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# Morphine-tolerant longitudinal muscle strip from guinea-pig ileum

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## Summary

1. Implantation of morphine pellets in guinea-pigs produced a high degree of tolerance and dependence within 3 days.

2. The contractions of the longitudinal muscle induced by electrical stimulation of the myenteric plexus-longitudinal muscle preparations obtained from tolerant animals were less depressed by morphine than the contractions evoked in preparations from non-tolerant animals.

3. Naloxone did not alter the size of the evoked twitch but antagonized the depressant action of morphine in tolerant and in non-tolerant animals. When given to tolerant guinea-pigs, naloxone caused an increase in intestinal activity *in vivo*.

4. The contractile response of the longitudinal muscle to acetylcholine was the same in preparations obtained from tolerant and non-tolerant animals. Electrically evoked contractions of the myenteric plexus-longitudinal muscle preparations from tolerant animals showed reduced sensitivity to the depressant effects of adrenaline, isoprenaline, and particularly, dopamine.

## Introduction

In recent years the guinea-pig ileum has been used as model to study the basic mechanism of the acute effects of narcotic analgesics (Paton, 1957; Gyang & Kosterlitz, 1966). Although symptoms like tachyphylaxis and dependence were demonstrated in these isolated tissues (Paton, 1957; Fennessy, Heimans & Rand, 1969), morphine tolerance was never observed in electrically stimulated preparations isolated from tolerant guinea-pigs (Fennessy *et al.*, 1969; Ehrenpreis, Light & Schonbuch, 1972). Efforts to produce tolerance in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum appeared desirable because such a model could be useful in investigations of the molecular changes leading to tolerance.

Since the unsuccessful attempts used schedules of repeated administration of morphine, we tried to maintain continuous exposure of the tissue to the narcotic analgesic drug *in vivo*, by the implantation of morphine pellets first used in mice (Maggiolo & Huidobro, 1961; Way, Loh & Shen, 1969). The electrically stimulated myenteric plexus-longitudinal muscle strips from such guinea-pigs displayed tolerance when challenged with morphine *in vitro*.

We then tested such tolerant preparations for their response to acetylcholine (ACh), since morphine-like drugs modify the release of this neurotransmitter in the gut (Schaumann, 1957; Paton, 1957), and to catecholamines, since they also play

a role (Kosterlitz, Lydon & Watt, 1970) in the neuronal control of the gut. A preliminary account of the results was given at the 5th International Congress on Pharmacology (Schulz & Goldstein, 1972).

## Methods

### *Implantation of morphine pellets*

Male guinea-pigs (380–420 g) were used, maintained on food and water *ad libitum*. Under light ether anaesthesia, each animal was implanted subcutaneously with 4 pellets containing 75 mg morphine base each (Gibson & Tingstad, 1970), two in each flank. The incisions were closed with Michel clips.

### *Longitudinal muscle strip*

The animals were fasted overnight, then guillotined. The strip, consisting of myenteric plexus and longitudinal muscle, was prepared from a 5 cm piece of ileum, 10 to 15 cm proximal to the ileo-caecal valve by the method of Rang (1964). The strip was set up in a 5 ml organ bath containing Krebs solution (NaCl 118, KCl 4·75, $CaCl_2$ 2·54, $KH_2PO_4$ 1·19, $MgSO_4$ 1·20, $NaHCO_3$ 25·0 mM) with glucose (11 mM), choline chloride (20 $\mu$M), and mepyramine (pyrilamine) maleate (0·125 $\mu$M) at 37° C and bubbled with 95% $O_2$ and 5% $CO_2$. For maximal electrical field stimulation (Grass S 4 E stimulator, about 80V, 0·1 Hz, pulse duration 0·5 ms), a platinum ring anode was placed at the top of the bath and a platinum hook cathode at the bottom (Kosterlitz *et al.*, 1970). The resting tension was 0·5–1 g and the isometric twitches were recorded by means of a force displacement transducer (Grass FT .03C) and a Grass Polygraph. The strips were allowed to equilibrate after mounting for at least 1 h with washes every 5 to 10 minutes.

