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THE INHIBITORY EFFECTS OF PRESYNAPTIC α-ADRENOCEPTOR AGONISTS ON CONTRACTIONS OF GUINEA-PIG ILEUM AND MOUSE VAS DEFERENS IN THE MORPHINE-DEPENDENT AND WITHDRAWN STATES PRODUCED *in vitro*

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1 Isolated ilea from guinea-pigs implanted with morphine pellets were stimulated coaxially, either with or without morphine present in the bath fluid, and the longitudinal contractions recorded.

2 In the absence of morphine the inhibitory effects of the presynaptic α-adrenoceptor agonists, clonidine and oxymetazoline were much reduced and the dose-response curve was flat. This state of 'withdrawal' was readily reversed by morphine and levorphanol but not its inactive (+)-isomer, dextrorphan.

3 The κ-agonists, ketazocine and ethylketazocine, also restored the effects of clonidine as did the opioid peptides Tyr-D-Ala-Gly-Phe-D-Leu, acting preferentially on δ-receptors, and Tyr-D-Ala-Gly-MePhe-Met(O)-ol, acting mainly on μ-receptors.

4 The inhibitory effects of adrenaline and adenosine 3',5'-diphosphate were reduced at low but not at high concentrations.

5 In contrast, the inhibitory effect of clonidine on the electrically evoked contractions of vasa deferentia from mice implanted with morphine pellets was not abolished by the lack of morphine in the bath fluid or by addition of naloxone.

6 A possible explanation is suggested for the loss of the inhibitory effects of presynaptic α-adrenoceptor agonists in the withdrawn state of the dependent ileum.

Introduction

Goldstein & Schulz (1973) showed that the inhibitory effects of adrenaline and dopamine on the electrically evoked contractions of the longitudinal muscle are smaller in myenteric plexus–longitudinal muscle preparations from guinea-pigs implanted with morphine pellets than in those from control animals. Since in those experiments the preparations were suspended in Krebs solution without morphine, it may be assumed that they were in a state of withdrawal from morphine.

In order to test this hypothesis, we examined the inhibitory effects of adrenaline in similar preparations, both in the presence and absence of morphine. Since adrenaline has both pre- and postsynaptic effects, selective stimulation of the presynaptic receptors was effected in most experiments by clonidine and oxymetazoline. Some of the results have been presented to the British Pharmacological Society (Hughes, Kosterlitz, Robson & Waterfield, 1978).

Methods

Male guinea-pigs were implanted subcutaneously with 2 or 4 pellets, each containing 75 mg of morphine base. After 3 days, segments of ilea 10 cm proximal to the ileo–caecal junction were removed and washed with Krebs solution containing 0.5 to 2 μM morphine; a concentration of about 1 μM morphine in plasma of similarly treated guinea-pigs was observed by Goldstein & Schulz (1973). Segments of ileum (Paton, 1955) or longitudinal muscle strips with adherent myenteric plexus (Kosterliz, Lydon & Watt, 1970) were placed in organ baths containing Krebs solution with morphine. The former was stimulated coaxially and the latter by field stimulation (0.1 Hz, 0.5 ms, maximal voltage). The longitudinal contractions were recorded isometrically. Control preparations from naive, that is non-implanted, animals were prepared and set up in the same manner but without morphine in the Krebs solution.

The inhibitory effects of clonidine, oxymetazoline,

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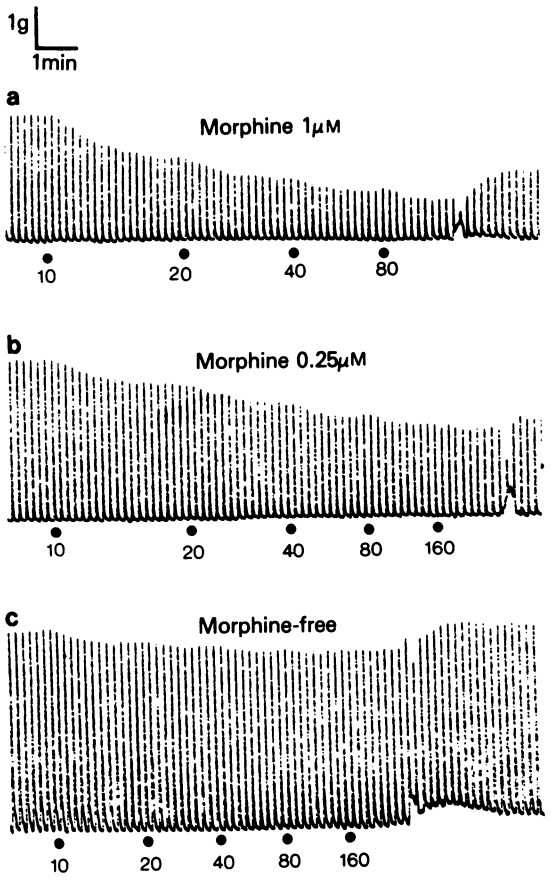

