Separation of Binding Affinity and Intrinsic Activity of the Potent \( \mu \)-Opioid 14-Methoxymetopon

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ABSTRACT
Receptor binding studies of 5,14-O-dimethyloxymorphine (14-methoxymetopon) in brain membranes have established its high affinity for \( \mu \)-binding sites, but its analgesic potency far exceeds the modest increase in binding affinity relative to other opioids. The current study has established the selectivity of \( ^3H \)14-methoxymetopon for \( \mu \) sites in calf striatal membranes and for a number of full-length splice variants of the cloned murine \( \mu \)-opioid receptor 1 (mMOR-1) in transfected cell lines. The binding affinity of \( ^3H \)14-methoxymetopon for the variants expressed in Chinese hamster ovary cells was quite high, with \( K_D \) values around 0.2 nM for all of the variants with the exception of mMOR-1F (\( K_D \) of 1.2 nM). The affinity for most of the expressed variants was greater than that seen in the brain membranes (\( K_D \) of 0.99 nM). Functionally, in guanosine 5'-O-[(3-[\( ^{35} \)S]thio)triphosphate ([\( ^{35} \)S]GTP\( \gamma \)S) binding assays with the MOR-1 variants, 14-methoxymetopon and the \( \mu \)-opioid peptide [\( \alpha \)-Ala\( ^2 \),\( N \)-Me-Phe\( ^4 \),Gly\( ^{\beta} \)-ol]-enkephalin (DAMGO) showed similar efficacies, as determined by maximal stimulation, but 14-methoxymetopon was up to 65-fold more potent than DAMGO. The greatest difference was seen with mMOR-1E and the least with mMOR-1C, which displayed only a 10-fold difference. These potency differences in the stimulation of [\( ^{35} \)S]GTP\( \gamma \)S binding far exceeded the differences in binding affinity. The differences between 14-methoxymetopon and DAMGO remained after normalizing the potency shifts based upon receptor binding affinities and varied from 1.2-fold with mMOR-1C to 21-fold for mMOR-1 and 42-fold with mMOR-1F. Thus, 14-methoxymetopon is a potent agonist against all of the mMOR-1 splice variants, but its potency ranged widely despite similar binding affinities for most of the variants and may give insight into its unusual pharmacological profile.

5,14-O-Dimethyloxymorphine (14-methoxymetopon) is a highly potent and selective \( \mu \)-opiate agonist (Schmidhammer et al., 1990; Fürst et al., 1993; Freye et al., 2000; Zernig et al., 2000; King et al., 2003; Bilevicute-Ljungar et al., 2006) with a unique pharmacology and extraordinary analgesic potency that sets it apart from traditional \( \mu \) agonists. Despite its high analgesic potency, it displays little respiratory depression, bradycardia, or sedation compared with sufentanil. It decreases gastrointestinal transit far less than morphine and reportedly develops lower levels of tolerance and physical dependence and a diminished propensity to cause convulsions in mice. Supraspinal 14-methoxymetopon analgesia is antagonized by 3-O-methylnalbexmone at a dose that antagonizes both heroin and MG6, but not morphine (King et al., 2003). Antisense mapping of MOR-1 also distinguishes 14-methoxymetopon from other opioids, with 14-methoxymetopon analgesia blocked by MOR-1 antisense probes against exons 1, 2, and 8, a pattern, however, different from that of either morphine or MG6 (King et al., 2003). Yet, the selectivity of 14-methoxymetopon for \( \mu \)-opioid receptors is well established, based on receptor binding experiments and its sensitivity in vivo to \( \mu \)-selective opioid antagonists, such as \( \beta \)-funtaloxamine and naloxonazine, but not \( \kappa \) or \( \delta \) antagonists.