Inhibitors of the twitch, like morphine and catecholamines, were added to the bath during continuous stimulation at 0·1 Hz. After the maximal depressant effect had been obtained, the drug was removed by washing. ACh was added to the bath 10 s after stopping electrical stimulation, and washed out after the contraction had reached its peak. Electrical stimulation was started again after relaxation of the strip to baseline. All washes were done at intervals of 2 to 10 minutes.

The intervals between successive tests with morphine and naloxone were 45–60 min, with isoprenaline 30 min, and 15–20 min with adrenaline, dopamine and ACh. The depressant effect of a drug on the twitch tension is expressed as % of the tension of the electrically induced twitches just before the drug was added to the bath ; the stimulant effect of ACh was expressed in the same way.

### *Tests for antinociceptive activity and dependence*

The antinociceptive effect was tested by the hot-plate method. A guinea-pig was placed in an aluminium pan (18×24×6 cm) installed in a water bath of 61 ± 0·5° C. The criterion for determining the reaction time was lifting of one of the forepaws. In controls this occurred after 9·2 ± 2·6 s ($n$=13). The guinea-pigs were removed immediately after a positive reaction or if they failed to react within 30 seconds.

Dependence was tested by injecting naloxone hydrochloride (50 mg/ml) intraperitoneally, as described in detail under **Results**. All experiments were carried out in the presence of the implanted morphine pellets.

*Rectal temperature*

The temperature was measured with a thermistor probe (Yellow Springs Instrument Co., Inc.), inserted 6 cm for at least 2 minutes. For intravenous injection morphine sulphate solutions were used (50 mg morphine base/ml).

*Morphine assay in plasma and pellets*

Morphine was measured by a modification of the spectrofluorometric method of Kupferberg, Burkhalter & Way (1964). Three ml of plasma and about 0·5 g of sodium bicarbonate were mixed and 10 ml of a 10% solution (v/v) of n-butanol in chloroform was added. The tubes were shaken for 5 min, centrifuged at 3,000 *g* for 2 min and the aqueous phase was removed by aspiration. Eight ml of the organic phase was transferred to a tube containing 1·2 ml of 0·01 M HCl. After shaking for about 5 min, and centrifuging at 1,000 *g*, 1 ml of the acid phase was removed by pipetting. The morphine of the acid phase was oxidized by adding 1 ml of 0·5 M Tris-HCl-buffer (pH 8·5) and 0·1 ml of a 1 in 10 dilution of potassium-ferri-ferrocyanide reagent (57·7 mg potassium ferricyanide + 5 mg potassium ferrocyanide dissolved in 100 ml distilled water). The fluorescence was measured after 10 min (excitation 250 nm, emission 440 nm, calibration: 100% = 0·1 $\mu$g quinine/ml 0·05 M $H_2SO_4$) with an Aminco-Bowman fluorometer.

For measuring the residual morphine in implanted pellets, the remaining substance of the pellets and the surrounding tissue were excised and homogenized in 50 ml of M HCl. After centrifugation, the supernatant was diluted 1,000-fold with 0·01 M HCl and 100 to 500 $\mu$l was assayed for morphine as described. Both for the plasma assay and for the pellet assay, it was shown by thin-layer chromatography (Eastman 6061, silica gel ; solvent system, n-butanol : acetone : acetic acid : 5% $NH_4OH$ : water ; 45 : 15 : 10 : 10 : 20) that the material assayed was free morphine ; added morphine was recovered without loss.

*Reagents and drugs*

Acetone, acetic acid, ammonium hydroxide, n-butanol and chloroform (Baker Chemical Co., Phillipsburg, N.J., reagent grade). Morphine sulphate (Mallinckrodt Chemical Works), naloxone hydrochloride (Endo Laboratories, Inc.) acetylcholine chloride (Merck & Co.), (−)-adrenaline (C grade, Calbiochem), isoprenaline hydrochloride (Winthrop Laboratories), dopamine hydrochloride (A grade, Calbiochem), quinine (Sigma Chemical Co.), choline chloride (Baker grade, Baker Chemical Co.), mepyramine (pyrilamine) maleate (Merck & Co.).

The solutions were made in distilled water, except that adrenaline was made up in 0·01 M HCl, acetylcholine in 0·001 M acetic acid, and dopamine in 10 $\mu$M ascorbic acid. These concentrations of acids did not affect the twitch amplitude or the resting tension. Drug concentrations are given as $\mu$M or nM.

