RECEPTOR BINDING, ANTAGONIST, AND WITHDRAWAL PRECIPITATING PROPERTIES OF OPIATE ANTAGONISTS*

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SUMMARY

A number of opiate antagonists and the dextro isomers of some of these drugs were studied for antagonism of acute opiate effects on ilea isolated from opiate-naive guinea pigs, precipitation of a withdrawal contraction of ilea isolated from morphine-dependent guinea pigs, precipitation of withdrawal in morphine-dependent rhesus monkeys and stereospecific displacement of ^{3}H-etorphine binding to rat-brain membranes. With the exception of d-naloxone, all of the compounds displaced ^{3}H-etorphine. With the exception of d-naloxone, nalorphine, and quaternary nalorphine, all of the antagonists caused a contraction of ilea isolated from morphine-dependent guinea pigs. Moreover, the IC 50 values of the compounds for displacing ^{3}H-etorphine binding were well correlated with both their Ke values for antagonism in the ileum (r = 0.95) and with their EC 50 values for precipitating a contraction in this preparation (r = 0.92). Generally, the concentration of antagonist necessary to precipitate half maximal contracture was 30-fold greater than the Ke value of the antagonist. Most of the opiate antagonists also precipitated withdrawal when administered to morphine-dependent rhesus monkeys and their in vivo potencies were well correlated with their in vitro potencies in ileum (with Ke: r = 0.95; with EC 50: r = 0.99) and in displacing ^{3}H-etorphine (r = 0.95). The quaternary derivative of naltrexone, however, was an effective opiate antagonist only in vitro, and was ineffective in precipitating withdrawal in morphine-dependent rhesus monkeys. These results suggest that the receptor sites labeled by ^{3}H-etorphine are the same as those involved in antagonism of acute opiate actions and in precipitation of withdrawal.

Opiate antagonists can prevent or reverse the direct actions of opiate agonists and can precipitate a withdrawal syndrome in subjects dependent on opiates. The actions of these drugs in antagonizing effects of opiate agonists appear in many respects to involve competition at a single receptor, i.e., dose-effect curves for agonists are shifted in a parallel fashion by appropriate doses of antagonist (e.g., 1, 2). The actions of these drugs in precipitating a withdrawal syndrome, however, may not be due solely to competition at the same receptor at which the agonist acts. Some studies suggest that antagonists produce withdrawal signs directly, when

administered in the presence of a narcotic agonist (3, 4). Additionally, other studies have suggested that it is impossible to protect against antagonist-precipitated withdrawal by pretreatment with an agonist (5; but see, however, 6). Finally, studies of displacement of morphine by naloxone in dependent subjects have yielded equivocal results (7,8).

The present study compared actions of opiate antagonists in precipitating a contraction from ilea isolated from morphine-dependent guinea pigs, antagonizing inhibitory effects of opiate agonists on electrically-stimulated ilea from non-dependent guinea pigs, precipitating withdrawal in morphine-dependent rhesus monkeys, and displacing ^{3}H-etorphine from binding sites. Since the actions of these opiate antagonists in all of the above preparations were well correlated, these comparisons suggested that precipitation of withdrawal is due to actions at sites similar to those involved in antagonism of opiate agonists.

METHODS

The procedures for isolation of rat brain membranes and for assessment of opiate receptor binding have been described previously (9, 10). Male Sprague-Dawley rats weighing 200 g were decapitated and the brains were excised at 4°C. The tissue was washed briefly in 50 ml Tris HCl, pH 7.4, blotted and the cerebrum was dissected and weighed. The tissue was disrupted with 100 parts of cold buffer for 1 min by using a Polytron homogenizer, model PT-10, at output 6.5. The homogenate was centrifuged at 20,000 x g for 15 min in the cold and the obtained pellet was resuspended with the original amount of buffer, using a Dounce all-glass homogenizer. Aliquots of this suspension, sufficient for a set of experiments on one given day, were frozen at -70°C. Before use, the suspension of membranes was quickly thawed and dispersed in a Dounce homogenizer. The protein concentration of the homogenate was determined by the method of Lowry et al. (11).

