Different Effects of Opioid Antagonists on μ-, δ-, and κ-Opioid Receptors with and without Agonist Pretreatment

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ABSTRACT

Opioid receptors display basal signaling (constitutive, agonist-independent activity), which seems to be regulated by agonist exposure. Whereas agonist pretreatment desensitizes receptors to subsequent agonist stimulation, basal signaling of μ-opioid receptor (MOR) was shown to increase. Moreover, agonist pretreatment converts the neutral antagonists naloxone and naltrexone into inverse agonists, suppressing basal signaling, whereas analogs with reduced C6-position, e.g., 6β-naltrexol, remain neutral antagonists at MOR under any condition. This study compares the regulation of basal signaling of MOR, δ-(DOR), and κ-(KOR) opioid receptors after pretreatment with morphine or receptor-selective agonists, in transfected human embryonic kidney 293 cell membranes. Moreover, naloxone, naltrexone, and related antagonists were compared for binding potency and effect on basal and agonist-stimulated receptor signaling, measuring guanosine 5′-O-[32P]triphosphate binding. The results demonstrate basal activity for each opioid receptor, which is modulated by pretreatment with agonists. Even closely related opioid antagonists display distinct patterns of neutral and inverse effects before and after agonist pretreatment, including distinct efficacies between naloxone and naltrexone at agonist-pretreated DOR and KOR. Pretreatment with different agonists has varying effects on inverse and neutral activities of some analogs tested. These results demonstrate that antagonist efficacy is context-dependent, possibly accounting for paradoxical pharmacological effects. Activity profiles at the three opioid receptors under different conditions could lead to antagonists with optimal clinical properties in treatment of addiction and adverse opioid effects.

G protein-coupled receptors (GPCRs) have diverse physiological functions, and they are important pharmacological targets. Although a GPCR typically requires activation by an agonist, many GPCRs also display basal or spontaneous signaling activity in the absence of agonist (constitutive activity). The identification of inverse agonists that block basal signaling of a GPCR further confirmed the existence of basal activity, and it was subsequently suggested that a majority of currently known GPCR antagonists are inverse agonists (Kenakin, 2004). Antagonists with inverse agonist property potentially have distinct treatment outcomes compared with neutral antagonists, i.e., antagonists that block agonist activation but do not affect basal activity. For example, the classification of “typical” and “atypical” antipsychotics may in part be related to inverse agonism (atypical) and neutral antagonism (typical) at 5-hydroxytryptamine_{2C} receptors (Herrick-Davis et al., 2000).

The opioid receptors belong to GPCR family and consist of three genes encoding μ-, δ-, and κ-opioid receptors (or MOR, DOR, and KOR, respectively). Although the basal signaling activity for DOR is readily detectable (Costa and Herz, 1989), being the first GPCR found to display basal signaling activity, this was more difficult to demonstrate for MOR, possibly due to the masking of basal MOR activity by interacting regulatory proteins, such as calmodulin (Wang et al., 1999). Our laboratory has demonstrated the presence of basal MOR signaling activity in various tissues in cell culture (Wang et al., 1994, 1999, 2000; Burford et al., 2000) and in mouse brain tissue (Wang et al., 2004), which was typically more prominent in opioid agonist-pretreated (“dependent”) tissues. This was at first an unexpected finding, because MOR is thought
to desensitize during agonist pretreatment; yet, we have shown that release of calmodulin from the receptor by agonist stimulation uncovered the innate basal activity of MOR in the dependent state (Wang et al., 1999, 2000). We have further identified several inverse agonists and neutral antagonists, the latter blocking both opioid agonist and inverse agonist effects, a strong indication that the observed effects are indeed elicited by binding to MOR (Bilsky et al., 1996; Wang et al., 2001, 2004). Constitutive MOR activity was independently confirmed (Liu et al., 2001), as was the usual regulation of constitutive activity of MOR and DOR receptors by chronic agonists pretreatment (Liu and Prather, 2001, 2002). Moreover, basal opioid activity has been implicated in appetite (Emmerson et al., 2004), morphine tolerance (Heinzen et al., 2005), and methamphetamine-induced behavioral sensitization (Chiu et al., 2006).

An important finding came from the observation that some neutral antagonists, such as naloxy and naltrindole, turned into inverse agonists after agonists pretreatment, suggesting that the receptor has been modified in the dependent state. The conversion of naloxy and naltrindole from neutral antagonist to inverse agonist seems to contribute to precipitated withdrawal symptoms in opioid addicts (Wang et al., 2004; Raehal et al., 2005; Sadee et al., 2005). This was supported by our finding that neutral antagonist 6β-naltrexol and β-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH2, which remain neutral in the dependent state, precipitate less withdrawal than naloxy in morphine-dependent mice, at equipotent pharmacological doses in blocking morphine-induced antinociception (Bilsky et al., 1996; Wang et al., 2004; Raehal et al., 2005). Others also reported that basal activity of MOR was related to conditioned aversive effect of naloxy in morphine-dependent mice (Shoblock and Maidment, 2006), opioid antagonists with different inverse agonist properties have different effects in precipitating withdrawal in acute morphine-dependent mice (Walker and Sterious, 2005), and constitutive opioid receptor activation is critically involved in acute opioid withdrawal (Freys and Levy, 2005).

