Br. J. Pharmac. (1977), **61**, 271–278

OPIATE BINDING AND EFFECT IN ILEUM PREPARATIONS FROM NORMAL AND MORPHINE PRETREATED GUINEA-PIGS

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1 Dose-response curves for normorphine in the absence and presence of naloxone have been obtained from myenteric plexus-longitudinal muscle strip preparations from normal and morphine pretreated guinea-pigs. In addition, the high affinity stereospecific binding of [^{3}H]-etorphine has been measured in homogenates of the same tissue.

2 Higher concentrations of normorphine were required to produce 50% inhibition of the electrically stimulated contractions of strip preparations from morphine pretreated animals. There was also an increase in the slope of linearized dose-response curves in opiate-tolerant preparations. Maximum opiate effect was unchanged, and responses to exogenous acetylcholine were not affected by the pretreatment.

3 There was a slight increase in the apparent equilibrium constant for naloxone after morphine pretreatment.

4 Tolerance to opiate effect was not accompanied by a change in the affinity or number of stereospecific binding sites for [^{3}H]-etorphine. Hill plots of [^{3}H]-etorphine binding in both control and morphine pretreated preparations gave slopes close to unity.

5 Most of these results can be explained by the assumption that in tolerant preparations, a certain fractional opiate receptor occupation threshold must be exceeded before opiate effects become apparent. It is suggested that the tissue adapts toward a threshold equivalent to the mean receptor occupancy attained during the period of opiate drug pretreatment.

Introduction

Opiate drugs inhibit the output of acetylcholine from neurones in the myenteric plexus of guinea-pig ileum (Paton, 1957). The receptors mediating this effect are similar to opiate receptors in the central nervous system with respect to (i) the relative potencies of opiate agonists (Paton, 1957; Cox & Weinstock, 1966; Kosterlitz, Lord & Watt, 1972), (ii) antagonism by appropriately substituted opiate drugs (Cox & Weinstock, 1966; Gyang & Kosterlitz, 1966; Kosterlitz & Watt, 1968) and (iii) stereospecificity, both for agonists and antagonists (Cox & Weinstock, 1966). Pretreatment of the guinea-pig with morphine results in a reduced sensitivity to opiates of the subsequently isolated ileum (Haycock & Rees, 1973; Goldstein & Schulz, 1973; Ward & Takemori, 1976). Recently, a high affinity stereospecific component of the binding of opiate drugs to homogenates of longitudinal muscle-myenteric plexus preparations from guinea-pig ileum has been shown to possess the expected characteristics of opiate receptor binding (Terenius, 1973; Creese & Snyder, 1975). The preparation thus permits an analysis of the relationship between receptor binding and drug effect. In this paper we describe experiments on opiate agonist and antagonist sensitivity, and opiate receptor binding, in ileum preparations from normal and morphine-tolerant guinea-pigs.

Methods

Pretreatment of guinea-pigs

Male Hartley guinea-pigs weighing from 250 to 400 g were used. Control and morphine-treated guinea-pigs were selected at random from the same batch, and were housed in adjacent cages with unlimited access to food and water. Morphine tolerance was induced by the subcutaneous implantation of five morphine pellets (each containing 75 mg morphine base; Goldstein & Schulz, 1973) under light ether anaesthesia, at about 15 h 30 min on day 1. On day 4, the animal was either killed by decapitation at 09 h 30 min (i.e. about 66 h after pellet implantation; designated '3 day morphine treatment') and the ileum removed, or a further five pellets were implanted at a different site, and the animal was not killed until day 6 at 09 h 30 min (i.e. about 114 h after the first morphine implantation;

designated '5 day morphine treatment'). About half of the control guinea-pigs underwent a sham implantation procedure (laparotomy, under light ether anaesthesia). This practice was discontinued since the results from these animals were not different from untreated controls.