**Results**

*Plasma morphine concentration and rate of absorption*

After implantation of 4 pellets (300 mg morphine base) a fast initial absorption of about 25% of the total morphine occurred within the first 24 h (Figure 1). Then the rate per day declined to about 5% between the 6th and 9th day after implanta-

tion. The morphine content of the pellets remaining after 9 days was 90 mg, about 30% of the total.

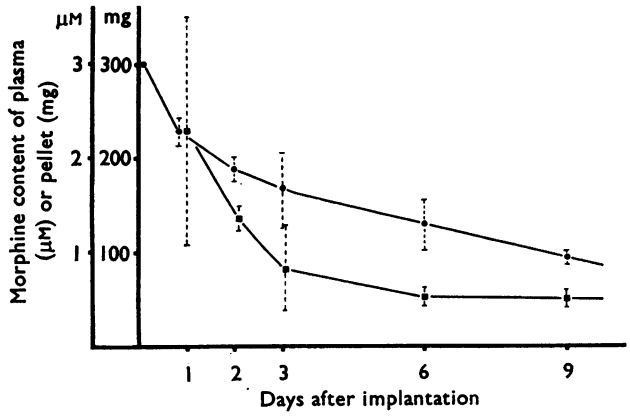



FIG. 1. Morphine absorption from pellet implants in guinea-pigs. Time-course of morphine concentration in plasma (■) and residual morphine contents of implanted pellets (●). Abscissae, time after implantation of 4 pellets of 75 mg morphine base each. Ordinates, mean morphine content of pellet (mg) or plasma (μM). In this and the following figures the vertical bars represent S.E. of the means ($n=3$–6).

The plasma morphine concentration was highest ($2{\cdot}3 \pm 1{\cdot}3$ μM) on the first day after implantation. Then it decreased rapidly to $0{\cdot}8 \pm 0{\cdot}5$ μM on the 3rd day and about $0{\cdot}5 \pm 0{\cdot}1$ μM subsequently.

*Effects of pellet implantation*

Following implantation of the morphine pellets the guinea-pigs were obviously tranquillized for 24 hours. The time-course of the antinociceptive effect is shown in Figure 2. The greatest effect occurred during the first 2 h, reflecting the fast absorption and high blood concentration. Then sensitivity to the hot-plate returned gradually, but the reaction time was significantly increased ($P<0{\cdot}01$) for 4 h as compared with sham-operated animals. The rectal temperature declined in the first hour by about 1° C and then progressively increased to more than 40° C in the

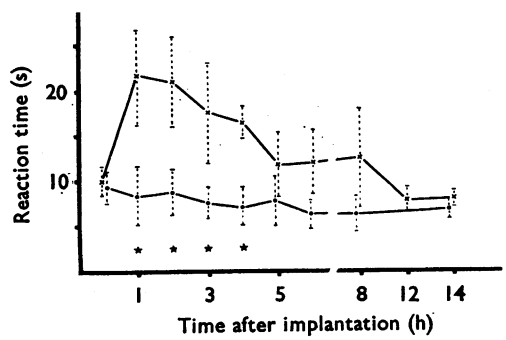



FIG. 2. Antinociceptive effect of morphine pellet implants. Mean reaction times (hot-plate test), in guinea-pigs ($n=5$) after implantation of morphine pellets (×), and in sham-operated animals ($n=3$ (●). Asterisks indicate significant differences ($P<0{\cdot}01$) between the groups.

next 3 h (Figure 3). The body temperature decreased slowly to normal at about 8 hours.

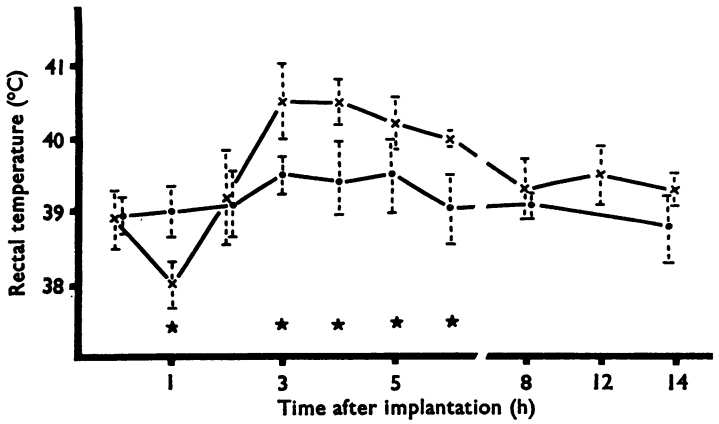



FIG. 3. Rectal temperature after implantation of morphine pellets. Mean rectal temperature in guinea-pigs ($n=5$) following implantation of 4 morphine pellets (×), and in sham-operated animals ($n=3$) (●). Asterisks indicate significant differences ($P<0{\cdot}01$) between the groups.

