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Interaction of Loperamide with [^{3}H]Naloxone Binding Sites in Guinea Pig Brain and Myenteric Plexus

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SUMMARY

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The effects of morphine, naloxone, and loperamide on the binding of [^{3}H]naloxone to the opiate receptor site(s) in guinea pig brain and myenteric plexus homogenates in the presence of sodium were studied. Results obtained from morphine and naloxone binding studies were consistent with the hypothesis of a single population of receptor sites. Scatchard plots prepared from the data for loperamide binding, however, indicated two possible populations of binding sites, only one of which was readily accessible to loperamide. Loperamide- and morphine-induced inhibition of the electrically stimulated contracting guinea pig ileal longitudinal muscle indicated only one site of action. The results of these experiments suggest that loperamide is bound stereospecifically to two populations of binding sites, both of which may be involved in producing opiate-like pharmacological effects in the longitudinal muscle preparation.

INTRODUCTION

Our laboratory has recently reported (1) on the binding of loperamide, an antidiarrheal agent, to opiate receptors in the brain and gut of the guinea pig. We showed that the displacement of [^{3}H]naloxone from the opiate receptor by loperamide differed significantly from that observed with other agonists. Hill plots of the data indicated that the binding of loperamide to [^{3}H]naloxone binding sites was anticooperative. The present report describes the results of experiments which were designed to investigate further the interaction of loperamide with [^{3}H]naloxone binding sites.

METHODS

[^{3}H]Naloxone binding in brain. Albino guinea pigs of either sex, weighing 500–600 g (Scientific Small Animals, Arlington Heights, Il.), were used. The animals were killed by cervical dislocation or decapitation, their brains were quickly removed, and the cerebella were discarded. The remainder of the brains was homogenized in Krebs-Tris buffer (2). Opiate binding activity of the homogenates was measured by the methods of Pert and Snyder (3) and Creese and Snyder (2).

[^{3}H]Naloxone binding in myenteric plexus. Albino guinea pigs of either sex, weighing 500–600 g, were killed by decapitation and the small intestine was quickly removed. The terminal 5–10 cm of ileum and the duodenum were discarded. The remainder of the intestine was flushed out and cut into 15-cm segments which were rinsed in 0.9% NaCl. Longitudinal muscle strips were obtained by the method of Rang (4), placed in Krebs-Ringer-bicarbonate solution with glucose added (5) at

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23°, and gassed with 95% O_2–5% CO_2. Muscle strips from 10–50 guinea pigs were collected, blotted, weighed, and placed in a beaker of ice-cold Krebs-Tris buffer (2), at a concentration of 1 g of tissue per 20 ml of buffer. The tissue was very finely minced with scissors and subjected to 2 min of sonication (7.0 g of sonic pressure; measured with the 0.5-in. disruptor horn immersed 1.0 in. into a 250-ml beaker containing 150 ml of distilled water). The resulting suspension was centrifuged for 10 min at 600 × g at 4°, the pellets were discarded, and the supernatants were recentrifuged at 49,000 × *g* at 4°. The supernatants of this second centrifugation were discarded, and the pellets were resuspended in Krebs-Tris buffer by means of a hand-held ground glass tissue grinder so that residue from 1 g of longitudinal muscle was contained in 10 ml. This final homogenate was divided into 50-ml aliquots and stored at −70°. Opiate antagonist and agonist binding activity of the homogenate was not altered by freezing and subsequent storage during the course of the experiments (4 weeks), but repeated freezing and thawing caused a marked loss of activity.

Binding activity was measured by the methods of Pert and Snyder (3) and Creese and Snyder (2). Aliquots of homogenate and buffer (final volume, 2 ml) were incubated at 37° for 10 min in the presence of levorphanol, dextrorphan, or unlabeled drugs at several concentrations, after which [^{3}H]naloxone was added and incubation was continued for an additional 20 min. After incubation samples were cooled on ice, filtered through Whatman GF-B or GF-C glass fiber filters under reduced pressure, and washed twice with 8 ml of ice-cold buffer. Tissue-bound [^{3}H]naloxone was solubilized with "NCS" tissue solubilizer (Amersham/Searle) and counted in "PCS" scintillation fluid (Amersham/Searle). Specific binding was determined as described by Pert and Snyder (3).

