Evidence for functional dissociation of dependence and tolerance in guinea-pig isolated ileal segments following 20 hour exposure to morphine *in vitro*

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1 In the present study we have examined the relationship between tolerance and dependence in isolated ileal segments from the guinea-pig under three different conditions: fresh preparations not previously exposed to morphine (fresh/morphine naive); preparations stored overnight at 4°C in modified Krebs-Henseleit saline (overnight-stored/morphine-naive); preparations stored overnight at 4°C in Krebs-Henseleit saline containing 10 µM morphine and extensively washed with modified Krebs-Henseleit saline to remove residual morphine (overnight-stored/morphine-exposed).

2 Morphine produced a concentration-dependent inhibition of the response of ileal segment to 0.1 Hz, 1 ms and 10 V transmural field stimulation in fresh/morphine-naive, overnight-stored/morphine-naive and overnight-stored/morphine-exposed preparations. The maximum effect observed was similar in all three preparations – approximately 80% inhibition. Although, morphine was significantly more potent in the fresh/morphine-naive preparations (pD_2 6.72 ± 0.05 , $n = 8$) than either the overnight-stored/morphine-naive (pD_2 6.42 ± 0.11 , $n = 8$) or the overnight-stored/morphine-exposed (pD_2 6.44 ± 0.14 , $n = 8$), there was no significant difference between the overnight models in their sensitivity to morphine. The latter observation indicates that overnight exposure to ileal segments to 10 µM morphine at 4°C failed to induce tolerance to morphine.

3 The µ opiate receptor antagonist, naloxone (10 µM), produced contractions in both fresh/morphine-naive and overnight-stored/morphine-naive ileal segments following acute exposure to 10 µM morphine. Naloxone (10 µM) also produced contractions in 2/9 fresh/morphine-naive, 1/9 overnight-stored/morphine-naive and 7/9 overnight-stored/morphine-exposed preparations in the absence of morphine. The greater incidence of naloxone-induced contractions in overnight-stored/morphine-exposed preparations, suggests that dependence in this model is the product of adaptive changes that outlive the presence of morphine.

4 The selective α₂-adrenoceptor agonists, clonidine (0.3 µM) and 5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate (UK-14304, 1 µM), inhibited naloxone-induced contractions in overnight-stored/morphine-exposed preparations of ileal segments ($n = 4$ preparations for each agonist), suggesting that the response is due to transmitter release from the myenteric plexus.

5 The findings in the present study indicate that tolerance and dependence to morphine in ileal segments of the guinea-pig can be functionally dissociated by overnight exposure to morphine at 4°C. The development of tolerance to morphine, unlike dependence, appears to be a temperature-dependent process. This also raises the possibility that naloxone possesses intrinsic negative agonism at morphine-sensitive receptors, which is manifested as a functional response only after adaptive changes in the myenteric plexus following exposure to morphine.

Keywords: Morphine; naloxone; tolerance; dependence; guinea-pig ileum; withdrawal contraction

Introduction

Opiate dependence is generally viewed as the 'physiological abnormalities when morphine is removed from its receptors either by discontinuing its administration or by using a specific receptor antagonist' (Johnson & Fleming, 1989), and there are numerous examples consistent with this proposal. For example, a contraction of the guinea-pig isolated ileum is observed following short-term exposure to morphine (minutes) and subsequent washout (Chahl, 1986; 1990). Similarly, either short-term or chronic exposure of the guinea-pig isolated ileum to morphine, followed by a challenge with naloxone, is also associated with a contraction (Miguel & Rodriguez, 1981; Johnson *et al.*, 1987; Chahl, 1990).

There are, however, examples of naloxone-induced contractions of guinea-pig isolated ileum which clearly do not involve removal of morphine from its receptor. For example, Alfaro *et al.* (1990) reported that isolated ileal segments from morphine-treated guinea-pigs responded to naloxone with a

contraction even after repeated washing of the preparation with opiate-free solution. Similarly, Collier *et al.* (1981a) noted that naloxone produced a contraction in guinea-pig isolated ileal segments exposed to normorphine for 22 h at 22°C which had also been exposed to an opiate-free solution for 90 min prior to the addition of the antagonist. Significantly, in both of these reports the ileal segments also exhibited evidence of tolerance, as judged by the reduction in either the potency of, and/or the maximum inhibition produced by, opiates against the electrically-evoked twitch response. Based upon these findings, and earlier reports of a close association between the development of opiate dependence and tolerance (see Collier, 1980), Johnson & Fleming (1989) proposed that the ability of naloxone to produce a contraction in the guinea-pig isolated ileum is a consequence of non-specific 'supersensitivity' within the myenteric plexus, which is associated with the development of tolerance to agents that inhibit acetylcholine release from cholinergic neurones (see also, Taylor *et al.*, 1988).