The opiate receptor assay mixture consisted of 400 ul of the membrane suspension, 50 ul of H$_2$O or NaCl, 50 ul of either dextrorphan, levorphanol, or the drug being investigated, and 25 ul of ^{3}H-etorphine. The final concentrations of NaCl, dextrorphan, levorphanol, and etorphine in the medium were 1.5 x 10^{-1} M, 6 x 20^{-7} M and 3 x 10^{-9} M, respectively. Appropriate dilutions of the drugs were made daily from stock solutions kept either at -20°C (dextrorphan, levorphanol, or test drugs) or at 4°C (^{3}H-etorphine). During pipetting of the assay mixture, the tubes were kept on ice. After addition of NaCl or an appropriate aliquot of water to the membrane suspension, the tubes were incubated for 10 min at 25°C. Subsequently, either dextrorphan, levorphanol, or the test drug was added and the incubation was continued for 15 min, at which time radiolabelled etorphine was added. The tubes were incubated for an additional 30 min, then placed in ice and their contents filtered through Whatman GF/C filters previously washed in H$_2$O. Before filtering the sample, the glass disc was washed on the assembly with 3 x 5 ml of water saturated with n-amyl alcohol. The samples on the filter were quickly washed three times with 4 ml of ice-cold 50 mM Tris-HCl, pH 7.4. Subsequently, the filters were placed in counting vials, 1 ml of absolute ethanol and 10 ml of a dioxane-xylene-napthalene scintillation mixture were added and the vials then subjected to liquid scintillation counting. The counting efficiency was determined by the use of ^{3}H$_2$O as an internal standard.

The binding of ^{3}H-etorphine in the presence of a given drug was related to the maximum stereospecific etorphine binding, obtained as the difference between binding in the presence of an appropriate excess of dextrorphan and

excess of levorphanol. The IC 50 values were obtained graphically from log-probit plots of the binding data. Each drug was investigated at five concentrations, run in duplicates.

Male albino guinea pigs weighing between 300 and 500 g were sacrificed and the terminal portion of the ileum was removed after discarding 10-15 cm closest to the ileocaecal junction. The tissue was placed in a Kreb's physiological solution containing NaCl, 118 mM; KCl, 4.74 mM; $CaCl_2.2H_2O$, 2.54 mM; $MgSO_4.7H_2O$, 1.19 mM; KH_2PO_4, 1.19 mM; glucose, 11 mM; $NaHCO_3$, 25 mM; pyrilamine maleate, 0.125 uM; and hexamethonium bromide, 0.07 mM. The solution was maintained at 37°C and saturated with 95% O_2 - 5% CO_2. Segments of approximately 3 cm in length were tied to the base of a platinum electrode that was inserted through the lumen. The opposite end of the tissue was attached to a Grass FTO3C force displacement transducer. Tissues were stimulated coaxially by an AEL Laboratory Simulator, Model 104 A, with single pulses of 0.1 Hz, 0.5 msec duration and a voltage that was 1.5 times that necessary to elicit a maximum contraction of the tissue. Tension was recorded isometrically on a Grass model 7 polygraph. Tissues were equilibrated at 0.5 g resting tension for at least 30 to 60 min and until the contraction remained stable.

Cumulative concentration-response relationships were determined for morphine in the absence and presence of opiate antagonists by increasing the concentration of morphine by three-fold increments until a maximum inhibition of the contraction was obtained. To determine the opiate antagonist potency for certain drugs, the antagonists were added to the organ bath 5 min before the addition of morphine. The EC 50 values for morphine in the presence and absence of the antagonists were determined by probit analysis. The Ke, or antagonist potency, was determined by the method of Arunlakshana and Schild (12).

To determine the potency for precipitating a withdrawal sign in vitro, ilea were isolated from animals that were injected s.c. with morphine (300 mg/kg) in a slow release suspension of mannide monooleate, mineral oil, and saline (1:6:8), 48 hours before sacrifice. The tissues were equilibrated in the absence of electrical stimulation for 30 min with washes every 10 min. Cumulative concentration-response relationships for the antagonists were determined in these preparations. After washing the preparations, acetylcholine, 5 x 10^{-6} M, was added to the organ bath to determine the maximum contraction of the tissue; effects of antagonists were calculated as a percentage of the response to acetylcholine. The EC 50 values of the antagonists in producing a contraction of ilea isolated from morphine-dependent guinea pigs were calculated by probit analysis.

Groups of rhesus monkeys were trained to routinely receive morphine injections and were maintained on a regular schedule of injections (3 mg/kg/6 hr) that produces dependence in this species (13). After at least three months stabilization on the above schedule, antagonists were studied for precipitation of a withdrawal syndrome. Two hrs after the last morphine injection doses of the antagonist under study were injected, s.c., and the subjects were graded by trained observers for intensity of the withdrawal syndrome precipitated, and for side effects. For further details of the procedure, and details on the grading of the various signs, see Seevers and Deneau (13) and Villarreal (14).