Measurement of opiate drug effect

Myenteric plexus—longitudinal muscle strips were set up in 5 ml organ baths in Krebs-bicarbonate buffer composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.20, KH_2PO_4 1.19, $NaHCO_3$ 25.0, glucose 11.1 and choline chloride 20 μM, mepyramine maleate 125 nM) oxygenated with 95% O_2 and 5% CO_2, at 37°C as described by Goldstein & Schulz (1973). Contractions were induced by field stimulation at 0.1 Hz, 0.25 ms pulse duration at maximal voltage (80 V). Responses were recorded isometrically. After a 1 h period of equilibration, during which the bath fluid was changed frequently, a dose-response curve to the standard opiate normorphine hydrochloride was obtained. The drug was left in contact with the tissue for 3 min before the bath fluid was changed. The next normorphine dose was applied to the tissue 3 min after the second of two further washes given at 3 min intervals (i.e. the interval between normorphine doses was 12 minutes). At least four, and usually five or six normorphine doses, covering the range 15% to 75% of maximal effect, were used to construct each dose-response curve; the doses were not administered in an ascending or descending concentration sequence. On completion of the initial dose-response curve, naloxone hydrochloride (10 nM) was added to the bathing fluid and the tissue equilibrated for 10 min before a further dose-response curve was established in the presence of naloxone.

Linear plots of the dose-response curves were constructed by plotting the $logit_{10}$ transformation of the percentage inhibition of contractile tension [$\log (y/100-y)$ where y=% inhibition] produced by each normorphine dose against log normorphine concentration (nM). This plot is analogous to the Hill plot for ligand—macromolecular interactions. Normorphine IC_{50} values were read from the plot at $\log (y/100-y)=0.000$, and the slope was recorded. The apparent naloxone equilibrium constant (K_e) was calculated from the Schild equation, essentially as described by Kosterlitz & Watt (1968), $K_e=a/(DR-1)$, where a=molar concentration of antagonist (in these experiments 10 nM) and DR=the ratio of normorphine IC_{50} (nM) in presence of naloxone to the normorphine IC_{50} in the absence of naloxone.

On some strips, acetylcholine dose-response curves were also obtained before the administration of naloxone; 30 s before each acetylcholine dose, the stimulator was turned off and acetylcholine was left in contact with the tissue for 20 s after which time the bath fluid was changed. After 40 s the stimulator was turned on for 2 min, and the cycle was then repeated (i.e. time between successive acetylcholine doses was 3.5 minutes). On each tissue, four or five submaximal contractions were obtained and the maximum response to acetylcholine was determined. The submaximal responses, expressed as the $logit_{10}$ transformation of the percentage of maximal contraction were plotted against log acetylcholine concentration (nM).

Measurement of high affinity stereospecific opiate binding

Myenteric plexus—longitudinal muscle preparations from normal and morphine-treated guinea-pigs were washed in Krebs-Tris buffer (mM: NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.20, Tris-HCl 100 (pH 7.4) and choline chloride, 20 μM, mepyramine maleate, 125 nM) at 0°C for 20 min, then blotted, weighed, and chopped with scissors. The chopped tissue was homogenized with a Tissuemizer (Kunkel, GFR) in 10 ml Krebs-Tris buffer and made up to a final tissue concentration of 0.02 g/ml. The homogenate was centrifuged at 10,000 *g* for 15 min, the supernatant discarded, and the pellet resuspended in the same volume of Krebs-Tris buffer. The centrifugation was repeated and the tissue was finally resuspended by hand homogenization in a glass—glass homogenizer in Krebs-Tris buffer at a tissue concentration of 0.02 g/ml. Aliquots (250 μl) of this homogenate were incubated at 37°C for 30 min with 250 μl Krebs-Tris buffer containing either [^{3}H]-etorphine or levorphanol (final concentration 1 μM) plus [^{3}H]-etorphine. The incubates were cooled on ice for 10 min before bound and free radioactivity were separated by filtration through Whatman GF/C glass fibre filters under vacuum. The filter was washed with 4 × 4 ml of ice cold Krebs-Tris buffer, and transferred to a counting vial where it was shaken for 60 min with 1 ml Soulene (Packard, Ill., USA). Finally 10 ml of Dimilume Scintillation Cocktail (Packard) or Neutralizer Cocktail (RPI Corp., Ill., USA) was added, and radioactivity determined in a Packard TriCarb liquid scintillation spectrometer. Counting efficiency was determined by external standardization. The protein content of each homogenate was measured (Lowry, Rosebrough, Farr & Randall, 1951).

