Br. J. Pharmac. (1975), 53, 131-138

AN ANALYSIS OF THE PHENOMENON OF ACUTE TOLERANCE TO MORPHINE IN THE GUINEA-PIG ISOLATED ILEUM

H.W. KOSTERLITZ & ANGELA A. WATERFIELD
Unit for Research on Addictive Drugs, University of Aberdeen AB9 1AS

1 The observations which Paton (1957) interpreted as 'acute tolerance' and 'dependence' have been confirmed for coaxially stimulated segments of guinea-pig ileum and extended to the contractions evoked by field stimulation in the myenteric plexus-longitudinal muscle preparation. Evidence is adduced that the morphine receptors of the myenteric plexus are not involved in the two phenomena.

2 The contraction of the longitudinal muscle depressed by low concentrations of morphine, or levorphanol, can be restored to control level not only by high concentrations of morphine but also by levorphanol and equally well by its (+)-isomer, dextrorphan, which does not fulfil the stereospecific requirements of the morphine receptor. Acetylcholine output was not increased.

3 When, after restoration of the twitch by high concentrations of morphine, the drug is washed out, contractions become depressed. This effect cannot be due to 'dependence' because either morphine or its antagonist, naloxone, restore the twitch again.

4 In the concentrations used, morphine, levorphanol and dextrorphan inhibit the cholinesterase of homogenates of the myenteric plexus-longitudinal muscle preparation by 10-15%, Since a concentration of physostigmine which causes a similar inhibition also restores the twitch, it is concluded that the described phenomena are best explained by the anti-cholinesterase effects of the drugs.

Introduction

When Paton (1957) showed that morphine-like drugs depressed the evoked contraction of the longitudinal muscle of the guinea-pig isolated ileum, he also described two phenomena which he interpreted as 'acute tolerance' and 'dependence'. A depression of contraction was first obtained with a relatively low concentration of morphine (2.6 μM); increases in the concentration to 5.3 and 58 μM did not lead to any further depression of the twitch. When the morphine was then washed out, there was a decrease in the twitch height which could, however, be restored to the control or pre-morphine level by successive additions of morphine (up to 530 μM) to the bath fluid. Paton proposed that the decrease in sensitivity to morphine after exposure to a relatively low concentration may be likened to 'tolerance' and the depression of the twitch after washing out of the high concentration and its restoration by a similar or higher concentration may be an expression of 'dependence'. Similar findings were obtained with nalorphine. These observations have been confirmed in our and other laboratories (Fennessy, Heimans & Rand, 1969).

The concentrations of morphine that counteract the depression of lower concentrations, are very high indeed when compared with the concentration of 0.07 μM that is required to depress the twitch by 50% (Kosterlitz & Watt, 1968). Since Gyang & Kosterlitz (1966) have shown that morphine has also weak antagonist activity, the possibility has to be considered that the high concentrations of morphine antagonize the depressant effects of the low concentrations. When Paton made his original observations, the only available morphine-antagonists were nalorphine and levallorphan, both of which have also considerable agonist activity. With the introduction of naloxone, which is a morphine antagonist without significant agonist activity, it has become possible to test whether or not the proposed interpretation is correct. A preliminary report of the results was given to a joint meeting of the British and German

