British Journal of Pharmacology (2000) 131, 893–902

www.nature.com/bjp

Pharmacological examination of contractile responses of the guinea-pig isolated ileum produced by μ-opioid receptor antagonists in the presence of, and following exposure to, morphine

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1 We have assessed the potential of several μ-opioid receptor antagonists to elicit a response in the guinea-pig isolated ileum in the presence of, and following overnight exposure to, morphine.

2 Naloxone, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP), (−)-5,9α-diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan (MR2266), but not D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP), produced a transient inhibition of electrically-evoked contractions of the guinea-pig ileum. The effect of 1 μM CTOP, but not that to MR2266, was inhibited by 1 μM somatostatin.

3 Naloxone (0.3 μM), CTOP (3 μM), CTAP (3 μM) and MR2266 (0.3 μM) antagonized the inhibitory effect of morphine on electrically-evoked contractions of the guinea-pig to a similar degree and, following 60 min exposure to morphine, produced non-sustained contractions. The response to 3 μM CTOP was significantly smaller than that to 3 μM CTAP. None of the antagonists produced a response in the absence of morphine.

4 Following overnight exposure of the ileum to 0.3 μM morphine (4°C), and repeated washing to remove the agonist, all four antagonists elicited non-sustained contractions. However, the responses to 3 μM CTOP and 0.3 μM MR2266 were significantly smaller than those elicited by 0.3 μM naloxone and 3 μM CTAP. Somatostatin (1 μM) significantly reduced naloxone-induced contractions, but not those to CTAP.

5 While all four μ-opioid antagonists elicited contractions in the presence of, and following prolonged exposure to, morphine, differences between them were noted which may be a consequence of non-opioid actions.

British Journal of Pharmacology (2000) **131,** 893–902

Keywords: Naloxone; CTOP; CTAP μ-opioid receptors; guinea-pig isolated ileum

Abbreviations: CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, MR2266, (−)-5,9α-diethyl-2-(3-furyl-methyl)-2′-hydroxy-6,7-benzomorphan; U50488H, *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrorolidinyl)cyclohexyl]-benzenacetamide methane sulphonate

Introduction

Evidence is accumulating that many receptor antagonists not only occupy the active sites to block responses to agonists, but under certain conditions have the potential to elicit biological responses functionally opposite to that of agonists, so-called 'inverse agonism' at constitutively-activated receptors (Milligan *et al*., 1995; Kenakin 1999). In the majority of instances, however, the effects of putative inverse agonists have been reported in cultured cells with the pharmacological significance of the findings unclear.

For example, acute exposure of SH-SY5Y cells to the μ-opioid receptor agonist morphine caused a reduction in forskolin-stimulated cyclic AMP which was abolished by the antagonist naloxone (Yu & Sadee, 1988; Yu *et al*., 1990). Naloxone alone produced very little effect on cyclic AMP levels. However, following prolonged exposure to morphine and extensive washing, resting level of cyclic AMP was elevated and, under this condition, many μ-opioid receptor antagonists, including naloxone, caused a further increase in the level of the cyclic nucleotide (Wang *et al*., 1994). In marked contrast, the somatostatin derivatives D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP), which are recognized as potent antagonists of μ-opioid receptors (Hawkins *et al*., 1989; Kramer *et al*., 1989), failed to influence cyclic AMP levels but abolished both the stimulatory effect of naloxone and the inhibitory response to morphine (Wang *et al*., 1994). These authors concluded that prolonged exposure of SH-SY5Y cells to morphine induced a constitutively activated state of the μ-opioid receptor, at which naloxone then exhibited inverse agonist activity while CTOP and CTAP behaved as conventional 'neutral' antagonists. Qualitatively similar responses to naloxone have also been reported in HEK 293 cells previously exposed to morphine (Wang *et al*., 1999).

With respect to the action of naloxone, there are interesting parallels with observations in a physiologically-intact tissue, the guinea-pig isolated ileum. It is well documented that while naloxone *per se* exerts little effect on electrically-evoked contractions in this preparation, the reversal of morphine-induced inhibition of these responses is associated with the appearance of a 'withdrawal' contraction due to the release of myenteric neurotransmitters (Collier *et al*., 1981; Johnson *et al*., 1987; David *et al*., 1993; Mundey *et al*., 1998). Furthermore, we have reported that following 20 h incubation of the guinea-pig ileum in morphine at 4°C, and subsequent washout to remove the agonist, naloxone was still capable of eliciting a withdrawal contraction (David *et al*., 1993), *prima facie* evidence for the induction of an activated state of μ-opioid receptors.

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The principal aim of this experiment was to compare the ability of structurally diverse μ-opioid receptor antagonists, naloxone, CTOP, CTAP and (−)-5,9α-diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan (MR 2266) (Portoghese *et al*., 1987; Standifer *et al*., 1994), to elicit withdrawal contractions of the guinea-pig isolated ileum in the presence of, and also following prolonged prior exposure to, morphine. These findings were expected to provide greater insight into the pharmacological significance of 'inverse agonism' in a physiologically-intact system and shed light on the reported inability of CTAP to precipitate withdrawal symptoms in morphine-dependent mice (Bilsky *et al*., 1996). During the course of our investigation it also became necessary to (i) optimize the conditions required for prolonged exposure to morphine to induce naloxone-induced contractions, (ii) establish equieffective concentrations of the antagonists for inhibition of the acute effects of morphine at μ-opioid receptors and (iii) examine the pharmacological significance of 'non-opioid' actions of putative antagonists.

Methods

Male Dunkin-Hartley guinea-pigs (500–900 g) were killed by a blow to the head and exsanguination. The ileum was exteriorized, the ileo-caecal junction located and approximately 70 cm removed. A 10 cm segment of the terminal portion was then discarded before the contents of the remaining ileum was flushed out with modified Krebs-Henseleit solution (previously gassed with 95% O_2/ 5% CO_2 and maintained at 37°C). The ileum was then divided into either 5–6 cm segments which were used on the day of the experiment ('fresh') or into 12–15 cm segments which were stored overnight at 4°C in a sealed conical flask containing 50 ml Krebs-Henseleit solution ('overnight'). For the 'overnight' tissues, morphine (0.03, 0.1, 0.3, 1, or 3 μM) was often included in the Krebs-Henseleit solution 1 h after equilibration in the refrigerator and the vessel resealed. Preparations not exposed to morphine overnight were called 'overnight, morphine-naive', while those incubated with morphine were considered to be 'overnight, morphine-exposed'. The total incubation time with morphine at 4°C was 18–22 h. The following day the incubation medium was exchanged for fresh Krebs-Henseleit solution (also maintained at 4°C and previously gassed with 95% O_2/CO_2), gently swirled and replaced in the refrigerator. This washout protocol was repeated four times over 60 min and the segments were then left for a further 60 min at room temperature to increase removal of morphine. For the 'overnight' preparations, direct exposure to morphine was only conducted at 4°C.