Early studies of multiple \( \mu \)-opioid receptors used the antagonists naloxonazine and naloxazone to selectively block a \( \mu \)-binding subtype and then define the pharmacological con-

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ABBREVIATIONS: MG6, morphine-\( \delta \)-glucuronide; MOR, \( \mu \)-opioid receptor; DAMGO, [\( \alpha \)-Ala\( ^2 \),N-Me-Phe\( ^4 \),Gly\( ^{\beta} \)-ol]-enkephalin; GTP\( \gamma \)S, guanosine 5'-O-[(3-[\( ^{35} \)S]thio)triphosphate; CHO, Chinese hamster ovary; Gpp(NH)p, guanosine 5'-[(\( \gamma \)-imido)triphosphate; U50,488H, (1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-ly]-benzeneacetamide.

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sequences of this blockade (Pasternak et al., 1980a,b; Wolo-
zin and Pasternak, 1981). In these early studies, naloxo-
zine selectively blocked morphine analgesia without inter-
fering with respiratory depression or gastrointestinal
transit, raising the possibility of distinct receptor mecha-

The dissociation between analgesia and both respira-
tory depression and gastrointestinal inhibition is similar to the pharmacology reported with 14-methoxymeto-
pon.

Since the initial descriptions of the cloning of MOR-1 (Chen et al., 1993; Eppler et al., 1993; Thompson et al., 1993; Wang et al., 1993), a large number of MOR-1 splice variants have been isolated from mice (Pan et al., 1999, 2000, 2001, 2005b), rats (Zimprich et al., 1994; Pasternak et al., 2004), and hu-
mans (Bare et al., 1994; Pan et al., 2003, 2005a). The full-
length variants all contain identical binding pockets that are
defined by exons 1, 2, and 3 and differ only at the tip of the C

Materials and Methods

[3H]14-Methoxymetopon (15.9 Ci/mmol) was prepared as de-
scribed previously (Spetea et al., 2001). [3H]DAMGO (51 Ci/mmol)
and [35S]GTPγS (1250 Ci/mmol) were purchased from PerkinEl-
mer Life and Analytical Sciences (Boston, MA), and [3H]naloxone (63
Ci/mmol) was from GE Healthcare (Little Chalfont, Bucking-
hamshire, UK). Unlabeled 14-methoxymetopon was synthesized as de-
scribed previously (Schmidhammer et al., 1990). All other opioids
were obtained from the Research Technology Branch of the National
Institute of Drug Abuse (Bethesda, MD). GDP sodium salt and
peptidease inhibitor cocktail reagents (bestatin, leupeptin, pepstatin
A, and aprotinin) were purchased from Sigma-Aldrich (St. Louis,
MO).

Membranes were prepared from fresh tissue, with the exception of
guinea pig cerebellum, which was obtained frozen (Harlan Biopro-
ducts for Science Inc., Indianapolis, IN). Cell membrane preparations
from CHO cells stably transfected with MOR-1 splice variants were
obtained as described previously (Pan et al., 1999, 2000, 2001). In
brief, tissue or cell pellets were homogenized in 50 volumes of Tris
buffer (50 mM Tris, pH 7.4, at 25°C) containing 10 μM phenylmeth-
ysulfonyl fluoride, 100 mM NaCl, and 1 mM K+ EDTA. The homog-
enate was incubated for 15 min at 25°C and centrifuged at 49,000g
for 45 min. The pellet was resuspended in 0.32 M sucrose and frozen
at −70°C until use. Saturation binding assays used varying concen-
trations of ligand, whereas competition assays used a fixed amount of
[3H]14-methoxymetopon (1 nM) or [3H]DAMGO (1 nM).

Binding in brain membranes was carried out using 2 ml samples (10
mg/ml wet weight tissue) or 1 ml of cell homogenate (0.2 mg/ml
protein) in 50 mM potassium phosphate buffer, pH 7.4. Assays using
[3H]DAMGO or [3H]methoxymetopon also contained 5 or 10 mM
MgSO4, respectively. [3H]14-Methoxymetopon binding was per-
formed at 25°C for 150 min. [3H]DAMGO and [3H]naloxone incuba-
tions were carried out at 25°C for 60 min. Levalloprphan (1 μM) was
used to define nonspecific binding. Reactions were terminated by
rapid filtration over glass fiber filters using a Brandel cell harvester
(Brandel Inc., Gaithersburg, MD) and subjected to liquid scintilla-
tion counting. All values are presented as the means ± S.E.M. Ki,
Bmax, and Kp values were computed from nonlinear regression anal-
ysis using the program Prism (GraphPad Software Inc., San Diego,
CA). Only specific binding is reported, unless otherwise stated.