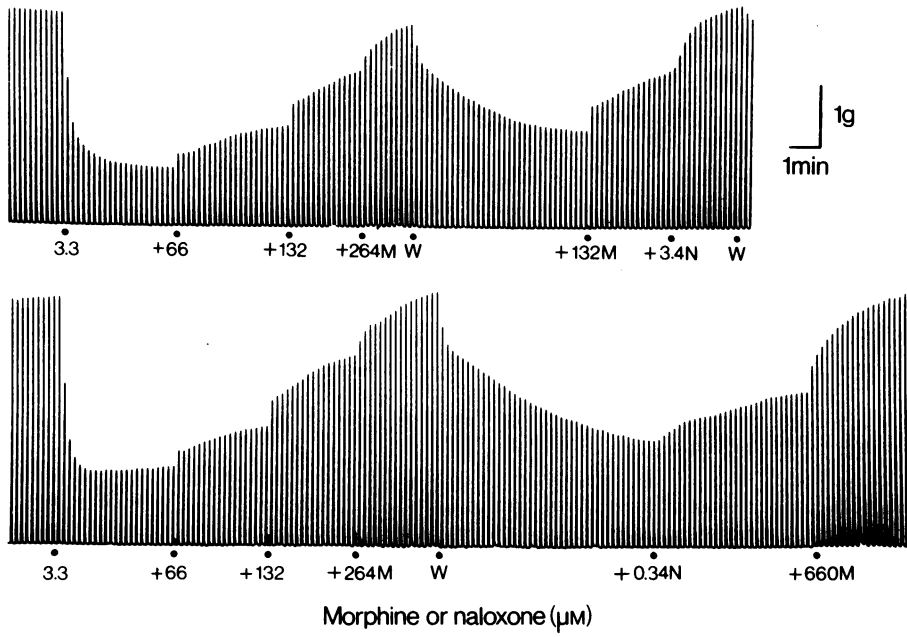

Figure 1 The effects of low and high concentrations of morphine and naloxone on the evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. Field stimulation (0.1 Hz, 0.5 ms, supramaximal voltage) and isometric recording. The figures under the tracing indicate concentrations (μM) of morphine (M) or naloxone (N). W denotes washing out of drugs.

Pharmacological Societies (Waterfield & Kosterlitz, 1973).

Methods

Experimental procedure

All experiments were performed on the guinea-pig isolated ileum; the terminal section was used after the 10 cm nearest to the ileo-caecal junction had been discarded. The techniques used were those described earlier (Kosterlitz, Lydon & Watt, 1970). The bath fluid was a modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.20, $NaHCO_3$ 25, glucose 11; it also contained mepyramine maleate (0.125 μM) and, for the experiments on the myenteric plexus-longitudinal muscle preparation, choline chloride (20 μM). It was bubbled with 95% O_2 and 5% CO_2. The volume of the bath fluid was 40 ml when contractions of a segment of ileum were recorded, and 3 ml in all other experiments. The temperature was 36°C. The stimuli were 1.3 times maximal rectangular pulses of 0.5 ms duration at a frequency of 0.1 Hz; the whole segment of ileum was stimulated coaxially and the myenteric plexus-longitudinal muscle preparation by field stimulation. The twitch-like contractions of the longitudinal muscle were recorded isometrically by a mechano-electrical transducer and displayed on a pen oscillograph or a potentiometric recorder.

The acetylcholine released from the electrically stimulated myenteric plexus-longitudinal muscle preparation in the presence of physostigmine sulphate (7.7 μM) was assayed on the guinea-pig ileum (Kosterlitz *et al.*, 1970).

For the estimation of total cholinesterase, weighed strips of longitudinal muscle with attached myenteric plexus (about 350 mg) were ground by hand in a ground glass homogenizer containing 7 ml of ice-cold incubation medium (NaCl 130 mM, $MgCl_2$ 35 mM, $NaHCO_3$ 31 mM, gassed with 5% CO_2 and 95% N_2; pH 7.4). Cholinesterase was determined in a Gilson respirometer, at 37°C, with acetylcholine perchlorate (14 mM) as substrate. The mean cholinesterase activity of homogenized tissue was 148.5 ± 5.3 μl

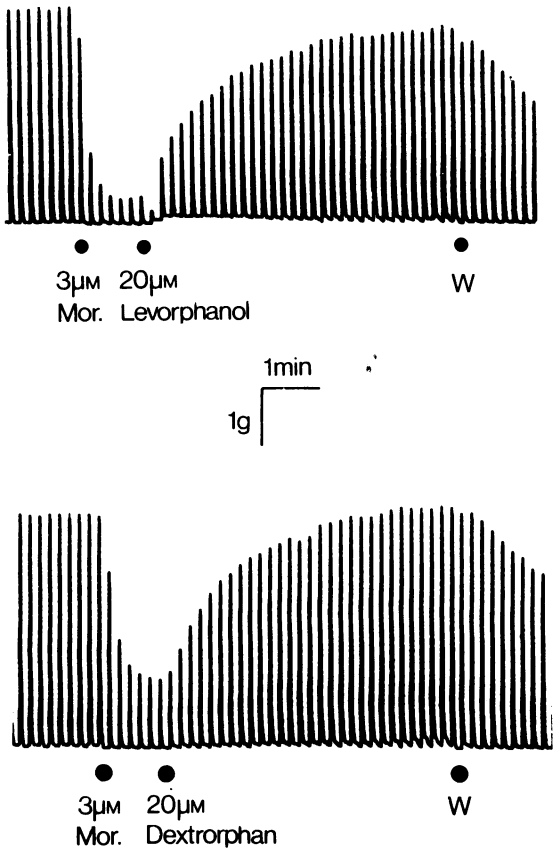

Figure 2 The effects of high concentrations (20 μM) of levorphanol and dextrorphan on the height of the contractions of a myenteric plexus-longitudinal muscle preparation depressed by morphine (Mor.). Stimulation and recording as in Figure 1. W, washing out of drugs.