For both the 'fresh' and 'overnight' tissues, 5–6 cm segments were secured to a perspex holder with two parallel platinum wires and then placed in a 20 ml isolated organ bath containing Krebs-Henseleit solution (maintained at 37°C and gassed with 95% O_2/5% CO_2). The upper end of the segment was attached by cotton to a Grass FT03C isometric transducer connected to a CED 1902 amplifier (Cambridge Electronic Devices, Cambridge, U.K.) and, *via* a 1401 Laboratory interface to a 486 PC running Spike 2 software (CED). After 30 min equilibration, 2 g wt resting tension was applied (which usually declined to approximately 0.5 g wt.). The preparations were stimulated transmurally (0.1 Hz, 0.3 ms, 200 mA; D330-multisystem stimulator, Digitimer Ltd, U.K.) and a minimum of 45 min allowed to establish reproducible responses. All preparations were washed twice with fresh Krebs-Henseleit solution 30 min into the period of electrical stimulation.

Assessment of the effect of naloxone on 'fresh' and 'overnight-stored' preparations

For the 'fresh' preparations, at the end of the control period of electrical stimulation morphine (0.03, 0.1, 0.3, or 3 μM) was added for a further 60 min. The stimulator was then switched off and 1 μM naloxone added. Once the associated withdrawal contraction had declined to baseline, the stimulator was again turned on for 5 min and finally each preparation was exposed to 60 mM KCl. For the 'overnight' preparations ('morphine-naive' and 'morphine-exposed') a similar protocol was adopted except that morphine was not added to the organ bath. In a separate series of experiments, the effect of morphine on electrically-evoked contractions of 'overnight, morphine-naive' and 'overnight, morphine-exposed' preparations was determined. Cumulatively increasing concentrations (0.5 log unit) of morphine (0.003–3 μM) were added at intervals of 5 min, or until a plateau had been reached.

μ-Opioid receptor antagonists and morphine-induced inhibition of electrically-evoked contractions

After stable electrically-evoked contractions were obtained, 'fresh' segments were exposed to various concentrations of either naloxone, MR 2266, CTOP or CTOP and 40 min later the effect of cumulatively increasing concentrations of morphine determined. In each experiment the effect of morphine on electrically-evoked contractions was also examined in the absence of the antagonist. Only one concentration response curve to morphine was determined in each preparation. The effect of 20 nM norbinaltorphimine was also determined against morphine- and *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrorolidinyl)cyclohexyl]-benzenacetamide methane sulphonate (U50488H)-induced inhibition of the electrically-evoked contractions. In a separate series of experiments the effect of 3 μM CTOP and 0.3 μM MR2266 on electrically-evoked contractions was examined in the presence and absence of either 1 μM somatostatin or 1 μM naloxone.

μ-Opioid receptor antagonists and withdrawal contractions of 'fresh' and 'overnight, morphine-exposed' preparations

The effect of equieffective concentrations of the antagonists was examined in 'fresh' preparations of the guinea-pig ileum in the presence of 0.3 μM morphine and also following overnight exposure to morphine (1 μM).

Data analysis

Electrically-evoked contractions have been expressed as either force (g wt.) or as a percentage of the contraction to 60 mM KCl, while the inhibitory effect of drugs has been calculated as a percentage of the control response. Antagonist-induced contractions have been calculated as a percentage of the contraction caused by 60 mM KCl. All data are shown as the mean ± s.e.mean. The potency of the opioid receptor agonists in the absence and presence of the antagonists was assessed as the negative logarithm of the concentration required to cause 50% of the maximum response (pD_2) using the logistic equation described by DeLean *et al*. (1978) with Kaleidagraph software (Synergy) on a Macintosh LC II computer. For the antagonist experiments, the ratio of the concentration of the agonist producing 50% of the maximum response, in the presence and absence of the antagonist, the agonist concentration-

ratio, was determined in each experiment. Differences between mean pD_2 values and the effect of drugs on the electrically-evoked contractions were assessed by a Student *t*-test with $P<0.05$ considered statistically significant. In many experiments, the withdrawal contractions to the antagonists were variable and, therefore, differences were assessed by using either a Kruskal Wallis test followed by Dunn's test for individual comparisons or by a Mann-Whitney U-test.

Drugs

The following compounds were used: morphine sulphate (Evans, Speke), (−)-5,9α-diethyl-2-(3-furyl-methyl)-2′-hydroxyl-6,7-benzomorphan (MR 2266; Boehringer Ingelheim KG), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP; Bachem) and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP; Bachem) norbinaltorphimine dihydrochloride (Tocris, Bristol, U.K.) naloxone HCl (Sigma), somatostatin (Bachem), *trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrorolidinyl)cyclohexyl]-benzenacetamide methane sulphonate (U50488H; Sigma), tetrodotoxin citrate (Sigma), phentolamine mesylate ('Rogitine', Ciba-Geigy), clonidine hydrochloride (Sigma). All other drugs were dissolved in distilled water and added to the organ baths in a volume of 0.1 ml or less. The composition of the modified Krebs-Henseleit saline was (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11.1.

Results

The effect of naloxone on 'fresh' and 'overnight' preparations of the guinea-pig ileum

As shown in Figure 1a, 3 μM morphine caused a 82.4±6.1% ($n=9$) reduction of electrically-evoked contractions of 'fresh'