In each experiment, four concentrations of [^{3}H]-etorphine between 0.1 and 3.0 nM were used. At each concentration, four samples were incubated without levorphanol, and four samples with the unlabelled ligand. Stereospecific binding was calculated by subtracting mean bound radioactivity with levorphanol from mean bound radioactivity without levorphanol. For each experiment, Scatchard plots

were prepared, and the dissociation constant and maximum binding determined by extrapolation.

Materials

[^{3}H]-etorphine was purchased from Amersham/Searle. Naloxone was a gift from Endo Laboratories; levorphanol and levallorphan from Hoffmann-La Roche; normorphine from Merck, Sharpe and Dohme. The pituitary endorphin sample was provided by Dr Susan Gentleman.

Results

Normorphine and naloxone effects in normal and morphine-treated ileum

The strip preparations were washed frequently immediately after being mounted since strips from morphine-treated animals showed a rapidly increasing sensitivity to normorphine during this period, a phenomenon also observed by Schulz & Herz (1976). About 1 h after mounting in the organ bath, consistent responses to normorphine could be obtained in both control and pretreated tissues. Normorphine IC_{50} was 235 nM in untreated strips; after the 3 day morphine treatment this value was increased to 679 nM (Table 1). There was no indication that the maximum response to opiates was reduced by morphine pretreatment; inhibitions of greater than 90% were recorded from both control and morphine treated strips.

Naloxone antagonized normorphine resulting in a parallel shift of the normorphine dose-response curve to the right in both control and pretreated preparations. Figure 1 shows log normorphine concentration-logit$_{10}$ response plots for typical control and morphine-treated preparations before and after exposure to naloxone. A small increase in the apparent naloxone equilibrium constant as measured by the shift of the normorphine concentration-response curve was consistently observed following morphine treatment (Table 1). There was no evidence of the increase in sensitivity to the antagonist effects of naloxone that is seen in intact animals after opiate pretreatment (Tulunay & Takemori, 1974). The low concentration of naloxone used in these experiments did not induce the sustained contractures that have been reported to occur when the antagonist was applied to ileum preparations that had recently been removed from guinea-pigs given high doses of opiates

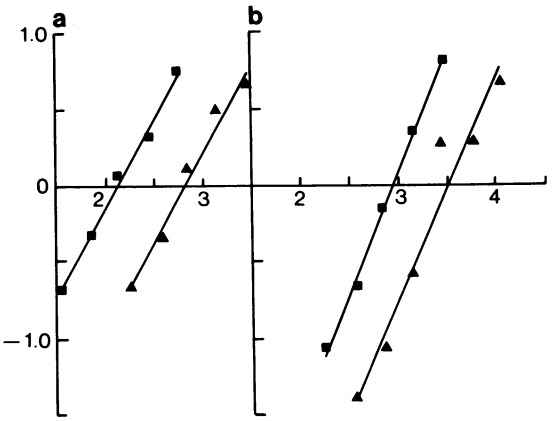

Figure 1 Normorphine concentration-response curves before and after exposure to naloxone. Abscissa scale: log normorphine concentration (nM). Ordinate scale: logit$_{10}$ response (i.e. $\log_{10}(y/100-y)$, where y=% inhibition of contraction). (■) Normorphine responses without naloxone; (▲) normorphine responses in the presence of naloxone (10 nM). (a) Control strip; normorphine IC_{50}=135 nM, naloxone K_e=2.68, mean slope=1.15. (b) Three day morphine-treated strip; normorphine IC_{50}=881 nM, naloxone K_e=3.57, mean slope=1.51.