Figure 1 Contractions of isolated ileum from guinea-pig, implanted with 2 pellets each containing 75 mg morphine base 3 days before the experiment. Co-axial stimulation, 0.5 ms, 0.1 Hz, maximal voltage. (a) Ileum suspended in Krebs solution with 1 μM morphine; (b) with 0.25 μM morphine; (c) in morphine-free Krebs solution. At the dots, clonidine was added cumulatively to give the indicated concentrations (nM). Calibration: horizontal 1 min, vertical 1 g.

adrenaline or adenosine 3',5'-diphosphate on the contractions of the longitudinal muscle of ilea from pellet-implanted animals were tested by cumulative dose-response curves, first in Krebs solution containing morphine ('dependent' state) and then in morphine-free Krebs solution ('withdrawn' state). Morphine or other opioids were then added to test whether it was possible to reverse the 'withdrawn' state. Since adrenaline acts not only on presynaptic α-adrenoceptors but also on postsynaptic β-receptors (Paton & Vizi, 1969; Kosterlitz *et al.*, 1970) 0.85 μM (±)-propranolol or 0.4 μM (−)-propranolol was added to the Krebs solution when adrenaline was used.

Male albino mice were implanted with 2 pellets each containing 75 mg of morphine base. After 3 days the vasa deferentia were removed and mounted for field stimulation (0.1 Hz, 0.5 to 1 ms, maximal voltage) in Krebs solution containing 0.5 to 2 μM morphine. The inhibitory effects of clonidine were tested by cumulative dose-response curves in the presence and absence of morphine or in the presence of both morphine and naloxone.

The composition of the Krebs solution (mM) was as follows: NaCl 118, $CaCl_2$ 2.55, KCl 4.70, KH_2PO_4 1.19, $NaHCO_3$ 25, glucose 11 and mepyramine maleate 0.00013; for segments of guinea-pig ileum, $MgSO_4$ 0.59 mM and hexamethonium bromide 69 μM were also present and, in addition, for longitudinal muscle strip preparations, choline chloride 20 μM. The solutions were maintained at 36°C and bubbled with 95% and 5% CO_2.

Drugs

The drugs used were: adrenaline bitartrate, choline chloride (BDH), clonidine hydrochloride (C.H. Boehringer Sohn, Ingelheim), oxymetazoline (Allen & Hanbury Research Laboratories), adenosine 3',5'-diphosphate (Sigma Chemical Co.), morphine hydrochloride (Macfarlan Smith), naloxone hydrochloride (Endo Laboratories), levorphanol tartrate, dextrorphan free base (Roche Products), ethylketazocine and ketazocine as free bases (Sterling Winthrop Research Institute), Tyr-D-Ala-Gly-Phe-D-Leu (Wellcome Research Laboratories), pellets containing 75 mg of morphine base (Wellcome Foundation), Tyr-D-Ala-Gly-MePhe-Met(O)-ol (Sandoz), propranolol (ICI), mepyramine maleate and hexamethonium bromide (May & Baker). Stock solutions of the salts and peptides were made in distilled water, and those of the free bases after the addition of the calculated amounts of HCl, and kept at −25°C. Solutions of peptides were stored in plastic vials. The concentrations are expressed as mM, μM or nM.