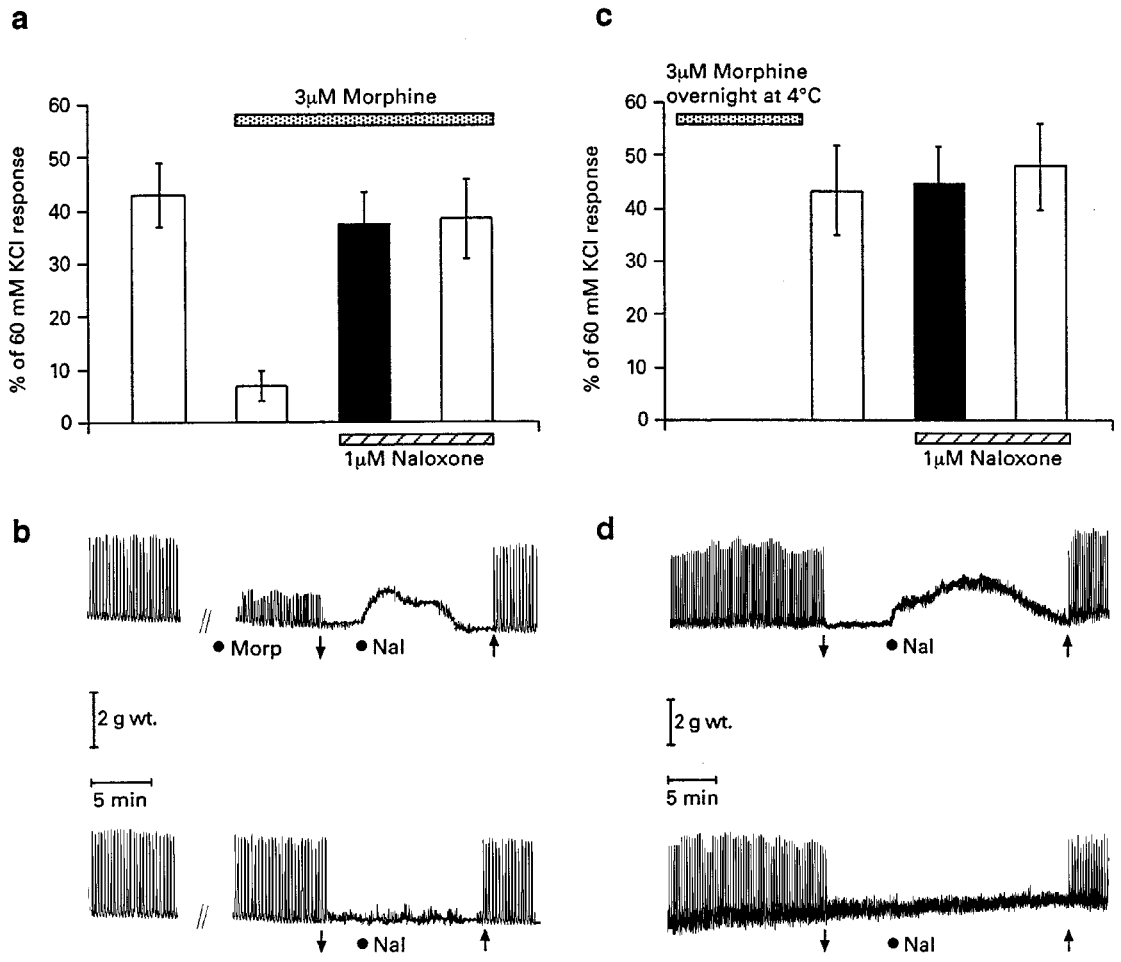

Figure 1 The effect of morphine and naloxone on the guinea-pig isolated ileum. (a) A graph of electrically-evoked contractions (open columns) in the absence of morphine, the presence of 3 μM morphine and a combination of 3 μM morphine and 1 μM naloxone. Also shown is the withdrawal contraction (closed column) observed upon addition of 1 μM naloxone. Responses have been expressed as a percentage of the contraction to 60 mM KCl and shown as the mean±s.e.mean of nine (electrically-evoked contractions) and eight out of 9 (withdrawal contractions) observations. (b). A representative digitized trace recording of the effect of 1 μM naloxone on 'fresh' segments of guinea-pig isolated ileum in the presence (upper, morphine-present) and absence (lower, morphine-naive) of 3 μM morphine. Note that 1 μM naloxone only produced a withdrawal contraction in the presence of morphine and that this was associated with a reversal of the inhibitory effect exerted by the agonist on electrically-evoked contractions. (c) A graph of electrically-evoked contractions (open columns) of preparations previously exposed to 3 μM morphine overnight (4°C), and subsequently washed to remove the agonist, in the absence and presence of 1 μM naloxone. Also shown is the withdrawal contraction (closed column) observed upon addition of 1 μM naloxone. Responses have been expressed as a percentage of the contraction to 60 mM KCl and shown as the mean±s.e.mean of seven (electrically-evoked contractions) and seven out of seven (withdrawal contractions) observations. (d). A representative digitized trace recording of the effect of 1 μM naloxone on segments of the guinea-pig isolated ileum previously stored overnight (4°C) in either the presence (upper; morphine-exposed) or absence (lower; morphine-naive) of 3 μM morphine, and subsequently washed repeatedly to remove the agonist. Note that 1 μM naloxone only produced a withdrawal contraction in the preparation previously exposed to morphine, but that this was *not* associated with a change in the magnitude of the electrically-evoked contractions.

segments of the guinea-pig isolated ileum which was reversed by the addition of 1 μM naloxone. In eight out of nine of these preparations ('fresh, morphine-present') the addition of naloxone was associated with a non-sustained contraction equivalent to 37.5±6.1% ($n=8$) of the contraction to 60 mM KCl (see Figure 1a,b). In the absence of morphine ('fresh, morphine-naive') 1 μM naloxone failed to elicit a contraction ($n=7$; Figure 1b). In a separate series of experiments, 1 μM naloxone also failed to elicit a withdrawal contraction in the presence of 3 μM morphine if 0.3 μM tetrodotoxin was included in the medium ($n=4$).

Following overnight storage of the ileum at 4°C in modified Krebs-Henseleit solution, either with ('overnight, morphine-exposed') or without ('overnight, morphine-naive') 3 μM morphine, and subsequent washing with morphine-free Krebs-Henseleit solution, the responses to 60 mM KCl and electrical field stimulation were not significantly different from 'fresh' preparations (Table 1). As shown in Figure 1c, while 1 μM naloxone did not alter the electrically-evoked contraction of overnight, morphine-exposed preparations, it caused large, non-sustained contractions (Figure 1c,d) equivalent to 44.6±5.0% of the response to 60 mM KCl ($n=7$). In overnight, morphine-naive preparations, however, naloxone neither elicited a contraction (Figure 1d) nor did it affect responses following the resumption of electrical stimulation ($n=7$). In a separate series of experiments, 1 μM naloxone also failed to elicit a withdrawal contraction of preparations previously exposed to 3 μM morphine if 0.3 μM tetrodotoxin was included in the medium ($n=5$).

The α-adrenoceptor antagonist phentolamine (1 μM) failed to elicit a contraction of the ileum either in the presence of, or following exposure to, morphine ($n=5$ for both conditions). However, phentolamine (1 μM) caused a withdrawal contraction (20.6±4.2% of 60 mM KCl, $n=11$) of the ileum in the presence of 0.1 μM clonidine (60 min exposure), while naloxone (1 μM) was devoid of activity under these conditions ($n=5$).

