Multiple opiate receptors in the guinea pig enteric nervous system: Unmasking the copresence of receptor subtypes

(oPIOIDS/MYENTERIC PLEXUS/µ, δ, and κ receptors)

ALAN R. GINTZLER AND DAVID HYDE

Departments of Biochemistry and Psychiatry, Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203

Communicated by Chandler McC. Brooks, December 19, 1983

ABSTRACT Experiments were performed in order to obtain physiological evidence for the presence of δ opioid receptors in the guinea pig isolated ileum. The nonequilibrium narcotic antagonists β-chlornaltrexamine (β-CNA) and β-funaltrexamine (β-FNA) differentially affected inhibitory responses to µ- and δ-specific opiate ligands (g value). These results indicate that these agonists do not act through identical populations of receptor in the ileum. In agreement with the similarity of the naloxone Ks values (apparent naloxone dissociation constant determined physiologically). Moreover, elimination of myenteric µ receptors resulted in a 10-fold increase in the naloxone Ks for the highly selective δ agonist Tyr-d-Ser-Gly-Phe-Leu-Thr, suggesting the involvement of a second receptor subtype. It is suggested that this receptor is δ in nature.

A variety of experimental approaches have suggested the existence of a multiplicity of classes of opioid receptor in the central and peripheral nervous system that have been classified as µ, δ, and κ (1, 2). Although it is well established that the guinea pig myenteric plexus contains the µ and κ classes, the presence and function of δ opioid receptors in this nervous system is controversial. In vitro radioligand binding experiments indicated the presence of discrete δ binding sites in the guinea pig myenteric plexus (3). Consistent with these binding data were the observations from our laboratory (4) that the guinea pig ileum manifests differential tolerance to the inhibitory effects of agonists that bind differentially to the µ and δ receptors (µ and δ agonists), suggesting that, at least in tolerant/dependent preparations, the inhibitory effects of these two classes of opioid agonist are mediated through distinct populations of receptor. However, both of these observations are in striking contrast to data obtained from naive ilea, where the naloxone Ks (apparent naloxone dissociation constant determined physiologically) for µ and δ agonists are almost identical (2, 3), indicating that in these preparations µ and δ agonists act through the same class of receptor, the µ receptor.

One possible explanation for this discrepancy is that, in the guinea pig ileum, several classes of opioid receptor such as µ and κ are associated with the same function, inhibition of release of acetylcholine. Because most receptor-specific agonists do have considerable crossreactivity with one or more of the other classes, the naloxone Ks for a receptor-specific ligand might indicate an interaction with only the class of receptor that is present in largest numbers or that is most efficiently coupled to the process being regulated. Consequently the presence of one class of receptor may prevent the demonstration of the copresence of others, resulting in the masking of differential naloxone sensitivity. The present experiments were performed in order to clarify the apparent discrepancies concerning myenteric δ receptors and to determine whether the presence of functional δ receptors in naive ilea are obscured by the copresence of the µ subtype.

MATERIALS AND METHODS

Bioassay for Opioids. An intact segment of guinea pig small intestine was secured in a 3.5-ml jacketed organ bath containing Krebs buffer that was maintained at 37°C and bubbled continuously with 95% O2/5% CO2 (4). With resting tension fixed at 0.5 g, the ileum was stimulated by transmural electrical stimulation with platinum electrodes (4).

Isometric contractions of the preparation were recorded on a Brush polygraph with a Grass FT-03 transducer.

Calculation of g Value. The g value was derived by using the equation 1/A = 1/qA' + 1/qAKA, in which A and A' are equieffective concentrations of the agonist before and after inactivation of a fraction of the population of receptor (q) and Ks is the apparent dissociation constant (5). This equation predicts that a plot of 1/A vs. 1/A' should give a straight line for which the slope is 1/q (5).

Dose–response curves to morphine and [D-Ala², D-Leu⁵]enkephalin (DADLE) or normorphine and Tyr-d-Ser-Gly-Phe-Leu-Thr ([D-Ser², Leu⁵]-enkephalin-Thr; DSLET) were constructed in parallel on contiguus segments of intestine before and after exposure to the nonequilibrium opioid antagonists β-chlornaltrexamine (β-CNA) at 40 nM for 20 min (6, 7) or β-funaltrexamine (β-FNA) at 1 µM for 30 min (8–11), respectively. The concentration of β-CNA and β-FNA were chosen such that the maximal response to each opioid agonist was depressed between 20% and 80% (5). From each pair of curves, A and A' were obtained, the reciprocals of which were plotted against each other. These data were analyzed by linear regression, and q was obtained by calculating the inverse of the slope. In all cases, the coefficient of linearity was 0.95 or greater.

