Subsensitivity to Opioids Is Receptor-Specific in Isolated Guinea Pig Ileum and Mouse Vas Deferens after Obstructive Cholestasis

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ABSTRACT

The rate and degree of subsensitivity development to morphine (μ-opioid receptor, preferred, but not selective agonist) and U50488H (highly selective κ-opioid receptor agonist) were assessed in vitro on guinea pig ileum (GPI) of cholestatic animals 2, 5, and 7 days after bile duct ligation. In addition to this phenomenon of morphine, the effects of U50488H and SNC 80 (highly selective δ-opioid receptor agonist) were studied in vitro on mice vas deferens (MVD) of cholestatic animals 2, 5, and 7, 10, and 15 days after bile duct ligation. The IC50 for each compound was determined in these preparations. The ratio of the IC50 in bile duct-ligated animals to sham and control animals provides a quantitative index for the degree of subsensitivity development to each agonist. For any given time, the highest degree of subsensitivity to morphine was observed in GPI of cholestatic animals, whereas in MVD obtained from the cholestatic animals, the highest degree of subsensitivity developed to inhibitory effect of SNC 80. The subsensitivity development in cholestatic animals was time dependent; in GPI the maximum subsensitivity developed after 7 days of the operation, whereas the maximum subsensitivity in MVD developed 15 days after bile duct ligation. Moreover, subsensitivity to exogenous acetylcholine and norepinephrine in GPI and MVD, respectively, did not develop in the presence of subsensitivity to opioids in cholestatic animals. Significant accumulation of endogenous opioids in plasma of cholestatic animals has been shown in several studies and this may account for a significant development of subsensitivity to inhibitory effects of opioid agonists.

Endogenous opioids are known to circulate in low levels in the plasma of many mammals, including humans and rats (Clement-Jones et al., 1980; Swain et al., 1992). Many researchers have documented a marked elevation of endogenous opioid levels in plasma of animals models of acute cholestasis (Bergasa et al., 1992, 1994; Swain et al., 1992, 1994). Also, it has been suggested that endogenous opioids are implicated in the pathophysiology of cholestasis (Jones and Bergasa, 1990; Dehpour et al., 1999). Observations compatible with this hypothesis include precipitation of an opioid withdrawal-like syndrome in patients with chronic cholestatic liver disease by administration of an opioid antagonist (Thornton and Losowsky, 1988a) and a global down-regulation of μ-opioid receptors in the brain of rats with cholestasis due to bile duct resection (Bergasa et al., 1992). The opioid peptides have multiple actions that are exerted via at least three classes of receptors: μ, κ, and δ (Imura et al., 1985). It is well established that chronic administration of an opioid agonist such as morphine invariably leads to tolerance that may be seen at the level of the receptor and its second messenger systems (Kolesnikov et al., 1993). The isolated guinea pig ileum (GPI) and the mouse vas deferens (MVD) have been extensively used for the assessment of acute, as well as chronic effects of opioids. For example, it has been shown that the excised GPI from animals implanted with morphine pellets exhibits a high degree of tolerance (Goldstein and Schulz, 1973; Schulz and Herz, 1976). Likewise, implantation of mice with morphine pellets (Cox, 1978) or mini-infusion pumps containing opioids resulted in the development of tolerance in MVD (Schulz et al., 1980). The GPI contains μ- and κ-type opioid receptors (Ward and Takemori, 1976; Lord et al., 1977) in addition to receptors associated with mucosal transport (Fogel and Kaplan, 1984) and MVD is a tissue that possesses δ-opioid receptors in addition to μ- and κ-opioid receptors (Lord et al., 1977). Although the role of cholestasis in the expression of subsensitivity to opioids has already been determined in vivo (Bergasa et al., 1994), the current study evaluates whether subsensitivity to exogenous

ABBREVIATIONS: GPI, guinea pig ileum; MVD, mouse vas deferens; β-FNA, β-funaltrexamine; nor-BNI, nor-binaltorphimine; BDL, bile duct ligated.
opioids in cholestatic models is produced in tissues such as GPI and MVD that are shown to have opioid receptors. We also tried to determine the specific opioid receptor types involved in the rate and degree of subsensitivity development to opioids in these models. A preliminary account of this work has been given at the 13th International Congress of Pharmacology (July 26–31, 1998, Munchen, Germany).