Weight loss was also observed, between 20 and 30% during the first two days after implantation. Thereafter, there was a slow return to normal body weight.

*Development of physical dependence*

Naloxone was employed to precipitate withdrawal symptoms at various times after pellet implantation. Intraperitoneal injection of 10 mg/kg of naloxone hydrochloride on the third day after pellet implantation caused characteristic withdrawal symptoms. Initially, there was a phase of intensive grooming activity lasting about 5 minutes. This was interrupted by excited states during which the animals raked the shavings, urinated, defaecated, appeared to gnash their teeth and ran about the cage (47·5×25·5×15·5 cm). An increase in intestinal motility could be readily palpated through the abdominal wall. A normal guinea-pig placed in the same cage was subjected to aggressive attacks (including biting) by a naloxone-treated animal. About 10–20 min after naloxone administration, the guinea-pig tried to leave the cage, stood on its hind legs, looked over the cage wall and finally leapt out. This ' jumping syndrome ' was never observed outside the cage. If an animal was placed back in the cage, it jumped out repeatedly at intervals of 1–5 minutes. In this phase, grooming activity was less frequent, whereas the gnashing of teeth became more violent. After 45–60 min, the animal became quiet, the jumping stopped and lacrimation increased. Rectal temperature dropped by about 1° C during the first 60 min following naloxone administration and returned to normal within the next 2 hours. In control animals, no reactions were seen after injection of naloxone, even in doses of up to 100 mg/kg.

About the same sequence of withdrawal symptoms was observed on the 6th and 9th days after implantation, with addition of a reaction characterized by stretching of the hindlegs or motions at the hip. These effects were very severe on the 9th day, with the hindlegs appearing to be paralyzed in some animals. At low doses (8 mg/kg) they failed in the jumping attempts, but higher doses (32 mg/kg) elicited successful jumping.

Naloxone treatment (4 mg/kg) of two guinea-pigs 14 days after implantation produced dramatic results. Both animals stretched the hindlegs and failed in their attempts to jump. A tremor of the hindlegs appeared, and the animals ran inside the cage using only the forelegs during the first 15 min after injection. One guinea-pig recovered, but the other tumbled on its side with the forelegs paralyzed. Convulsions were seen, paralysis continued for the following two days and the animal died on the third day after naloxone administration.

The jumping behaviour evoked by naloxone has been used for measuring the degree of physical dependence on morphine-like drugs in mice (Way *et al.*, 1969). Using the same technique, the median effective dose ($ED_{50}$) of naloxone hydrochloride for jumping out of the cage was determined according to the 'up-and-down-method' of Dixon (1965). The $ED_{50}$ and 95% confidence limits were 3·1 (0·5–6·7) mg/kg of naloxone hydrochloride on the 3rd day, 4·3 (0·7–7·9) mg/kg on the 6th day, and 16·1 (12·7–19·9) mg/kg on the 9th day after inplantation. Six guinea-pigs were used for each determination.

*Development of tolerance*

The highest degree of physical dependence was found on the third day after implantation. Therefore, the same period was chosen to investigate whether tolerance developed at this time. Attempts to demonstrate tolerance by the hot-plate method were unsuccessful. The $ED_{50}$ for analgesia according to the 'up-and-down-method' 30 min following intraperitoneal administration of morphine was 45·3 mg/kg in both control and implanted guinea pigs ($n=6$ in each group).

Another attempt to demonstrate tolerance was made in which the criterion for tolerance was the response of body temperature to morphine administration. The hypothermic action of morphine appears to be due to a specific effect in the anterior hypothalamus (Lotti, Lomax & George, 1965). The intravenous injection of 10 mg/kg of morphine depressed the rectal temperature in controls by about 1° C over a period of nearly 2 h (Figure 4). The same dose failed to elicit a depression three days after implantation of morphine pellets. To get the same response in these tolerant animals as in controls, it was necessary to increase the dose to 50 mg/kg.

