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MODEL OF OPIATE DEPENDENCE IN THE GUINEA-PIG ISOLATED ILEUM

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1 Segments of ileum, incubated for 2–24 h at 22°C with normorphine (0.01–1.0 μM), in the presence of hexamethonium, contracted when challenged with naloxone (0.03 μM). No response to this dose of naloxone was induced either by incubation in control solution without opiate for 2–24 h or by exposure of the preparation to opiate for 30 min at 37°C.

2 When segments were incubated for 24 h, the size of the response to naloxone was directly related both to the normorphine concentration in the incubation fluid (0.01 to 0.1 μM), and to the concentration of naloxone applied (0.03 to 0.1 μM).

3 A spontaneous withdrawal contracture was elicited in ilea that had been incubated with normorphine (1.0 μM), when the normorphine-containing bathing fluid was exchanged for one without opiate.

4 Normorphine restored to resting level the tension of the withdrawal contracture, whether it had been elicited spontaneously or by naloxone challenge.

5 Addition of naloxone (1.0 μM) to normorphine (1.0 μM) in the incubation fluid abolished the withdrawal contracture to subsequent challenge with naloxone.

6 Naloxone elicited a contracture from segments incubated for 24 h at 22°C with levorphanol (0.1 μM) but not from those incubated with dextrorphan.

7 Application of (+)-naloxone (0.03 μM) to segments previously incubated with normorphine (0.1 μM) did not elicit a contracture.

8 The contracture elicited by naloxone in preparations incubated with morphine (10 μM) was associated with a reduction in sensitivity to the acute inhibitory effect of morphine on the electrically-evoked response.

9 Addition of hyoscine (0.5 μM) immediately after challenge with naloxone restored the tension of the withdrawal contracture to resting level.

10 Tetrodotoxin (3.0 μM) given before challenge, prevented naloxone from eliciting a withdrawal contracture.

11 The inclusion of 5-hydroxytryptamine (10 μM) with morphine (10 μM) inhibited the induction of tolerance to morphine.

12 These experiments, together with those described earlier, indicate that incubation with opiate induces a dependence in the final cholinergic motor neurones of the myenteric plexus, manifested as a contracture of the longitudinal muscle on removal of opiate or administration of an antagonist. This dependence is associated with tolerance, expressed as a decrease in sensitivity to inhibition by morphine of the electrically-evoked contracture. Tolerance and dependence are induced and withdrawal precipitated through specific and stereospecific opiate receptors.

Introduction

Several authors have found that incubation of the guinea-pig isolated ileum in Krebs solution containing opiate induces a responsiveness to naloxone, expressed as a contracture of the longitudinal muscle on challenge with this antagonist (Ehrenpreis, Light & Schonbuch, 1972; Hammond, Schneider & Collier, 1976; Villarreal, Martinez & Castro, 1977; North & Karras, 1978; Luján & Rodriguez, 1978). This effect is accompanied by tolerance to the acute action of opiate on the ileum (Hammond *et al.*, 1976; Collier, Cuthbert & Francis, 1980a). Since responsiveness to naloxone and diminished response to opiates parallel two of the basic characteristics of opiate dependence in the whole animal, it has been

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thought that the ileum may provide a new and simpler model of opiate dependence, occurring in an isolated preparation of normal neurones. Because such a model should provide new possibilities for studying the molecular mechanism of opiate dependence, we have investigated three questions about it. First, in what conditions can quantitatively consistent reactions be obtained, with suitable controls, from ilea incubated with opiates? Second, since suitable experimental conditions for consistent reactions can be established, to what extent does the isolated ileum provide an experimental model of a dependence that is comparable to that in the whole animal? Third, since the model proves to be valid, what is the site of opiate dependence in the isolated ileum? Some of these results have been presented to the British Pharmacological Society (Collier, Cuthbert & Francis, 1980b, 1981a) and to the International Narcotic Research Club (Collier *et al.*, 1980a).

Methods

Preparation of ileal segments

Pieces of ileum 10 cm long removed from drug-naïve male guinea-pigs (Dunkin-Hartley; 300–500 g) were prepared for incubation in sets of 2–6 from each animal as previously described (Collier, Cuthbert & Francis, 1981b).

Incubation procedure

Single segments of ileum were incubated for 2–24 h at 5 ± 2, 22 ± 2 or 37 ± 2°C, each in a 10 ml bath containing pre-gassed (95% O_2 and 5% CO_2) modified Krebs solution containing hexamethonium (70 μM) (incubation fluid) with or without normorphine and/or other test substance. The bath contents were changed at 30 min intervals by means of a multichannel peristaltic pump (Collier *et al.*, 1981b). The opiate used in the incubation fluid in these experiments was normorphine because it is more readily washed out of the preparation than is morphine, with which it is equipotent (Kosterlitz, Lord & Watt, 1972).

Test procedures

Two to four segments of ileum from the same animal, but incubated under different conditions, were set up in parallel for comparative tests. Ilea were set up in 40 ml organ baths at 37°C as for transmural stimulation (Gyang & Kosterlitz, 1966; Hammond *et al.*, 1976). The bath solution was continuously gassed. The ilea were suspended, under a resting tension of 1 g from an isometric transducer (Statham Gold Cell) and contractures were recorded on a potentiometric recorder (Devices). A negative pressure of 1 cm saline was applied to the aboral end of the segment to remove excess mucus. When appropriate, the preparation was stimulated transmurally with a stimulator (Grass S88) set to give square wave electrical pulses of 40 V which was approximately 1.5 times the voltage producing maximal contracture in fresh preparations. Pulses were of 0.5 ms duration and 0.1 Hz frequency.