Electrically induced contractions of longitudinal muscle. Longitudinal muscle segments from guinea pig ileum with myenteric plexus attached were obtained by the method of Rang (4), mounted vertically in 3-ml organ baths containing Krebs-Ringer-bicarbonate solution with glucose added (5) at 37°, and gassed with 95% O_2–5% CO_2. Isotonic contractions were measured by means of a lever system connected to an isotonic myograph transducer (Physiograph MKII, E and M Instrument Company, Houston); the signal was amplified and recorded on a physiograph (E and M Instrument Company). Resting tension was adjusted to 200–300 mg, and contractions were evoked by electrical stimulation (2). Square-wave monophasic pulses of 5-msec duration at rates of 20–25 pulses/min were applied at supermaximal voltage through electrodes positioned at the top and bottom of the baths. Muscle strips were allowed to equilibrate under continuous stimulation with intermittent washes until constant contractile amplitude was obtained. Drugs were added during stimulation and were allowed to remain in contact with the tissue until contractions were maximally inhibited. The tissues were washed after a drug response was obtained, and contractility was allowed to return to baseline before another addition was made.

Drugs. Drugs were obtained from the following sources: Hoffmann-La Roche (levorphanol), Janssen Pharmaceutica (loperamide), Endo Laboratories (naloxone), and Mallinckrodt Chemical Works (morphine sulfate). [^{3}H]Naloxone (23.6 Ci/mmole) was purchased from New England Nuclear.

RESULTS

Effects of morphine, loperamide, and naloxone on [^{3}H]naloxone binding to brain homogenates. Previous results from our laboratory (1) have shown that loperamide binds to the guinea pig brain and gut opiate receptor, but in a markedly different manner from that reported for other opiates. In order to determine the nature of this binding, loperamide, morphine, and naloxone were studied for their effects on the specific binding of 8 nM [^{3}H]naloxone to guinea pig homogenates (Fig. 1). Both morphine and naloxone inhibited [^{3}H]naloxone binding in a parallel and linear dose-dependent manner. With loperamide,

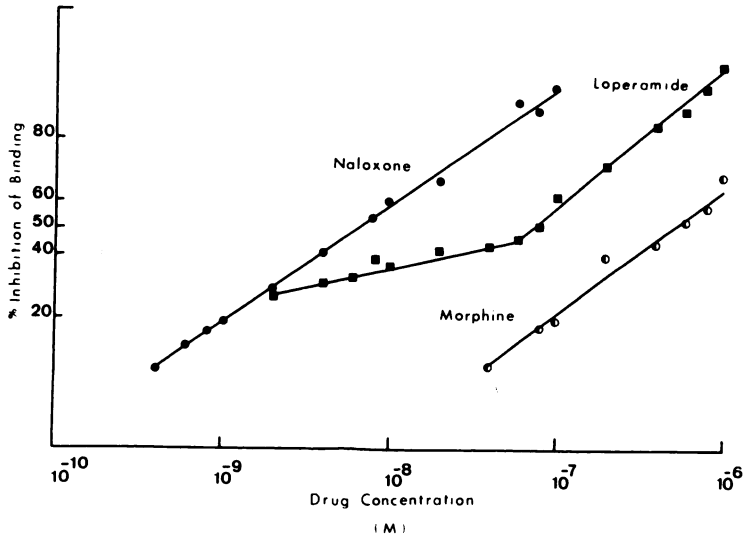

FIG. 1. *Inhibition of [3H]naloxone binding to homogenates of guinea pig brain by morphine, naloxone, and loperamide*

Homogenates of brain in Krebs-Tris buffer (pH 7.4) were incubated for 10 min at 37° with various concentrations of loperamide (■), morphine (◐), or naloxone (●) in the presence and absence of 1 μM levorphanol or dextrorphan. After cooling and the addition of 8 nM 3H-naloxone, incubation was continued for an additional 20 min and binding was assayed as described in METHODS. Varying the incubation time from 10 to 40 min (data not shown) had no significant effect on the displacement of [3H]naloxone by loperamide. In a typical experiment, approximately 2200 cpm of [3H]naloxone were bound to brain homogenates, while about 650 cpm of [3H]naloxone were bound to brain homogenates in the presence of 1 μM levorphanol. Dextrorphan had no significant effect on specific or nonspecific binding in our system. Also, neither morphine nor loperamide altered nonspecific binding in our assay procedures. The data are plotted on log probit paper.