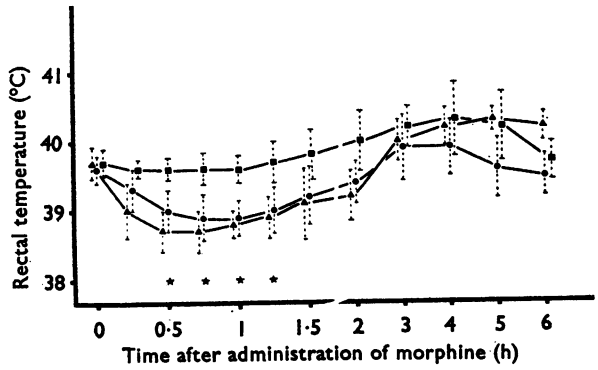



FIG. 4. Effect of morphine on rectal temperature of nontolerant and tolerant guinea-pigs. Morphine (10 mg/kg) was given intravenously to controls (●) and to animals on the 3rd day after implantation of 4 morphine pellets (■). A higher dose of morphine (50 mg/kg) was given to another group of animals on the 3rd day after pellet implantation (▲). Each point is the mean of 5 observations. Asterisks indicate significant difference ($P<0·01$) between controls and implanted animals.

*Response of the myenteric plexus-longitudinal muscle preparation to morphine*

In control strips, a 50% depression of the twitches evoked by electrical stimulation required a final bath concentration of 85 nM of morphine (median effective concentration), which confirms the range of sensitivity reported for the whole ileum by Paton (1957) and by Cox & Weinstock (1966). In longitudinal muscle strips taken from guinea-pigs at various times after pellet implantation, tolerance to the action of morphine was observed (Figure 5). As early as 24 h after pellet implantation, the preparation was less sensitive to morphine ; the median concentration for 50% depression was 250 nM. The highest degree of tolerance was found between the 3rd ($ED_{50}$ 590 nM) and the 6th day ($ED_{50}$ 750 nM) after implantation. In some tolerant preparations the dose-response curves were very flat, not reaching 50%