In preliminary experiments we observed that overnight

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incubation of the guinea-pig isolated ileum with morphine at 4°C, rather than 22°C, and subsequent washing to remove residual morphine, was not associated with any evidence of tolerance to the effects of morphine against electrically-evoked contractions. Hence, the possibility exists that the development of tolerance to opiates is a temperature-sensitive process. In the present study we have examined this model in greater detail to determine whether the development of dependence to morphine is also a temperature-sensitive phenomenon. Our results indicate the tolerance to and dependence on morphine in the guinea-pig isolated ileum are differentially affected by a reduction in temperature and, therefore, are not mutually-dependent phenomena.

Methods

Male or female guinea-pigs, 350–450 g, were killed by cervical dislocation and the intestines exteriorized. The ileocaecal junction was located and 5 cm of the terminal ileum discarded. Approximately 70 cm of the ileum was removed and divided into four segments and the lumen flushed with modified Krebs-Henseleit saline. Two of the segments were placed in Krebs-Henseleit saline which were gassed with 95% O₂/5% CO₂ and maintained at room temperature ('fresh/morphine-naive' preparations) until required for experimentation (less than 4 h). The remaining two segments were placed in 250 ml of either Krebs-Henseleit saline ('stored/morphine-naive') or Krebs-Henseleit saline with 10 µM morphine ('stored/morphine-exposed') and stored for 20–24 h at 4°C. Both solutions were pre-gassed with 95% O₂/5% CO₂ before receiving the ileal segments.

Five to six cm length segments of the ileum were secured onto a glass holder supporting a wire electrode which protruded into the lumen of the preparation. The tissue and electrode were placed in a 50 ml isolated organ bath containing modified Krebs-Henseleit saline which was maintained at 37°C and gassed with 95% O₂/5% CO₂. The upper end of the tissue was attached to a Washington isotonic transducer which was counterbalanced with a 1 g weight. Contractions of the tissue were elicited by transmural stimulation (1 ms duration, 0.1 Hz, 10 V) with a SRI stimulator. Responses were recorded on a George Washington pen recorder.

After 15 min equilibration, all ileal segments were stimulated for a minimum of 45 min to establish reproducible responses to electrical field stimulation. At the end of this period the stimulus intensity was adjusted to achieve a 'just submaximal' response.

Fresh/morphine-naive preparations

Agonist concentration-response curves were constructed by cumulative addition of either morphine (1 nM–30 µM), clonidine (1 nM–3 µM), or UK-14304 (1 nM–3 µM) with approximately 0.5 log₁₀ unit increments. A minimum of 3 min was allowed between individual concentrations. Only one agonist was used per tissue. In another series of experiments, the effect of 10 µM naloxone was examined in either the absence or after a 3 min exposure to 10 µM morphine, with the stimulator turned off 1 min prior to the addition of naloxone.

Stored/morphine naive and stored/morphine-exposed preparations

On the day of experimentation, the lumen of ileal segments stored overnight were gently flushed through with 10 ml of Krebs-Henseleit saline. Segments were then placed in 250 ml of fresh Krebs-Henseleit saline stored at room temperature and gassed with 95% O₂/5% CO₂. This washout procedure was repeated a further 3 times over an 80 min period prior to setting up the ileal segment in isolated organ baths.

Agonist concentration-response curves were constructed by cumulative addition of morphine (1 nM–30 µM) with approx-

imately 0.5 log₁₀ unit increments, allowing a minimum of 3 min between individual concentrations. In a separate series of experiments with stored/morphine/naive preparations, the effect of 10 µM naloxone was examined in either the absence or after a 3 min exposure to 10 µM morphine. The effect of 10 µM naloxone was also examined in stored/morphine-exposed ileal segments in the presence and absence of either 0.3 µM clonidine or 1 µM UK-14304.