Results

Effects of clonidine on the contractions of ilea of guinea-pigs implanted with morphine pellets

Segments of ilea obtained from guinea-pigs implanted with 2 pellets, containing 75 mg morphine base each, for 3 days showed a decreased sensitivity to the inhibitory action of morphine; the IC_{50} values obtained 60 to 90 min after removal of morphine from the bath fluid were 195 ± 24 nM ($n = 7$) compared with 52.2 ± 5.3 nM ($n = 9$; P of difference <0.001) found in preparations from non-implanted control animals. These findings confirmed that our

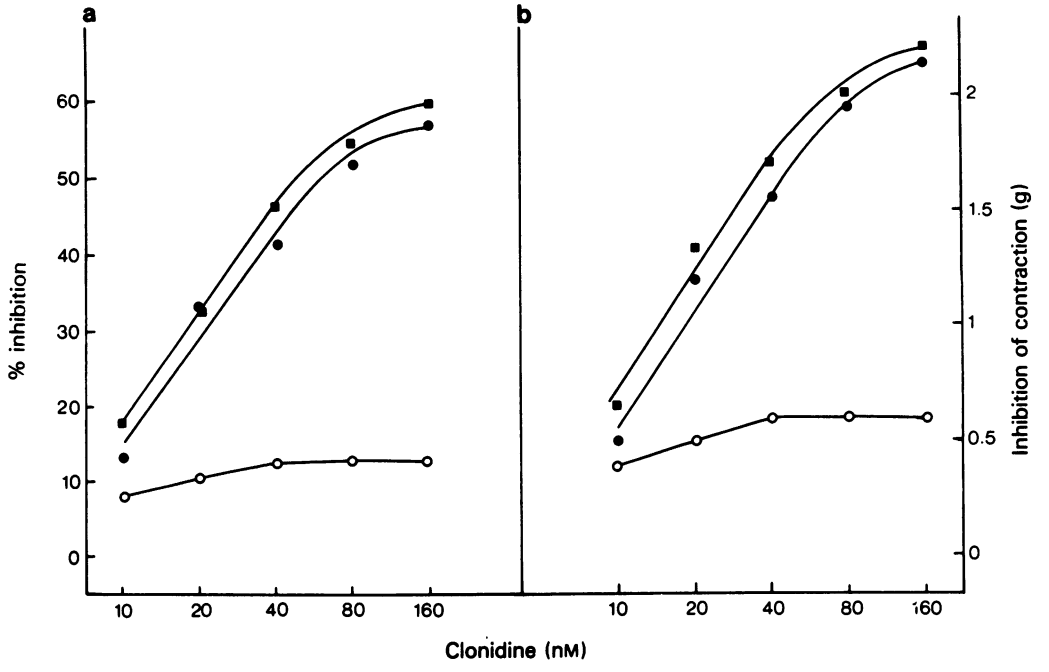

Figure 2 The inhibitory effect of clonidine on the longitudinal contractions of the coaxially stimulated ileum from guinea-pigs implanted with 2 pellets of morphine 3 days before the experiment. Depression of this inhibitory effect by removal of morphine from the bath fluid and its restoration by replacement of morphine. Abscissa scale: concentration of clonidine (nM). Ordinate scale: (a) inhibition of contraction expressed as % of the contraction in the absence of clonidine; (b) inhibition of contraction expressed in decrease of tension (g). Typical results of 3 experiments: (●) in the presence of 0.5 μM morphine, tension of control twitch, 3.7 g; (○) 20 min after removal of morphine, tension of control twitch, 4.8 g; (■) 17 min after replacement of 0.5 μM morphine, tension of control twitch, 3.7 g.

regimen of implantation of morphine pellets caused tolerance to morphine.

When the presynaptic α-adrenoceptors in ileal segments from pellet implanted guinea-pigs were stimulated by clonidine, it was found that, as long as morphine (1 μM) was present in the bath fluid, the sensitivity to the inhibitory effect of clonidine (IC_{50} of 16.3 ± 1.0 nM; $n = 14$) was not significantly different from that obtained in ileal segments from non-implanted control animals (IC_{50} of 16.8 ± 1.4 nM; $n = 19$). However, when the morphine concentration in the bath fluid was reduced, the dose-response curve to clonidine flattened progressively and was almost parallel to the abscissa when morphine was absent (Figures 1, 2a). The maximal inhibitory effect of clonidine on the contraction was now only 13.8 ± 1.1% compared to 71.0 ± 2.9% ($n = 14$; $P < 0.001$) in the presence of morphine (1 μM). This reduction in sensitivity to clonidine was complete within 10 min after removal of morphine. The depressant activity of clonidine was readily restored when morphine (0.25 to 1 μM) was added again to the bath fluid (Figure 2a).