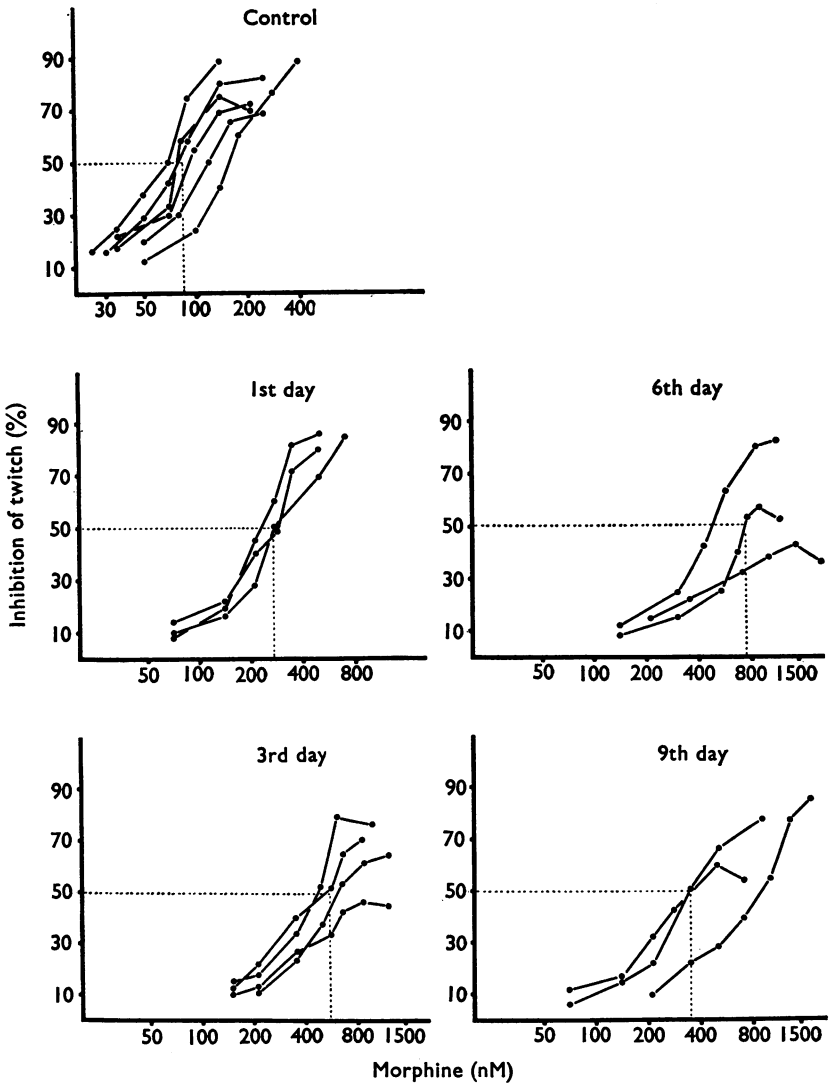



FIG. 5. Morphine tolerance in the electrically stimulated isolated longitudinal muscle strip with attached myenteric plexus at various times after implantation of 4 morphine pellets in guinea-pigs. The dotted lines indicate the median effective dose for a 50% twitch depression. Each line represents one experiment.

inhibition. Thus, during this state, morphine was about 12% as potent as in controls. At later times the tissue recovered partially; a 50% depression required about 350 nM morphine on the 9th day after implantation. The maximum twitch tension of tolerant strips was unchanged as compared with control strips. Attempts to influence the degree of tolerance by washing the strip every 15 min over a period of 8 h before the challenge with morphine were without success.

*Effect of naloxone on the longitudinal muscle strip*

This narcotic antagonist had the same effect in strips from control and tolerant animals. It blocked the inhibition of twitches when added before the morphine challenge and reversed the inhibition when added after morphine.

The effect of naloxone by itself was tested in control strips and on the 3rd, 6th and the 9th day after implantation. Final bath concentrations from 0·5 nM up to 1500 nM did not alter the twitch tension in control or morphine-tolerant strips.

*Response of the longitudinal muscle to acetylcholine*

ACh causes contraction of the longitudinal muscle strip. The sensitivity to ACh of strips isolated from untreated guinea-pigs was compared with those taken from animals implanted with morphine pellets. As shown in Fig. 6, the responses to ACh (0·05–24 nM) were about the same in controls and in strips prepared on the 3rd, 6th and 9th day after pellet implantation, as also shown by Haycock & Rees (1972).

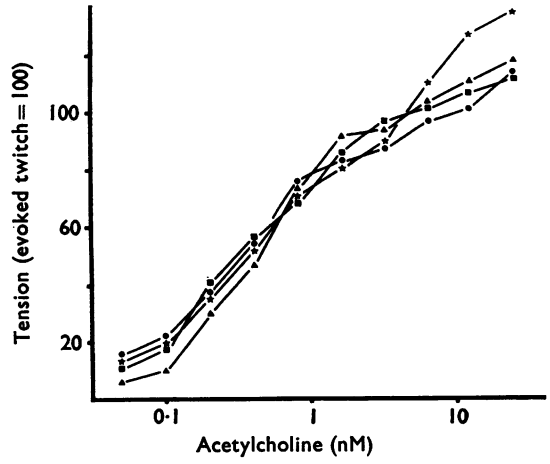


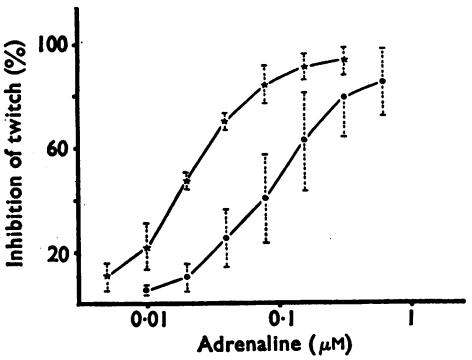

FIG. 6. Dose-response curves for acetylcholine (ACh). Strips from controls (*) and from morphone-tolerant guinea-pigs on the 3rd (■), 6th (▲) and 9th day (●) after pellet implantation. ACh was added to the bath 10 s after stopping the electrical stimulation. The ordinates represent tension in % of the tension induced by maximal electrical stimulation 10 s prior to the addition of ACh. Each point is the mean of 3–6 tests, on tissues from different animals.