Table 1 Normorphine and naloxone activity on ileum from normal and morphine-treated guinea-pigs

Treatment	*Normorphine IC_{50} (nM)*	*Apparent naloxone K_e (nM)*	*Mean slope of log concentration-logit$_{10}$ response curve*
Controls (20)	235±22	2.17±0.12	1.16±0.04†
Morphine, 3 days (28)	679±65*	4.08±0.39*	1.49±0.06*
Morphine, 5 days (12)	428±45*	3.11±0.23*	1.45±0.06*

Figures in parentheses indicate the number of preparations in each treatment group. Normorphine IC_{50} values (±s.e. mean) were determined as described in Methods for each tissue. The apparent naloxone K_e (±s.e. mean) was computed (see: Methods) from the shift of the normorphine concentration-response curve in the presence of naloxone (10 nM). The slope of the normorphine log concentration-logit$_{10}$ response curve for each preparation was determined both before and during exposure to naloxone. Naloxone did not significantly alter the slope; the Table therefore presents mean values (±s.e. mean). Morphine treatments are described in Methods.
* Significantly different from control values ($P<0.01$); † not significantly different from 1.00 ($P>0.05$).

(Ehrenpreis, Greenberg & Comarty, 1975; Schulz & Herz, 1976).

The slope of the linearized normorphine dose-response curve was increased following morphine pretreatment (Table 1, and Figure 1). This increase in slope was evident not only in the transformed response measurements, but also by visual inspection of the effects of normorphine doses on the strip preparations. Furthermore, the increase in slope was also apparent after antagonism of the opiate effect by naloxone (Figure 1). Increasing the duration of prior exposure to morphine did not result in a further increase in tolerance to normorphine, but the changes observed in preparations from 3 day morphine-treated animals were also seen after 5 day morphine treatment (Table 1).

Cross tolerance to pituitary endorphin

In a second series of experiments concentration-response curves for normorphine and a pituitiary endorphin preparation were compared in strips from control and morphine-treated animals (Table 2). Exposure to morphine resulted in a lower sensitivity to both normorphine and the pituitary extract, and in each case there was a significant increase in the slope of the log concentration-logit$_{10}$ response curves.

Effects of acetylcholine on normal and morphine-treated ileum

The concentrations of acetylcholine giving 50% maximal contractions, and the slopes of the log concentration—logit$_{10}$ response plots for acetylcholine are presented in Table 3. Clearly the effects of acetylcholine on the preparations were not affected by morphine pretreatment of the guinea-pigs. Similar results have previously been reported (Goldstein & Schulz, 1973).

High affinity stereospecific opiate binding

At least two classes of opiate binding sites can be discerned in preparations of guinea-pig ileum (Creese & Snyder, 1975; Cox, Opheim & Goldstein, 1976); in the present experiments, drug concentrations have been selected so that the measured binding is predominantly to the high affinity sites that have been

Table 2 Effects of a pituitary endorphin on ileum preparations from normal and morphine-treated guinea-pigs

Drug and function	*(a) Control strips*	*(b) 3 day morphine strips*	*Ratio (b)/(a)*
Normorphine IC_{50} (nM)	172 ± 40	459 ± 54	2.67
Slope	1.10 ± 0.07	1.65 ± 0.07	1.50
n	12	10	–
Pituitary endorphin IC_{50} (μl)	3.82 ± 1.12	8.05 ± 0.89	2.11
Slope	0.96 ± 0.07	1.52 ± 0.17	1.59
n	5	6	–

Results are means ± s.e. mean. IC_{50} values and slopes were obtained from log concentration-logit$_{10}$ response plots. *n* = number of preparations in each group. Preparation of the partially purified pituitary endorphin sample is described elsewhere (Gentleman, Ross, Lowney, Cox & Goldstein, 1976). The morphine treatment is described in Methods.

Table 3 Effects of acetylcholine on normal and morphine-treated ileum

Treatment	*Acetylcholine concentration giving 50% of maximum effect (nM)*	*Slope of log conc-logit$_{10}$ response curve*
Controls (8)	53 ± 15	1.14 ± 0.09
3 day morphine (8)	64 ± 11	0.98 ± 0.05
5 day morphine (12)	60 ± 14	1.02 ± 0.10

Results are mean values ± s.e. mean. The number of preparations in each treatment group is indicated in parentheses. Acetylcholine concentrations giving 50% of the maximum contraction were read from the log concentration-logit$_{10}$ response curve. Differences between the treatments are not significant (*t* test; $P > 0.05$). Morphine treatments are described in Methods.