CO_2 min^{-1} g^{-1} wet weight. Drugs were added 5 min before the start of measurements which were continued for 1 hour.

Drugs

Drugs used were: acetylcholine chloride, choline chloride, physostigmine sulphate, hexamethonium bromide, mepyramine maleate, histamine acid phosphate, bradykinin, morphine hydrochloride, nalorphine hydrobromide, levorphanol tartrate, dextrorphan tartrate, naloxone hydrochloride, cyclazocine ({±}α-2-cyclopropylmethyl-5,9-dimethyl-2′-hydroxy-6,7-benzomorphan).

Stock solutions were made up in distilled water; cyclazocine was dissolved after addition of the required amount of HCl. Concentrations are given as μM or nM with respect to base.

Results

Paton (1957) obtained his original findings on a segment of ileum. We confirmed these observations on the myenteric plexus-longitudinal muscle preparation (Figure 1). A concentration of 3.3 μM of morphine depressed the twitch by 72%. When the morphine concentration was raised to about 70 μM, the twitch height increased; at 200 μM morphine, the depression was reduced to 26.5% and at 464 μM it was only 4%. On washing out morphine, the twitch height decreased immediately and reached a minimum after 5 to 6 min, a phenomenon likened by Paton to 'dependence'. If this interpretation is correct, additions of morphine and of the antagonist naloxone should have opposite effects, morphine should increase the twitch height and naloxone decrease it by evoking an 'abstinence syndrome'. This expectation, however, was not fulfilled, since both drugs caused an increase in twitch height. The order of applying morphine and naloxone did not affect the results (Figure 1).

Similar results were obtained when the initial depression of the longitudinal contraction of a segment of ileum was caused by compounds which had not only agonist but also antagonist activity. Thus, the depressant effects of nalorphine (0.48 μM) or of cyclazocine (0.009 μM) were reversed by morphine (660 μM).

If the increase in twitch height caused by high concentrations of morphine were due to interaction with morphine receptors, this effect would be expected to be stereospecific. Therefore, the actions of the (−)-isomer of 3-hydroxy-*N*-methylmorphinan, levorphanol and of its (+)-isomer, dextrorphan, were investigated. It was found that both isomers were effective in increasing the twitch height depressed by 3 μM morphine (Figure 2).

The similarity of the effects of morphine and naloxone and the lack of stereospecificity suggest that the restoring effects of high concentrations of morphine are not due to an action on morphine receptors which, on activation, decrease acetylcholine release from the myenteric plexus of the guinea-pig ileum. This view was tested by determining the output of acetylcholine evoked by electrical stimulation before and after addition of low and high concentrations of morphine, levorphanol, dextrorphan and naloxone.