Materials and Methods

Drugs

Drugs were obtained from the following sources: morphine HCl (Macfarlan Smith Ltd., Edinburgh, UK) and U50488H, β-funaltrexamine (β-FNA), nor-binaltorphimine (nor-BNI), and SNC 80 (Tocris Cookson Ltd., Bristol, UK). SNC 80 was dissolved in dimethyl sulfoxide and other drugs were dissolved in deionized water.

Animals

Male albino guinea pigs (350–500 g) and male Naval Medical Research Institute mice (20–30 g) were used. Animals were housed in temperature-controlled room (25 °C ± 1 °C) and exposed to 12-h light/dark cycle. The animals had free access to food and water. There were three experimental groups: unoperated controls (control), sham-operated controls (sham), and bile duct-ligated (BDL) animals. Laparotomy was performed under general anesthesia induced by i.p. injection of ketamine (50 mg/kg) and promazine (10 mg/kg). In the sham-operated controls, the bile duct was left in situ after its manipulation with forceps. In the BDL animals, the bile duct was doubly ligated and then the abdominal wound was closed in two layers (Bergasa et al., 1994). Operation mortality rate was <10%. Experiments were conducted 2, 5, and 7 days after surgery in guinea pigs and 2, 5, 7, 10, and 15 days after operation in mice. The experiments were performed in accordance with the recommendations of the ethics committee on animal experimentation of the Medical School.

Preparation of Excised Tissues

GPI. Animals that had been fasted overnight were sacrificed by a blow on the head. Isolated segments of guinea pig distal ileum, 1.5 to 2 cm in length, were vertically suspended in oxygenated Krebs’ solution (113 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 5.5 mM glucose) in 20 ml of organ bath. The temperature of the bath was maintained at 37°C. Before the administration of any drug, the tissues were fixed at a resting tension of 1 g and equilibrated for 60 min washing out every 15 min. The electrical stimulation was applied by platinum ring electrode at supramaximal rectangular pulses in all preparations (70 V of 0.5-ms duration at a frequency 0.1 Hz) and twitches were recorded isometrically with a Narco Grass force transducer connected to a Narco Grass polygraph. To indicate the time dependence of tolerance development in cholestasis, dose-response curves of opioid agonists after 2, 5, and 7 days of surgery were determined in cholestatic groups.

Assessment of Degree of Subsensitivity in GPI. Subsensitivity was assessed by the exposure of the tissues to various concentrations of morphine or U50488H cumulatively. The half-maximal concentration that inhibited electrically induced contraction (IC₅₀) for each compound was determined in this preparation and the IC₅₀ values were used for comparison between treatments. To calculate the degree of subsensitivity to agonists, the ratio of the IC₅₀ of each drug in cholestatic groups to the IC₅₀ in control groups was compared (Rezvani et al., 1983).

Determination of Involvement of Opioid Receptor Types in GPI. To determine the involvement of opioid receptor subtypes on development of tolerance in GPI in the 7-day cholestatic group, the tissue was incubated with nor-BNI, a highly selective κ-opioid receptor antagonist, at 20 nM for 30 min and the IC₅₀ of morphine was determined (Portoghese et al., 1987). Likewise, to assess κ-receptor involvement, the IC₅₀ of U50488H was obtained in tissues preincubated with β-FNA, a selective irreversible μ-opioid receptor antagonist, at 200 nM for 30 min (Ward et al., 1982, 1986).

MVD. Animals were sacrificed by cervical dislocation and the vasa deferentia were removed and suspended in organ bath containing oxygenated Krebs’ solution (118 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl₂, 0.93 mM KH₂PO₄, 24 mM NaHCO₃, and 11 mM glucose). After equilibration for 1 h under a tension of 0.5 g, the vas deferentia were stimulated electrically (70 V of 0.5-ms duration at a frequency 0.1 Hz). The induced twitches were recorded as described for GPI. Concentration-response curves of morphine, U50488H, and SNC 80 were determined by adding cumulative doses of each compound to organ bath. The degree of subsensitivity to agonists was calculated as mentioned above. To indicate the time dependence of subsensitivity development in cholestasis, the dose-response curve to SNC 80 after 2, 5, 10, and 15 days of surgery was determined in the cholestatic group. To identify the type of opioid receptors involved in subsensitivity development in 5-day cholestatic mice, the isolated vas deferentia were incubated with β-FNA at 200 nM or nor-BNI at 20 nM for 30 min, and the IC₅₀ values of SNC 80 and morphine were determined, respectively.