Assessment of tolerance Tolerance was measured by comparison, between segments incubated with or without opiate, of the dose-response curves for opiate inhibition of electrically-evoked contractures. Following incubation, segments were set up in Krebs solution in the presence of mepyramine maleate (0.125 μM) and hexamethonium bromide (70 μM), to eliminate respectively the histamine releasing and preganglionic effects of morphine. The first challenge dose of morphine was applied 45 min after the segment was set up (15 min after the third wash) and increasing doses were given, at 15 min intervals, until maximal inhibition of the electrically-evoked contracture had been reached. The preparation was washed after the opiate had been in contact for 60 s, by which time the maximal inhibitory effect had occurred. The bath contents were changed at least once every 15 min. In all experiments, pairs of segments derived from the same animal were tested simultaneously, one of which had been incubated with test compound, the other with control or vehicle. Inhibition of contracture was expressed as a percentage of the pre-challenge contracture tension. The concentration of morphine necessary to inhibit the contracture by 50% (IC_{50}) and the 95% fiducial limits were calculated by a least-squares linear regression analysis.

Assessment of dependence Dependence was measured in non-stimulated preparations in terms of the contracture induced either by application of naloxone or by removal of opiate. In experiments in which tolerance was determined, the stimulator was switched off 15 min after the last wash; at least 5 min later when resting tone had stabilized, the segment was challenged with naloxone (0.038 μM); unless stated otherwise, 'naloxone' means (−)-naloxone. At the time of naloxone challenge the preparations had been maintained in opiate-free Krebs solution for approximately 90 min. In experiments in which tolerance was not determined, segments were set up for test at 37°C in fluid equivalent to that used for incubation and allowed to equilibrate for 30 min with washes at 15 min intervals, without electrical stimulation. After equilibration, a tension of 1 g was applied to the transducer for calibration purposes. Four

minutes later, 4–6 electrical pulses were given, followed by a wash. Five minutes later, the challenge dose of naloxone, other test drug or vehicle was applied and left in contact with the tissue for 2 min. This was followed, after washing, by challenge with acetylcholine (ACh) as either a single dose (0.01 μM) or a series of discrete ascending doses (0.01, 0.1, 1.0, 3.0, 10 μM) until the maximal contracture of the longitudinal muscle had been reached. Since experiments had shown (see below) that hyoscine suppressed the withdrawal contracture precipitated with naloxone we considered that the contracture was largely due to release of ACh from the final motor neurone. In view of this and to allow for variation between tissues in responsiveness (maximal and submaximal) to ACh, which an analysis of variance showed to be unrelated to treatment, response to antagonist challenge was expressed as a ratio of the peak tension elicited by antagonist to that elicited by ACh (0.01 μM) (tension ratio). Tensions and tension ratios are expressed as the mean ± s.e.mean.

Statistical analysis of the significance of differences between values from comparable pairs of preparations was performed by Student's *t* test or by the Wilcoxon matched-pairs signed-rank test. Mean slopes ± s.e.mean and IC_{50} values, with 95% fiducial limits, were determined by least squares linear regression analysis.

Materials

Substances used were acetylcholine iodide (Sigma), dextrorphan (Roche), hexamethonium bromide (Sigma), 5-hydroxytryptamine creatinine sulphate (Sigma), hyoscine hydrochloride (Sigma), levorphanol (Wellcome), mepyramine maleate (May & Baker), morphine hydrochloride (Macfarlan Smith), (+)-naloxone (Dr K.C. Rice), naloxone hydrochloride (Endo), normorphine (Wellcome), tetrodotoxin (Sigma). Normorphine was dissolved in 0.3 ml of 0.5 N HCl and diluted with distilled water. All other drugs were dissolved in distilled water. Concentrations given for each drug refer to the final bath concentration. No drug was applied to the bath in a volume exceeding 0.4 ml. The incubation fluid was modified Krebs solution of the following composition (mM): NaCl 117.5, KCl 4.75, $CaCl_2$ 2.6, KH_2PO_4 1.19, $MgSO_4$ 1.2, $NaHCO_3$ 24.5 and glucose 11; it also contained hexamethonium, 70 μM.

Results

Effects of conditions of incubation

Incubation procedure The effects of the incubation procedure on the viability of preparations were determined by comparing three parameters in fresh and incubated ilea: (1) contracture induced by 40 V electrical stimulation; (2) sensitivity to morphine inhibition of electrically-evoked contracture; (3) contracture to ACh. The mean tension (2.2 ± 0.2 g, $n = 6$) elicited by 40 V stimulation of segments that had been incubated in incubation medium for 24 h at 22°C was not significantly different from that elicited by stimulating fresh ilea (2.3 ± 0.2 g, $n = 5$). Likewise, the IC_{50} (0.1 μM; range 0.04–0.2 μM, $n = 13$) for inhibition by morphine of electrically-evoked contracture for preparations incubated for 24 h at 22°C was not significantly different from that for fresh ilea (0.03 μM, range 0.02–0.05 μM, $n = 10$).

A later, more detailed study compared the effects of viability of incubation for 2, 4, 6, 8 and 10 h at 5, 22 and 37°C. An analysis of variance showed that there were no consistent changes in sensitivity to 40 V stimulation or to application of ACh that were due to the incubation procedure for up to 8 h at all three temperatures; but responsiveness both to 40 V stimulation and to application of ACh was significantly lower in segments incubated for 10 h at 37°C. The inclusion of normorphine (0.03 μM) in the incubation fluid did not modify the effect of incubation on sensitivity to 40 V stimulation or to ACh application over the 8 h period at all three temperatures.

Temperature Segments of ileum, taken in pairs from the same animal, were incubated for 2–8 h in incubation fluid with or without normorphine (0.03 μM) at either 5 and 22°C or 22 and 37°C. Figure 1 shows that 0.03 μM naloxone elicited a marked contracture from all ilea incubated with normorphine for 2–8 h at 5, 22 or 37°C, whereas it was without effect in ilea incubated in the absence of opiate. The degree of dependence induced was largely unaffected by the incubation temperature, although the mean tension ratio determined in ilea incubated for 4 h at 37°C was significantly greater than that in segments incubated for 4 h at 5 or 22°C ($P < 0.05$).