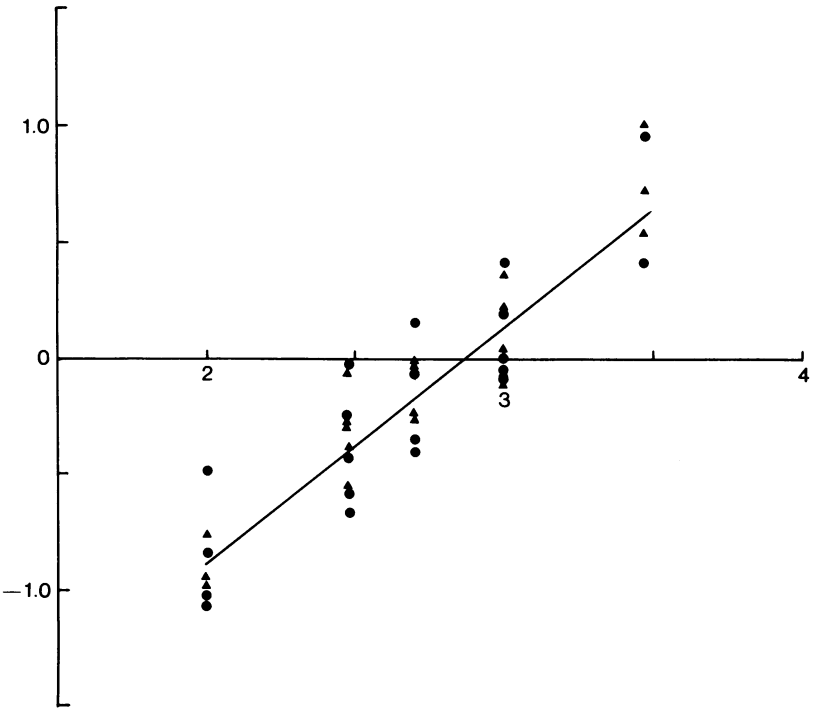

Figure 2 Hill plot of [^{3}H]-etorphine binding by ileal preparation homogenates; accumulated data from experiments shown in Table 4. Abscissa scale: log concentration (pM) of [^{3}H]-etorphine. Ordinate scale: $\log_{10}(Y/1-Y)$, where Y=stereospecifically bound [^{3}H]-etorphine expressed as a fraction of the maximum binding capacity calculated for each each experiment by extrapolation of the Scatchard plot. (●) Control preparations; (▲) 3 day morphine preparations. The line of best fit for the 3 day morphine preparations is drawn; slope=1.01. (Control slope=1.09.)

shown to be stereospecific. The tissues were extensively washed before use to ensure removal of residual morphine in pretreated preparations. The stereospecific binding of [^{3}H]-etorphine to ileum homogenates from control and morphine pretreated guinea-pigs was measured. Dissociation constants and binding capacity were estimated from Scatchard plots for each experiment (Table 4). Neither the dissociation constants nor the number of binding sites were changed by pretreatment with morphine. A Hill plot was constructed for the accumulated [^{3}H]-etorphine binding data (Figure 2). From this plot the mean etorphine dissociation constant was estimated at 0.68 nM in control preparations and 0.73 nM in preparations from 3 day morphine-treated animals. The slopes of the Hill plot for homogenates from

Table 4 [^{3}H]-etorphine binding to homogenates of ileum preparations from normal and morphine-treated guinea-pigs

Treatments	*[^{3}H]-etorphine K_D (nM)*	*[^{3}H]-etorphine binding capacity (fmol/5 mg tissue)*
Controls (6)	0.65 ± 0.08	14.2 ± 3.1
Morphine, 3 days (6)	0.79 ± 0.16	11.7 ± 1.6

Figures are mean values ± s.e. mean. The morphine treatment is described in Methods. The figures in parentheses indicate the number of separate experiments. K_D values, and the capacity of high affinity binding sites were computed from Scatchard plots of [^{3}H]-etorphine binding to homogenates of ileum at concentration between 0.1 and 3.0 nM, in each experiment. Differences between morphine-treated and control groups were not statistically significant (t test; $P > 0.05$).

untreated animals was 1.09, and for homogenates from 3 day morphine-treated animals was 1.01.