Analysis of data

All responses have been calculated as a percentage of the response to electrical field stimulation and expressed as the mean ± standard error of the mean (s.e.mean). The logarithm of the concentration of morphine producing 50% of the maximum response (pD₂) was calculated using the logistic equation (DeLean *et al.*, 1978).

Differences between mean values were considered statistically significant when $P < 0.05$ using either a paired or unpaired Student's *t* test.

Drugs and solutions

The composition of the modified Krebs-Henseleit saline was as follows (mM): NaCl 118, KCl 4.8, MgSO₄·7H₂O 1.2, KH₂PO₄ 1.2, CaCl₂ 1.25, NaHCO₃ 25 and glucose 11.1. The following drugs were used: morphine sulphate (Evans), naloxone (Endo Laboratories), clonidine HCl (Sigma); UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer). All drugs were dissolved in distilled water and added in volumes less than 0.1% of the organ bath volume.

Results

The effect of morphine against electrically-evoked contractions of the guinea-pig isolated ileum

Following 20–24 h storage at 4°C, and repeated washing in fresh Krebs-Henseleit saline at room temperature for a further 3 h, morphine-naive and morphine-exposed preparations of the guinea-pig ileum responded to electrical field stimulation (10 V, 1 ms, 0.1 Hz) with stable contractions.

Morphine produced a concentration-dependent inhibition of the electrically-evoked twitch response in fresh/morphine-naive, stored/morphine-naive and stored/morphine-exposed segments of the guinea-pig isolated ileum (Figure 1). As shown in Table 1, morphine was 2 fold more potent in the

Figure 1 The effect of morphine against electrically-evoked contractions of fresh/morphine-naive (□), overnight-stored/morphine-naive (●) and overnight-stored/morphine-exposed (○) segments of the guinea-pig isolated ileum. The inhibition produced by morphine has been expressed as a percentage of the electrically-evoked twitch response and the values shown are the mean (± s.e.mean) of eight observations.

fresh/morphine-naive preparations, than in the two overnight storage models. However, there was no significant difference in either the potency of, or the maximum effect produced by, morphine in the stored/morphine-naive and stored/morphine-exposed preparations.

The effect 10 μ M naloxone on the guinea-pig isolated ileum in the presence of 10 μ M morphine

Following acute exposure to 10 μ M morphine, the electrically-evoked contractions of fresh/morphine-naive and stored/morphine-naive ileal segments were inhibited by $83.5 \pm 5.6\%$ ($n = 7$) and $82.4 \pm 5.9\%$ ($n = 6$), respectively. Subsequent addition of 10 μ M naloxone produced contractions in both preparations ($131.3 \pm 10\%$ and $209 \pm 32\%$ of the twitch response in fresh/morphine-naive ($n = 7$) and stored/morphine-naive ($n = 6$) ileal segments, respectively).

The effect of 10 μ M naloxone on the guinea-pig isolated ileum in the absence of morphine

In a separate series of experiments, fresh/morphine-naive, stored/morphine-naive and stored/morphine-exposed ileal segments (not used to determine the potency of acute exposure to morphine) were challenged with 10 μ M naloxone after stable electrically-evoked responses were established. Contractions to 10 μ M naloxone were observed in 2/9 fresh/morphine-naive preparations, 1/9 stored/morphine-naive preparations and 7/9 stored/morphine-exposed preparations. Responses in the stored/morphine-exposed preparations varied between 75% and 363% of the electrically-evoked twitch response. Because of the highly variable nature of the responses, the results have been represented graphically in Figure 2 in the form of a scattergram.

Table 1 pD_2 and E_{max} values for morphine against electrically-evoked contraction of isolated ileal segments of the guinea-pig ($n = 8$)

	pD_2	E_{max}
Fresh/morphine-naive	$6.72 \pm 0.05^*$	0.80 ± 0.04
Overnight-stored/morphine-naive	6.42 ± 0.11	0.82 ± 0.03
Overnight-stored/morphine-exposed	6.44 ± 0.14	0.77 ± 0.04

*Significantly different from overnight-stored/morphine-naive and overnight-stored/morphine-exposed preparations ($P < 0.05$, paired Student's t test).

Figure 2 The effect of 10 μ M naloxone in fresh/morphine-naive (Fresh), overnight-stored/morphine-naive (O/N K-H) and overnight-stored/morphine-exposed (O/N Mor) segments of the guinea-pig isolated ileum. Responses have been expressed as a percentage of the electrically-evoked contractions and are represented by (●). The values in parentheses indicate where a number of individual responses overlap.