Statistical Analysis

Data are expressed as means ± S.E. ANOVA was used to compare mean IC₅₀ values obtained in various experiments. P < .05 was considered as the significance level between groups.

Results

Two days after bile duct ligation, the animals revealed signs of cholestasis (jaundice, dark urine, steatorrhea, and yellow plasma) and the signs persisted beyond two days.

Determination of IC₅₀ of Morphine and U50488H in GPI. Morphine inhibited the twitches of stimulated ileum in a dose-dependent manner in all groups. The IC₅₀ values of morphine in ilea from cholestatic, sham, and control animals obtained from dose-response curves were 1.44 × 10⁻⁶, 1.81 × 10⁻⁹, and 2.36 × 10⁻⁹ M, respectively (Table 1), indicating a significant subsensitivity to morphine in the cholestatic group (Fig. 1). The degree of subsensitivity was 610 times greater in cholestatic animals compared with control animals (Table 1). Incubation of tissues with nor-BNI, a κ-selective antagonist (20 nM), produced a slightly parallel shift to the right of the dose-response curve of morphine in the cholestatic group, and the development of subsensitivity to morphine was still higher than in the sham and control groups (Fig. 1).

U50488H, a κ-selective agonist, also produced a concentration-dependent inhibition in the height of the electrically induced twitch responses in all groups. The IC₅₀ values of U50488H in ilea from cholestatic, sham, and control animals were 1.94 × 10⁻⁹, 5.69 × 10⁻¹⁰, and 6.37 × 10⁻¹⁰ M, respectively (Table 1). The degree of subsensitivity was three times greater in the cholestatic group and mean IC₅₀ for the drug was significantly different from that of the control. As shown in Table 1, β-FNA, as a selective irreversible μ-opioid receptor antagonist, did not significantly alter the response to U50488H. Therefore, U50488H in isolated GPI selectively affects κ-receptors.

Time-Dependent Effect of Cholestasis on Subsensitivity Development to Morphine in GPI. Prolongation of cholestasis increased significantly the IC₅₀ of morphine (Ta-
ble) after 5 and 7 days of bile duct ligation compared with
2-day cholestatic animals. The dose-response curve of mor-
phine markedly shifted to the right as shown in Fig. 2.

Determination of IC50 of Opioid Receptor Agonists in
MVD. In all groups, morphine, U50488H, and SNC 80 pro-
duced concentration-dependent inhibition of electrically
stimulated twitch contractions in MVD. Calculated IC50 val-
ues for these agonists 5 days after the surgery are shown in
Table 2. The degree of subsensitivity in BDL mice to SNC 80
and morphine were 244 and 152, respectively (Fig. 3 and 4).
There was no significant difference in dose-response curve of
U50488H between the control and the cholestatic groups
(Table 2). It also was observed that the effect of cholestasis
on the subsensitivity development to SNC 80 in MVD was time-
dependent (Fig. 5). The IC50 of the agonist in 5-, 10-, and
15-day cholestatic mice were 8, 27, and 38 times greater than
that of 2-day cholestatic mice, respectively (Table 2). There-
fore, the dose-response curve of SNC 80 showed increasing
rightward parallel shifts by prolongation of cholestasis. In-
cubation of isolated MVD for 30 min with the selective
\( \mu \)-antagonist \( b\)-FNA (200 nM) had no effect on the SNC 80
dose-response curve (Fig. 3). Preincubation of the preparations
with \( \kappa \)-selective antagonist (20 nM), produced a parallel
rightward shift in dose-response curve of morphine
in cholestatic animals (Fig. 4).

The excitatory responses of the GPI and MVD of choles-
tatic animals to exogenous acetylcholine and norepinephrine,
respectively, did not show any significant changes. EC50 val-
ues of acetylcholine in control and cholestatic guinea pig were
1.73 \( \times \) 10^{-2} and 2.41 \( \times \) 10^{-2}, respectively, and
EC50 values of norepinephrine in the control and cholestatic
mice were 3.41 \( \times \) 10^{-6} and 3.18 \( \times \) 10^{-6}, respectively.

Discussion

There has been no previous demonstration of opioid sub-
sensitivity with respect to the cholestasis in isolated tissues
in vitro. The purpose of this study was to find out whether
cholestasis subsensitivity to exogenous opioids is induced in
tissues such as GPI and MVD, which are known to have
opioid receptors. If so, we wanted to determine what opioid
receptor types were involved and how the time course of
subsensitivity was achieved.