Receptor activation was assessed using agonist-induced stimula-
tion of [35S]GTPγS binding. Cell membrane homogenates (0.1 mg/ml)
were incubated with 30 μM GDP, 0.05 nM [35S]GTPγS, 1 μg/ml
peptidease inhibitor cocktail, and varying concentrations of unlabeled
ligand in 1 ml of 50 mM Tris buffer, pH 7.4, containing 0.2 mM
EGTA, 100 mM NaCl, and 3 mM MgCl2 for 60 min at 30°C, as
described previously (Bolan et al., 2004). Unlabeled 100 μM GTPγS
in the absence of ligand was used as an internal control to assess
nonspecific binding. The reaction was terminated by rapid filtration
over glass fiber filters using a Brandel cell harvester and subjected to
liquid scintillation counting. Individual data points were tested in
triplicate, and each assay was repeated at least three times. Results
are presented as the maximal stimulation over 10 μM DAMGO,
normalized to 100%.

Results

General Characterization of [3H]14-Methoxymeto-
pon Binding. 14-Methoxymetopon is a unique μ opioid.
Competition binding studies have documented its high affin-
ity for μ-binding sites, but even this affinity does not explain
its exceedingly high potency as an analgesic. We therefore

Fig. 1. [3H]14-Methoxymetopon binding in calf striatal membrane. A and
B, association (A) and dissociation (B) of 1 nM [3H]14-methoxymetopon
binding to calf striatal membranes was determined at 25°C. Association
studies involved incubating the radioligand for the indicated time. For
the dissociation studies, [3H]methoxymetopon binding was allowed to
proceed for 2.5 h. At that time, 1 μM levalloprphan was added, and binding
was assessed at the time following the addition of the competitor. Incu-
bations were terminated by rapid filtration at the indicated time. The
half-life of dissociation was 38.6 ± 4.8 min. Results in A and B are the
means ± S.E.M. of three experiments. C, binding was carried out on calf
striatal membranes at the indicated concentration of tissue. Results are
the means ± S.E.M. of three independent experiments. D, binding was
carried out at the indicated pH. Results are the means ± S.E.M. of three
independent experiments.
directly examined its receptor binding and stimulation of GTPγS binding with a series of MOR-1 splice variants.

First, we established the binding assay in calf striatal membranes. Binding was temperature-dependent, with levels at 0°C approximately one-half those seen at 25°C (data not shown). Although binding increased further at 37°C, we chose 25°C to facilitate comparisons with the literature. Binding approached equilibrium by 150 min and remained stable for up to 3 h (Fig. 1A). It was linear with tissue up to 20 mg wet weight (Fig. 1C) and was optimal at pH 7 (Fig. 1D). Saturation studies showed that [3H]14-methoxymetopon labeled sites in the calf striatal membranes with high affinity (K_D of 1 nM) (Fig. 2). The linear Scatchard plot (inset) implies a similar affinity of all of the labeled sites, which was further supported by the linear semilog dissociation curve (Fig. 1B).

[3H]14-Methoxymetopon binding displayed agonist characteristics. Divalent cations enhance radiolabeled agonist binding (Pasternak et al., 1975), whereas sodium ions decrease it (Pert et al., 1973). We saw a similar effect with [3H]14-methoxymetopon binding (Fig. 3). Sodium chloride lowered binding by approximately 75% at 100 mM, a result similar to other [3H]-opioid agonists. Conversely, magnesium increased binding and was used in subsequent assays. Radiolabeled opioid agonist binding is sensitive to GTP and its stable analogs (Childers and Snyder, 1978, 1980; Childers et al., 1979). The stable GTP analog Gpp(NH)p decreased [3H]14-methoxymetopon binding in a dose-dependent manner (Fig. 3B). The inclusion of sodium chloride potentiated this decrease, as shown previously for other radiolabeled opioid agonists. The distribution of binding within calf brain was similar to that seen previously with other μ-opioid ligands, with highest levels in the striatum, followed by lower levels in the periaqueductal gray, thalamus, frontal cortex, and brain stem (Fig. 4).