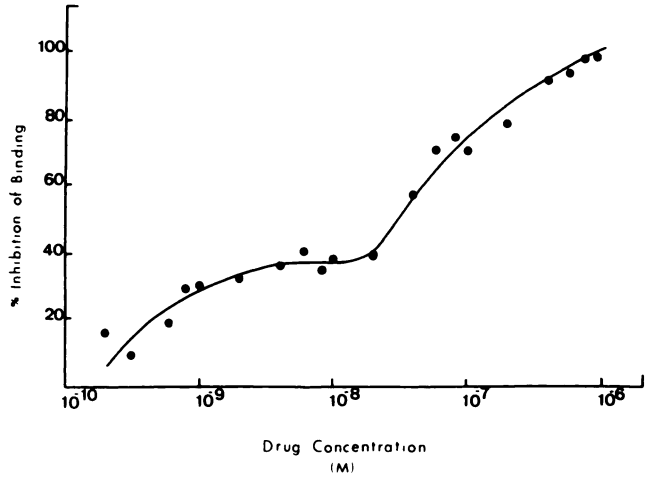

FIG. 2. *Inhibition of [3H]naloxone binding to homogenates of guinea pig brain by loperamide*

The experimental procedure is described in the legend to Fig. 1 and METHODS. The data are plotted on semilog paper.

displacement was dose-dependent, but the curve derived from the displacement data is composed of two distinct portions, possibly describing low- and high-affinity binding sites, as shown also in Fig. 2.

In order to determine whether more

than one binding site is involved in loperamide binding, Scatchard plots were made from these data. The basic assumption made in applying the Scatchard equation (6) in our studies was that the drug bound was considered to be equivalent to the [^{3}H]naloxone displaced. Therefore the ratio of percentage of [^{3}H]naloxone displaced to drug concentration was plotted on the ordinate, and the percentage of [^{3}H]naloxone displaced was plotted on the abscissa. Scatchard plots prepared from the data in Fig. 1 are shown in Fig. 3. These plots yielded straight lines for the binding of morphine and naloxone. However, the plot for loperamide binding had two distinct slopes, indicating that at least two populations of loperamide binding sites exist in brain homogenate. The K_D values calculated for the binding of naloxone, morphine, and loperamide to common sites are shown in Table 1.

Effects of morphine, loperamide, and naloxone on [^{3}H]naloxone binding to myenteric plexus homogenates. The inhibition of binding of 2.5 nM [^{3}H]naloxone produced by loperamide, morphine, and naloxone was studied in homogenates of guinea pig myenteric plexus (Fig. 4). Loperamide was displaced by [^{3}H]naloxone

TABLE 1

Binding affinities and biological activity

Binding affinities are the K_D values obtained from binding studies with guinea pig brain and myenteric plexus homogenates in the presence of sodium ion. Biological activity (ID_{50}) refers to the concentrations necessary to inhibit contractions of electrically stimulated longitudinal muscle by 50%. The K_D value calculated for the interaction of naloxone with binding sites associated with the contracting muscle was between 0.98 and 1.85 nM. Naloxone binding affinities were determined in the presence of 8 nM [^{3}H]naloxone in brain homogenates and 2.5 nM [^{3}H]naloxone in myenteric plexus homogenates. As can be seen from the displacement curve (Fig. 1), 50% of the [^{3}H]naloxone is displaced by 8 nM unlabeled naloxone in brain; K_D values were determined from the data shown in Figs. 3 and 5. K_D values previously determined for [^{3}H]naloxone binding (1) were 0.478 nM for brain and 1.27 nM for myenteric plexus in the presence of sodium.

