INTERACTION OF MORPHINE-EPoxide WITH MULTIPLE OPIATE RECEPTORS

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We examined the interaction of morphine-epoxide, which was assumed to be a new metabolite of morphine, with opiate receptor subtypes using pharmacological and biochemical techniques. Morphine-epoxide was about 3 to 4 times less potent than morphine to the interactions of opiate receptors. However, the ratio of IC_{50} values for the guinea pig ileum and mouse vas deferens to electrical field stimulation and the ratio of IC_{50} values for the [3H]-dihydromorphine and [3H]-D-alα2-leu enkephalin binding to rat brain membrane preparations of morphine-epoxide was similar to those of morphine. Morphine-epoxide had virtually no effect on the twitch responses of rabbit and rat vas deferens. Furthermore, "sodium ratio" and "GTP ratio" of morphine-epoxide were similar to those of morphine and differed from naloxone. These results suggest that morphine-epoxide as well as morphine behaves as the agonist on the mu type opiate receptor notwithstanding that the affinity of morphine-epoxide is slightly less than that of morphine.

Keywords—morphine-epoxide; morphine; guinea pig ileum; mouse vas deferens; rat brain membrane; twitch response; binding assay; multiple opiate receptor

INTRODUCTION
Morphine-7,8-oxide (morphine-epoxide) was found to be a potent inhibitor of electrically stimulated ileum from guinea pig and its inhibitory effect was antagonized by naloxone.1) Furthermore, it has been reported that the antinociceptive action induced by morphine-epoxide was 0.62 times as potent as morphine2) and the ability of morphine-epoxide to displace specific binding of [3H]-dihydromorphine to membrane preparation from rat brain was slightly less potent than morphine.3)

Recently, the concept of multiple opiate receptors has been demonstrated on the basis of pharmacological and biochemical results. It has been shown pharmacologically that the guinea-pig ileum contains mu- and kappa-receptors while mouse vas deferens contains mu-, kappa- and delta-receptors.4) Furthermore, rabbit vas deferens and rat vas deferens has kappa and epsilon type receptors.5,6) Biochemically, the subtypes of opiate receptors have been suggested from the binding studies.7-10) However, the interactions of morphine-epoxide with subtypes of opiate receptors remain to be elucidated. Therefore, we examined the interaction of morphine-epoxide with opiate receptors using pharmacological and biochemical techniques.

MATERIALS AND METHODS
Pharmacological Studies — Pieces (about 3—4 cm) of ileum from male Hartley guinea pigs, weighing 250—350 g, were removed and mounted in a 20 ml of organ bath containing Krebs solution (NaCl: 118, KCl: 4.75, CaCl_2: 2.54, NaHCO_3: 25, KH_2PO_4: 1.19, MgSO_4: 1.2,
glucose: 11 mM) at 37°C and gassed with carbon. The twitch responses of the ileum to electrical stimulation were recorded isometrically under an initial tension of 0.5 g. The electrodes were made of platinum and the intraluminal electrode was the anode. The field stimulation was carried out by passing rectangular pulses (duration: 0.1 ms, frequency: 0.1 Hz, supramaximal voltage) between two electrodes.

Isolated vasa deferentia from 25–35 g of male ddY mice were mounted in a 10 ml of organ bath at 37°C in Mg²⁺-free Krebs solution. Vasa were subjected to field stimulation through platinum electrodes (duration: 1 ms, frequency: 0.1 Hz, supramaximal voltage). The responses to stimulation were recorded isometrically under an initial tension of 0.5 g. Other experimental conditions were as reported by Lemaire et al.⁶ for the rat vas deferens and by Oka et al.⁷ for the rabbit vas deferens. In order to obtain a dose inhibitory response curves of drugs, the drugs were applied cumulatively.

Biochemical Studies — Male Wistar rats, weighing 200–300 g, were decapitated and their brains were rapidly removed. The cerebellum was removed from each brain. Crude mitochondrial fraction was suspended in ice-cold 50 mM Tris/HCl buffer, pH 7.4, and centrifuged at 17000 × g for 10 min. The pellet was resuspended in ice-cold 50 mM Tris/HCl buffer and incubated for 40 min at 37°C to facilitate dissociation of endogenous inhibitors of ligands binding.¹¹ The suspensions were centrifuged twice at 17000 × g for 10 min.

The measurement of labeled ligand binding in rat brain membrane preparation was performed using a filtration method. Potencies of competing ligands were determined by coincubating unlabeled ligands with [³H]-dihydromorphine (DHM; 0.3 nM), [³H]-d-Ala²-D-Leu⁵ enkephalin (DADLE; 0.5 nM) and [³H]-naloxone (0.5 nM). The binding reaction was performed at 25°C for 60 min. Non-specific binding was determined in the presence of 1 μM of naloxone. To determine the “Na” or “GTP” effect on the ligand binding, the binding reactions was performed in the presence and absence of 100 mM of NaCl or 50 μM of GTP with [³H]-naloxone. The binding reaction was stopped by rapid filtration through a Whatman glass fiber filter (GF/C). The filters were rinsed twice with 5 ml of ice-cold 50 mM Tris/HCl buffer. They were then completely dried and placed in liquid scintillation counting vials containing 10 ml of a toluene scintillator and counted in an ALOKA LSC-900 liquid scintillation counter. The protein concentration in the reaction medium was 1 mg/ml which was determined by the method of Lowry et al.¹² using bovine serum albumin (F-V) as the standard.