*Effects of adrenaline, isoprenaline and dopamine on the responses of electrically stimulated myenteric plexus-longitudinal muscle preparation*

Adrenaline, isoprenaline and dopamine inhibited the electrically induced twitch (Figures 7–9). The most effective compound was adrenaline ($ED_{50}$–0·022 μM),

which blocked the response to electrical stimulation almost completely within 60 s at sufficiently high concentrations.

Considerably less effective was isoprenaline; the maximal depressant effect was established slowly (2–3 min), and the required concentration for a 50% inhibition of the twitch was 0·32 $\mu$M. The maximal inhibition was not more than 70%, at a concentration of 3 $\mu$M.



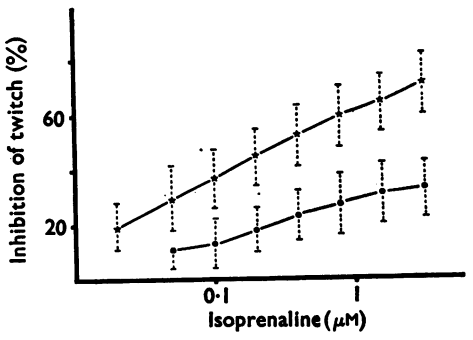




FIG. 7. (Above left.) Dose-response curves for adrenaline. Control strips (*) and preparations from morphine-tolerant guinea-pigs (●), on the 3rd day after pellet implantation. Each point represents the mean depression of the twitch of the electrically stimulated preparation ($n$=6–8).

FIG. 8. (Above right.) Dose-response curves for isoprenaline. For symbols and explanation, see Fig. 7.

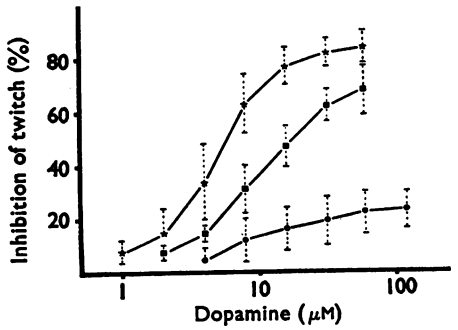



FIG. 9. Dose-response curves for dopamine. Control strips (*) and preparations from morphine-tolerant guinea-pigs, on the 1st day (■) ($n$=3) and the 3rd day (●) ($n$=8) after pellet implantation. For further explanation, see Fig. 7.

The weakest activity was seen with dopamine. A 50% depression of tension required a concentration of 5·8 $\mu$M, but the onset was as fast as with adrenaline. It was possible to inhibit the twitches by 85% (60 $\mu$M).

The depressant activity of these three catecholamines was greatly reduced in morphine-tolerant strips (3rd day after implantation) (Figures 7–9). The adrenaline concentration required for a 50% depression of the twitch was 5 times higher (0·11 $\mu$M) than in control strips. A similar shift to the right of the dose-response curve was obtained with isoprenaline, but even a concentration of 3 $\mu$M depressed the twitch by only 30%.

Dopamine showed the greatest loss of sensitivity in morphine-tolerant tissues. Even on the first day after pellet implantation, when tolerance of the strip to morphine was only moderate (Fig. 5), the dopamine concentration required for 50% inhibition of the twitch was 18 $\mu$M, nearly three times the concentration required in control strips. The weakest activity of dopamine was seen in strips taken from animals on the 3rd day after implantation. The high concentration of 120 $\mu$M resulted in maximal inhibition which, however, was only 25%. Naloxone, tested in concentrations up to 1 $\mu$M, did not block or reverse the inhibitory effect of dopamine in control or tolerant strips.