As has been shown previously for segments of

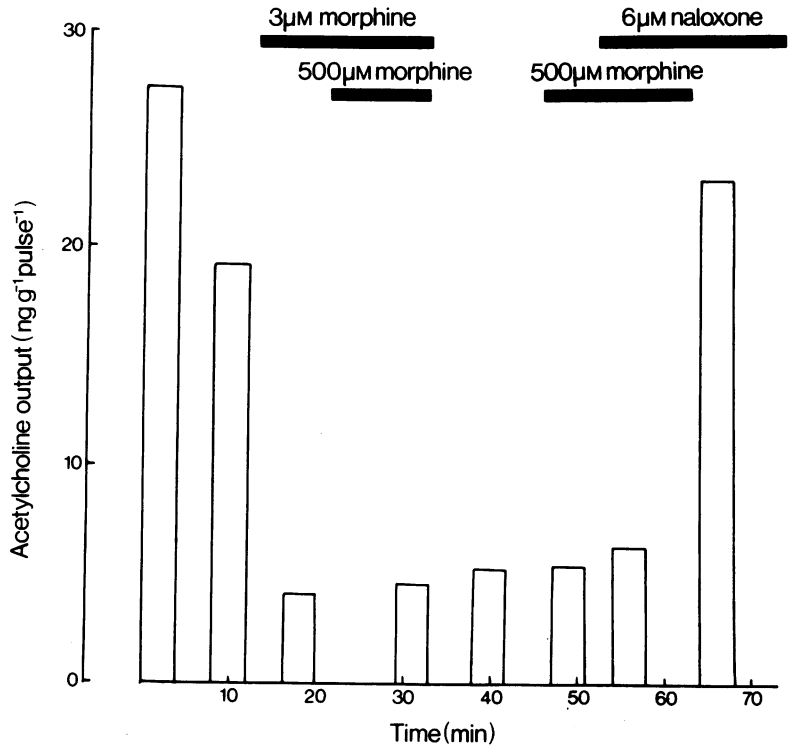

Figure 3 The effects of low and high concentrations of morphine and naloxone on the acetylcholine output of the myenteric plexus-longitudinal muscle preparation in the presence of physostigmine (7.7 μM). Stimulation as in Figure 1. Abscissae, time (min); ordinates, acetylcholine output (ng g^{-1} $pulse^{-1}$). The horizontal bars indicate presence of drugs.

ileum (Paton, 1957) and for the myenteric plexus-longitudinal muscle preparation (Cowie, Kosterlitz & Watt, 1968; Paton & Zar, 1968), a low concentration of morphine (3 μM) reduced acetylcholine output evoked by electrical field stimulation (Figure 3). When the concentration of morphine was raised to 500 μM, there was no restoration of acetylcholine output; after washing out this high concentration of morphine, acetylcholine output did not change for up to 5 minutes. Thus, when the twitch height was increased by raising the morphine concentration or when it was decreased immediately after washing out morphine (Figure 1a, b), there was no change in acetylcholine output. When a relatively small amount of naloxone (6 μM) was added in presence of the high concentration of morphine, there was, in this particular experiment, no increase in acetylcholine output which, however, returned to the control level when morphine was washed out while naloxone remained in the bath fluid. In a series of 5 experiments, morphine (3 μM) lowered the control acetylcholine output (ng g^{-1} $pulse^{-1}$) from 20.5 to 6.6 ($P < 0.01$). When the morphine concentration was raised to 500 μM, the acetylcholine output was 6.5; addition of naloxone (6 μM) raised the output to 8.5 ($P < 0.025$). When morphine was washed out while naloxone was still present, the output rose to 20.2 ($P < 0.01$).

The effects of levorphanol and dextrorphan on evoked contractions of the longitudinal muscle and on acetylcholine output are shown in Figure 4. The results were obtained in two separate series; in the experiments on the contraction, no physostigmine was added to the bath fluid while for the determination of acetylcholine output physostigmine had to be used. A low concentration of levorphanol (0.4 μM) decreased the contraction and lowered acetylcholine output. When levorphanol or dextrorphan was then added to give a high concentration (70 μM), the size of the contraction became larger than that obtained