The drugs used included morphine sulfate (Mallinckrodt Chemical Works, St. Louis, MO), naltrexone hydrochloride, naloxone hydrochloride (Endo Laboratories, Garden City, NY), d-naloxone hydrochloride (A.E. Jacobson, NIH), nalorphine hydrochloride (Eli Lilly and Company, Indianapolis, IN), GPA 1843

((-)-N-allyl-alpha-9-methyl-5-phenyl-2-hydroxy-6,7-benzomorphan, CIBA Geigy, Summit, NJ), and WIN 44,441 ((2 ,6 ,11S)-(-)-1-cyclopentyl-5-(1,2,3,4,5,6-hexahydro-8-hydroxy-3,6,11-trimethyl-2,6 methano-3-benzazocin-11-yl-3-penta none methanesulfonate, Sterling Winthrop Laboratories, Rensselaer, NY). Dr. H. Merz of Boehringer Ingelheim Sohn, Federal Republic of Germany provided MR 2266 ((-)-5,9-diethyl-2-(3-furylmethyl)-2'-hydroxy-6,7-benzomorphan), MR 2267 (the (+)-isomer of MR 2266), MR 1452 ((-)-5,9-dimethyl-2-(3-furyl-methyl)-2'-hydroxy-6,7-benzomorphan methanesulfonate), MR 1453 (the (+)-isomer of MR 1452), quaternary naltrexone (naltrexone methobromide), quater-nary naloxone (naloxone methobromide) and quaternary nalorphine (N-diallynor-morphine methobromide). The benzomorphan optical isomers had a resolution purity of greater than 99.7 percent (H. Merz, personal communication). The [15,16(n)-^{3}H] etorphine (specific activity 31 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL).

<div align="center">RESULTS</div>

All of the compounds studied except d-naloxone displaced ^{3}H-etorphine with varying potencies (Table I). d-Naloxone was without effect up to concentrations of 15 uM. The EC 50 values for active compounds ranged from 1.8 to 11,000 nM in the presence of 150 mM NaCl. All of the sodium response ratios were less than 1.0 which is consistent with the antagonist activity of the compounds.

With the exception of d-naloxone, nalorphine, and quaternary nalor-phine, all of the compounds studied precipitated a contraction of the ileum isolated from morphine-dependent guinea pigs. The inactive compounds were tested up to concentrations of 30 uM. The maximum responses produced by the active compounds were similar and ranged between 35 and 50 percent of the contraction produced by adding acetylcholine, 5 x 10^{-6} M, to the organ bath. The EC 50 values for precipitation of a contraction ranged from 17.4 nM (naltrexone) to 6,510 nM (MR 2267).

Like their tertiary analogues, quaternary naltrexone and quaternary naloxone antagonized the agonist actions of morphine on the electrically-stimulated ileum (Table I). Interestingly, while the d-isomer of naloxone was without antagonist or binding activity, the d-isomers of the benzomor-phan antagonists retained antagonist activity, albeit with markedly reduced potency. While it is possible that the activity of these isomers in displac-ing ^{3}H-etorphine might be accounted for by the 0.3% impurity, this cannot account for the activity of the isomers in the guinea pig ileum.

With the exception of quaternary naltrexone, all of the antagonists studied in the morphine-dependent rhesus monkey promptly precipitated a withdrawal syndrome. Quaternary naltrexone was inactive up to doses of 32.0 mg/kg. The d-isomers of the benzomorphans were the least potent, and at high doses produced ataxia; the highest doses (MR 1453: 13, 26 mg/kg; MR 2267: 18, 26 mg/kg, s.c.) produced convulsions. Due to these additional actions, with MR 2267, a maximally graded withdrawal syndrome was not obtained at any of the doses tested. All of the other compounds produced similar maximal effects and had dose-effect curves with similar slopes. Both nalorphine and quaternary nalorphine produced a maximally graded withdrawal, however, both compounds produced pupil dilation, an effect typically produced by narcotic agonists in the rhesus monkey. Pupil dila-tion produced by nalorphine (1.0 mg/kg, s.c.) in nondependent monkeys, however, was not antagonized by naloxone (1.7 mg/kg, s.c.) suggesting that this effect is mediated by non-narcotic mechanisms. There was an approxi-mate 3000-fold range of potencies for precipitation of withdrawal from the most potent compound, naltrexone, to the least potent compound, MR 2267.