Competition studies in striatal membranes confirmed the selectivity of [3H]14-methoxymetopon for μ sites. All of the μ opioids competed binding in a monophasic manner (Fig. 5). Morphine and DAMGO, two highly selective μ agonists, and the highly selective μ antagonist δ-Phe-Cys-Tyr-d-Trp-Arg-Thr-NH2 all competed binding very potently, whereas the δ agonist δ(Pen²,Pen⁵)-enkephalin and the κ₁ agonist U50,488H were quite weak. Compared with [3H]DAMGO binding, the various ligands showed similar K_i values (Table 1). The only exception seemed to be naloxone. Although it retained high affinity against both radioligands, its K_i values against [3H]14-methoxymetopon were approximately 14-fold higher than against [3H]DAMGO.

Three μ-opioid agonists were examined in CHO cells to a series of MOR-1 splice variants expressed in CHO cells, comparing the results to both the μ-opioid agonist [3H]DAMGO and the antagonist [3H]naloxone (Table 2). Since the binding of the radioligands for a specific variant
used identical membrane preparations, comparisons among them for a single variant are relatively simple. However, these differences in level of expression among the variants make it difficult to compare one variant with another. [3H]14-Methoxymetopon displayed high affinity for all of the MOR-1 variants. Its $K_D$ values against all of the MOR-1 variants ranged between 0.1 and 0.3 nM and were far lower than in brain, with the exception of MOR-1F which displayed an affinity (1.17 nM) that more closely approximated the value in striatal membranes. The reasons underlying this difference are not clear, particularly since [3H]DAMGO and [3H]naloxone both labeled MOR-1F membranes with affinities similar to the other variants.

The $B_{max}$ values for [3H]14-methoxymetopon were generally higher than [3H]DAMGO (Table 2). Although they labeled approximately the same number of sites in MOR-1C and MOR-1D, the $B_{max}$ values for [3H]methoxymetopon far exceeded those of [3H]DAMGO in the others. The most prominent difference was MOR-1F, where the [3H]DAMGO $B_{max}$ was more than 4-fold lower than [3H]naloxone and approximately 8-fold lower than [3H]methoxymetopon. G protein-coupled receptors are thought to exist in agonist and antagonist conformations. Thus, it is not unusual to see far greater levels of binding with a radiolabeled antagonist, such as [3H]naloxone. The higher $B_{max}$ values for [3H]naloxone compared with [3H]DAMGO were not unexpected. However, the differences between [3H]DAMGO and [3H]14-methoxymetopon were striking. The similar binding levels of [3H]naloxone and [3H]14-methoxymetopon raised the possibility that 14-methoxymetopon may be labeling both agonist and antagonist receptor conformations.

Stimulation of GTP$\gamma$S Binding in MOR-1 Variants. Although 14-methoxymetopon has a slightly higher affinity than DAMGO in receptor binding studies, these differences cannot explain its strikingly higher analgesic potency. Therefore, we compared 14-methoxymetopon and DAMGO functionally by examining their ability to stimulate [35S]GTP$\gamma$S binding in CHO cell lines expressing the variants (Fig. 6; Table 3). Both 14-methoxymetopon and DAMGO maximally stimulated [35S]GTP$\gamma$S binding to a similar extent within each variant, suggesting that the two drugs had comparable efficacies. However, their potencies, as defined by EC$_{50}$ values, differed markedly (Table 3). 14-Methoxymetopon was 10- to 65-fold more active than DAMGO on the basis of EC$_{50}$ values. However, a portion of this difference may reflect the greater receptor occupancy at a given concentration of 14-methoxymetopon due to its higher binding affinity. Therefore, the data were normalized to account for the differences in binding site affinity by examining the EC$_{50}$/$K_D$ ratio, which should provide an indication of the intrinsic activity of

**TABLE 1**

Comparison of [3H]14-methoxymetopon and [3H]DAMGO binding by various opioid agonists and antagonists.