Elevated plasma levels of enkephalins have been reported

TABLE 1 Effect of bile duct ligation on the development of subsensitivity in the isolated GPI
Each value represents mean inhibitory concentration that inhibits electrically induced twitches \( \pm \) S.E. (n = 6).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC50 Morphine</th>
<th>IC50 U50488H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.36 ( \times ) 10^{-2} ( \pm ) 0.28</td>
<td>6.37 ( \times ) 10^{-10} ( \pm ) 0.57</td>
</tr>
<tr>
<td>Sham</td>
<td>1.81 ( \times ) 10^{-2} ( \pm ) 0.33</td>
<td>5.69 ( \times ) 10^{-10} ( \pm ) 0.76</td>
</tr>
<tr>
<td>BDL (day 2)</td>
<td>8.07 ( \times ) 10^{-8} ( \pm ) 1.10 ( a )</td>
<td>8.59 ( \times ) 10^{-7} ( \pm ) 0.66 ( a )</td>
</tr>
<tr>
<td>(day 5)</td>
<td>8.95 ( \times ) 10^{-5} ( \pm ) 0.68 ( a,b )</td>
<td>1.44 ( \times ) 10^{-6} ( \pm ) 0.17 ( a,b )</td>
</tr>
<tr>
<td>BDL (day 7) + nor-BNI</td>
<td>4.79 ( \times ) 10^{-6} ( \pm ) 0.69 ( a,b,c )</td>
<td>2.47 ( \times ) 10^{-9} ( \pm ) 0.26 ( a )</td>
</tr>
<tr>
<td>BDL (day 7) + b-FNA</td>
<td>610</td>
<td>3</td>
</tr>
</tbody>
</table>

\( a \) P < .001 compared with control or sham animals.
\( b \) P < .001 compared with day 2 of cholestasis.
\( c \) P < .001 compared with cholestatic group in the absence of antagonist.
in patients with primary biliary cirrhosis, and it has been suggested that increased plasma methionine-enkephalin levels may be a predictor of reduced survival in patients with cholestasis (Thornton and Losowsky, 1988a,b; Swain et al., 1992). Observations compatible with elevation of opioid levels include precipitation of an opioid withdrawal-like syndrome in patients with cholestasis as well as in the mouse model of cholestasis by administration of an opioid antagonist and a global down-regulation of \( \mu \)-opioid receptors in the brain of BDL rats (Thornton and Losowsky, 1988a; Bergasa et al., 1992; Ghafourifar et al., 1997; Dehpour et al., 1998). It has been shown that cholestasis in rats is associated with naloxone-reversible antinociception (Bergasa et al., 1994).

Our results show that in acute cholestasis, the subsensitivity to morphine has developed significantly in the ileum (degree of subsensitivity is \( 0.600 \)). Subsensitivity to exogenous acetylcholine did not develop in cholestatic animals. This shows that cholestasis, however, has not altered the sensitivity of GPI to the excitatory effect of acetylcholine. Because morphine is a \( \mu \)- and \( \kappa \)-opioid agonist in this preparation, an attempt was made to determine the predominant type of opioid receptor that mediates the decreased effect of morphine during cholestasis. To show this, nor-BNI was used. Because nor-BNI increased the IC\(_{50}\) of morphine in the cholestatic animals only three times, it may be concluded that in cholestatic guinea pigs, the majority of decreased effects of morphine could be mediated by \( \mu \)-opioid receptors. This could not be achieved by directly using a selective \( \mu \)-receptor antagonist because we had already used a high dose of morphine, and any higher concentration would have led to nonspecific responses and possible tissue damage. Therefore, nor-BNI was used to determine the responsible opioid receptor indirectly. Moreover, we studied the effect of U50488H on GPI obtained from the cholestatic animals. Because results showed a 3-fold increase in subsensitivity to the inhibitory effect of this agent, it means that the different type of opioid receptor could not have the same role in the development of subsensitivity. The development of this phenomenon to inhibitory effects of morphine appeared to be time-dependent. The result showed that the degree of subsensitivity to morphine was lowest on day 2 and highest on day 7 of cholestasis, respectively. There are several reports that show that im-