TABLE I

Potencies of Narcotic Antagonists in Various Preparations

Compound	IC 50 (nM) in Displacing ^{3}H-Etorpine		Guinea-Pig Ileum		Dose (mg/kg,s.c.) for Precipitating Withdrawal in Morphine-Dependent Monkeys
	+Na	+/-	Ke[1] (nM)	EC 50 (nM)	
Win 44,441	1.8	0.80	0.5 (15)	89.0	0.03
Naltrexone	2.5	0.27	0.4 (16)	17.4	0.004
Cyclazocine	3.5	0.55	1.5 (16)	NT[2]	0.03
MR 2266	5.1	0.68	1.5 (17)	73.4	0.04
Naloxone	9.0	0.29	1.2 (16)	34.0	0.01
Nalorphine	20.0	0.39	4.5 (16)	IA[3]	0.09
MR 1452	20.9	0.67	6.0 (17)	454	0.13
GPA 1843	63.9	0.37	19.8 (17)	355	NT
O-Naltrexone	130.7	0.43	15.0	444	IA[4]
O-Naloxone	252.9	0.41	52.8	879	1.1
O-Nalorphine	950	0.55	37.2 (18)	IA[3]	7.3
MR 2267	2240	0.94	41.0 (17)	6510	10.9
MR 1453	11100	0.50	132.0 (17)	3250	10.6
d-Naloxone	IA[5]	–	IA[3]	IA[3]	NT

[1] Numbers in parentheses following Ke values are the references from which the values were obtained.

[2] NT – not tested

[3] IA – inactive up to 30 uM

[4] IA – inactive up to 32 mg/kg

[5] IA – inactive up to 15 uM

The correlations among the Ke or EC 50 values for actions in the guinea-pig ileum and the IC 50 values for displacement of ^{3}H-etorphine are shown in Figure 1. Values for ^{3}H-etorphine displacement were well correlated with values for Ke (r = 0.95) and EC 50 (r = 0.92), however, the line describing the relationship between ^{3}H-etorphine displacement and precipitation of a contraction in the ileum is approximately one and a half log units to the right of the line describing the relationship between ^{3}H-etorphine displacement and the Ke value. Thus, the EC 50 value of a drug for precipitating a contraction in the guinea-pig ileum was roughly 30-fold greater than its Ke value. The sodium ratio was not well correlated with potency in any of the functional assays of antagonist activity. Coefficients for correlations of sodium ratio with Ke value (r = -0.11), EC 50 value (r = 0.46), and dose producing an intermediate grade of withdrawal in the monkey (r = 0.40), were all less than 0.5. Additionally, since with few exceptions all of the antagonists produce similar maximal effects, sodium ratio was not well correlated with antagonist efficacy.
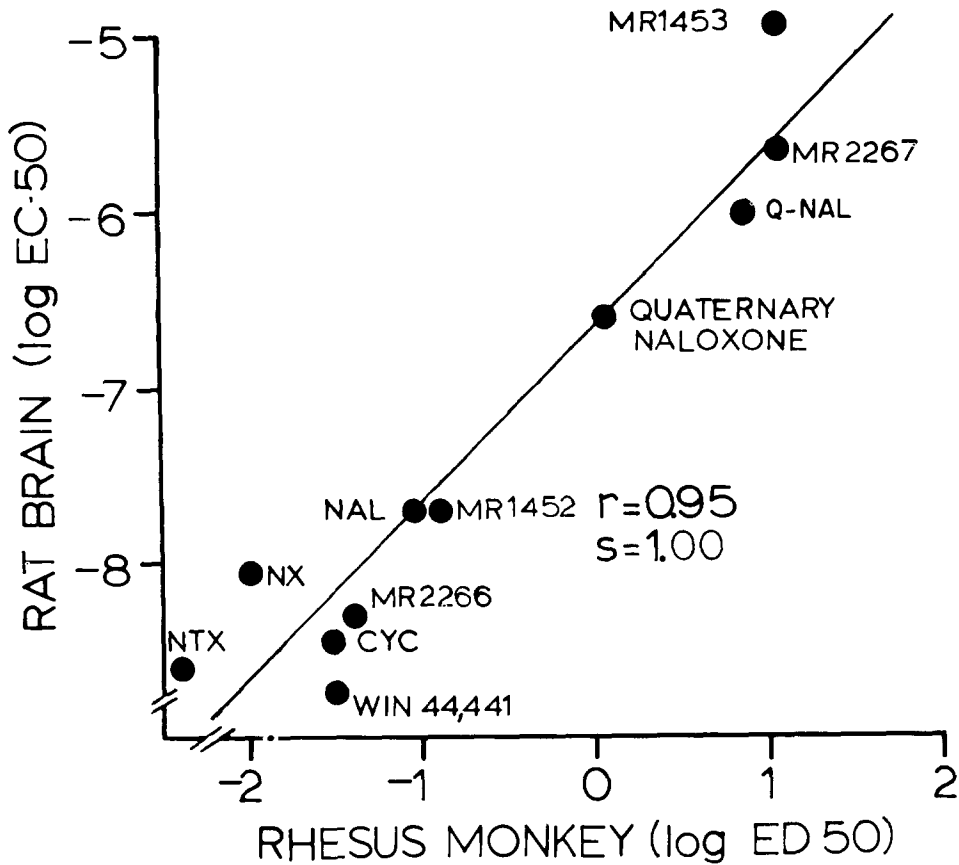
Figure 1.