In view of the apparent lack of effect of temperature over the range 5–37°C, when preparations were incubated for 6 h or more, we chose 22°C for the majority of experiments, because at this temperature it was possible to incubate a large number of segments overnight, with automated changes of incubation fluid, in the small air-conditioned laboratory used for this work.

Characteristics of dependence in the isolated ileum

Effect of drug concentration Figure 2 shows the effects of normorphine (0.01, 0.1 and 1.0 μM) on the response to three challenge doses of naloxone after 24 h incubation at 22°C. The dose-response lines at 0.03 μM and 0.1 μM naloxone had significant slopes

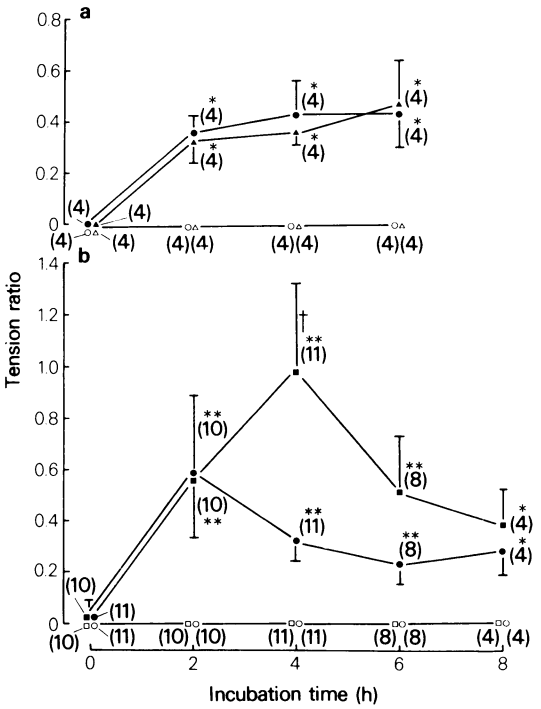

Figure 1 Effect of temperature of incubation on the induction of responsiveness to naloxone. Segments of ileum, taken in fours from the same animal, were incubated for 2, 4, 6 and 8 h at either 5°C (▲/△) and 22°C (●/○) (a) or 22°C (●/○) and 37°C (■/□) (b) in baths containing pre-gassed incubation fluid (modified Krebs solution containing hexamethonium 70 μM) with (closed symbols) or without (open symbols) normorphine (0.03 μM). After incubation, the ileal segments were set up, as for transmural stimulation, at 37°C in 40 ml baths containing fluid equivalent to that used for incubation and allowed to equilibrate for 0.5 h. The segments tested without prior incubation (0 h) were taken from freshly killed guinea-pigs, and set up at 37°C in incubation fluid with or without normorphine and allowed to equilibrate for 0.5 h. After 4–6 pulses (40 V) and a wash, a challenge dose of 0.03 μM naloxone was applied. After several washes, a series of doses of ACh (0.01–10 μM) was applied until maximal contracture was elicited. The tension ratio expresses the maximum contracture elicited by 0.03 μM naloxone as a ratio of that elicited by 0.01 μM ACh. Points give the mean and vertical bars the s.e.mean of at least 4 experiments (number in parentheses). For significance of difference from segments incubated without normorphine: * $P<0.05$; ** $P<0.01$; for significance of difference of segments incubated with normorphine at 22°C, † $P<0.05$.

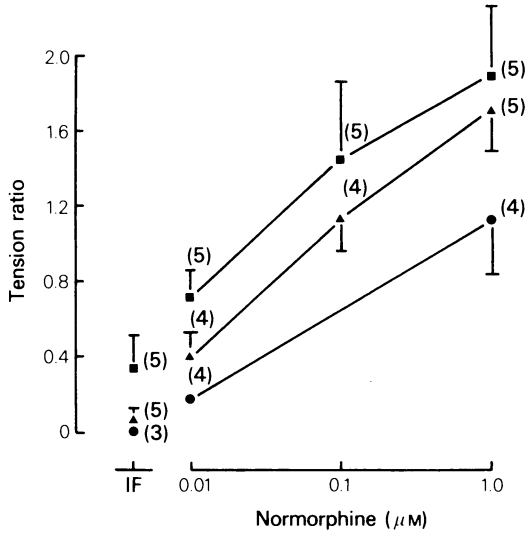

Figure 2 Effect of incubation concentration of normorphine on the response elicited by naloxone. Segments of ileum were incubated for 24 h at 22°C in either incubation fluid (IF) or in this fluid with the addition of 0.01, 0.1 or 1.0 μM normorphine. After incubation and equilibration for 0.5 h at 37°C, the preparations were challenged with 0.03 μM (●), 0.1 μM (▲) or 0.3 μM (■) naloxone. Other details as in Figure 1.

(0.47 ± 0.14, $P<0.05$; 0.66 ± 0.13, $P<0.01$ respectively). Naloxone (0.03 μM) produced a significant contracture in segments incubated with each of the three concentrations of normorphine, whereas it was ineffective on those incubated with incubation fluid alone. However, at higher doses naloxone elicited a slight contracture from segments incubated without normorphine.

For each incubation concentration of normorphine, the slopes of the dose-response lines to naloxone challenge were significant ($P<0.01$). The dose of naloxone (with 95% fiducial limits) required to elicit a tension ratio of 0.8 was: incubation fluid alone, > 5.0 μM (extrapolated); 0.01 μM normorphine, 0.4 (0.1–1.5) μM; 0.1 μM normorphine, 0.03 (0–> 1) μM; 1.0 μM normorphine, 0.02 (0.002–0.2) μM. Since the higher doses of naloxone in this experiment elicited a slight contracture from control preparations incubated without normorphine (Figure 2), we used a challenge dose of 0.03 μM naloxone routinely in subsequent experiments. Whereas incubation with 0.01 μM normorphine induced a significant response to naloxone, this concentration was completely ineffective acutely in inhibiting the response to electrical stimulation in opiate-naïve preparations ($n=7$). In our experiments, the acute IC_{50} of normorphine was 0.09 (0.08–0.1) μM.