Since the maximal twitch tension of the ileum increased as the concentration of morphine in the Krebs solution was reduced (Figure 1), it became necessary to examine whether the changes in percentage inhibition were based on changes in absolute reductions in twitch tension. It was found that the mean absolute inhibition of the contraction was reduced from 1.66 ± 0.11 g in the presence of 1 μM morphine to 0.66 ± 0.04 g ($n = 14$, $P < 0.001$) after removal of morphine (Figure 2b).

Similar results were obtained in longitudinal muscle strip–myenteric plexus preparations prepared from the ilea of morphine-dependent guinea-pigs implanted with 2 pellets for 3 days. In the presence of 1 μM morphine, the IC_{50} of clonidine was 18.4 ± 2.0 nM ($n = 7$), a value not significantly different from that obtained with preparations from non-implanted animals examined in morphine-free Krebs solution (20.3 ± 1.8 nM; $n = 3$). The maximal inhibition obtained with clonidine was 47.7 ± 2.3% or 1.23 ± 0.13 g in the presence of morphine (1 μM) whereas after removal of morphine from the Krebs

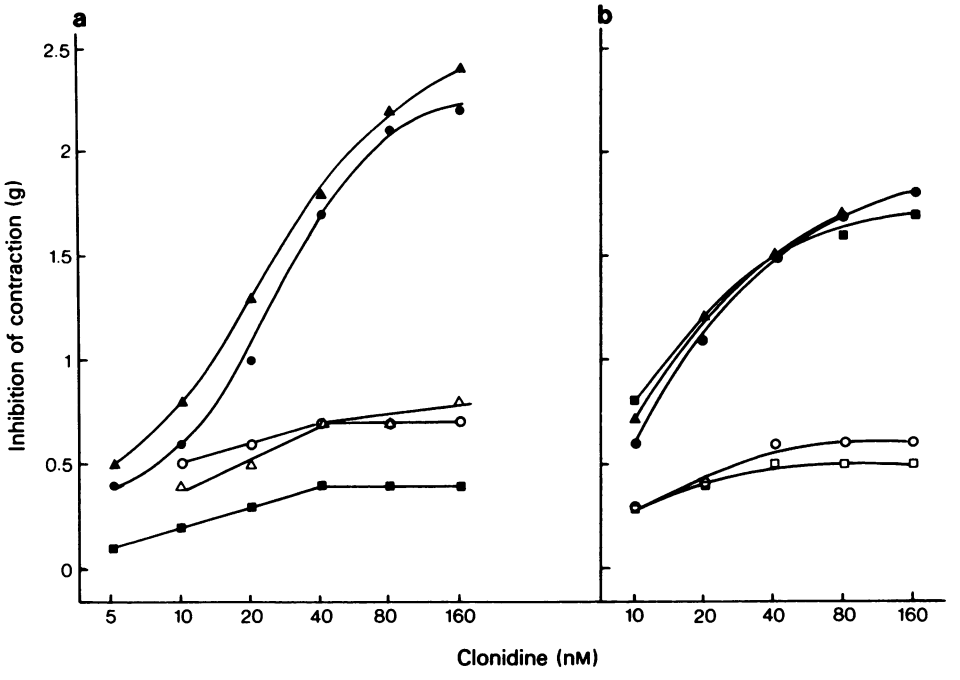

Figure 3 Depression of the inhibitory action of clonidine by withdrawal of morphine from the dependent ileum and its restoration by levorphanol, ketazocine and ethylketazocine. Experimental procedure as in Figure 2(b). Abscissa scale: concentration of clonidine (nM). Ordinate scale: reduction in twitch tension (g). Drugs in Krebs solution: (a) (●) morphine (0.25 μM); (○) 13 min after removal of morphine; (■) 10 min after addition of dextrorphan (0.35 μM); (▲) 13 min after addition of levorphanol (0.2 μM); (△) 20 min after removal of levorphanol. (b) (●) Morphine (0.25 μM); (○) 13 min after removal of morphine; (■) 15 min after addition of ethylketazocine (0.0016 μM); (□) 18 min after removal of ethylketazocine; (▲) 17 min after addition of ketazocine (0.0075 μM). Typical results of 3 experiments in (a) and 2 experiments in (b).

solution the corresponding values were 16.4 ± 1.6% or 0.54 ± 0.05 g ($n = 7$; $P < 0.001$).