The quantity of opiate drug bound has not been related to the protein content of the homogenate in these experiments since it was found that the protein content of strips from 3 day morphine-treated guinea-pigs (100 ± 8 μg protein as bovine serum albumin/5 mg tissue wet weight; mean ± s.e. mean, $n=6$) was significantly higher ($P<0.05$) than control strips, 66 ± 8 μg/5 mg, $n=6$), despite the fact that the morphine-treated animals lost about 18% of their total body weight following implantation. Only a small proportion of the tissue in the strip preparations is neuronal, and hence this increase in protein concentrations probably reflects an adaptive change in the smooth muscle; its relevance to opiate binding and effect is unclear.

Discussion

Our experiments confirm that pretreatment of guinea-pigs with morphine results in a reduction in the sensitivity of subsequently isolated ileal preparations to opiate drugs (Haycock & Rees, 1971; Goldstein & Schulz, 1973; Ward & Takemori, 1976). Cross tolerance to pituitary endorphin was also observed; Waterfield, Hughes & Kosterlitz (1976) have recently reported cross tolerance between morphine and methionine-enkephalin in this preparation. Schulz & Herz (1976) have shown that ileal strips display very great insensitivity to normorphine immediately after removal from the morphine-treated animals, or after being maintained in an opiate containing medium, but that extensive washing reduces that insensitivity. This paper is concerned with strips and homogenates of ileum that were repeatedly washed before use; normorphine responses were consistent over a period of several hours, and the IC_{50} values of tolerant strips were similar to those reported by Schulz & Herz (1976) for extensively washed preparations. It is possible therefore that there are two processes with differing decay times underlying the opiate tolerance seen in isolated ileum strips; a biphasic recovery pattern has also been observed when tolerance to the analgesic effects of opiates has been measured in intact rats (Cox, Ginsburg & Willis, 1975). We discuss here only the slowly decaying phenomena.

The etorphine dissociation constant measured in Krebs-Tris buffer with homogenates of ileum preparation (Table 1) was similar to the etorphine dissociation constant measured in intact strip preparations in Krebs-bicarbonate buffer (Cox *et al.*, 1976), where the estimated etorphine dissociation constant (0.4 nM) and the etorphine concentration giving 50% inhibition of the electrically stimulated contractions of the ileum preparation (0.5 nM) were shown to be similar. It was also shown in these experiments and by Creese & Snyder (1975) that the high affinity component of opiate binding was stereospecific. Thus these binding sites display the expected properties of receptors mediating the characteristic opiate effects on guinea-pig ileum preparations. Furthermore, the data suggest that there are no spare receptors (Stephenson, 1956) in this system (Creese & Snyder, 1975).

The three day morphine pretreatment resulted in a 2.5 to 3-fold increase in the normorphine IC_{50}. However, there was no change in measured opiate agonist binding affinity for the receptors. On the basis of the variances seen in these experiments even a two-fold increase in K_D would have been readily detected. There was also no change in the number of high affinity binding sites following morphine pretreatment. Hill plot slopes of close to unity indicate that there was neither positive nor negative cooperativity of binding in control or morphine treated preparations. The failure of opiate pretreatment to alter binding characteristics of opiate receptors in rat brain (Klee & Streaty, 1974) and in neuroblastoma X glioma hybrid cells in culture (Sharma, Klee & Nirenberg, 1975) has previously been described.