Statistical significance was evaluated by the paired t-test.

Drugs Used: Morphine-epoxide was synthesized according to the method of Miyata et al.¹³ Morphine hydrochloride from Sankyo. GTP and D-alaa²-D-leu⁵ enkephalin from Sigma. [³H]-dihydromorphine (specific activity: 70.7 Ci/mmol), [³H]-D-alaa²-D-leu⁵ enkephalin (specific activity: 39.5 Ci/mmol) and [³H]-naloxone (specific activity: 43.9 Ci/mmol) from New England Nuclear. Other chemicals were of analytical grade.

RESULTS

Pharmacological Studies

In a preliminary experiment, tetrodotoxin (10⁻⁷ M) completely abolished the electrically evoked contractions, indicating that the contractions are not due to direct electrical stimulation of the smooth muscle.

Morphine-epoxide as well as morphine dose-dependently inhibited the twitch responses of guinea pig ileum and mouse vas deferens to electrical field stimulation (Fig. 1-A and B). The IC₅₀ values calculated from their dose-response curves were shown in Table I. Morphine-epoxide was significantly less potent than morphine on the inhibitions of twitch response of guinea pig ileum and mouse vas deferens. However, the ratio of IC₅₀ values for the guinea pig ileum and mouse vas deferens of morphine-epoxide was similar to that of morphine. The inhibition of twitch response of guinea pig ileum by morphine-epox-
ide as well as morphine was more potent than that of mouse vas deferens. $10^{-6}$ M of morphine-epoxide, however, had virtually no effect on the twitch responses of rabbit and rat vas deferens (data not shown).

**Binding Assay**

The bindings of $[^3H]$-DHM and $[^3H]$-DADLE to membrane preparation from rat brain were dose-dependently displaced by the simultaneous addition of non-radioactive morphine, morphine-epoxide and DADLE (Fig. 2-A and B). The IC$_{50}$ values calculated from their displacement curves were shown in Table II. The abilities of morphine-epoxide to displace $[^3H]$-DHM and $[^3H]$-DADLE (IC$_{50}$ values) were about 3 to 4 times less potent than those of morphine. However, the ratio of IC$_{50}$ values of morphine-epoxide for $[^3H]$-DHM and $[^3H]$-DADLE binding was similar to that of morphine, and morphine-epoxide and morphine are more potent in inhibiting the binding of $[^3H]$-DHM than $[^3H]$-DADLE. On the other hand, DADLE is more potent in inhibiting the binding of $[^3H]$-DADLE than $[^3H]$-DHM.

In order to further characterize the interaction of morphine-epoxide with opiate receptors, we also examined its "sodium ratio" and "GTP ratio". because various opiates are differently affected by sodium ions and GTP. The IC$_{50}$ values calculated from their displacement curves

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**FIG. 1. Inhibitory Effects of Morphine and Morphine-Epoxide on the Twitch Responses of Guinea Pig Ileum (A) and Mouse vas Deferens (B)**

Each mark represents the mean with S.E. of 4 to 5 experiments. Ordinate: twitch response (%), Abscissa: negative log of dose (M). ○: morphine •: morphine-epoxide.
in the presence and absence of 100 mM of NaCl or 50 μM of GTP were shown in Table III. In the absence of 100 mM of NaCl or 50 μM of GTP, the IC₅₀ values of morphine-epoxide for [³H]-naloxone binding were about 3 to 4 times less than that of morphine. These results were in good agreement with the ratios of IC₅₀ values of morphine and morphine-epoxide for [³H]-DHM and [³H]-DADLE binding. The displacement of [³H]-naloxone by morphine-epoxide as well as morphine, a mu-receptor agonist, reduced in the presence of 100 mM of NaCl and 50 μM of GTP by a factor of about 34 and 27 to 29, respectively. On the other hand, naloxone, an opiate antagonist, displace none in the presence of 100 mM of NaCl and slightly decreased in the presence of 50 μM of GTP by a factor of about 4.

DISCUSSION

The existence of multiple opiate receptors in the brain and in the peripheral tissues has been well-documented on the basis of biochemical and pharmacological studies.⁴⁻⁵⁻⁹⁻¹¹) Therefore, we examined the interaction of morphine-epoxide, which was assumed to be a new metabolite of morphine,¹³) with opiate receptor subtypes using pharmacological and biochemical techniques.