<table>
<thead>
<tr>
<th>Compound</th>
<th>[3H]14-Methoxymetopon $K_D$ (nM)</th>
<th>[3H]DAMGO $K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-Methoxymetopon</td>
<td>0.30 ± 0.03</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Morphine</td>
<td>3.9 ± 0.7</td>
<td>1.25 ± 0.29</td>
</tr>
<tr>
<td>DAMGO</td>
<td>1.32 ± 0.10</td>
<td>0.60 ± 0.09</td>
</tr>
<tr>
<td>M6G</td>
<td>6.9 ± 1.6</td>
<td>5.46 ± 1.42</td>
</tr>
<tr>
<td>[d-Pen^2,d-Pen^5]-enkephalin</td>
<td>&gt;400</td>
<td>&gt;250</td>
</tr>
<tr>
<td>U50,488H</td>
<td>&gt;400</td>
<td>&gt;350</td>
</tr>
<tr>
<td>Naloxone</td>
<td>1.65 ± 0.24</td>
<td>0.35 ± 0.15</td>
</tr>
<tr>
<td>D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH$_2$ (CTAP)</td>
<td>1.84 ± 0.28</td>
<td>7.24 ± 3.24</td>
</tr>
<tr>
<td>Naloxonazine</td>
<td>2.53 ± 0.28</td>
<td>1.84 ± 6.48</td>
</tr>
<tr>
<td>3-Methoxynaltrexone</td>
<td>&gt;200</td>
<td>&gt;600</td>
</tr>
</tbody>
</table>

**TABLE 2**

[3H]14-Methoxymetopon, [3H]DAMGO, and [3H]naloxone binding to calf striatum and CHO/MOR-1 splice variants.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>[3H]14-Methoxymetopon $B_{max}$ (fmol/mg)</th>
<th>[3H]DAMGO $B_{max}$ (fmol/mg)</th>
<th>[3H]Naloxone $B_{max}$ (fmol/mg)</th>
<th>14-Methoxymetopon $B_{max}$ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td>365 ± 12</td>
<td>254 ± 14</td>
<td>423 ± 102</td>
<td>1.43 (0.79)</td>
</tr>
<tr>
<td>MOR-1</td>
<td>321 ± 14</td>
<td>244 ± 21</td>
<td>28 ± 2.3</td>
<td>1.32 (1.15)</td>
</tr>
<tr>
<td>MOR-1A</td>
<td>579 ± 20</td>
<td>390 ± 12</td>
<td>461 ± 40</td>
<td>1.48 (1.26)</td>
</tr>
<tr>
<td>MOR-1B1</td>
<td>405 ± 17</td>
<td>281 ± 6.9</td>
<td>479 ± 69</td>
<td>1.44 (0.85)</td>
</tr>
<tr>
<td>MOR-1C</td>
<td>154 ± 3.4</td>
<td>129 ± 14</td>
<td>177 ± 11</td>
<td>1.04 (0.76)</td>
</tr>
<tr>
<td>MOR-1E</td>
<td>48 ± 6.6</td>
<td>42.3 ± 2.7</td>
<td>81.4 ± 7.3</td>
<td>1.14 (1.69)</td>
</tr>
<tr>
<td>MOR-1F</td>
<td>159 ± 28</td>
<td>18.7 ± 2.7</td>
<td>81.4 ± 7.3</td>
<td>8.53 (1.96)</td>
</tr>
</tbody>
</table>
the compound. Taking the binding affinity differences into consideration, the differences between the two drugs were lower, but still present. Indeed, there was little difference between the drugs for MOR-1C and only a modest difference for MOR-1D. However, we continued to see a 42-fold difference between the drugs for MOR-1F and a 21-fold difference with MOR-1 itself.