Drug	Binding affinity (K_D)		Biological activity (ID_{50})
	Brain	Myenteric plexus	
	nM	*nM*	*nM*
Naloxone	0.438	1.27	
Morphine	37.3	119	140
Loperamide			
K_{D_1}	0.1	1.0	7.4
K_{D_2}	13.6	120	

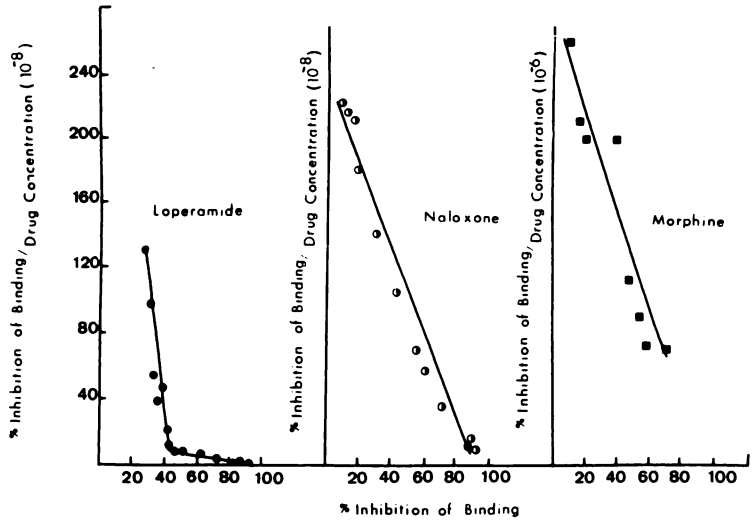

FIG. 3. *Scatchard plots of data obtained for inhibition of [^{3}H]naloxone binding to homogenates of guinea pig brains*

The experimental procedure is described in the legend to Fig. 1 and METHODS. In the absence of a competing substrate for the receptor, [^{3}H]naloxone (8 nM) was bound to the receptor at 60 pmoles/mg of brain.

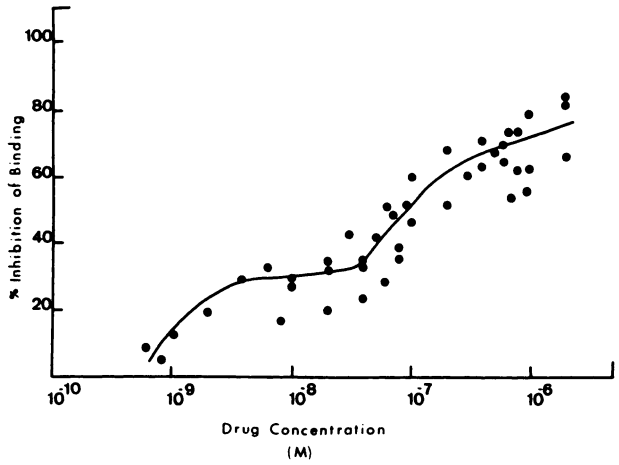

FIG. 4. *Inhibition of [^{3}H]naloxone binding to homogenates of guinea pig myenteric plexus by loperamide*
The experimental procedure is described in METHODS. Homogenates were incubated with various concentrations of loperamide in the presence and absence of 1 μM levorphanol or dextrorphan, and the inhibition of specific binding of 2.5 nM [^{3}H]naloxone was determined. The data are plotted on semilog paper.