Morphine-epoxide was significantly less potent than morphine on the inhibitions of the twitch responses of guinea pig ileum and mouse vas deferens to electrical field stimulation and 3 to 4 times less potent than morphine to the interactions of opiate receptors, which were determined by the binding assay. However, the ratios of IC₅₀ values in the guinea pig ileum to that in the mouse vas deferens to electrical field stimulation and the ratios of IC₅₀ values in inhibiting [³H]-DHM binding (mu type) to that in inhibiting [³H]-DADLE binding (delta type) of morphine-epoxide are similar to those of morphine, a repre-

![Figure 2](image-url)  
**FIG. 2. Inhibitory Effects of Morphine, Morphine-Epoxide and DADLE on the Specific Binding of [³H]-DHM (A) and [³H]-DADLE (B) to Rat Brain Membranes**  
Each mark represents the mean with S.E. of 4 separate experiments. Ordinate: specific binding of [³H]-ligands (%). Abscissa: negative log of dose (M). ○ : morphine ● : morphine-epoxide ▲ : DADLE.
sentative mu-agonist. Since morphine-epoxide had virtually no effect on the twitch responses of rabbit (kappa type) and rat vas deferens (epsilon type), morphine-epoxide preferentially act on mu type receptor. Furthermore, “sodium ratio” and “GTP ratio” of morphine-epoxide were similar to those of morphine, an opiate agonist, and differed from those of naloxone, an opiate antagonist. There results suggested that morphine-epoxide as well as morphine behaved as the agonist on the

**TABLE I. Inhibitory Effects (IC$_{50}$) of Morphine and Morphine-Epoxide on the Twitch Responses of Guinea Pig Ileum and Mouse vas Deferens**

<table>
<thead>
<tr>
<th></th>
<th>Guinea pig ileum</th>
<th>Bioassay IC$_{50}$ (nM)</th>
<th>Illeum/Vas deferens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>34±10</td>
<td>1100±500</td>
<td>0.031</td>
</tr>
<tr>
<td>Morphine-epoxide</td>
<td>90±2</td>
<td>3300±1200$^{a}$</td>
<td>0.027</td>
</tr>
</tbody>
</table>

*Each value represents the mean with S.E. of 4 to 5 experiments. a) significantly different from the morphine treated group at $p < 0.05$ and $p < 0.01$, respectively (paired t-test).*

**TABLE II. Inhibitory Effects (IC$_{50}$) of Morphine, Morphine-Epoxide and DADLE on the Binding of $[^3$H$]$-DHM and $[^3$H$]$-DADLE to Rat Brain Membranes**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>1.58±0.20</td>
<td>36.9±2.8</td>
<td>0.043</td>
</tr>
<tr>
<td>Morphine-epoxide</td>
<td>5.99±0.91$^{a}$</td>
<td>131.0±3.0$^{a}$</td>
<td>0.046</td>
</tr>
<tr>
<td>DADLE</td>
<td>4.43±0.44$^{a}$</td>
<td>1.8±0.4$^{a}$</td>
<td>2.46</td>
</tr>
</tbody>
</table>

*Each value represents the mean with S.E. of 4 separate experiments.  
a) significantly different from the morphine treated group at $p < 0.01$ (paired t-test).*

**TABLE III. Effects of Sodium and GTP on the Potencies of Morphine, Morphine-Epoxide and Naloxone in Competing with the Binding of $[^3$H$]$-Naloxone to Rat Brain Membranes**

<table>
<thead>
<tr>
<th></th>
<th>−Na</th>
<th>+Na</th>
<th>+Na/−Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>1.1±0.1</td>
<td>37.8±14.4</td>
<td>34.4</td>
</tr>
<tr>
<td>Morphine-epoxide</td>
<td>4.0±0.3$^{a}$</td>
<td>135.0±46.2$^{a}$</td>
<td>33.8</td>
</tr>
<tr>
<td>Naloxone</td>
<td>0.6±0.3</td>
<td>0.6±0.2$^{a}$</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>−GTP</th>
<th>+GTP</th>
<th>+GTP/−GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>1.5±0.5</td>
<td>43.0±22.5</td>
<td>28.7</td>
</tr>
<tr>
<td>Morphine-epoxide</td>
<td>4.7±0.5$^{a}$</td>
<td>125.0±65.5$^{a}$</td>
<td>26.6</td>
</tr>
<tr>
<td>Naloxone</td>
<td>0.8±0.6</td>
<td>3.0±1.1$^{a}$</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*Each value represents the mean with S.E. of 4 separate experiments.  
a) Significantly different from the morphine treated group at $p < 0.01$ (paired-test).*
mu type receptor, notwithstanding that the affinity of morphine-epoxide on the mu type receptor was slightly less than that of morphine. The difference of the affinities of morphine-epoxide and morphine on the opiate receptor sites may be a reflection of the difference of the antinociceptive action induced by morphine-epoxide and morphine (0.62 times as potent as morphine) which reported by Takayanagi et al.2)

REFERENCES