Time-course of induction Segments of ileum were incubated at 22°C for up to 24 h in incubation fluid with or without normorphine (0.01–1.0 μM). After incubation, segments were set up for test at 37°C in

fluid equivalent to that used for incubation and, 30 min later, challenged with naloxone (0.03 μM). The segments tested without prior incubation (0 h) were taken from freshly killed guinea pigs, and set up at 37°C in incubation fluid with or without normorphine and challenged 30 min later.

Figure 3 shows the effect of duration of incubation on the responsiveness to naloxone challenge in ilea incubated in incubation fluid alone, or in this fluid with normorphine 0.01, 0.03, 0.1 or 1.0 μM. Incubation with 0.03, 0.1 or 1.0 μM normorphine induced a rapid increase in responsiveness to naloxone, and after 2 h incubation this effect appeared unrelated to the normorphine concentration. Segments incubated with 0.01 μM normorphine showed a more gradual increase in responsiveness to naloxone and the maximum level reached was much lower than that seen after prolonged incubation with 0.1 or 1.0 μM normorphine.

These data were subsequently analysed using an analysis of variance (Winer, 1971). Segments incubated for 2 h in 0.03, 0.1 or 1.0 μM normorphine were significantly ($P < 0.001$) more responsive to naloxone than those tested 30 min after being set up (0 h); but there was no effect of normorphine concentration over this period and dose-range. For periods up to 6 h there was a significant ($P < 0.001$) effect of time on the increase in responsiveness to naloxone in segments incubated with 0.1 or 1.0 μM normorphine; but, again, there was no effect of normorphine concentration. Periods greater than 6 h did not significantly alter the level of responsiveness reached and the difference between the effects of normorphine concentrations of 0.1 and 1.0 μM were not significant. Segments incubated with 0.03 μM normorphine were not tested after incubation periods greater than 6 h, by which time a plateau of responsiveness had been reached. Over the 24 h incubation period, 0.01 μM normorphine induced a significant ($P < 0.01$) responsiveness, compared with that of ilea incubated in incubation fluid alone. The induction of this was time-related ($P < 0.01$); but its level was lower than that in preparations incubated with high concentrations of normorphine ($P < 0.01$).

Since we were expressing the responsiveness to naloxone as a ratio of the tensions produced by

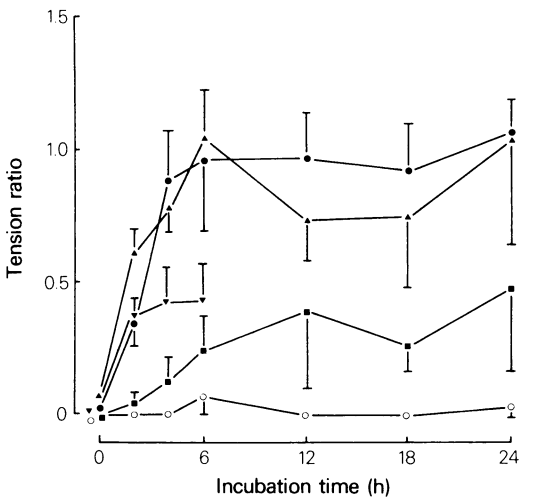

Figure 3 Effect of duration of incubation with normorphine on the responsiveness to naloxone. Segments of ileum were incubated for 2, 4, 6, 12, 18 and 24 h at 22°C in baths containing pre-gassed incubation fluid either alone (○) or with normorphine (0.01 μM, ■; 0.03 μM, ▼; 0.1 μM, ▲; 1.0 μM, ●). Points indicate the mean for the response elicited by 0.03 μM naloxone in at least four preparations. Vertical lines are s.e.mean. Other details as in Figures 1 and 2.

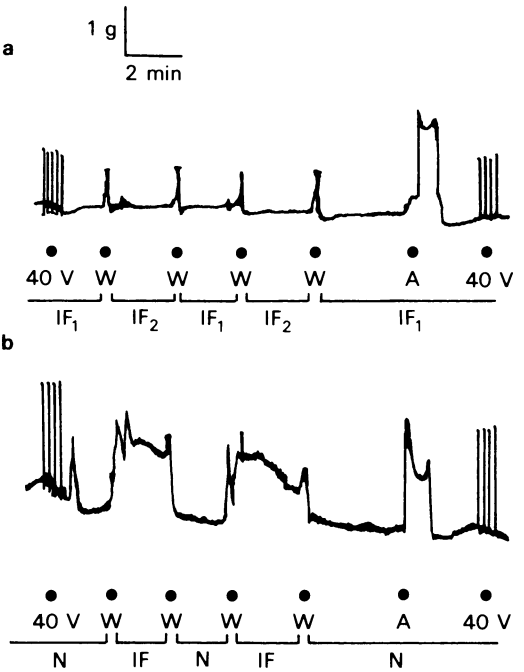

Figure 4 Spontaneous withdrawal contracture and its suppression. Tracings from one of five experiments in which segments were incubated for 24 h at 22°C in incubation fluid (IF) either alone (a) or with 1.0 μM normorphine (b). After incubation, ilea were set up at 37°C for transmural stimulation in fluid equivalent to that used for incubation. After 30 min equilibration, several 40 V pulses were applied. In (a) are shown the effects of several washes (W) when at each wash the bath was refilled with incubation fluid from alternate reservoirs (IF_1, IF_2); (b) shows the effect of replacing 1.0 μM normorphine (N) with incubation fluid (IF) and then refilling the bath with normorphine. The sensitivity to acetylcholine was determined by application of 0.01 μM (A). Other details as in Figures 1 and 2.

naloxone and by ACh (tension ratio), an analysis of variance was performed to determine the effects of normorphine concentration and duration of incubation on sensitivity to 0.01 μM ACh and on the dose of ACh required to produce a half-maximal contracture. This analysis showed that there were no consistent effects of treatment or duration of treatment on sensitivity of ileal segments to ACh. This supported the use of the tension ratio as a measure of increase in responsiveness to naloxone.