As shown in Figure 2, the magnitude of the naloxone-induced contractions in 'overnight' preparations was dependent upon the concentration of morphine, but the response in 'fresh' preparations (morphine-present) was largely independent of the concentration of the agonist. The maximum response to naloxone in 'fresh' preparations (28.0±5.1%, $n=15/17$) was obtained in the presence of 0.3 μM morphine, while the maximum response in overnight preparations was observed following exposure to 1 μM morphine (68.9±7.4%, $n=5/5$). Interestingly, morphine exhibited similar potency as an inhibitor of electrically-evoked contractions in overnight, morphine-naive (max response 68.4±6.3%; pD_2 7.13±0.03, $n=8$) and overnight morphine-exposed (1 μM) preparations (max response 60.7±5.8%; pD_2 7.23±0.06, $n=8$) (Figure 3).

Thus, naloxone produces a contraction of overnight, morphine-exposed (1 μM) preparations despite evidence that the washout procedure had ensured removal of the agonist from the receptor (no change in magnitude of the electrically-evoked contractions following naloxone), and there being no change in the sensitivity of the preparation to morphine.

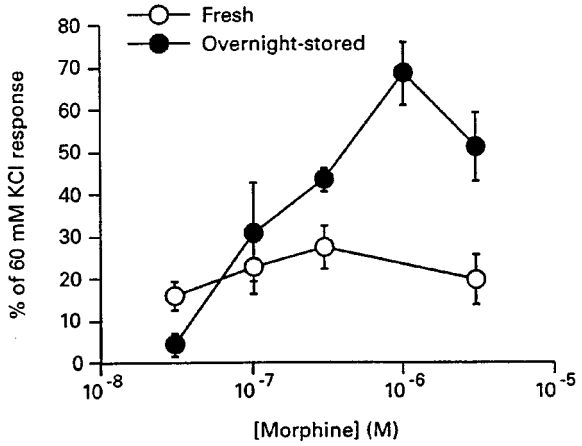

Figure 2 A graph of the relationship between morphine concentration and the magnitude of withdrawal contraction of the guinea-pig isolated ileum elicited by naloxone. Preparations of the guinea-pig ileum were exposed to various concentrations of morphine and then exposed to 1 μM naloxone ('fresh') or stored overnight (4°C) in the presence of morphine, washed repeatedly to remove the agonist, and challenged with 1 μM naloxone (overnight-morphine-exposed). Each preparation was exposed to only a single concentration of morphine and naloxone. Responses have been expressed as a percentage of the contractions to 60 mM KCl and shown as the mean±s.e.mean of the preparations that responded to naloxone. The number of experiments conducted are given in parenthesis: fresh, 0.03 μM (17), 0.1 μM (eight), 0.3 μM (17) and 3 μM (nine) morphine; overnight, morphine-exposed, five experiments for each concentration.

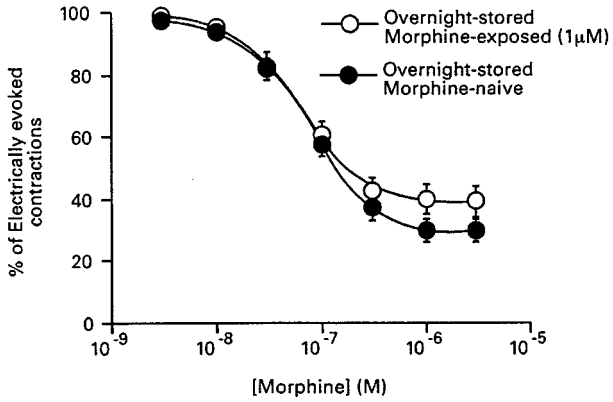

Figure 3 A comparison of the effect of morphine on electrically-evoked contractions of the guinea-pig isolated ileum following overnight storage (4°C) in either the absence ('morphine-naive') or presence of 1 μM morphine ('morphine-exposed') and subsequently washed repeatedly to remove the agonist. The electrically-evoked contractions have been expressed as a percentage of the response prior to the addition of morphine and are shown as the mean± s.e.mean of eight observations.

Table 1 Mean contractions (g. wt.) of the guinea-pig isolated ileum under various conditions

	60 mM KCl	*Electrically-evoked responses*
Fresh, morphine-naive	4.40±1.60	2.17±0.89 ($n=7$)
Fresh, morphine (3 μM) present	4.74±0.50	2.00±0.29 ($n=9$)
Overnight, morphine-naive	4.47±0.49	2.65±0.50 ($n=6$)
Overnight, morphine (3 μM) exposed	4.37±0.43	1.87±0.31 ($n=7$)

The contractions are shown as the mean±s.e.mean with 'n' indicating the number of experiments conducted.

The effect of 'selective' antagonists against morphine-induced inhibition of neurogenic responses in 'fresh' preparations of the guinea-pig ileum

As shown in Figure 4, naloxone (0.01 – 1 μM), MR-2266 (0.01 – 1 μM), CTOP (0.3 and 3 μM) and CTAP (0.3 and 3 μM) caused a concentration-dependent, rightward displacement of morphine-induced inhibition of neurogenic contractions, without altering the maximum inhibition. For naloxone, CTAP and CTOP the magnitude of the displacement of the morphine concentration response curve was related to the concentration of the antagonist. For MR-2266, however, the inhibition produced by 0.1 μM MR2266 (morphine pD_2 – 5.85 ± 0.16, $n = 5$) was only 3 fold greater than that produced by 0.01 μM MR-2266 (morphine pD_2 – 6.40 ± 0.14, $n = 5$) (see also Figure 4b). Since MR 2266 also possesses high affinity for κ-opioid receptors (Miller *et al.*, 1986), we sought to determine the contribution of this receptor to the action of morphine. Norbinaltorphimine (20 nM), a κ-selective opioid receptor antagonist (Portoghese *et al.*, 1987), failed to affect morphine-induced inhibition of neurogenic contractions (control pD_2 7.51 ± 0.09 ($n = 5$); norbinaltorphmine pD_2 7.45 ± 0.15 ($n = 5$)), but produced a 200 fold rightward displacement of the concentration response curve for U50488H, a κ-selective opioid receptor agonist (control pD_2 8.90 ± 0.07 ($n = 6$), norbinaltorphmine pD_2 6.70 ± 0.09 ($n = 6$)). Thus, κ-opioid receptors do not make a significant contribution to the effect of morphine in the guinea-pig ileum.