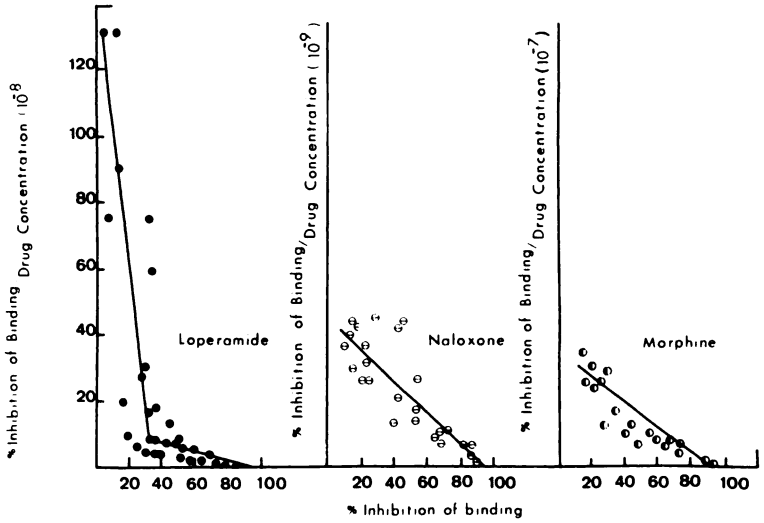

FIG. 5. *Scatchard plots of data obtained for inhibition of [^{3}H]naloxone binding to homogenates of guinea pig myenteric plexus*
The experimental procedure is described in the legend to Fig. 3 and METHODS. In the absence of a competing substrate for the receptor, [^{3}H]naloxone (2.5 nM) was bound to the receptor at 0.47 pmoles/mg of longitudinal muscle.

in a dose-dependent manner, but the response was not linear and was composed of two distinct portions. Figure 5 shows the Scatchard plots derived from the data obtained with morphine, naloxone, and loperamide. As was found with the brain homogenates, morphine and naloxone were displaced by [^{3}H]naloxone in a dose-dependent manner, and the Scatchard plots obtained for both drugs are straight lines. However, the Scatchard plot for loperamide indicates that loperamide binds to at least two distinct populations of binding sites. The K_D values obtained for naloxone, morphine, and loperamide binding to common sites are shown in Table 1.

Effects of morphine and loperamide on electrically induced contractions of guinea

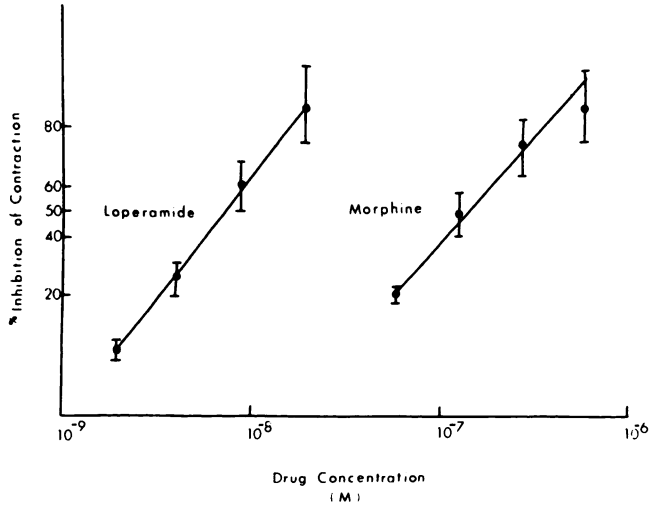

FIG. 6. *Inhibitory effect of morphine and loperamide on electrically stimulated contractions of longitudinal muscle from guinea pig ileum*

Drugs were administered at the indicated molar concentrations.

pig ileum. Dose-response curves demonstrating the inhibitory effects of morphine and loperamide on electrically induced contractions of the guinea pig ileum are shown in Fig. 6. The two-phase dose-response curve obtained with loperamide in the [^{3}H]naloxone binding studies was not apparent in the contracting longitudinal muscle preparation. The dose-response curves for morphine and loperamide are parallel. It has been reported previously (1) that naloxone shifted both morphine and loperamide curves in a parallel fashion toward higher drug concentrations.

Effects of varying opiate receptor density on loperamide binding to guinea pig brain homogenates. The inhibition of [^{3}H]naloxone binding produced by loperamide was studied in guinea pig brain homogenates containing varying densities of opiate receptor sites. Using the cerebellum, a tissue known to contain few opiate receptors (3), brain homogenates were adjusted to vary the density of opiate receptors per milligram of tissue. All assays were then carried out as described in METHODS. For these experiments, [^{3}H]naloxone (19.1 Ci/mmole, New England Nuclear) was used at a concentration of 2.5 nM. Decreasing the opiate receptor density did not significantly alter either low- or high-affinity binding. At a 1:1 dilution of whole brain minus cerebellum and cerebellum, the high-affinity binding data in these experiments were similar for both tissue homogenates, and the apparent K_{D_1} value was 0.46 nM. The K_{D_2} for the whole brain minus cerebellum preparations and for the 1:1 mixture of brain and cerebellum was 240 nM.