Withdrawal contracture and its suppression The results so far presented have involved precipitated-withdrawal where opiate activity is abruptly reduced by application of the competitive antagonist, naloxone. Since the cessation of chronic opiate treatment *in vivo* leads to the spontaneous development of withdrawal effects, as blood and tissue levels fall below those necessary to maintain dependence without withdrawal, we also attempted to demonstrate spontaneous withdrawal in our *in vitro* model.

Segments of ileum were incubated for 24 h at 22°C with incubation fluid either alone or with normorphine (1.0 μM). They were set up at 37°C in fluid equivalent to that used for incubation and allowed to equilibrate for 30 min. Following 4 pulses at 40 V and a wash, the bath was emptied, and refilled with incubation fluid without normorphine. This was left in contact with the preparation for 2–3 min and produced a pronounced contracture (0.9 ± 0.1 g) in the segments ($n = 5$) that had been incubated with normorphine (Figure 4b). Refilling the bath with fluid containing normorphine (1.0 μM) returned the tension to baseline. As a control, the bathing fluid of segments incubated with incubation fluid alone (IF_1) was exchanged for fluid from a separate reservoir (IF_2). This procedure did not modify the base-line (Figure 4a). These results show that withdrawal can be elicited by lowering the concentration of opiate at the opiate receptor and that replacement of opiate abolishes the withdrawal contracture. This resembles the characteristics of dependence *in vivo*, where a simple withdrawal of opiate elicits withdrawal effects and application of fresh opiate alleviates these effects.

That the precipitated withdrawal contracture of the ileum is also suppressed by opiate was shown by experiments in which normorphine (10 μM) was applied to preparations ($n = 3$) that had been incubated with normorphine (1.0 μM) either 1 min before or 1 min after challenge with naloxone (0.03 μM) (Figure 5). Figure 5 also shows that repeated applications of naloxone are capable of eliciting a strong contracture from segments maintained in 1.0 μM normorphine. In other experiments, up to eight successive challenges with naloxone induced a contracture.

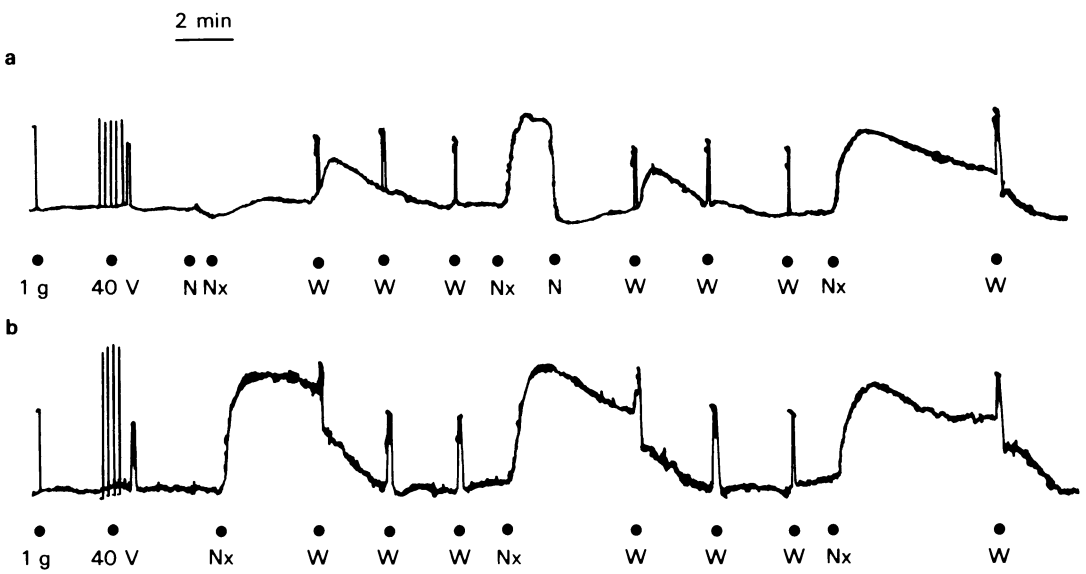

Figure 5 Effect of close application of normorphine on the naloxone-elicited withdrawal contracture. Tracings from one of three experiments in which paired segments of ileum were incubated for 24 h at 22°C in incubation fluid containing 1.0 μM normorphine. After incubation, segments were set up at 37°C for transmural stimulation in incubation fluid containing normorphine (1.0 μM) and equilibrated for 0.5 h at this temperature. Panel (a) shows the effect of application of normorphine (10 μM) (N) either 1 min before or 2 min after challenge with naloxone (0.03 μM) (Nx). Panel (b) shows the effect of repeated challenges with naloxone (0.03 μM). Other details as in Figure 4.

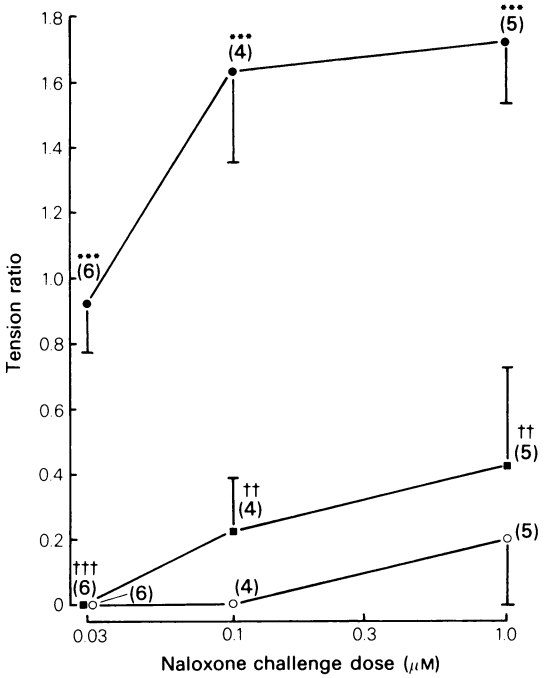

Figure 6 Effect of inclusion of naloxone with normorphine in the incubation fluid. Segments of ileum taken in threes from the same animal were incubated for 24 h at 22°C with incubation fluid, either with no addition (○), or with normorphine (1.0 μM) (●), or with normorphine (1.0 μM) and naloxone (1.0 μM) (■). After incubation, segments were challenged with 0.03 μM naloxone. For significance of difference from segments incubated in fluid alone, ***, $P < 0.001$. For significance of difference between segments incubated with normorphine and normorphine plus naloxone, ††, $P < 0.01$; †††, $P < 0.001$. Other details as in Figure 1.