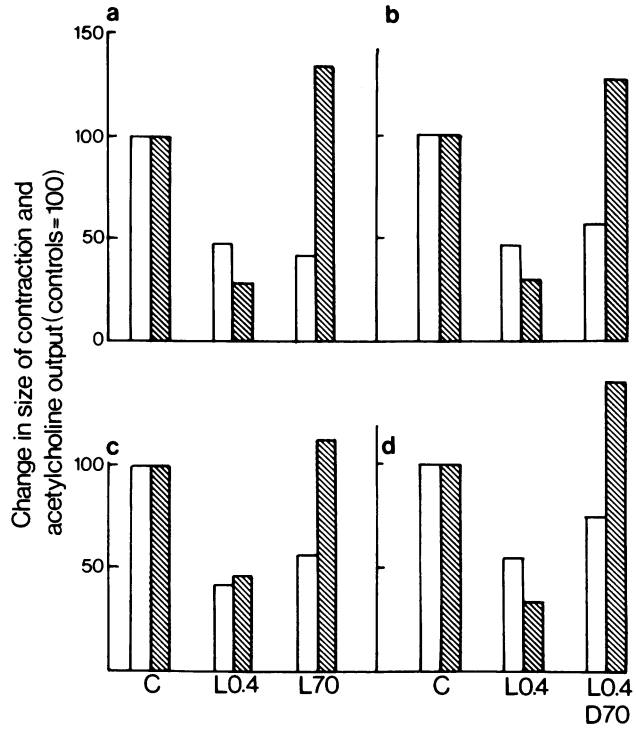

Figure 4 Myenteric plexus-longitudinal muscle preparation. The effects of low and high concentrations of levorphanol and dextrorphan on the size of contractions in the absence of physostigmine and the evoked acetylcholine output measured in separate preparations in the presence of physostigmine. Stimulation and recording as in Figure 1. Abscissae, C, controls; L 0.4, 0.4 μM levorphanol; L 70, 70 μM levorphanol; D 70, 70 μM dextrorphan. Ordinates, changes in acetylcholine output (clear columns) or contractions (shaded columns) (control values = 100) (a and b) control output 23 ng g^{-1} $pulse^{-1}$; (c and d) hexamethonium (140 μM) present throughout, control outputs, 19 (c) and 13 (d) ng g^{-1} $pulse^{-1}$.

before any drug had been added although the output of acetylcholine was unaltered or raised only very little. Addition of hexamethonium which lowered the control output of acetylcholine, did not alter the described effects of levorphanol or dextrorphan on either contraction or acetylcholine output.

Since the findings so far reported indicate that in this preparation the phenomena likened by Paton (1957) to tolerance and dependence are not due to prejunctional effects, the possibility of changes in the sensitivity of the smooth muscle cells to acetylcholine was examined. The dose-response curve for acetylcholine was reversibly shifted to the left by morphine (500 μM) and levorphanol (70 μM) but there was no increase in the sensitivity to histamine or bradykinin in the presence of hyoscine (Figure 5). Similar results were obtained with dextrorphan (70 μM).

These observations suggest that the increase in the size of contraction caused by high concentrations of morphine, levorphanol or dextrorphan is possibly due to a partial inhibition of cholinesterase present in the preparation particularly since it is known that these compounds inhibit acetylcholinesterase in brain homogenates (e.g. Harris & Dewey, 1972) and that acetylcholinesterase is present in the myenteric plexus and butyrylcholinesterase in the longitudinal muscle (Ambache, Freeman & Hobbiger, 1968). The inhibitory effects of physostigmine, levorphanol, dextrorphan and morphine on total cholinesterase are shown in Figure 6. The following ED_{50} values (μM) were obtained from calculated regression equations: physostigmine 0.96, levorphanol 59, dextrorphan 141 and morphine 3500. Although these values are higher than those found by Harris & Dewey (1972), who used rat