Figure 5 shows the Schild plot for the effect of the antagonists on morphine-induced inhibition of neurogenic contractions of the guinea-pig ileum, and indicates that 0.3 μM

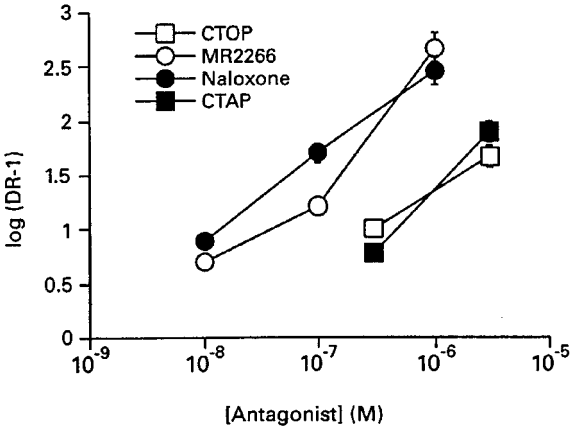

Figure 5 Schild plot of the effect of various opioid receptor antagonists on morphine-induced inhibition of electrically-evoked contractions of the guinea-pig isolated ileum. Each log (DR-1) value represents the mean ± s.e.mean of 5 – 10 observations.

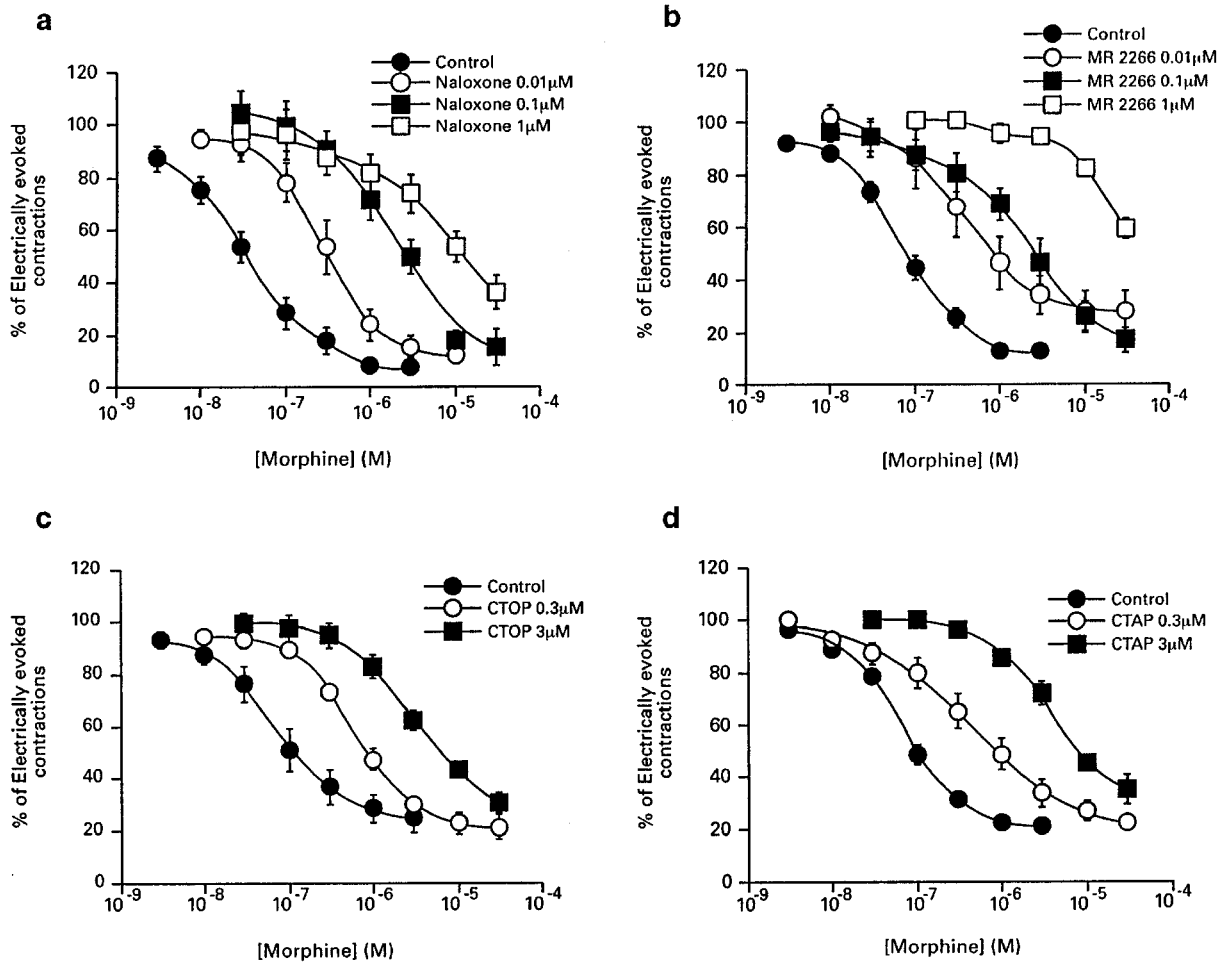

Figure 4 Comparison of the effect of (a) naloxone, (b) MR 2266, (c) CTOP and (d) CTAP on morphine-induced inhibition of electrically-evoked contractions of the guinea-pig isolated ileum. The electrically-evoked contractions have been expressed as a percentage of the response prior to the addition of morphine and are shown as the mean ± s.e.mean of 5 – 10 observations.

naloxone, 0.3 μM MR2266, 3 μM CTOP and 3 μM CTAP can be considered equieffective concentrations, producing between a 50–100 fold displacement of the agonist concentration response curve. These concentrations of the antagonists were used for the final series of experiments.

During the course of these experiments we noted that a number of the antagonists caused a transient reduction in the electrically-evoked contractions. Figure 6a shows that the addition of 3 μM CTOP produced a significant reduction of neurogenic contractions of the guinea-pig ileum which returned towards control values over the following 5–10 min. As indicated in Table 2, MR2266 and, to a lesser extent, naloxone produced qualitatively similar effects on neurogenic contractions. In marked contrast, 3 μM CTAP did not significantly affect neurogenic contractions (Table 2). Since this study involves a comparison of the ability of receptor antagonists to elicit transient biological responses, it was considered necessary to determine the basis of the inhibitory effect of CTOP and MR2266.