Role of the opiate receptor In several series of experiments in which segments of ileum were incubated for 24 h at 22°C, we investigated how far dependence in the isolated ileum was mediated through the opiate receptor. Figure 6 shows that, in segments incubated with normorphine (1.0 μM), the presence of naloxone (1.0 μM) in the incubation fluid depressed the induction of dependence ($P < 0.01$). In other experiments, ($n = 3$) segments of ileum were incubated with either levorphanol (0.1 μM) or dextrorphan (0.1 μM). Upon subsequent challenge with naloxone, a significantly ($P < 0.01$) larger contracture was elicited in segments incubated with levorphanol than with either dextrorphan or incubation fluid alone (Figure 7). Further experiments ($n = 2$) showed that preparations incubated with normorphine (0.1 μM) responded to the (−)-enantiomer of naloxone (0.03–0.1 μM) with the expected contracture (1.0 and 0.8 g respectively), whereas there was no response to the (+)-enantiomer (Figure 8).

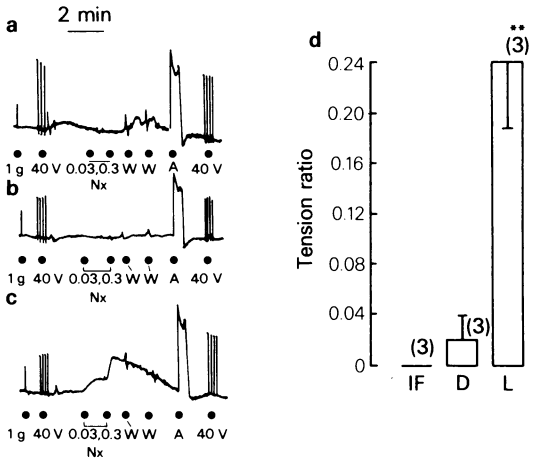

Figure 7 Effect of incubation with either levorphanol or dextrorphan on responsiveness to naloxone. Panels (a), (b) and (c) show tracings, obtained with three segments of ileum from the same animal, of the effect of incubation for 24 h at 22°C in incubation fluid either with no addition (a; column IF in d), or with dextrorphan (0.1 μM) (b; column D in d) or with levorphanol (0.1 μM) (c; column L in d) on the response to 0.03 and 0.3 μM naloxone (Nx). Panel (d) illustrates the mean results for naloxone (0.03 μM) challenge from three experiments. For significance of difference from preparations incubated with incubation fluid alone, **, $P < 0.01$. Other details as in Figures 1 and 4.

Association of tolerance with dependence Since the development of dependence on opiates *in vivo* is accompanied by the development of tolerance to the acute effects of the opiate, we investigated this association in the isolated ileum, by determining the degree of tolerance followed by that of dependence in the same preparation. The IC_{50} for inhibition by morphine of electrically-evoked contracture in ilea

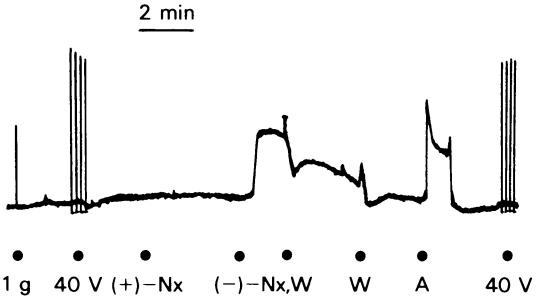

Figure 8 Effect of challenge with (+)- and (−)-naloxone in segments incubated with normorphine. Tracing from one of two experiments in which segments of ileum were incubated for 24 h at 22°C with normorphine (0.1 μM) and subsequently challenged with 0.03 μM (+)-naloxone ((+)-Nx) and 0.03 μM (−)-naloxone ((−)-NX). Other details as in Figures 1 and 4.

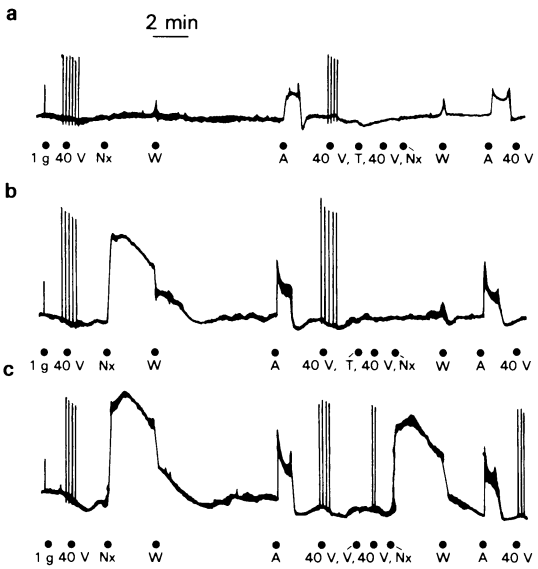

Figure 9 Effect of tetrodotoxin on the contracture elicited by naloxone. Tracings from one of three experiments in which segments of ileum were incubated for 24 h at 22°C with incubation fluid either with no addition (a) or with normorphine (1.0 μM) (b and c). After the demonstration of strong contractures to electrical stimulation (40 V), or 0.03 μM naloxone (Nx) or 0.01 μM ACh (A), the test procedure was repeated in the presence of either 3 μM tetrodotoxin (T in a and b) or vehicle (V in c). Other details as in Figures 1 and 4.

incubated with normorphine (10 nM) for 18–22 h at 22°C was 1.7 (0.9–4.6) μM ($n = 11$), which was significantly ($P < 0.001$) greater than that (0.1 (0.04–0.2) μM; $n = 13$) in ilea incubated without normorphine. Subsequently, each preparation was challenged with naloxone (0.038 μM), which elicited a significantly greater ($P < 0.001$) mean tension in the ilea incubated with normorphine (1.8 ± 0.2 g) than in the control ilea (0.4 ± 0.2 g). Thus, in the isolated ileum, incubated with normorphine, there was a strong association between the development of dependence and of tolerance.