Figure 3 Representative trace recordings of the effect of 10 μ M naloxone in overnight-stored/morphine-exposed preparations of the guinea-pig isolated ileum in (a) the absence of clonidine and (b) the presence of 0.3 μ M clonidine.

Figure 4 The effect of 10 μ M naloxone in overnight-stored/morphine-exposed segments of the guinea-pig isolated ileum in the presence of saline (Con), 1 μ M UK-14304 (UK) and 0.3 μ M clonidine (Clon). Responses have been expressed as a percentage of the electrically-evoked contractions and are represented by the closed circles. The values in parentheses indicate where a number of individual responses overlap.

The effect of α_2 -adrenoceptor-activation on naloxone-induced contractions of stored/morphine-exposed preparations

The α_2 -adrenoceptor agonists clonidine (0.3 nM–1 μ M) and UK-14304 (1 nM–1 μ M) produced concentration-dependent inhibition of the twitch responses in stored/morphine-naive preparations of the guinea-pig ileum. The maximum inhibition produced in both agonists was greater than 90%.

Clonidine (0.3 μ M, 100 fold greater than the EC_{50}) completely inhibited naloxone-induced contraction of stored/morphine-exposed preparations in 3 out of 4 preparations (Figure 3). As shown in Figure 4, 1 μ M UK-14304 (100 fold greater than the EC_{50}) was similarly effective against contractions evoked by 10 μ M naloxone.

Discussion

Functional dissociation of tolerance and dependence

In the present study we have shown that overnight incubation of the guinea-pig isolated ileum in Krebs-Henseleit saline at 4°C (stored/morphine-naive) does not impair the ability of the preparation to respond either to electrical field stimulation or to 10 μ M naloxone after acute exposure to 10 μ M morphine; this agrees with an earlier report by Collier and coworkers (1981a). However, compared to fresh/morphine-naive preparations, there was a significant reduction in the potency of morphine as an inhibitor of the electrically-evoked contractions even though the maximum inhibition

produced was unaffected. The basis of this difference in sensitivity to morphine is not known.

A comparison of stored/morphine-naïve and stored/morphine-exposed preparations failed to reveal any difference in either the potency or maximum inhibition produced by morphine against the electrically-evoked contractions. Hence, there was no evidence for the development of tolerance following overnight exposure of the guinea-pig isolated ileum to 10 μ M morphine at 4°C. These findings also indicate that the washout procedure adopted (4 washes over 3 h at room temperature) reduces the receptor biophase concentration of morphine in stored/morphine-exposed preparations to below 10 nM – the threshold concentration for inhibition of the electrically-evoked response. Our results contrast sharply with the earlier report that 18–22 h incubation of the guinea-pig isolated ileum with 10 nM normorphine at 22°C was associated with a 10 fold rightward displacement of the normorphine concentration-response curve (Collier *et al.*, 1981a). It seems likely that the adaptive processes responsible for receptor-specific tolerance, e.g. a reduction in receptor number and/or uncoupling of the receptor (Johnson & Fleming, 1989), are temperature-sensitive.

In spite of the lack of evidence for the development of tolerance to morphine in stored/morphine-exposed guinea-pig ileal preparations, seven out of nine segments responded to 10 μ M naloxone with a contraction. This represents a significant increase over the incidence of naloxone-induced contractions in fresh/morphine-naïve preparations (2/9) and stored/morphine-naïve preparations (1/9; see Figure 2). Since the naloxone-induced contractions in stored/morphine-exposed preparations were practically abolished by the α_2 -adrenoceptor agonists, clonidine and UK-14304, it seems likely that these contractions, like those observed following acute exposure to morphine and naloxone (Chahl, 1990), involves the release of acetylcholine from cholinergic neurones. Furthermore, these observations are also consistent with the known efficacy of α_2 -adrenoceptor agonists against withdrawal responses in opiate-dependent states in experimental models (Alfaro *et al.*, 1990; Coupar 1991) and in man (Gold *et al.*, 1978). Significantly, the effectiveness of clonidine against both the electrically-evoked contractile responses and the naloxone-induced contractile response (> 90% inhibition) also supports the view that overnight exposure to morphine at 4°C failed to induce tolerance. Taylor *et al.* (1988) and Alfaro *et al.* (1991) both reported that tolerance following long-term exposure to opiates was not receptor specific, and α_2 -adrenoceptors effects against the electrically-evoked contractions of the guinea-pig isolated ileum or the naloxone-induced contractions were similarly blunted.