The reduction in sensitivity to the inhibitory effect of clonidine was very long-lasting. In one experiment, the segment of ileum from a pellet-implanted guinea-pig was suspended after dissection in Krebs solution containing morphine (0.5 μM). A normal dose-response curve for clonidine was obtained; after removal of morphine the curve became flat and the clonidine effect remained depressed for the next 5 h when the IC_{50} for morphine was still as high as 200 nM.

When the contractions of preparations from 6 non-implanted control guinea-pigs were inhibited by 10 to 50% by the presence of low concentrations of morphine (10 to 50 nM), the mean IC_{50} value of clonidine was 16.9 ± 2.2 nM compared to that of 19.3 ± 1.9 nM before the addition of morphine. Paired analysis of the difference gave a value of 2.42 ± 2.68 nM. Two of these observations were on segments of ileum and the remainder on longitudinal muscle–myenteric plexus preparations. Thus, in normal control preparations the partial depression of the twitch by morphine did not affect the sensitivity to clonidine.

Other drugs acting on opiate receptors also restored the inhibitory effect of clonidine when it was depressed by the withdrawal of morphine. This was shown for the morphinan derivative, levorphanol, whereas its (+)-isomer, dextrorphan, was ineffective (Figure 3a). The benzomorphans, ketazocine and ethylketazocine, which interact with κ-receptors rather than μ-receptors, were also effective (Figure 3b).

It would have been of particular interest to test the ability of the natural enkephalins to restore the clonidine effect. However, since they are rapidly inactivated by peptidases, two enzyme-resistant analogues were used. One of them, Tyr-D-Ala-Gly-Phe-D-Leu (Beddell, Clark, Hardy, Lowe, Ubatuba, Vane, Wilkinson, Chang, Cuatrecasas & Miller, 1977), had a high affinity for the enkephalin binding site while the other, Tyr-D-Ala-Gly-MePhe-Met(O)-ol (Roemer, Buescher, Hill, Pless, Bauer, Cardinaux, Closse, Hauser & Hugenin, 1977), had a higher affinity for