Site of opiate dependence

The presence of hexamethonium in the incubation solution eliminates the possibility that the opiate dependence in the guinea-pig ileum occurred in a pre-ganglionic cholinergic neurone. To determine whether dependence occurred in a post-ganglionic neurone, we tested the effect of tetrodotoxin on the withdrawal-contracture. Figure 9 illustrates one of three experiments in which tetrodotoxin (3 μM) abolished the response to naloxone (0.1 μM) in ileal segments that had been incubated for 24 h at 22°C in normorphine (1.0 μM) and blocked the responses to electrical stimulation in control and normorphine-incubated tissue. Tetrodotoxin did not affect responsiveness to challenge with ACh.

We also tested whether hyoscine, which blocks the muscarinic receptors of the longitudinal smooth muscle, suppressed the precipitated withdrawal contracture of ileum that had been incubated in normorphine (1.0 μM) for 24 h at 22°C. In two experiments hyoscine (0.5 μM) abolished the contracture elicited by naloxone (0.03 μM). This dose of hyoscine reduced by 100 fold the sensitivity of the preparation to ACh.

Since there is evidence that 5-hydroxytryptamine (5-HT) acts at least partly on the final cholinergic motor neurone of the myenteric plexus (Gaddum & Picarelli, 1957; Dingledine, Goldstein & Kendig, 1974; Costa & Furness, 1979), we tested, by incubating segments with morphine (10 μM) for 18–22 h at 22°C, whether 5-HT inhibited the induction of morphine tolerance. Table 1 shows that, compared with

Table 1 Effect of 5-hydroxytryptamine (5-HT) on the induction of opiate tolerance *in vitro*

Addition to incubation fluid (μM)	*Morphine challenge* IC_{50} (μM) *with limits*	*Slope*	n
None	0.08 (0.02–0.2)	50§	6
Morphine, 10	0.9 (0.7–1.3)*	34§	8
Morphine, 10 + 5-HT, 10	0.6 (0.4–0.8)†	31§	11
5-HT, 10	0.1 (0.06–0.2)	66§	3

Segments of ileum were incubated for 18–22 h at 22°C with incubation fluid either alone or with the addition of morphine (10 μM), morphine (10 μM) plus 5-HT (10 μM) or 5-HT (10 μM). After incubation, segments were set up for transmural stimulation (40 V) in Krebs solution containing hexamethonium and mepyramine. The IC_{50} for inhibition by morphine of the electrically-evoked contracture was determined for segments receiving each treatment. For significance of differences from segments incubated with incubation fluid alone (no addition); *, $P < 0.001$. For significance of difference from segments incubated with morphine; †, $P < 0.01$. For significance of slope; §, $P < 0.001$.

segments incubated in morphine (10 μM) alone, those incubated with morphine (10 μM) plus 5-HT (10 μM) showed less tolerance to the acute inhibitory action of morphine ($P < 0.01$). The acute response to morphine of segments incubated in incubation fluid alone did not differ from that of those incubated in this fluid to which 5-HT had been added.

Discussion

Effects of incubation temperature and opiate concentration

The absence of an effect of differences in incubation temperature between 5 and 37°C on the rate of induction of dependence by normorphine (0.03 μM) over the first 2 h (Figure 1) was unexpected. So also was the comparable lack of effect of different normorphine concentrations, between 0.03 and 1.0 μM, on the rate of induction over the same period (Figure 3). After 2 h, the effects of different temperatures and, still more, of different opiate concentrations tended to separate. These observations suggest two possibilities that are not mutually exclusive. First, that two distinct processes of dependence may operate as has been previously proposed (see Cox, 1978). Second, that one of these processes is the unmasking, by exposure to opiate, of a state of dependence that already exists in potential form in the neurone. Such a process, under the name of 'kindling', has already been proposed on other grounds (Villarreal & Castro, 1979; Castro & Villarreal, 1980).

Validity of the model

To validate the present model of opiate dependence *in vitro*, it would be convincing to establish, if possible, that it possesses the essential characteristics of opiate dependence in intact animals and that it is distinct from dependence on α-adrenoceptor agonists, which also induce dependence in the ileum (Collier, Cuthbert & Francis, 1980c, 1981b).

Opiate dependence in intact animals has the following essential characteristics. (1) Dependence does not appear immediately on first treatment with opiate, but develops during continued exposure to drug. (2) Withdrawal of opiate elicits from dependent animals a behavioural disturbance that can be suppressed by retreatment with opiate. (3) A withdrawal disturbance can be precipitated with a specific opiate antagonist, the necessary dose of which is inversely related to the intensity of dependence. (4) Induction of dependence requires the activation of a specific and stereospecific opiate receptor, since induction is inhibited by specific opiate antagonists and requires the active member of a pair of stereoisomeric opiate enantiomers. (5) Precipitation of withdrawal also involves this specific and stereospecific receptor, since it requires a specific opiate antagonist and the active member of a pair of antagonist enantiomers. (6) Dependence is accompanied by tolerance to the acute effects of opiates.

The results described in this paper together with the observation that phentolamine did not elicit a contracture from segments incubated with normorphine (Collier *et al.*, 1981b) show that the isolated ileum exhibits all of these characteristics. It is interesting to note that, insofar as they have been tested, these characteristics are also exhibited by opiate dependence in neuroblastoma × glioma hybrid cells possessing opiate receptors (Sharma, Klee & Nirenberg, 1975; 1977; Lampert, Nirenberg & Klee, 1976; Klee & Streaty, 1981).