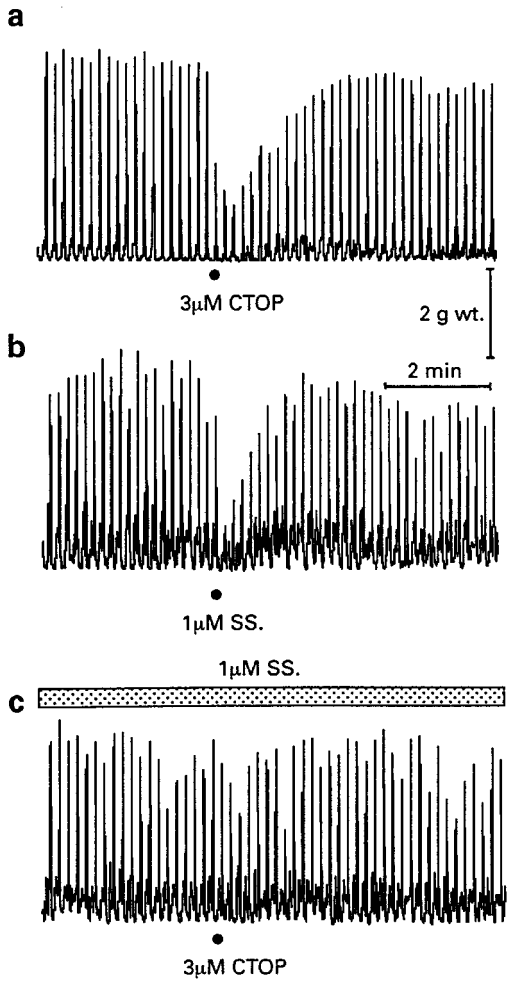

Figure 6 Representative digitized recordings of the effect of (a) 3 μM CTOP, (b) 1 μM somatostatin (SS) and (c) 3 μM CTOP in the presence of 1 μM somatostatin (stippled bar), on electrically-evoked contractions of the guinea-pig isolated ileum. Note that CTOP and somatostatin caused a transient inhibition of electrically-evoked contractions but that CTOP failed to do in the presence of somatostatin.

Somatostatin (1 μM), the endogenous peptide upon which CTAP and CTOP are based (Hawkins *et al*., 1989; Kramer *et al*., 1989), also caused a significant, short-lived inhibition of neurogenic responses (Figure 6b) and significantly reduced the inhibitory effect of 3 μM CTOP (Table 2; Figure 6c). In contrast, the inhibitory effect of MR 2266 and CTOP were not altered by the presence of naloxone (Table 2). Thus, the antagonists MR 2266 and CTOP possess transient, inhibitory activity in the guinea-pig ileum that appears to involve a naloxone-insensitive mechanism. In the case of CTOP this effect may be mediated via somatostatin receptors.

Comparison of the antagonists in fresh and overnight, morphine-exposed ileal segments

Figure 7a shows that equieffective concentrations of naloxone, MR 2266, CTOP and CTAP produced a contraction of fresh segments of the ileum in the presence of 0.3 μM morphine. In each instance the contractions were not sustained but lasted 3–5 min. For CTOP (3 μM), only four of the eight preparations examined responded with a contraction and the mean response was significantly less ($P<0.05$) than that produced by CTAP (eight out of eight preparations). In the four 'CTOP-unresponsive' preparations the displacement of the agonist from the receptor was indicated by reversal of the effect of morphine on electrically-evoked contractions; an effect also observed in the four 'CTOP-responsive' preparations and those exposed to CTAP.

Figure 7b shows that the antagonists also produced contractions of overnight, morphine-exposed preparations but the response to 3 μM CTOP (36.1±6.1% of 60 mM KCl, $n=12$) and 0.3 μM MR 2266 (35.9±7.2% of 60 mM KCl, $n=12$) were significantly smaller than that elicited by 3 μM CTAP (69.7±7.2% of 60 mM KCl, $n=12$). In each instance the magnitude of the electrically-evoked contractions was not significantly altered by the antagonist. Naloxone (0.3 μM) produced similar responses to 3 μM CTAP, both in the presence of, and following exposure to, morphine (Figure 7).

Table 2 The effect of opioid receptor antagonists and somatostatin on neurogenic contractions of the guinea-pig isolated ileum in the absence and presence of either 1 μM naloxone or 1 μM somatostatin

	Control		*Naloxone*		*Somatostatin*	
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*
Naloxone (1 μM)	80.3±3.7	91.9±6.2	–	–	n.a.	n.a.
MR2266 (1 μM)	46.4±7.7	100.6±5.0	55.4±8.1	71.0±3.5*	35, 48	85, 93 ($n=2$)
CTOP (3 μM)	38.0±9.0	87.0±7.1	29.6±7.1	78.3±3.5	85.4±7.0*	94.6±8.0
CTAP (3 μM)	87.9±6.0	83.0±7.1	98.2±2.0	91.2±4.6	n.a.	n.a.
Somatostatin (1 μM)	20.7±6.8	78.0±10.4	n.a.	n.a.	–	–

The 'initial' response was measured as the peak effect following addition of the antagonist, while the 'final' response is that measured after 10 min incubation. Responses to electrical field stimulation have been expressed as a percentage of the response prior to the addition of the agent under investigation and are shown as the mean±s.e.mean of 5–10 observations, unless indicated otherwise. *denotes a statistically significant difference ($P<0.05$) from the corresponding value under control conditions (Student's *t*-test). n.a. denotes not applicable.