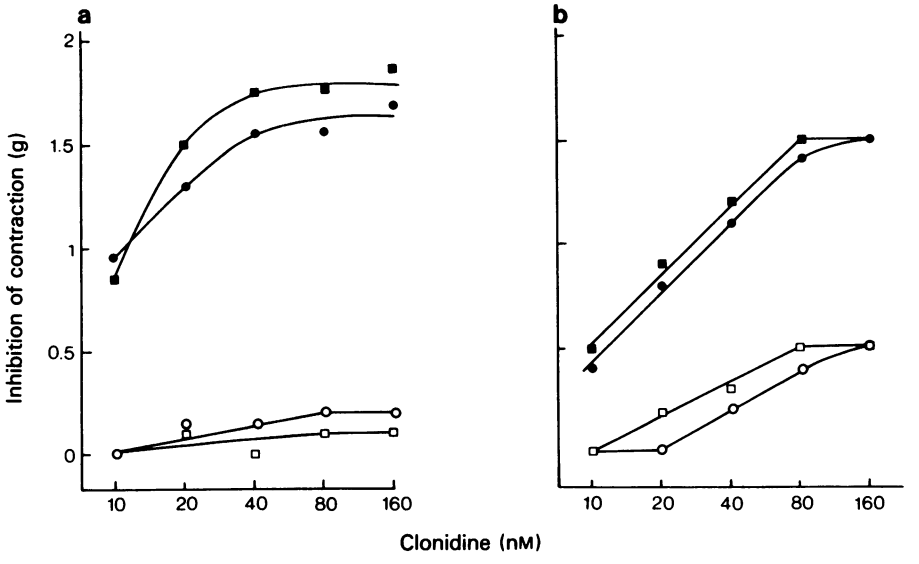

Figure 4 Depression of the inhibitory action of clonidine by withdrawal of morphine from the dependent ileum and its restoration by opioid peptides. Experimental procedure and abscissa and ordinate scales as in Figure 3. Drugs in Krebs solution: (a) (●) morphine (1 μM); (○) 15 to 20 min after removal of morphine; (■) 50 min after addition of Tyr-D-Ala-Gly-Phe-D-Leu (0.09 μM); (b) (●) morphine (0.5 μM); (○) 15 to 20 min after removal of morphine; (■) 13 min after addition of Tyr-D-Ala-Gly-MePhe-Met(O)-ol (0.014 μM); (a) and (b) (□) 15 to 20 min after removal of peptides. Typical results of 2 experiments in (a) and 3 experiments in (b).

the naltrexone than for the enkaphalin binding site (Kosterlitz, McKnight, Waterfield, Gillan & Paterson, 1978). Both analogues restored the depressant effect of clonidine after morphine had been removed from the bath fluid (Figure 4).

Effects of oxymetazoline

Oxymetazoline, another agonist acting on presynaptic α-adrenoceptors, also depressed the contractions of the coaxially stimulated guinea-pig isolated ileum. As was found for clonidine, morphine had no effect on the potency of oxymetazoline in preparations from non-implanted animals. In preparations from morphine-dependent guinea-pigs, the dose-response curve of oxymetazoline was flattened when morphine was removed from the bath fluid and restored when morphine was added again (Figure 5).

Effects of adrenaline

The results obtained with adrenaline differed qualitatively and quantitatively from those found with clonidine or oxymetazoline. Dose-response curves for adrenaline for preparations from pellet-implanted guinea-pigs were first obtained in the presence of morphine (1 μM). After removal of morphine, the inhibitory effects of low concentrations were reduced but those of high concentrations were almost unchanged (Figure 6a). The depressant action of adrenaline at high concentrations was not mediated by β-adrenoceptors in the longitudinal muscle since (−)-propranolol (0.4 μM) was present throughout the experiment and an increase in the concentration to 2 μM did not modify the responses to adrenaline after removal of morphine. Moreover, the responses of the longitudinal muscle to standard concentrations of acetylcholine (5 to 160 nM) were not affected by any of the concentrations of adrenaline.

In preparations from non-implanted guinea-pigs morphine (50 nM) did not alter the IC_{50} values of adrenaline.

Effects of adenosine 3',5'-diphosphate

To examine whether the inhibitory effects of clonidine, oxymetazoline and adrenaline were shared by compounds whose action was not mediated by α-adrenoceptors, the effects of adenosine 3',5'-diphosphate were studied. They were found to be similar to those found with adrenaline rather than those with clonidine or oxymetazoline (Figure 6b). In preparations from normal guinea-pigs, morphine did not affect the IC_{50} value of adenosine 3',5'-diphosphate.

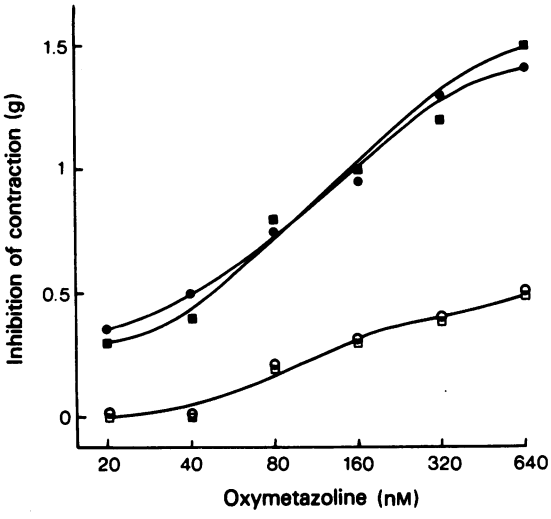

Figure 5 Depression of the inhibitory action of oxymetazoline by withdrawl of morphine and its restoration by the addition of morphine. Experimental procedure and abscissa and ordinate scales as in Figure 3. Drugs in Krebs solution: (●) morphine (1 μM); (○) 12 min after removal of morphine; (■) 9 min after addition of morphine (0.25 μM); (□) 11 min after removal of morphine. Typical results of 2 experiments.