## Discussion

Morphine tolerance in guinea-pigs has been produced by long-term treatment with daily injections (Takagi, Takayanagi, Irikura, Nishino, Ichinoseki & Shishido, 1965 ; Mulé, Redman & Flesher, 1967 ; Ehrenpreis *et al.*, 1972). Using the technique of morphine pellet implantation (Maggiolo & Huidobro, 1961 ; Way *et al.*, 1969), we found that, as in mice (Goldstein & Sheehan, 1969 ; Cheney & Goldstein, 1971), the intensity of tolerance and dependence is related to the total exposure to physiologically effective drug concentrations. Even in three days a high degree of both tolerance and dependence was produced. Whereas many withdrawal effects, elicited by naloxone, were similar to those reported in other species (Fichtenberg, 1951 ; Seevers & Deneau, 1963 ; Huidobro & Maggiolo, 1965 ; Herz, Teschemacher, Albus & Ziegelgaensberger, 1972), we also observed a severe ' stretching syndrome ' after the longer periods of pellet implantation. This reaction probably interferes with the ability to jump, so that naloxone-precipitated jumping may lose its value as a quantitative measure of dependence in the presence of this phenomenon.

Mattila (1962) found that the 5-hydroxytryptamine-induced contractions of the longitudinal muscle of gut from tolerant animals were less sensitive than normal to inhibition by morphine. On the other hand, using electrically stimulated gut preparations, Fennessy *et al.* (1969) and also Ehrenpreis *et al.* (1972) were unable to demonstrate morphine tolerance. We found that in longitudinal muscle strip preparations obtained from tolerant and dependent guinea-pigs on the third day after pellet implantation, morphine was only one-sixth as potent as in normal strips ; at the same time, the hypothermic potency of morphine in the intact animal was reduced to the same extent.

The relationship of our findings to the acute tolerance and ' dependence ' described by Paton (1957) bears discussion. Paton found that, when the isolated normal gut was exposed continuously to morphine, the twitch amplitude gradually returned to normal, was then reduced on removal of the drug and was restored by again adding morphine to the bath. This phenomenon evidently requires the presence of morphine initially, a condition that did not exist in our experiments. The ' withdrawal effects ', moreover, were entirely unlike those displayed *in vivo* by the gut of a tolerant and dependent animal ; the response to morphine withdrawal or naloxone is increased intestinal activity, not inhibition of contraction.

Our findings with putative neurotransmitters can probably be interpreted in several ways. When the ileum or longitudinal muscle-myenteric plexus preparation is stimulated electrically, acetylcholine (ACh) is released (Paton, 1957 ; Paton & Zar, 1968) and a muscle twitch results. Since the response is blocked by atropine, it is likely that a cholinergic neurone acts directly on the smooth muscle. Morphine

inhibits the twitch, and at the same time reduces the output of ACh (Paton, 1957; Cox & Weinstock, 1966), but there is no evidence to indicate that morphine acts on the cholinergic terminals. On the contrary, morphine, at reasonable concentrations, does not interfere with ACh release at cholinergic terminals in other tissues (sympathetic ganglia, some parasympathetic endings, skeletal neuromuscular junctions). We suppose, therefore, that morphine acts in the plexus, on synapses other than the nerve-smooth muscle junction, e.g., at a more proximal synapse in the excitatory pathway.

We have shown here that the catecholamines (adrenaline, isoprenaline and dopamine) act in the same way as morphine in decreasing the twitch amplitude of the electrically stimulated strip. Moreover, it is known that catecholamines decrease ACh output in this preparation (Paton & Vizi, 1969; Kosterlitz *et al.*, 1970). Morphine probably does not act by releasing noradrenaline, since its action is not affected by $\alpha$- and $\beta$-adrenoceptor blocking agents (Gyang & Kosterlitz, 1966); but the possibility that it releases dopamine cannot yet be ruled out. It is possible, therefore, that a catecholamine acts as an inhibitory modulator of transmission in the excitatory pathways (Vander Wende & Spoerlein, 1972), in addition to the known direct action on $\beta$-receptors in the muscle (Kosterlitz *et al.*, 1970). Morphine could block transmission at an excitatory synapse in the pathway leading to ACh release, either by preventing the release or the postsynaptic action of an excitatory neurotransmitter, or by causing release of an inhibitory modulatory transmitter which may possibly be dopamine. A feedback regulatory mechanism could increase the effectiveness of the excitatory pathway (Goldstein & Goldstein, 1961; Shuster, 1961; Collier, 1965), causing tolerance to morphine and decreased sensitivity to catecholamines. Preliminary evidence indicates that this adaptation may be mediated by sensitization of postsynaptic receptors to 5-hydroxytryptamine (Schulz & Goldstein, 1973).

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