### TABLE 2 Effect of bile duct ligation on the development of subsensitivity in the isolated MVD

Each value represents mean inhibitory concentration that inhibits electrically induced twitches \( \pm \) S.E. \((n = 6)\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SNC 80 ( \times 10^{-5} )</th>
<th>Morphine ( \times 10^{-6} )</th>
<th>U50488H ( \times 10^{-6} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.97 \pm 2.22 ( \times 10^{-10} )</td>
<td>5.60 \pm 1.35 ( \times 10^{-9} )</td>
<td>1.33 \pm 0.21 ( \times 10^{-6} )</td>
</tr>
<tr>
<td>Sham</td>
<td>4.33 \pm 1.61 ( \times 10^{-10} )</td>
<td>5.48 \pm 0.53 ( \times 10^{-9} )</td>
<td></td>
</tr>
<tr>
<td>BDL (day 2)</td>
<td>2.12 \pm 0.32 ( \times 10^{-8} )</td>
<td>8.50 \pm 0.95 ( \times 10^{-7} )</td>
<td>1.84 \pm 0.32 ( \times 10^{-6} )</td>
</tr>
<tr>
<td>(day 5)</td>
<td>1.70 \pm 0.25 ( \times 10^{-7} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(day 10)</td>
<td>5.65 \pm 0.75 ( \times 10^{-7} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDL (day 5) + ( \beta )-FNA</td>
<td>8.02 \pm 1.25 ( \times 10^{-7} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDL (day 5) + nor-BNI</td>
<td>2.22 \pm 0.10 ( \times 10^{-6} )</td>
<td>3.51 \pm 0.14 ( \times 10^{-6} )</td>
<td>1.95 \pm 0.12 ( \times 10^{-6} )</td>
</tr>
<tr>
<td>Degree of subsensitivity (day 5)</td>
<td>244</td>
<td>152</td>
<td></td>
</tr>
</tbody>
</table>

\( *P < .001 \) compared with control or sham animals.

\( \#P < .001 \) compared with day 2 of cholestasis.

\( \&P < .001 \) compared with day 5 of cholestasis.

\( \&\&P < .001 \) compared with cholestatic group in the absence of antagonist.

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**Fig. 3.** Mean dose-response curves for SNC 80 inhibition of electrically induced twitches in the isolated MVD in control (○), sham (○), BDL (□), and BDL in presence of \( \beta \)-FNA (200 nM) (■). Each point represents the mean \( \pm \) S.E.

**Fig. 4.** Mean dose-response curves for morphine inhibition of electrically induced twitches in the isolated MVD in control (○), sham (○), BDL (□), and BDL in presence of nor-BNI (20 nM) (■). Each point represents the mean \( \pm \) S.E.
plantation of morphine pellets in guinea pigs promotes tolerance to opioids (Ward and Takemori, 1976; Johnson et al., 1978; Vaught and Takemori, 1978; Vaught, 1981; Chavkin and Goldstein, 1982, 1984; Leedham et al., 1989; Johnson, 1991; Taylor et al., 1991; Garaulet et al., 1994). A well described function of the opioid neurotransmitter system in the inhibition of electrically induced twitches in GPI is the result of the interaction between opioid agonists and specific receptors in this preparation. By analogy, increased opioidergic tone in cholestasis is in the absence of exogenously administrated opioids would be associated with development of subsensitivity to opioids in GPI.

The reason that subsensitivity is μ-specific may be because in cholestasis enkephalins are the only endogenous opioid ligands that are accumulated in plasma and these ligands have no specific affinity for κ-receptors (Reisine and Pasternak, 1996). In addition, our results show that MVD obtained from cholestatic animals exhibits a considerable subsensitivity to SNC 80 (selective μ-agonist) when compared to GPI. This subsensitivity to SNC 80 is time-dependent. Thus, a significant subsensitivity to opioids in MVD preparations to opioids induced by cholestasis is opioid receptor-type-specific, and the effect of cholestasis is time-dependent. The identity of the mechanisms involved or how they are involved in the subsensitivity to opioids in the cholestatic animals remains to be elucidated.

Finally, we can draw some tentative conclusions based on the data presented herein: 1) cholestasis induces the development of a high degree of subsensitivity to opioid agonists in GPI and MVD, 2) the effect of cholestasis on subsensitivity is opioid receptor-type-specific, and 3) the effect of cholestasis is time-dependent. The identity of the mechanisms involved or how they are involved in the subsensitivity to opioids in the cholestatic animals remains to be elucidated.

References


Fig. 5. Comparison of tolerance subsensitivity to SNC 80 at 2 (■), 5 (□), 10 (∆), and 15 (▲) days after BDL in the isolated MVD. Each point represents the mean ± S.E.


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