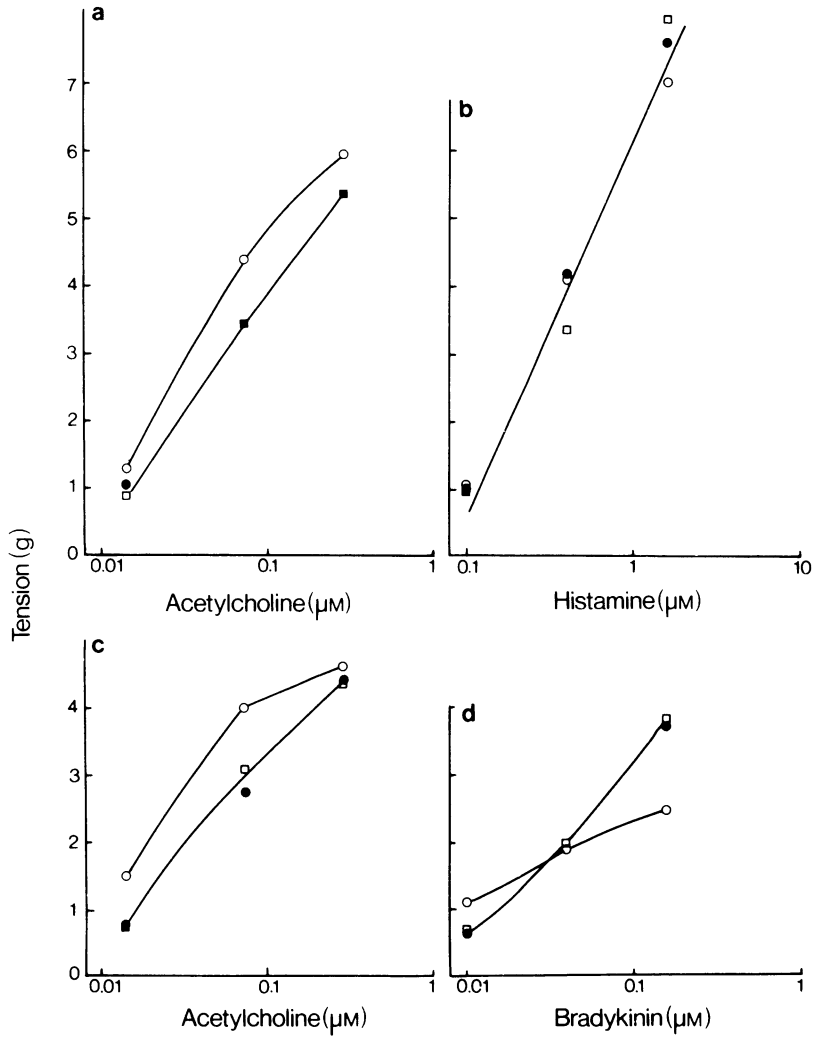

Figure 5 The effects of morphine (a and b, 500 μM) and levorphanol (c and d, 70 μM) on the size of contractions produced by acetylcholine (a and c), and, in the presence of hyoscine 0.76 μM, histamine (b) and bradykinin (d). Isometric recording. Abscissae, concentration (μM); ordinates, tension (g). Controls (●); in presence of drug (○); after washing out (□).

brain homogenates as the source of enzyme and thiocholine as substrate, the rank order is the same as in their experiments.

Finally, it was found that 0.1 μM physostigmine, which inhibited cholinesterase to the same extent as 500 μM morphine, also restored the twitch depressed by morphine 3 μM.

Discussion

The observations which Paton (1957) interpreted as 'acute tolerance' and 'dependence' have been confirmed for segments of the guinea-pig ileum and similar findings have now been obtained on the myenteric plexus-longitudinal muscle preparation. The analysis of these observations has shown, however, that the morphine receptors present in the myenteric plexus are not responsible for these phenomena.

First, the (+)-isomer, dextrorphan which is not a narcotic analgesic, is as effective as the (−)-isomer, levorphanol, in restoring the contraction depressed by morphine. Secondly, morphine and the antagonist naloxone should have opposite

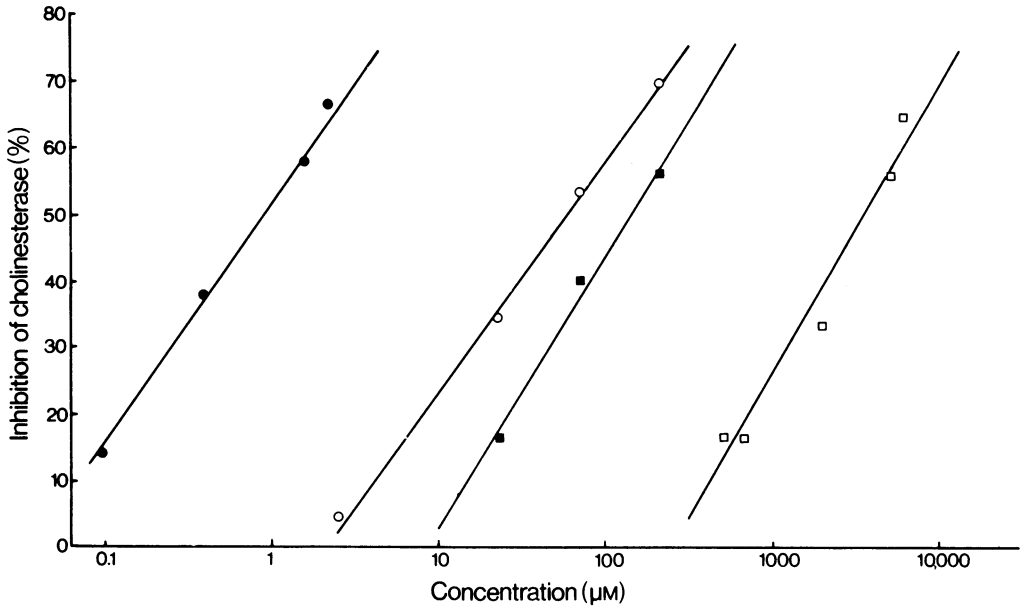

Figure 6 Inhibition of cholinesterase of homogenates of the myenteric plexus-longitudinal muscle preparation. Abscissae, concentration of drug (μM); ordinates, inhibition of cholinesterase (%). The lines have been drawn from regression equations; the number of observations were for physostigmine 41 (●), levorphanol 21 (○), dextrorphan 14 (■), morphine 11 (□).

effects but, in fact, they both improve the contraction, the former without and the latter with a concomitant increase in acetylcholine output.