Discussion

[^H]14-Methoxymetopon is a highly selective µ-opioid agonist with an analgesic potency well more than 100-fold that of morphine (Fürst et al., 1993; Freye et al., 2000; Zernig et al., 2000; King et al., 2003). Although radioligand binding studies confirm the µ selectivity of the ligand, they do not explain its extraordinary analgesic potency in vivo, which far exceeds its higher affinity for the receptors. Other aspects of its pharmacological profile also illustrate unusual characteristics, with ceiling effects on a number of other opioid actions, including respiratory depression and the inhibition of gastrointestinal transit (Freye et al., 2000; King et al., 2003). Thus, this compound is quite unique. One possible explanation for this difference between the analgesic and other actions of 14-methoxymetopon may involve the concept of multiple µ-opioid receptors, as first formally proposed 25 years ago (Wolozin and Pasternak, 1981). This proposal was initially based upon studies with naloxazone and naloxonazine, which selectively blocked morphine analgesia without interfering with respiratory depression or gastrointestinal transit (Hahn et al., 1982; Ling et al., 1983, 1984, 1985; Lutz et al., 1984; Heyman et al., 1988; Paul and Pasternak, 1988). This dissociation between analgesia and both respiratory depression and gastrointestinal inhibition is similar to the pharmacology reported with 14-methoxymetopon.

Binding was first carried out in calf brain membranes to validate the assay conditions and to create a detailed binding profile of 14-methoxymetopon, controlling for tissue linearity, pH, temperature, and ions. Binding criteria were similar to other ^3H-opioid ligands.[^H]14-Methoxymetopon labeled calf striatal membranes with a $K_D$ of 0.99 nM and a distribution similar to that observed previously with other ^3H-opioid ligands. The binding responded to temperature, divalent cations, guanine nucleotides, and sodium ions as reported previously for ^3H-opioid agonists (Pert et al., 1973; Creese et al., 1975; Pasternak et al., 1975; Childers and Snyder, 1978). Saturation studies revealed linear Scatchard plots in the brain membranes. However, with a multitude of MOR-1 variants present in the tissue, the radioligand was clearly labeling more than one receptor. Thus, the linear plot implies a similar affinity of the radioligand for the sites labeled. This is supported by the linear semilog dissociation curve. These observations were similar to those previously reported in rat brain, which also showed high affinity for the ligand ($K_D$ of 0.43 nM) and a linear Scatchard plot (Spetea et al., 2003a).

In rat brain,[^H]methoxymetopon reportedly labels only µ sites (Spetea et al., 2003). Our results in calf brain were similar, with all of the binding effectively competed by µ-selective ligands. The competition curves were monophasic and showed no evidence of a binding site that was insensitive to traditional µ opioids. In addition, the $K_I$ values were similar to those determined in the same tissue preparations with[^H]DAMGO. Yet, we observed interesting $B_{max}$ differences between[^H]14-methoxymetopon and[^H]DAMGO. Although both are µ-selective agonists,[^H]14-methoxymetopon labeled approximately 45% more sites than[^H]DAMGO.