Calculation of Naloxone Ks. Concentrations of agonist producing a 50% inhibition (IC₅₀) of the 0.1-Hz contraction were determined by testing at three or more concentrations giving 20–80% inhibition and interpolating by log-linear regression analysis. The apparent naloxone dissociation constant, Ks, was calculated from the equation Ks = C/(DR − 1) (12) in which the concentration (C) of naloxone is 10 nM and DR is the ratio of IC₅₀ values for the agonist in the presence and absence of antagonist. In order to obtain preparations that were functionally devoid of µ receptors, segments of ileum were treated with the µ-receptor-selective nonequilibrium antagonist β-FNA (1 µM for 60 min) as described (11) after which dose–response curves to the above agonists were constructed. The naloxone Ks was determined as described above except that, because of the diminished responsiveness to µ-receptor (normorphine) and δ-receptor (DSLET) ago-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DADLE, [D-Ala², D-Leu⁵]enkephalin; DSLET, [D-Ser², Leu⁵]enkephalin-Thr; β-CNA, β-chlornaltrexamine; β-FNA, β-funaltrexamine; naloxone Ks, apparent naloxone dissociation constant.

2252
Table 1. q value after exposure to β-CNA

<table>
<thead>
<tr>
<th>Agonist</th>
<th>q</th>
<th>% depression of maximum response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>0.12 ± 0.02 (3)*</td>
<td>72.0 ± 4.9</td>
</tr>
<tr>
<td>DADLE</td>
<td>0.43 ± 0.06 (3)</td>
<td>34.7 ± 10.2</td>
</tr>
</tbody>
</table>

Dose–response curves to morphine and DADLE were constructed in parallel on contiguous segments of intestine before and after exposure to β-CNA at 40 nM for 20 min from which q values were calculated as described. Numbers in parentheses are the numbers of experiments.

*P < 0.05 vs. DADLE.

RESULTS

Differential Sensitivity of Receptor-Subtype-Specific Ligands to β-CNA and β-FNA. The q value indicates the percentage of receptors remaining functional after irreversible inactivation provided the agonist interacts with a single homogeneous population of receptor (5). In the case of agonists that interact with a heterogeneous population of receptor, differences in their respective q values would reflect differential inactivation of the receptor types through which each ligand acts (5). Accordingly, populations of receptor mediating responses to μ (morphine) and δ (DADLE) agonists were compared on the basis of their reactivity with the nonequilibrium antagonist β-CNA by calculating their respective q values (Table 1). In addition, the q values for the somewhat more selective ligands normorphine and DSLET (14) were calculated and compared after exposure to the μ-receptor-selective nonequilibrium antagonist β-FNA (Table 2). Exposure of segments of intestine to 40 nM β-CNA resulted in a differential inactivation of the receptors mediating responses to morphine and DADLE (Table 1). Similar results were obtained for normorphine and DSLET in ilea treated with 1 μM β-FNA (Table 2). In both cases the q values obtained for each μ-receptor-prefering ligand differed significantly (P < 0.05) from that obtained for the corresponding δ specific agonist that was determined in parallel. This indicates that, despite the similarity of the naloxone K<sub>e</sub> for morphine, normorphine, DADLE, and DSLET (see Table 3), the population(s) of receptor mediating responses to these μ- and δ-receptor-preferring agonists are not identical (i.e., they can be distinguished on the basis of their sensitivity to β-CNA and β-FNA).

Effect of Exposure to β-FNA on the DSLET Naloxone K<sub>e</sub>. It is well established that ligands such as DADLE and DSLET have considerable crossreactivity with μ receptors. Thus, it seemed plausible that the presence of μ receptors might obscure the demonstration of functional δ receptors in naïve ilea, resulting in similar naloxone K<sub>e</sub> values for μ- and δ-receptor-prefering ligands. In order to investigate this possibility, responses to DSLET were analyzed in ilea functional-

Table 2. q value after exposure to β-FNA

<table>
<thead>
<tr>
<th>Agonist</th>
<th>q</th>
<th>% depression of maximum response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normorphine</td>
<td>0.24 ± 0.05 (7)*</td>
<td>47.9 ± 3.2</td>
</tr>
<tr>
<td>DSLET</td>
<td>0.1 ± 0.04 (7)</td>
<td>56.0 ± 3.7</td>
</tr>
</tbody>
</table>

Dose–response curves to normorphine and DSLET were constructed in parallel on contiguous segments of intestine before and after exposure to β-FNA at 1 μM for 30 min from which q values were calculated as described. Values in parentheses are the numbers of experiments.