In control strips the log normorphine concentration-$logit_{10}$ response curve had a slope that was not significantly different from unity. This plot is analogous to the Hill plot for receptor-ligand interactions. Since the events linking receptor occupation and inhibition of acetylcholine release are unknown, a slope of unity may be coincidental. Nevertheless, the data are consistent with the hypothesis that opiates inhibit a rate-limiting step in processes coupling electrical stimulation to transmitter release, the magnitude of opiate effect in control preparations being determined solely by simple mass action equilibria at the opiate receptor. A more complex situation must exist in the tolerant state since the slope of the linearized concentration-response curve increased to 1.5 following morphine pretreatment.

Some postulated mechanisms for tolerance development are inconsistent with the phenomena we have observed. Thus there is no supersensitivity to the relevant neurotransmitter, acetylcholine (Goldstein & Schulz, 1973), as proposed by Collier (1966). There is no increase in the number of 'silent receptors' for opiates (Collier, 1966), or in the number of molecules of an enzyme directly inhibited by opiate drug (Goldstein & Goldstein, 1968); both of these hypotheses require that there should be an increase in the number of opiate binding sites. It is possible that the general principle of de-repression of enzyme synthesis following drug-induced inhibition (Goldstein & Goldstein, 1961, 1968) might be applied to enzymes indirectly inhibited by opiates, as proposed by Sharma *et al.* (1975). However this type of model, without

modification, does not appear to predict an increase in the slope of the linearized concentration-response curve. One possible interpretation of the increase in slope arises from theoretical formulations by Goldstein, Aronow & Kalman (1974). If the opiate inhibited process loses its rate-limiting property as a result of morphine pretreatment, then no opiate effect would be observed until a certain fractional receptor occupancy had been exceeded, although the maximal response (complete inhibition) would be unchanged. A consequence of the existence of a receptor occupancy threshold is that the concentration-response curve is steeper than predicted by simple mass action equilibria (Goldstein *et al.*, 1974). Thus tolerance would result, and the concentration-response curve would increase in slope, if the system adapted to the continued presence of opiate by introducing an opiate receptor occupancy threshold. An adaptive response of this type offers the potential advantage that during chronic drug administration, exogenous opiate occupies a proportion of the receptors corresponding to the threshold, allowing endogenous opioid peptides to continue to exert some control on the transmitter release process by increasing receptor occupancy above threshold.

In tolerant strips the antagonistic effects of naloxone were slightly reduced. A similar reduction of naloxone effect in ileum preparations from morphine pretreated guinea-pigs has been observed by Ward & Takemori (1976), although the absolute values of estimated equilibrium constants (as calculated from the pA_2 values they report) are four to six-fold higher than we have found. (The control naloxone K_e value in our experiments is close to that found by Kosterlitz & Watt, 1968). The significance of this reduction of naloxone sensitivity is uncertain. *In vivo* pretreatment with opiates results in an increase in the ability of naloxone to antagonize opiate-induced analgesia (Tulunay & Takemori, 1974). However, recent work has suggested that this increase in naloxone sensitivity is not necessarily associated with, and may be unrelated to, the tolerant state (Harris, Loh & Way, 1976).

Levorphanol tartrate was given by Hoffman La-Roche, normorphine hydrochloride by Merck, Sharpe and Dohme, and naloxone hydrochloride by Endo Laboratories. We thank Dr A. Goldstein for helpful discussion and advice on the preparation of the manuscript. This work was supported by National Institute on Drug Abuse grants 972 and 1199.

References

COLLIER, H.O.J. (1966). Tolerance, physical dependence, and receptors. *Adv. Drug. Res.*, **3**, 171–188.

COX, B.M., GINSBURG, M. & WILLIS, J. (1975). The offset of morphine tolerance in rats and mice. *Br. J. Pharmac.*, **53**, 383–391.

COX, B.M., OPHEIM, K.E. & GOLDSTEIN, A. (1976). Photoaffinity labelling of opiate receptors. In *Tissue Responses to Addictive Drugs*, ed. Ford, D.H. & Clouet, D.H. pp. 373–389. New York: Spectrum Press.