Effects of clonidine on the contractions of the mouse vas deferens

When dose-response curves for clonidine were constructed for vasa deferentia obtained from 9 mice implanted with morphine pellets, it was found that they were identical whether morphine (1 μM) was present in or absent from the bath fluid. Similarly, no change occurred when in 5 experiments the morphine present in the bath fluid was antagonized by addition of 100 to 500 nM naloxone to the bath fluid; no contracture of the vasa deferentia was produced by naloxone. Vasa deferentia from pellet-implanted animals were less sensitive to morphine (IC_{50} of 1106 ± 215 nM; $n = 9$) than those from normal mice (IC_{50} of 526 ± 65 nM; $n = 6$, $P < 0.05$). After intraperitoneal injection of 10 mg/kg naloxone, implanted mice displayed jumping and wet shakes, which indicated that the pellets had led to dependence.

Discussion

The experiments on ilea obtained from guinea-pigs implanted with morphine pellets have shown that there is an interaction between presynaptic α-adrenoceptors and presynaptic opiate receptors, both of which cause a depression of the contraction of the longitudinal muscle evoked by electrical stimulation of the myenteric plexus. It has been previously established (Kosterlitz & Watt, 1968) that the presynaptic opiate receptors are selectively blocked by naloxone and the presynaptic α-adrenoceptors by phentolamine. It is therefore likely that the described interaction occurs at a point between the recognition sites of the two agonists and the mechanism responsible for the release of the transmitter, acetylcholine, which mediates the contraction of the longitudinal muscle.

This interaction which leads to a decreased sensitivity to stimulation by agonists acting on presynaptic α-adrenoceptors, occurs only in the 'withdrawn' state when morphine has been removed from the bath fluid; it is absent in the 'dependent' state when morphine is present. This view is supported by a number of observations. First, in naive non-dependent ilea, addition or removal of morphine does not change the sensitivity to stimulation by α-adrenoceptor agonists. Secondly, the 'withdrawn' state can be readily restored to the 'non-withdrawn' state by the addition of various opioid agonists. This restoration is stereospecific since compared with levorphanol the (+)-isomer dextrorphan is ineffective. When after restoration the opiate is removed, 'withdrawal' appears again. Thirdly, the 'withdrawn' state is of long duration; it has been observed to persists for 5 h after removal of morphine from the bath fluid; at that time a dose-response curve to morphine showed that the preparation was still 'tolerant' to morphine.

It would appear then, that the reduced sensitivity to the effect of stimulation of presynaptic α-adrenoceptors is an important and reversible sign of 'withdrawal' in the morphine-dependent guinea-pig ileum. It may be related to the increased sensitivity of such preparations to naloxone observed by Ehrenpreis, Light & Schonbuch (1972), Ehrenpreis, Greenberg & Comaty (1975) and Schulz & Herz (1976).

The conversion from the 'withdrawn' to the 'non-withdrawn' state can be brought about by diverse opiate agonists. Morphine, levorphanol, ketazocine and ethylketazocine are all effective. The latter two compounds act in the guinea-pig ileum on κ-receptors which are different from those which interact with morphine (Hutchinson, Kosterlitz, Leslie, Waterfield & Terenius, 1975). Opioid peptides counteract the 'withdrawn' state; one of the peptides, Tyr-D-Ala-Gly-Phe-D-Leu, has a pharmacological pattern similar to natural Leu-enkephalin whereas the other, Tyr-D-Ala-Gly-MePhe-Met(O)-ol, is more similar to morphine (Kosterlitz *et al.*, 1978).