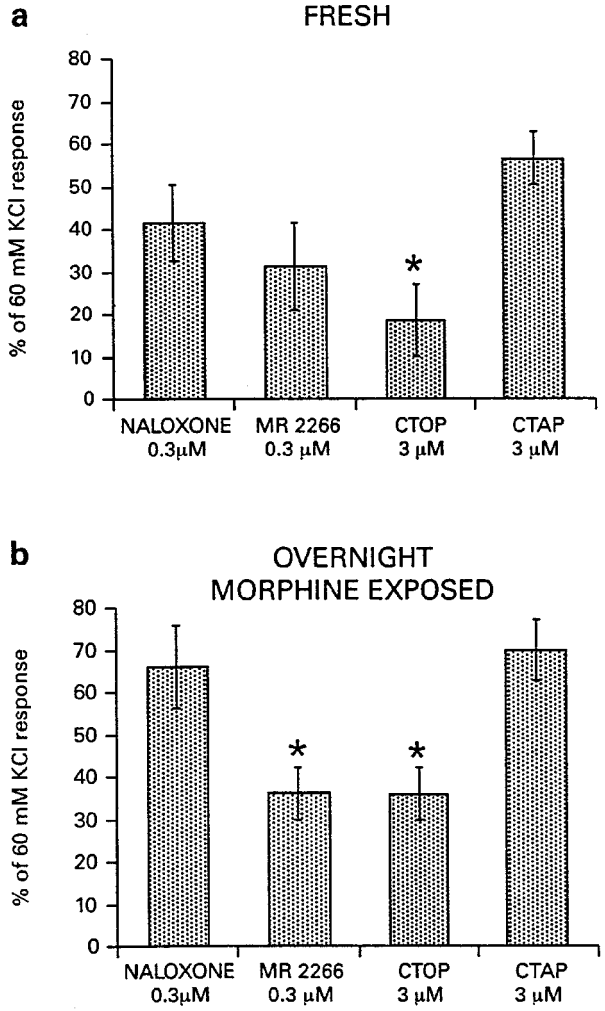

Figure 7 A comparison of equieffective concentrations of the opioid receptor antagonists to elicit withdrawal contractions of the guinea-pig isolated ileum (a) in the presence of 0.3 μM morphine and (b) following overnight exposure (4°C) to 1 μM morphine and subsequently washed repeatedly to remove the agonist. Responses have been expressed as a percentage of the contraction to 60 mM KCl and shown as the mean ± s.e.mean of eight (a) and 12 (b) observations. *Denotes a statistically significant difference from the effect of 3 μM CTAP.

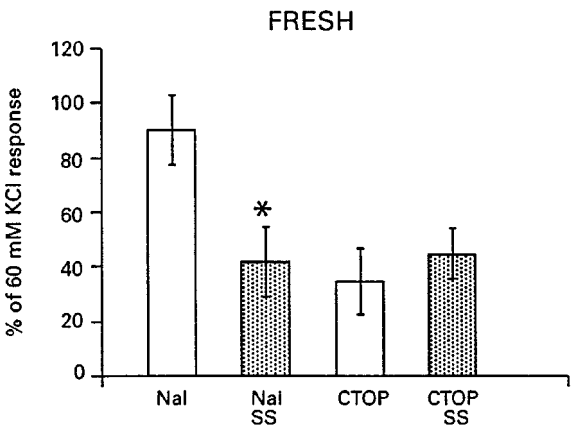

Figure 8 Comparison of withdrawal contractions of the guinea-pig isolated ileum elicited by 0.3 μM naloxone (Nal) and 3 μM CTOP in the presence and absence of 1 μM somatostatin (SS). All preparations were exposed to 0.3 μM morphine prior to the addition of the antagonists. Responses have been expressed as percentage of the contraction to 60 mM KCl and shown are the mean ± s.e.mean of eight observations. *Denotes a statistically significant difference (Mann Whitney U-test, $P<0.05$) for responses in the absence and presence of 1 μM somatostatin.

Figure 8 shows that the presence of 1 μM somatostatin (added 20 min before the antagonists but after 0.3 μM morphine) significantly reduced naloxone-induced contractions but failed to affect those elicited by 3 μM CTOP.

Discussion

Pharmacological characteristics of antagonist-induced contractions following exposure to morphine

We have confirmed our earlier observation that overnight exposure of the guinea-pig isolated ileum to morphine (at 4°C), followed by extensive washing to remove the agonist, induces a 'state' of μ-opioid receptors that renders naloxone capable of producing neurogenic contractions (David *et al*., 1993). Qualitatively, this finding is similar to that reported for naloxone on both adenylyl cyclase activity and cyclic AMP formation in SH-SY5Y cells previously exposed (48 h at 37°C) to morphine (Wang *et al*., 1994), and raises the possibility that a common mechanism may account for antagonist-induced responses in both systems. In addition, we have established that the response to naloxone is comparable in both magnitude and origin to 'withdrawal contractions' of the guinea-pig ileum elicited by naloxone in the presence of morphine (Collier *et al*., 1981; Mundey *et al*., 1998) and, as evidenced by the lack of effect of phentolamine, specific for opioid receptors.

The appearance of naloxone-induced contractions in the presence of, and following exposure to, morphine raises the possibility that the latter response simply arises from the presence of residual morphine due to inadequate washing. Several observations argue against this explanation. Firstly, the magnitude of the electrically-evoked contractions (and also that to KCl) was not altered by overnight exposure to morphine. Secondly, neither the sensitivity of the electrically-evoked contractions to inhibition by morphine nor the maximum effect produced were influenced by the experimental manipulation. These observations also provide evidence against the development of tolerance in the myenteric plexus to the effect of morphine (see David *et al*., 1993). Thirdly, and perhaps most revealing, is that while naloxone-induced contractions in the presence of morphine were associated with a reversal of the action of the agonist on electrically-evoked contractions, this was not the case in preparations previously exposed overnight to morphine (see Figure 1). It would appear, therefore, that prior to the addition of naloxone, morphine was either absent or present at a concentration below which any significant effect on electrically-evoked contractions could be detected (<10 nM). Interestingly, the conclusion regarding the relationship between naloxone-induced responses and agonist concentration is similar to that advanced by Kishioki *et al*. (1995) in morphine-dependent rats. It was noted that withdrawal symptoms precipitated by naloxone in this model were as pronounced at 24 h after the last dose of morphine (when plasma morphine was only 1% of steady state levels) as that observed 12 h earlier.

Clearly there are obvious parallels between our observations of antagonist-induced responses in the absence of an agonist and those reported for other receptors (e.g., Costa & Herz, 1989; Chidac *et al*., 1996; Jansson *et al*., 1998). However, the term 'constitutively-active' has not been used to describe the μ-opioid receptor in the ileum since the state of the receptor

under examination is not an inherent characteristic of the tissue, but a consequence of prolonged pre-treatment with an agonist. This stands in marked contrast to that reported recently for μ-opioid receptors expressed on HEK-293 cells, for example, which exhibits elevated signalling activity under basal conditions (Burford *et al*., 2000).

Although the myenteric plexus of the guinea-pig isolated ileum is endowed with μ and κ opioid receptors (Dhawan *et al*., 1996), the available evidence suggests that only the μ-opioid subtype is involved in responses elicited by antagonists. All four antagonists caused concentration-dependent inhibition of morphine-induced inhibition of electrically-evoked contractions. In the case of CTOP and CTAP, the potency (estimated $pK_B - 7.5$) was similar to that reported for interaction with μ-opioid receptors in the guinea-pig ileum (Wire *et al*., 1987; Tonini *et al*., 1998). Similarly, while MR 2266 possesses slightly higher affinity for κ opioid receptors than for μ-opioid receptors (Miller *et al*., 1986; Portoghese *et al*., 1987) the potency (estimated $pK_B - 8.5$) is consistent with an effect at the latter subtype. Also, norbinaltorphimine, a selective κ-opioid receptor blocker (Dhawan *et al*., 1996), inhibited responses to U50488H, a selective κ-opioid receptor agonist, but failed to alter morphine-induced inhibition of electrically-evoked contractions. Hence, it is unlikely that κ-opioid receptors are involved in mediating the effect of morphine and, therefore, the subsequent antagonist-induced contractions.

