant ileum to histamine which paralleled that seen to acetylcholine. The ED_{50} for histamine before and after morphine treatment, are shown in Table 1.

Discussion

In the present study, 'acute tolerance', or tachyphylaxis, to the depressant effect of morphine on the stimulated guinea-pig ileum was achieved by perfusing the tissue for 90 min with morphine solution. After such a perfusion, significant reductions in the twitch height could only be produced by increasing the dose of morphine 2 to 10-fold.

In the few experiments in which the morphine continued to depress the contractions, even after the 90 min perfusion, the responses to applied acetylcholine or KCl were also reduced by as much as 50%. It was noted that in such experiments, the ileum did not usually recover from the effects of individual doses of morphine, but tended to give progressively smaller contractions as more morphine was given. It is probable that in such preparations, morphine was depressing the ability of the muscle to contract, as well as reducing the amount of transmitter released. Such a depressant action of morphine has been demonstrated by Lewis (1960).

In the present experiments, morphine reduced the output of acetylcholine from the stimulated ileum by 60-70%, while depressing the contraction height by 30 to 40%. After chronic morphine treatment had resulted in a complete loss of the effect on the twitch height, the ability of morphine to reduce acetylcholine release remained unchanged.

On the other hand, morphine tachyphylaxis was accompanied in each experiment by a significant potentiation of the response of the ileum to applied acetylcholine. These experiments indicate that the apparent loss of effect of morphine on the twitch height could result from a sensitization of the muscle to the reduced amounts of transmitter released. It does not occur because morphine loses its ability to suppress the output of acetylcholine.

It was expected that a kind of 'withdrawal symptom' would occur on replacing the morphine solution by normal bathing fluid. This would be seen as an increase in the height of electrically-induced contractions above that of the pre-drug controls. However, repeated washing of the tissues with normal Krebs-Hensleit solution only resulted in the contractions exceeding the control height in 20% of experiments. There are at least two reasons for the difficulty in obtaining 'withdrawal symptoms'. Supramaximal stimulation was used in all experiments, so that in most cases the ileum was already responding with a maximal contraction before morphine was given. When the voltage was reduced in some experiments, a larger contraction was obtained after chronic morphine perfusion, than with the same voltage before the drug was given. Another reason was that in only 3 out of 8 experiments was the amount of acetylcholine released per stimulus restored to that in the initial controls. This suggests that morphine was not adequately removed from the tissue by the washing procedure, and therefore continued to suppress the release of acetylcholine. Thus, only when the amounts of acetylcholine released returned to their control level, would one have expected to see an exaggerated response.

The morphine-tolerant ileum also showed increased sensitivity to histamine, but the response

to KCl did not appear to be consistently potentiated when the effect of acetylcholine was enhanced. This suggests that sensitization of the ileum by morphine may be of a non-specific variety, resembling that produced in the vas deferens by reserpine treatment or decentralization. (Westfall, 1970). This author also found that reserpine treatment enhanced the responses of the guinea-pig vas deferens to acetylcholine and histamine more than it enhanced responses to KCl. It is possible that like denervation and reserpine treatment, morphine sensitizes the muscle of the ileum to various stimulants by increasing the efficiency of the mechanism responsible for mobilizing membrane-bound calcium.

Under certain conditions, morphine is also able to reduce the responses of guinea-pig ileum to acetylcholine, histamine and KCl, as seen in a few of the present experiments and as described by Lewis (1960). When this happened, acute tolerance to the effect of morphine on the coaxially stimulated contractions did not occur. It therefore seems likely that morphine can either increase or decrease the efficiency of the smooth muscle contractile mechanism under different circumstances. The type of responses produced by morphine in any given piece of ileum may depend on some property of the calcium-binding membranes in the muscle.

Since the completion of the present study, a report has appeared by Goldstein & Schulz (1973), in which guinea-pigs were pretreated with morphine in the form of depot pellet implantations, in an attempt to make them tolerant to the actions of morphine. Although the stimulated ilea of the morphine-implanted guinea-pigs were less sensitive to the depressant action of morphine on twitch height, these authors found no change in the sensitivity of the ileum to acetylcholine. They concluded that morphine tolerance does not result from supersensitivity of the muscle to acetylcholine.

It is possible that 'tolerance' produced in the whole animal differs from the acute variety described in the present study. However, Pollock, Muir, Macdonald & Henderson, 1972, who pretreated their rats with morphine in increasing doses for 24 days, found a significant increase in the response of the colon and vas deferens to acetylcholine, furmethide, noradrenaline and 5-hydroxytryptamine. It may be significant that the rats in this study were shown to be tolerant to the analgesic effect of morphine before the experiments on the isolated organs were carried out. On the other hand, the guinea-pigs in the experiments of Goldstein & Schulz (1973) did not show tolerance to morphine analgesia, but only to the hypothermic effect of the drug. Lotti, Lomax & George (1966) showed that tolerance to this latter action of morphine already occurred after the second injection of the drug, indicating that the mechanism involved may differ from that of analgesic tolerance.

Waterfield & Kosterlitz (1973) have also just published an abstract of their study in which they appear to confirm our *in vitro* findings that tolerance to the effect of morphine on the stimulated ileum is due to an increase in the sensitivity of the muscle to acetylcholine.

The results of the present study are consistent with the hypothesis of disuse supersensitivity adapted by Collier, (1965, 1968) to explain tolerance to morphine.

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