DISCUSSION

Several laboratories (3, 7, 8) have established the existence of a population of opiate receptor sites in neural tissue, and have shown that these receptors bind opiate agonists and antagonists with stereospecific selectivity. It has not been established, however, whether this receptor population is homogeneous. Currently the opiate receptor is thought to exist in two interconvertible forms (9, 10) that will bind opiate agonists and antagonists. Sodium ion, which markedly alters the affinity of the receptor for agonists and antagonists (11, 12), functions as an allosteric effector by increasing the affinity of the receptor for antagonists and decreasing receptor affinity for agonists. Most of the evidence presented to data supports the concept of a single opiate receptor. Recently Lee *et al.* (13) suggested that naloxone has two types of binding sites, one of which is not available to dihydromor-

phine. We now report the existence of two binding sites for the narcotic antidiarrheal loperamide in both the guinea pig brain and myenteric plexus homogenate.

In a previous study with loperamide (1), we reported that this drug binds competitively to the opiate receptor and has a decreased affinity for the receptor in the presence of sodium ion. The binding curves obtained, however, did not correlate well with data obtained from the electrically induced contracting guinea pig ileum. In the present study, binding experiments were carried out in the presence of sodium ion in order to compare the K_D values with those obtained in the intact myenteric plexus.

The results of binding studies with loperamide in both the brain and gut homogenates suggest that at least two populations of binding sites exist in these tissues. However, loperamide is displaced readily from only a portion of the total receptor sites available to [3H]naloxone. We have obtained this singularly characteristic binding pattern for several compounds displaying antidiarrheal characteristics, but varying in physical properties and chemical structure.[1]

In an attempt to determine whether the biphasic binding curve obtained for loperamide represents a transition in binding to two populations of binding sites, or whether one of the sites represents antifactual binding to other cellular constituents, loperamide binding to brain homogenates containing various densities of opiate receptor sites was studied. Opiate receptor density was varied by mixing cerebellum, known to contain few opiate receptor sites (3), with whole brain minus the cerebellum. No significant differences in binding affinities were detected with various concentrations of cerebellum added to the homogenate. K_D values for both high- and low-affinity binding in normal homogenate were in close agreement with those obtained for a 1:1 mixture of cerebellum and whole brain minus cerebellum. Preliminary experiments using highly purified brain homogenate membrane fractions have also given results very similar to those obtained in crude brain homogenates. Similarly, the myenteric plexus receptor preparation described here was highly purified, yet gave results almost identical with those obtained with the crude brain homogenate. It would appear, then, that loperamide binds with stereospecific selectivity to two populations of sites, and that neither altering opiate receptor density nor purification of receptor preparations significantly alters this binding pattern.

The data obtained from the electrically stimulated guinea pig ileum preparation did not display the complex binding pattern noted with the tissue homogenate binding studies. However, variations in individual tissue responsiveness were large enough so that we cannot state with any degree of certainty whether any complex interactions actually occur. Within the variation of experimental technique, dose-response curves for both morphine and loperamide were parallel, and naloxone blocked the inhibition caused by morphine and loperamide, shifting the curves in a parallel manner toward higher drug concentration (9). The ID_{50} for loperamide in this longitudinal muscle preparation fell between the K_D values obtained for low- and high-affinity binding in the guinea pig myenteric plexus homogenate binding studies, suggesting that both binding sites may be involved in producing the opiate-like pharmacological effects noted here.

It is not possible to speculate on the pharmacological relevance *in vivo* of the complex binding we have described *in vitro*. However, it is of interest that we have seen this complex binding only with antidiarrheal drugs with pharmacological properties similar to those of loperamide. These drugs do enter the brain (14) but appear to elicit only low levels of opiate-like activity beyond their antidiarrheal characteristics.

Further work in this area must be conducted using loperamide itself as the radiolabeled ligand, since the indirect method employed here allows us only to approximate K_D values, particularly in the

[1] G. A. Clay and C. R. Mackerer, unpublished observations.

high-affinity portion of the binding curve. Further resolution of the high- and low-affinity binding characteristics of these agents may provide us with a greater understanding of their unique separations of properties.

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