COX, B.M. & WEINSTOCK, M. (1966). The effects of analgesic drugs on the release of acetylcholine from electrically stimulated guinea pig ileum. *Br. J. Pharmac.*, **27**, 81–92.

CREESE, I. & SNYDER, S.H. (1975). Receptor binding and pharmacological activity of opiates in the guinea pig intestine. *J. Pharmac. exp. Ther.*, **194**, 205–219.

EHRENPREIS, S., GREENBERG, J. & COMARTY, J.E. (1975). Mechanism of development of tolerance to injected morphine by guinea pig ileum. *Life Sci.*, **17**, 49–54.

GENTLEMAN, S., ROSS, M., LOWNEY, L.I., COX, B.M. & GOLDSTEIN, A. (1976). Pituitary endormphins. In *Opiates and Endogenous Opioid Peptides*, ed. Kosterlitz, H.W., pp. 27–34. Amsterdam: North-Holland.

GOLDSTEIN, A., ARONOW, L. & KALMAN, S.M. (1974). *Principles of Drug Action*, 2nd edition, pp. 96–98. New York: Wiley.

GOLDSTEIN, D.B. & GOLDSTEIN, A. (1961). Possible role of enzyme inhibition and repression in drug tolerance and addiction. *Biochem. Pharmac.*, **8**, 48.

GOLDSTEIN, D.B. & GOLDSTEIN, A. (1968). Enzyme expansion theory of drug tolerance and physical dependence. In *The Addictive States*, ed. Wikler, A. Res. Publ. Ass. Neur. Ment. Dis., **46**, 265–267.

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.*, **27**, 514–527.

HARRIS, R.A., LOH, H.H. & WAY, E.L. (1976). Alterations in the efficacy of naloxone induced by stress, cyclic adenosine monophosphate and morphine tolerance. *Eur. J. Pharmac.*, **39**, 1–10.

HAYCOCK, V.K. & REES, J.M.H. (1973). The effect of morphine pretreatment on the sensitivity of mouse and guinea pig ileum to acetylcholine and morphine. In *Agonist and Antagonist Actions of Narcotic Analgesic Drugs*, ed. Kosterlitz, H.W., Collier, H.O.J. & Villareal, J.E., pp. 235–239. London: Macmillan.

KLEE, W.A. & STREATY, R.A. (1974). Narcotic receptor sites in morphine dependent rats. *Nature, Lond.*, **248**, 61–63.

KOSTERLITZ, H.W., LORD, J.A.H. & WATT, A.J. (1972). Morphine receptors in the myenteric plexus of the guinea pig ileum. In *Agonist and Antagonist Actions of Narcotic Drugs*, ed. Kosterlitz, H.W., Collier, H.O.J. & Villaneal, J.E. pp. 45–61. London: Macmillan.

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

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

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

SCHULZ, R. & HERZ, A. (1976). Aspects of opiate dependence in the myenteric plexus of the guinea pig. *Life Sci.*, **19**, 1117–1128.

SHARMA, S.K., KLEE, W.A. & NIRENBERG, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc. natn. Acad. Sci. U.S.A.*, **72**, 3092–3096.

STEPHENSON, R.P. (1956). A modification of receptor theory. *Br. J. Pharmac. Chemother.*, **11**, 379–393.

TERENIUS, L. (1973). Stereospecific uptake of narcotic analgesics by a subcellular fraction of the guinea pig ileum. *Upsala J. Med. Sci.*, **78**, 150–152.

TULUNAY, F.C. & TAKEMORI, A.E. (1974). The increased efficacy of narcotic antagonists induced by various narcotic analgesics. *J. Pharmac. exp. Ther.*, **190**, 395–400.

WARD, A. & TAKEMORI, A.E. (1976). Studies on the narcotic receptor in the guinea pig ileum. *J. Pharmac. exp. Ther.*, **199**, 117–123.

WATERFIELD, A.A., HUGHES, J. & KOSTERLITZ, H.W. (1976). Cross tolerance between morphine and methionine-enkephalin. *Nature, Lond.*, **260**, 624–625.

(Received February 21, 1977.
Revised March 25, 1977)