Relationship between displacement of ^{3}H-etorphine binding in rat brain membranes and antagonist activity in the guinea-pig ileum. Ordinates, log of the EC 50 for the antagonists in the opiate receptor binding assay. Abscissae, log of the Ke (O) for antagonism of the acute opiate effect in the guinea-pig ileum, or log of the EC 50 (O) for precipitation of a withdrawal contraction. Nalorphine, quaternary nalorphine, and d-naloxone were ineffective in precipitating the withdrawal contraction. The correlation coefficients and slopes of the two lines are given in the figure as r and s, respectively. Abbreviations: NTX = naltrexone, NX = naloxone, WIN = WIN 44,441, NAL = nalorphine, GPA = GPA 1843, Q-NTX = quaternary naltrexone, Q-NX = quaternary naloxone, Q-NAL = quaternary nalorphine, CYC = cyclazocine.

Figure 2.

Relationship between displacement of ^{3}H-etorphine binding in rat
brain membranes and precipitation of withdrawal in the morphine-
dependent rhesus monkey. Ordinates, log of the EC 50 for the
antagonists in the opiate receptor binding assay; Abscissae: log
of the dose producing an intermediate amount of withdrawal in
morphine-dependent rhesus monkeys. The correlation coefficient
and slope of the line are given in the figure as r and s, respect-
ively. Abbreviations are the same as those given in Figure 1.

The dose of antagonist necessary to precipitate withdrawal of inter-mediate severity in the monkey was closely correlated with the IC 50 value for displacing ^{3}H-etorphine from rat brain membranes (r = 0.95) as is illustrated in Figure 2. Additionally, the slope of the line describing this relation was close to unity, indicating that changes in potency in displacing ^{3}H-etorphine produced similar changes in potency in precipitating withdrawal in vivo. Finally, the doses precipitating an intermediate grade withdrawal in the monkey were also closely correlated with both the Ke (r = 0.95) and the EC 50 (r = 0.99) values obtained in the guinea-pig ileum preparations (Table I).

DISCUSSION

The physiological, opiate-receptor binding, and behavioral effects of a number of opiate antagonists were compared in order to determine whether precipitation of withdrawal was mediated by the same sites as the antagonism of acute opiate effects. The potencies with which the compounds precipitated a contraction of ilea from morphine-dependent guinea pigs, precipitated a withdrawal syndrome in morphine-dependent rhesus monkeys, and antagonized acute inhibitory effects of morphine in electrically-stimulated ilea, were well correlated with each other, and with the potency with which they displaced ^{3}H-etorphine from rat-brain membranes. Thus, the results suggest that these effects of antagonists are mediated by common binding sites. The present results replicate and extend previous studies of correlation between binding potencies and antagonist actions in guinea-pig ileum (16, 19, 20) and precipitation of withdrawal in morphine-dependent rats (21), as well as correlations of antagonist actions in the monkey and guinea-pig ileum (22). Additionally, these correlations among effects of opiate antagonists are similar to those also reported for opiate agonist actions (19, 22, 23). Close correlations such as these mutually validate each of the procedures as closely related effects having biological relevance.