*P < 0.05 vs. DSLET.

Table 3. Naloxone K<sub>e</sub> values before and after elimination of μ receptors

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Before exposure</th>
<th>After exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSLET</td>
<td>1.1 ± 0.3 (3)</td>
<td>9 ± 1.6 (5)</td>
</tr>
<tr>
<td>Normorphine</td>
<td>3.3 ± 0.6 (3)</td>
<td>16 ± 0.5 (4)</td>
</tr>
<tr>
<td>EKC</td>
<td>18 ± 0.3 (3)</td>
<td>16 ± 0.1 (3)</td>
</tr>
</tbody>
</table>

The apparent naloxone K<sub>e</sub> for the various agonists in untreated preparations was calculated from the equation K<sub>e</sub> = C/(DR – 1) in which the concentration (C) of naloxone is 10 nM and DR is the ratio of K<sub>e</sub> values for the agonist in the presence and absence of antagonist. Segments of ileum were treated with 1 μM β-FNA for 60 min as described (11) after which dose–response curves to the above agonists were constructed. The naloxone K<sub>e</sub> was determined as described above except that normorphine and DSLET concentrations producing a 10–30% inhibition were used. EKC, ethylketocyclazocine.

Fig. 1. Dose–response relationship to DSLET before and after β-FNA treatment. Dose–response curves to DSLET were constructed in untreated preparations (●) and in preparations that had been pretreated with 1 μM β-FNA for 60 min (○). Each response is the mean ± SEM of a minimum of five experiments. Exposure to β-FNA significantly attenuated but did not abolish inhibitory responses to DSLET.


**Fig. 2.** Effect of naloxone on β-FNA-resistant responses to DSLET. Preparations were exposed to 1 μM β-FNA for 60 min after which a dose–response curve to DSLET was constructed in the absence (•) and presence (○) of naloxone (10 nM). Naloxone produced a parallel shift to the right in the β-FNA-resistant dose–response curve to DSLET from which a naloxone $K_e$ value could be calculated.

κ agonist ethylketocyclazocine. After elimination of myenteric μ receptors, the naloxone $K_e$ for DSLET increased by approximately 1 order of magnitude. Thus, once myenteric μ receptors have been inactivated, one can directly discern the involvement of another class of receptor through which DSLET can inhibit 0.1-Hz ileal contractions. As reported previously (11), exposure to β-FNA also changed the naloxone $K_e$ for normorphine, but it did not alter the naloxone sensitivity to ethylketocyclazocine, a κ-receptor-prefering agonist (Table 3).

**DISCUSSION**

These results indicate that naloxone $K_e$ values by themselves are not sufficient criteria with which to conclude that a class of opioid receptor is not present in a particular tissue or associated with a particular function. Although the naloxone $K_e$ for μ- and δ-receptor-prefering agonists are virtually identical, the receptor populations mediating responses to each class of agonist can be distinguished on the basis of their reactivity with β-CNA or β-FNA (q value; Tables 1 and 2). Because occupation of several different classes of myenteric opioid receptor can produce similar physiological effects, the presence of one kind may obscure the demonstration of the others. Thus, it is necessary to eliminate myenteric μ receptors before one can discern the involvement of the other class through which DSLET can inhibit ileal contractions. Pretreatment for 1 hr with β-FNA at 1 μM substantially attenuates but does not abolish responses to DSLET. Moreover, the naloxone $K_e$ of the response remaining is approximately an order of magnitude higher than that observed in untreated ilea. Therefore, elimination of the μ receptor facilitates the demonstration of a second receptor through which DSLET inhibits 0.1-Hz contractions.

The precise nature of the class of opioid receptor mediating β-FNA-resistant responses to DSLET has not been unequivocally established because both δ and κ receptors remain functional after exposure to β-FNA (11). However, the extremely small crossreactivity between δ-receptor-prefering agonists (DADLE and DSLET) and the κ class strongly suggests, but does not prove, that the β-FNA-resistant inhibitory response to DSLET is mediated through myenteric δ receptors.

The authors would like to express their appreciation to Drs. Portoghese and Takemori for their generous gift of β-FNA, Dr. Blume for his generosity in supplying β-CNA, and Dr. Baron for her assistance in the preparation of this manuscript. This work was supported in part by National Institute on Drug Abuse Grant DA02893.