Equieffective concentrations of the antagonists were chosen in order to assess their ability to evoke contractions of the ileum following overnight exposure to, and in the presence of, morphine. This condition was deemed crucial to the objectives of the experiment, since we were particularly interested in establishing whether any of the agents behaved as a neutral antagonist, i.e. devoid of constrictor activity in either circumstance. Each antagonist was found to elicit contractions in both the presence of, and following exposure to, morphine, but those to CTOP were significantly smaller than those produced by CTAP. Following overnight exposure to morphine further significant differences between the antagonists were revealed, with both CTAP and naloxone producing larger responses than equieffective concentrations of CTOP or MR-2266. While these observations could be taken as preliminary evidence for CTOP and MR-2266 possessing weaker 'inverse agonist' properties at the agonist-free, activated μ-opioid receptor, the pharmacological profile of this effect is different from that described by Wang *et al*. (1994). In particular, both CTAP and CTOP were considered to act as neutral antagonists in SH-SY5Y cells, capable of antagonising the opposing effects of morphine and naloxone on cyclic AMP formation, yet in the ileum CTAP produced contractions as large as those elicited by naloxone.

Our finding that CTAP and naloxone produced quantitatively similar effects in the ileum is consistent with work by Maldonado *et al*. (1992) who demonstrated that both agents precipitated withdrawal symptoms in morphine-dependent rats. Although these results are at odds with those of Bilsky *et al*. (1996), in which CTAP failed to precipitate withdrawal symptoms in morphine-dependent mice, it is noteworthy that Maldonado *et al*. (1992) employed a 1000 fold dose range for the antagonist to ensure detection of responses. Similarly our experiments were designed on the basis of establishing equieffective concentrations of the antagonists prior to assessing their potential to elicit contractions

During the course of our experiments it became evident that both CTOP and MR-2266 caused a transient inhibition of the electrically-evoked contractions. In light of the report that CTOP (but not CTAP) possesses agonist activity at somatostatin receptors (Connor *et al*., 1997a), and that these receptors are present in the ileum (Fenuik *et al*., 1995), we investigated whether co-activation of a non-opioid receptor could account for the reduced responses to CTOP in overnight, morphine-exposed preparations of the ileum. Our results showed that somatostatin produced qualitatively similar responses to CTOP and that subsequent desensitization of the receptor reduced the effect of CTOP on electrically-evoked contractions. Moreover, in the presence of morphine somatostatin significantly reduced naloxone-induced contractions but failed to alter responses elicited by CTOP. It is noteworthy that Connor *et al*. (1997b) have reported the presence of somatostatin receptors (sst_2) on SH-SY5Y cells, which raises the possibility that an action on this receptor subtype contributed to the apparent neutral antagonist activity of CTOP in these cells (Wang *et al*., 1994). Although the basis of the non-opioid effect of MR-2266 was not examined in detail, it seems likely that the responses in overnight, morphine-exposed preparations was attenuated by this factor. Taken together, these observations indicate that caution is required when interpreting the action of putative inverse agonists in physiologically-intact systems, particularly in the absence of a neutral antagonist.

Thus, while we have provided convincing evidence that structurally dissimilar μ-opioid receptor antagonists can elicit neurogenic contractions of the guinea-pig ileum in the absence of an agonist, none of the data supports the view that the range of activity observed reflects intrinsic differences in their efficacy as 'inverse agonists'. Crucially, we were unable to identify an agent devoid of this activity (a neutral antagonist) which could provide unequivocal, pharmacological evidence that antagonist-induced responses are mediated *via* μ-opioid receptors. Also, it remains unclear whether a neutral antagonist may have reduced propensity to precipitate withdrawal symptoms in morphine-dependent rats, as has been suggested by Bilsky *et al*. (1996).

Cellular events associated with antagonist-induced contractions following exposure to morphine

The ability of prolonged exposure to morphine to alter the basal state of μ-opioid receptors in the guinea-pig myenteric plexus is similar to that reported for isoprenaline at β_2-adrenoceptors in Sf9 cells (Chidac *et al*., 1996). In this instance, a reduction in adenylyl cyclase activity was noted following exposure to isoprenaline, and the weak partial agonist dichloroisoprenaline acted as an inverse agonist at β_2-adrenoceptors, i.e. to elicit a reduction in basal adenylyl cyclase activity. Modulation of adenylyl cyclase activity and cyclic AMP has long been a major focus of attention for groups investigating subcellular mechanisms underlying opiate tolerance and dependence (see Nestler 1992). For example, chronic activation of μ-opioid receptors has been reported to elevate basal adenylyl cyclase activity in CHO cells transfected with μ-opioid receptor (Avidor-Reiss *et al*., 1995), SY-SH5Y cells (Ammer & Schulz, 1993; Wang *et al*., 1994), A431 cells (Ammer & Schultz, 1997) and even in the guinea-pig longitudinal muscle myenteric plexus preparation (Chakrabarti *et al*., 1998). However, with the exception of the observations by Wang *et al*. (1994), none of the above studies have shown that the alteration in basal adenylyl cyclase is accompanied by qualitative changes in the biological activity of μ-opioid receptor antagonists. Moreover, the significance to our study of any sustained alteration in enzyme activity by an agonist remains unclear for two reasons. First, it is not known whether similar changes occur when the ileum is exposed overnight to

morphine at 4°C. Second, μ-opioid responsive neurones in the myenteric plexus (S neurones) are not affected by agents that elevate cyclic AMP levels (Nemeth *et al*., 1986; Johnson & Pillai, 1990).

In summary, we have demonstrated that overnight exposure of the guinea-pig isolated ileum to morphine induces changes in myenteric μ-opioid receptors, qualitatively similar to those reported in SH-SY5Y and HEK 293 cells (Wang *et al*., 1994; 1999), that permit antagonists to elicit a biological response in the absence of an agonist. While differences between the antagonists were noted, it seems likely these are a consequence of non-opioid actions rather than evidence for partial inverse agonism at a constitutively-activated receptor.

MKM was supported by the BBSRC (ROPA Award) and we are grateful to Dr Steve Alexander for his helpful advice during the preparation of the manuscript.

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(Received May 22, 2000
Revised July 30, 2000
Accepted August 14, 2000)