Taken together, the findings in the present study clearly indicate that opiate dependence and tolerance are not mutually-dependent processes, a view consistent with earlier suggestions by Schulz and coworkers (1985). Since naloxone was able to elicit a contraction in the stored/morphine-exposed guinea-pig isolated ileum in the absence of both morphine and the development of tolerance, the view that this response is a consequence of adaptive processes associated with tolerance (Johnson & Fleming, 1989) is no longer tenable and an alternative explanation is required. One possibility is that 'dependence' in the guinea-pig ileum as judged by the appearance of a 'naloxone-induced' contraction, can be precipitated by concentrations of morphine that are sub-threshold for the inhibition of the electrically-evoked contractions (> 10 nM). Another possibility is that the antagonist, nalox-

one, possesses inverse agonist properties which are manifest only after receptor-specific adaptive changes in the myenteric plexus have been induced by morphine.

Does naloxone possess inverse agonist properties at morphine-sensitive receptors?

A number of recent observations indicate that naloxone may possess negative agonism at opiate receptors. Costa & Herz (1989) reported that antagonists for δ opiate receptors on the neuroblastoma/glioma NG-108-15 cell line can be divided into two groups based upon their effect on guanosine-triphosphatase (GTPase) activity. The antagonist (–)-5,9-diethyl-2-(3-furyl-methyl)-2'-hydroxybenzomorphane (MR 2266) had no effect on GTPase activity but produced a competitive inhibition of the GTPase stimulated by δ opiate receptor agonist [D-Ala², D-Leu⁵] enkephalin (DADLE). MR-2266 can be considered a pure δ receptor antagonist. In contrast, the antagonists ICI 174864 ([N,N'-diallyl-Tyr¹, Aib^{2,3}] Leu⁵ enkephalin), ICI 154129 ([N,N'-diallyl-Tyr¹, ϕ -(CH₂)S Phe⁴, Leu⁵] enkephalin) and naloxone produced a concentration-dependent inhibition of GTPase activity and, in the case of ICI 174854, the 'response' was sensitive to MR 2266. Thus, these antagonists can be considered as possessing negative agonist activity at δ opiate receptors. The essential difference between 'pure' antagonists and those possessing 'negative agonist' properties was underlined in studies by Millan *et al.* (1988) and Morris & Millan (1991), who showed that unlike naloxone long-term exposure to MR 2266 failed to up-regulate central μ , δ or κ opiate binding sites or to produce supersensitivity to opiate agonists.

If naloxone also possesses negative agonism at μ opiate receptors, this could account for the contractile activity in response to naloxone observed in both stored/morphine-exposed and in morphine-naïve preparations of the guinea-pig isolated ileum (see also Rodriguez *et al.*, 1980; Miguel & Rodriguez, 1981). An assumption underlying this suggestion is that μ opiate receptors undergo a 'temperature-insensitive' adaptive change following overnight exposure to morphine (that outlives the presence of the agonist) in order to account for the greater incidence of naloxone-induced contractions. Since there is no evidence for cross-dependence between μ opiate receptors and α_2 -adrenoceptors following overnight exposure to either morphine or clonidine (Collier *et al.*, 1991b), this adaptive change could be either at the receptor binding site or at some point in the receptor-coupling mechanism proximal to the point of interaction with neuronal α_2 -adrenoceptors. Support for this working model would be provided by the failure of both MR 2266 and an α_2 -adrenoceptor antagonist, e.g. phentolamine, to elicit a withdrawal response in stored/morphine-exposed preparations of the guinea-pig isolated ileum.

In conclusion, we have demonstrated that overnight exposure of the guinea-pig isolated ileum to morphine at 4°C results in the functional dissociation of dependence and tolerance, suggesting that the processes underlying the latter adaptive response are temperature-sensitive. Our findings also raise the possibility that naloxone-induced contractions in 'stored/morphine-exposed' segments of the guinea-pig ileum arise from either residual, 'subthreshold' concentrations of morphine or is a consequence of a combination of intrinsic inverse agonism of naloxone at μ opiate receptors on cholinergic neurones and receptor-specific adaptive changes.

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