**Fig. 6.** Stimulation of[^S]GTP binding in MOR-1 splice variants. Stimulation of[^S]GTP binding was determined for 14-methoxymetopon and DAMGO at the indicated concentrations in CHO cell stably expressing the indicated MOR-1 splice variant. EC$_{50}$ and $B_{max}$ values are in Table 3. Results are normalized to a percentage of the maximal stimulation by 10 µM DAMGO in each cell line. The maximal stimulation by DAMGO over basal[^S]GTP binding levels was MOR-1, 101 ± 6.5%; MOR-1A, 161 ± 8.3%; MOR-1B, 279 ± 10.2%; MOR-1C, 62.9 ± 21.5%; MOR-1D, 216 ± 24%; MOR-1E, 32.7 ± 1.4%; and MOR-1F, 86.9 ± 14.2%.
significantly more efficacious than DAMGO for MOR-1C (\textit{p} < 0.001). \textit{Stimulation} of \textsuperscript{3}H\textit{DAMGO} binding was performed in cell membranes isolated from CHO cells stably transfected with the indicated murine MOR-1 splice variant. \textit{EC}_{50} and maximal stimulation data (normalized to the percentage of stimulation of 10 \textmu M DAMGO) was computed using GraphPad Prism. Values represent the mean ± S.E.M. for at least three independent experiments. Two-way analysis of variance followed by Bonferroni’s post-tests showed a significant difference between the \textit{EC}_{50} of 14-methoxymetopon and DAMGO for MOR-1 (\textit{p} < 0.001), MOR-1A (\textit{p} < 0.001), MOR-1B (\textit{p} < 0.01), MOR-1C (\textit{p} < 0.05), and MOR-1F (\textit{p} < 0.001). 14-Methoxymetopon is significantly more efficacious than DAMGO for MOR-1C (\textit{p} < 0.001).

We considered the possibility that \textsuperscript{3}H\textit{14-methoxymetopon} was labeling a unique site unrelated to the \mu receptor, but we feel that this is unlikely since all of the binding was sensitive to \mu opioids. Radiolabeled antagonists oftentimes label more sites since they bind both agonist and antagonist receptor conformations, unlike agonists that typically label only the agonist conformation. The similar \textsuperscript{3}H\textit{14-methoxymetopon} and \textsuperscript{3}H\textit{naloxy} \textsubscript{Bmax} levels raised the interesting possibility that 14-methoxymetopon may label both conformations, a possibility supported by the binding studies with the expressed variants (see below). This would set the drug apart from traditional \mu agonists and might potentially help explain its unusual potency.

To more fully explore the mechanisms of 14-methoxymetopon, we examined its binding and functional activity in CHO cells expressing a series of full-length MOR-1 splice variants. \textsuperscript{3}H\textit{14-Methoxymetopon} displayed similar high affinities for all of the variants, except for MOR-1F, which was significantly lower (\textit{p} < 0.001). Since MOR-1F has the identical transmembrane domains as the other variants, the difference in affinity can only be due to the changes in amino acids at the C terminus.

The studies in brain tissue indicated that \textsuperscript{3}H\textit{14-methoxymetopon} labeled more sites than the \mu agonist \textsuperscript{3}H\textit{DAMGO}. In the cell lines, the \textsubscript{Bmax} levels for \textsuperscript{3}H\textit{14-methoxymetopon} in MOR-1A, MOR-1B, and MOR-1F-containing cells are significantly greater than that of \textsuperscript{3}H\textit{DAMGO} (\textit{p} < 0.001), with the largest increase with MOR-1F. MOR-1C, MOR-1D, and MOR-1E were exceptions, where all of the radioligands labeled similar number of sites within each transfected cell line. The differences between these variants and the others could not be simply due to expression levels, since the \textsubscript{Bmax} values of the three exceptions spanned a wide range. In contrast, \textsuperscript{3}H\textit{14-methoxymetopon} binding was quite similar to \textsuperscript{3}H\textit{naloxy} in all lines, except for MOR-1E and MOR-1F cells, where \textsuperscript{3}H\textit{naloxy} binding was actually lower. Thus, it seems that \textsuperscript{3}H\textit{14-methoxymetopon} can label all of the sites labeled by naloxone, suggesting that it binds to both agonist and antagonist conformations of the receptors.