An earlier study (21) reported some relation between efficacy of an antagonist in precipitating jumping in morphine-dependent rats and the sodium ratio in a binding assay. Interestingly, the compounds with the higher ratios produced a greater maximal effect. In the present study, all but one of the active antagonists produced a maximal grade of withdrawal in the monkey and all produced a similar maximal contraction of the guinea-pig ileum, however, there was a wide range of sodium ratios. Thus, the sodium ratio was not related to efficacy of the antagonists studied. Additionally, it was not related to potency of the antagonists in any of the assays. With the exceptions of nalorphine and cyclazocine, none of the compounds in the present study had appreciable agonist actions. On the basis of the findings by Blasig et al. (21), it is possible that a stronger relation between antagonist activity and sodium ratio would be found by including more antagonists with greater agonist activity. Alternatively, this discrepancy may involve the species or endpoint used in assessing withdrawal.

The correlation between precipitation of withdrawal in the monkey and precipitation of a contraction of the ileum suggests that the guinea-pig ileum can provide a useful model of dependence. A deviation from this correlation, however, was obtained with quaternary naltrexone which was active in vitro but not in vivo. The lack of antagonist activity has also been demonstrated for quaternary naltrexone in the pigeon (24). The differences in potencies in displacement of ^{3}H-etorphine for quaternary naltrexone and naltrexone are from about 36-fold (-Na) to 50-fold (+Na), indicating that the complete lack of activity of the quaternary analogue was

not due simply to a lack of affinity for the receptor. Rather, since charged quaternary analogues are thought to be distributed into the CNS less readily than their tertiary derivatives, the present results are consistent with earlier findings in the rat that indicated that the precipitation of withdrawal is due primarily to a central action of the antagonists (25, 26). In contrast, the quaternary analogues of naloxone and nalorphine were effective in precipitating withdrawal in the rhesus monkey at doses that would be predicted by their in vitro potency. It is not clear why these opiate antagonists were effective in vivo, although they may be distributed differently than quaternary naltrexone or perhaps metabolized to the tertiary analogues in vivo (see 27).

The Ke and EC 50 for precipitation of a contraction of the ileum were well correlated suggesting that precipitation of withdrawal and antagonism of acute morphine effects are intimately related. The EC 50, however, was approximately thirty times the Ke. Since the Ke for an opiate antagonist is similar in ilea isolated from normal and morphine-dependent guinea pigs (28), the presence of an antagonist in a concentration 30 times its Ke would result in a 31 fold shift to the right in the agonist concentration-response curve in either preparation. If the percentage occupancy of receptors by morphine in the dependent preparations were known, it would be possible to determine by how much the occupancy would have to be decreased by an antagonist such that 50% maximal withdrawal would result. The percentage occupancy, however, is difficult to determine and is complicated by the existence of spare receptors and changing numbers of spare receptors during dependence (28).

Nalorphine and its quaternary derivative antagonized the acute action of opiates in the guinea-pig ileum (18). In addition, nalorphine displaced ^{3}H–etorphine from rat–brain membranes and precipitated withdrawal when administered to morphine-dependent rhesus monkeys. Neither nalorphine nor its quaternary derivative, however, precipitated a contraction in the isolated guinea-pig ileum. These antagonists differ from others studied in the ileum for precipitating a contraction in that they also inhibit the electrically-evoked contraction of this preparation and are as efficacious as morphine in this action (18). Moreover, the agonist actions of nalorphine are antagonized by the relatively pure narcotic antagonist, naloxone (2). Thus, nalorphine, and probably its quaternary analogue, have narcotic agonist actions in this preparation. The EC 50 value of nalorphine as a narcotic agonist is 24 nM and its Ke value as an antagonist is 4.5 nM (18). Since the EC 50 for precipitating a contraction of ilea from dependent guinea pigs is about thirty times the Ke value, the concentration of nalorphine that would be necessary to produce a half maximal contraction would be predicted to be about 135 nM. This value is well beyond the EC 50 for the narcotic agonist actions of nalorphine. Thus, it is likely that the agonist actions of nalorphine, and probably quaternary nalorphine, interfere with the precipitation of a contraction of the isolated ileum. Precipitation of a contraction in the guinea-pig ileum, then, requires that an opiate antagonist be present at a concentration about 30-fold greater than its Ke value and lack agonist actions of its own at that concentration.

ACKNOWLEDGEMENTS

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