While clonidine and oxymetazoline are ineffective in the 'withdrawn' state, the action of adrenaline is depressed only at low concentrations. At higher concentrations of adrenaline, there may be a second component of its inhibitory action which clonidine or oxymetazoline appear not to possess. The nature of

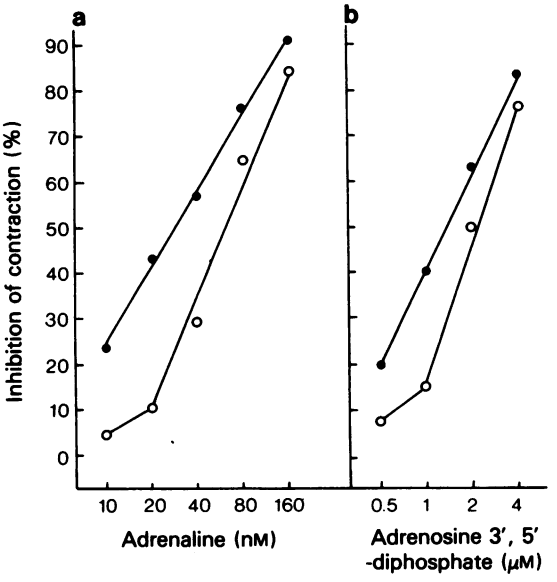

Figure 6 Depression of the inhibitory actions of adrenaline (a) and adenosine 3',5'-diphosphate (b) by withdrawal of morphine and their restoration by the addition of morphine. Experimental procedure as in Figure 2(a); (−)-propranolol (0.4 μM) was present in the bath fluid of the experiment with adrenaline. Abscissa scales: concentration of adrenaline (nM) and adenosine 3',5'-diphosphate (μM); ordinate scales: inhibition of contractions expressed as % of the contractions in the absence of adrenaline or adenosine 3',5'-diphosphate. (●) In the presence of morphine (a) 1 μM, tension of control twitch 2.1 g; (b) 2 μM, tension of control twitch 3.0 g; (○) 20 min after removal of morphine, tension of control twitch 3.7 g in (a) and 3.8 g in (b). Typical results of 2 experiments.

this difference is not known but it does not seem to reside in a postsynaptic action of adrenaline on β-receptors since propranolol was present throughout the experiment; moreover, the sensitivity to acetylcholine added to the bath fluid remained unchanged.

In cultures of mouse neuroblastoma × glioma hybrid NG 108-15 cells, Sharma, Klee & Nirenberg (1975) showed that during the development of morphine tolerance and dependence the activity of morphine-sensitive adenylate cyclase increased so that the cyclic adenosine 3',5'-monophosphate content was normal when morphine was present in the culture medium and increased when morphine was withdrawn. If similar changes may be assumed to occur in the myenteric plexus of the 'dependent' ileum, then the increase in adenylate cyclase activity due to 'withdrawal' of morphine would lead to such an increase in electrically evoked acetylcholine release that stimulation of the inhibitory presynaptic α-adrenoceptor would be less effective. The finding presented in this paper would be compatible with this interpretation but it has so far not been possible to test this view because preparations of myenteric plexus cannot be freed from smooth muscle.

It has recently been shown for human addicts (Gold, Redmond & Kleber, 1978) that during withdrawal from chronic methadone administration, clonidine ameliorates the withdrawal syndrome. In such patients, cooperation between the administered clonidine and the residual methadone may be possibly the basis of this beneficial effect.

It is of interest that the 'withdrawn state' cannot be provoked in vasa deferentia of mice made dependent by implantation with morphine pellets. The vasa deferentia which have a reduced sensitivity to morphine but do not lose their sensitivity to the inhibitory action of clonidine after removal of morphine from the bath fluid, do not respond to naloxone with a contracture as seen in dependent guinea-pig ilea. The reason for these differences between the two preparations is not known at present but it may be significant that, first, no detectable amounts of enkephalin are present in the mouse vas deferens in contrast to the guinea-pig ileum (Hughes, Kosterlitz & Smith, 1977). Secondly, the decrease in the sensitivity to morphine was four fold in the ileum and only two fold in the vas deferens. Thirdly, the receptor populations in the mouse vas deferens, in which δ-receptors are prevalent, are different from those of the guinea-pig ileum (Lord, Waterfield, Hughes & Kosterlitz, 1977). Finally, transmission in the mouse vas deferens is probably adrenergic (Hughes, Kosterlitz & Leslie, 1975; Jones & Spriggs, 1975) whereas that from the myenteric plexus to the longitudinal muscle is cholinergic (Cowie, Kosterlitz & Watt, 1968; Paton & Zar, 1968).

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