Like opiates, α-adrenoceptor agonists inhibit ACh release from the myenteric plexus (Paton & Vizi, 1969). This effect provided another means of demonstrating the specificity of opiate dependence in the isolated ileum. Incubation of the ileum for 18–24 h at 22°C with clonidine (Collier *et al.*, 1980c, 1981b) or with adrenaline (Collier *et al.*, 1980a) did not induce responsiveness to naloxone, although it did induce marked responsiveness to α-adrenoceptor antagonists.

Small responses to higher doses of naloxone were sometimes observed in segments incubated without opiate. These have also been described by Rodriguez and colleagues (Rodriguez, Lujan, Campos & Chorne, 1978; Rodriguez, Lujan & Vargas-Ortega, 1980). Again, when guinea-pigs were made to swim for 5 min before they were killed, ilea isolated from them were responsive to naloxone, whereas control ilea from unstressed guinea-pigs were not responsive (Bodycote & Chesher, 1979). It might be argued that such responses throw doubt on the validity of this experimental model. Responses to naloxone, resembling withdrawal effects, have, however, also been observed in opiate-naïve rats and mice (Collier, Francis & Schneider, 1972; Jacob, Tremblay & Columbel, 1974; Francis, Roy & Collier, 1975).

Opiate dependence in whole animals exhibits two characteristic interactions with drugs other than opiates or their antagonists. First, inhibitors of protein synthesis inhibit its induction (Loh, Shen & Way, 1969; Cox, 1972). Second, methylxanthines intensify precipitated withdrawal effects (Collier & Francis, 1975; Ho, Loh, Bhargava & Way, 1975; Francis, Cuthbert, Dinneen, Schneider & Collier, 1976). We have not yet explored these drug interactions with dependence in the isolated ileum, but preliminary observations have indicated that they may apply at least to tolerance in this preparation (Hammond *et al.*, 1976 and unpublished).

Ilea, prepared from guinea-pigs that had received

continued opiate-treatment *in vivo*, contract to naloxone challenge *in vitro* (Ehrenpreis *et al.*, 1972; Villarreal & Dummer, 1973; Ehrenpreis, Greenberg & Comaty, 1975; Frederickson, Hewes & Aiken, 1976; Schulz & Herz, 1976; Rodriguez *et al.*, 1978). Although these experiments cannot provide evidence on the induction of dependence *in vitro*, they support the use of the contracture to naloxone as a measure of dependence in the isolated ileum.

An earlier paper, explicitly aimed at validating the isolated ileum as a model of opiate dependence (Villarreal *et al.*, 1977), showed that, after 24 h incubation with morphine (0.48 μM) at 4°C, the isolated ileum contracted, in a dose-related way, to various concentrations of naloxone. Levorphanol and pethidine also effectively induced responsiveness to naloxone, whereas dextrorphan and mixed agonist-antagonist opiates had little or no effect. It was also found that high concentrations of levorphanol or pethidine did not suppress the response of dependent preparations to naloxone (0.3 μM). Apart from this last observation, in which a high concentration of naloxone was used, our observations described above confirm or are consistent with the findings of Villarreal *et al.* (1977). Our findings extend the comparison to several more essential properties of opiate dependence.

From the evidence discussed above, we conclude that incubation of the isolated ileum with opiates induces a state of dependence and associated tolerance that resembles in essential characteristics this state in experimental animals.

Site of dependence

It has been argued that dependence is a cellular phenomenon, developing within the neurone that possesses opiate receptors (North & Karras, 1978; Collier, 1978; 1979; 1980). This is consistent with our finding that the sensitivity of the preparation to ACh was not changed by incubation with normorphine.

Experiments, in which drugs were applied by iontophoresis to single neurones of the myenteric plexus and their effects recorded with microelectrodes, have shown that dependence occurs within these neurones (North & Karras, 1978). Such experiments, however, do not tell us which particular neurones of the plexus these are. For this information it is necessary to use drugs with specific actions.

The present studies confirm that opiate dependence and associated tolerance develop in the presence of hexamethonium (Hammond *et al.*, 1976; North & Karras, 1978). Furthermore, tolerance was of comparable intensity in ilea incubated with opiate in the presence or absence of hexamethonium (Hammond *et al.*, 1976). The present studies also confirm the findings that atropine or hyoscine and tetrodotoxin largely inhibit the withdrawal contracture (Schulz & Herz, 1976; North & Karras, 1978). They also indicate that 5-HT inhibits the induction of morphine tolerance in the ileum. Experiments with specific drugs therefore point to the final cholinergic motor neurone of the myenteric plexus as the site of dependence.

Advantages of the model

As a model of opiate dependence and associated tolerance, the ileum has several advantages. Experimentally, it has the advantage that test and control preparations can be obtained from the same ilea and their incubation and challenge can be conducted in parallel. Dependence can be recorded as a simple and readily quantitated response. Drugs at known concentrations can be applied during induction and removed before challenge or can be applied only at the time of challenge; and so the effects of drugs can be reliably measured and their influence on the induction or expression of dependence distinguished with confidence. Another advantage of the preparation is that the conditions of induction, such as temperature and the ionic composition of the incubation fluid, can be varied within wide limits. The relatively short duration of experiments in a field where these are often prolonged is another practical advantage.

A disadvantage of the preparation for biochemical studies is the difficulty of separating the neurones involved in dependence from other neurones of the plexus and from the smooth muscle that they supply. A factor to be taken into account is the circannual variation in the responsiveness of preparations to opiate (Shoham-Moshanov & Weinstock, 1977) and to naloxone (Rodriguez *et al.*, 1980). However, the latter authors have shown that variation in the response to naloxone is least at the lowest dose of this drug used by them, which was slightly higher than our routine dose.

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