Since high concentrations of morphine, levorphanol or dextrorphan increase the responses to acetylcholine but not to histamine or bradykinin the phenomena described by Paton are best explained by the anticholinesterase action of high concentrations of morphine.

These observations are probably of little importance for the explanation of tolerance and dependence *in vivo*. The concentrations of morphine required are two to three orders of magnitude higher than the plasma concentrations (1-2 μM) of morphine-dependent guinea-pigs (Goldstein & Schulz, 1973) or the brain concentrations (<1 μM) of morphine-dependent dogs (Mulé & Woods, 1962).

Supported by grants from the U.S. Committee on Problems of Drug Dependence, NRC-NAS, and the U.S. National Institute of Mental Health with funds from the Special Action Office for Drug Abuse Prevention (DA 00662). Drugs were generously supplied as follows: hexamethonium bromide and mepyramine maleate by May and Baker, levorphanol tartrate and dextrorphan tartrate by Roche Products, naloxone hydrochloride by Endo Laboratories and cyclazocine by Sterling-Winthrop.

References

AMBACHE, N., FREEMAN, M.A. & HOBBIGER, F. (1968). Localization of acetyl- and butyrylcholinesterases in the myenteric plexus and longitudinal muscle of the guinea-pig's ileum. *J. Physiol., Lond.*, **200**, 50-51P.

COWIE, A.L., KOSTERLITZ, H.W. & WATT, A.J. (1968). Mode of action of morphine-like drugs on autonomic neuro-effectors. *Nature, Lond.*, **220**, 1040-1042.

FENNESSY, M.R., HEIMANS, R.L.H. & RAND, M.J. (1969). Comparison of effect of morphine-like analgesics on transmurally stimulated guinea-pig ileum. *Br. J. Pharmac.*, **37**, 436-449.

GOLDSTEIN, A. & SCHULZ, R. (1973). Morphine-tolerant longitudinal muscle strip from guinea-pig ileum. *Br. J. Pharmac.*, **48**, 655-666.

GYANG, E.A. & KOSTERLITZ, H.W. (1966). Agonist and antagonist actions of morphine-like drugs on the

guinea-pig isolated ileum. *Br. J. Pharmac. Chemother.*, 27, 514-527.

HARRIS, L.S. & DEWEY, W.L. (1972). Role of cholinergic systems in the central action of narcotic agonists and antagonists. In: *Agonist and Antagonist Actions of Narcotic Analgesic Drugs*, ed. Kosterlitz, H.W., Collier, H.O.J. & Villarreal, J.E., pp. 198-206. London: Macmillan Press.

KOSTERLITZ, H.W., LYDON, R.J. & WATT, A.J. (1970). The effects of adrenaline, noradrenaline and isoprenaline on inhibitory α- and β-adrenoceptors in the longitudinal muscle of the guinea-pig ileum. *Br. J. Pharmac.*, **39**, 398-413.

KOSTERLITZ, H.W. & WATT, A.J. (1968). Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). *Br. J. Pharmac. Chemother.*, **33**, 266-276.

MULE, S.J. & WOODS, L.A. (1962). Distribution of N-C^{14}-methyl labelled morphine: I. In central nervous system of nontolerant and tolerant dogs. *J. Pharmac. exp. Ther.*, **136**, 232-241.

PATON, W.D.M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **12**, 119-127.

PATON, W.D.M. & ZAR, M.A. (1968). The origin of acetylcholine released from guinea-pig intestine and longitudinal strips. *J. Physiol., Lond.*, **194**, 13-33.

WATERFIELD, A.A. & KOSTERLITZ, H.W. (1973). Acute 'tolerance' and 'dependence' in guinea-pig ileum. *Naunyn-Schmiedeberg's Arch. exp. Pharmak.*, **279**, Suppl., 39.

(Received May 13, 1974.
Revised July 23, 1974)