Functionally, 14-methoxymetopon was a full agonist at each splice variant, with the maximal stimulation of \textsuperscript{35}S\textit{GTPG-S} binding, a measure of efficacy, virtually the same as DAMGO. However, the relatively potencies of the two agents varied far more than would be anticipated based upon binding affinities alone. The greatest differences were seen with MOR-1 and MOR-1E, where 14-methoxymetopon was over 50-fold more potent. To provide a comparison that takes into account the differences in their affinities in binding studies, we also compared the two agents using the ratio of the \textit{EC}_{50} and K\textsubscript{d}. The ratio should provide an indication of the relative potencies of the drugs at similar receptor occupancies, an indication of their relative intrinsic activities. As anticipated, the differences between the two drugs within a variant narrowed when using the ratios, but still varied markedly from variant to variant. Although the two drugs ratios showed little difference for MOR-1C and a very modest difference for MOR-1D, there was a 40-fold difference for MOR-1F. These observations illustrate that the intrinsic activity of 14-methoxymetopon differs from variant to variant as well as from that of DAMGO. These enhanced potencies, which were independent from efficacy, are consistent with the extremely high analgesic activity of the drug.

The reasons for these functional differences are not clear. The ability of 14-methoxymetopon to label both agonist and antagonist conformations of the receptor may help explain far greater potency than DAMGO in many of the assays. However, this does not explain the variability among the splice variants for 14-methoxymetopon itself. These differences are not unique to this agent. Prior studies revealed similar differences in the functional activation of the variants by a number of opioids in all species examined (Pan et al., 2003, 2005a; Bolan et al., 2004; Pasternak et al., 2004). The only structural differences among the full-length splice variants involve the amino acids at the tip of the C terminus. In MOR-1, exon 4 encodes 12 amino acids (LENLEAEATLPL), which are replaced in the other variants. These 12 amino acids are replaced by a wide range of sequences, from only two in MOR-1B to 58 in MOR-1F. How these differences at the C terminus may influence the activation of the receptor is still under investigation. Evidence suggests that the different sequences can influence the proteins associated with the receptor in the membrane and even which G protein \alpha subunit that associates with the receptor (Premkumar, Pan, and Pasternak, unpublished observations). However, the mechanism through which these sequences alter these associations has not been determined.

In conclusion, 14-methoxymetopon is a very unusual opioid

### Table 3

Stimulation of \textsuperscript{[35]S}\textit{GTPG-S} binding by 14-methoxymetopon and DAMGO

<table>
<thead>
<tr>
<th>14-Methoxymetopon</th>
<th>DAMGO</th>
<th>Relative Activity of 14-Methoxymetopon</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{EC}_{50}</td>
<td>Maximal Stimulation</td>
<td>\textit{EC}_{50}/K\textsubscript{d}</td>
</tr>
<tr>
<td>\textit{nM}</td>
<td>% DAMGO</td>
<td>\textit{EC}_{50}</td>
</tr>
<tr>
<td>MOR-1</td>
<td>1.1 ± 0.5</td>
<td>125.1 ± 4.9</td>
</tr>
<tr>
<td>MOR-1A</td>
<td>4.5 ± 1.9</td>
<td>99.0 ± 6.5</td>
</tr>
<tr>
<td>MOR-1B</td>
<td>1.6 ± 0.3</td>
<td>92.5 ± 3.6</td>
</tr>
<tr>
<td>MOR-1C</td>
<td>4.2 ± 0.9</td>
<td>125 ± 25</td>
</tr>
<tr>
<td>MOR-1D</td>
<td>1.1 ± 0.2</td>
<td>101.7 ± 8.4</td>
</tr>
<tr>
<td>MOR-1E</td>
<td>0.5 ± 0.1</td>
<td>109 ± 12</td>
</tr>
<tr>
<td>MOR-1F</td>
<td>2.7 ± 0.3</td>
<td>117 ± 14</td>
</tr>
</tbody>
</table>
analgesic. In vivo, its analgesic activity far exceeds that of morphine and most other μ opioids, despite very limited differences in binding affinities. The current study demonstrates the high binding affinity of 14-methoxymetopon for a series of mouse MOR-1 variants and an unexpectedly potent activation of [35S]GTPγS binding. However, the enhanced relative functional potency compared with DAMGO varied considerably from one variant to another, suggesting that the intrinsic activity of 14-methoxymetopon was dependent upon the variant being examined. This may help explain its unusual pharmacological profile.

References


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