The variable behavior of naloxy and naltrindole in naive and opioid-pretreated cells is consistent with the notion that ligand activity can vary with cellular context, i.e., the “protean” properties of the ligands (Gbahou et al., 2003). Thus, naltrindole and naloxy are protean antagonists at MOR, whereas 6β-naltrexol is neutral under all conditions studied (Wang et al., 2001; Raehal et al., 2005). A thorough understanding of the malleable protean properties of opioid antagonists is important because of the dramatic precipitated withdrawal caused by naloxy and naltrindole, effects that may be avoided or ameliorated with a neutral antagonist such as 6β-naltrexol. Moreover, these ligands also bind to DOR and KOR, but regulation of basal activity and protean ligand properties remain unknown at these subtypes.

In this study, we have investigated and compared the regulation of basal activity of MOR, DOR, and KOR, and the effects of naloxy, naltrindole, and naltrindole derivatives 6β-naltrexol (Raehal et al., 2005) and 6β-naltrexamide (Fig. 1) on MOR, DOR, and KOR receptor with and without agonists pretreatments. We were particularly interested in 6β-naltrexol and 6β-naltrexamide, because these neutral antagonists represent potential therapeutic agents in the treatment of opioid side effect. We used BNTX (Wang et al., 2001) and ICI 174,864 (Costa and Herz, 1989) as full MOR and DOR inverse agonists, respectively. For KOR, we have identified nor-binaltorphimine (nor-BNI) and 5’-guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5α-epoxy-3,14-dihydroxy-6,7-2’-3’-indolomorphinan dihydrochloride (GNTI) as inverse agonist.

Materials and Methods

Materials. Morphine sulfate, naloxy, naltrindole, 6β-naltrexol, and 6β-naltrexamide were obtained through the National Institute on Drug Abuse Drug Supply Program; U-69593, [d-Pen²,d-Pen⁶]-enkephalin (DPDPE), and [d-Ala²,N-Me-Phe⁵,Gly⁷-ol]-enkephalin (DAMGO) were purchased from Sigma-Aldrich (St. Louis, MO); U-50,488H, ICI 174,864, nor-BNI, and GNTI were purchased from Tocris Cookson Inc. (Ellisville, MO); [35S]GTPγS and [3H]diprenorphine were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Other reagents for cell culture were from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

Cell Culture and Treatment. Human embryonic kidney (HEK) 293 cells stably transfected with human MOR (HEK-MOR), mouse DOR (HEK-DOR), and human KOR (HEK-KOR) were maintained in Dulbecco’s modified Eagle’s medium H16/F-12 supplemented with 10% fetal bovine serum, 100 μM penicillin, 100 μg/ml streptomycin, and 200 μg/ml Geneticin (G-418; Invitrogen, Carlsbad, CA). The receptor expression levels were 1.2, 3.4, and 2.7 pmol/mg protein for HEK-MOR, HEK-DOR, and HEK-KOR, respectively (measured by [3H]diprenorphine saturation binding assays in cell membranes). For agonist pretreatment, 80% confluent cells were cultured in the presence MOR-, DOR-, or KOR-specific agonists DAMGO (1 μM), DPDPE (1 or 10 μM), U-50,488H (1 μM), or U-69593 (1 μM), or nonspecific agonist morphine (10 or 50 μM) for 24 h before harvest. Cells were then washed thoroughly with phosphate-buffered saline (PBS) to remove the treated drugs before membrane preparations.

[35S]GTPγS Binding. Membrane preparation and [35S]GTPγS binding assays were carried out as described previously (Wang et al., 2000). In brief, cells were harvested and washed with PBS, and then the cells were homogenized in buffer containing 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA and centrifuged at 30,000 g for 10 min. The pellets were resuspended in the same buffer and centrifuged again. The pellets from the second centrifugation were resuspended, aliquoted, and stored at −70°C. [35S]GTPγS binding assays were performed using different conditions. For agonist effects, cell membranes (10 μg of protein) were incubated with drugs in 100 μl of assay buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, and 10 μM GDP at 30°C for 5 min. For inverse agonist effects, cell membranes (50 μg of protein) were incubated with drugs in 500 μl of different assay buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, and 10 μM GDP at 30°C for 5 min. For inverse agonist effects, cell membranes (50 μg of protein) were incubated with drugs in 500 μl of different assay buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, and 10 μM GDP at 30°C for 5 min. After incubation, the reactions were stopped by adding 500 μl of ice-cold PBS followed by centrifugation at 13,000g for 10 min at 4°C. The pellets were washed once with 1 ml of PBS, and radioactivity was measured by liquid scintillation counter.

[3H]Diprenorphine Binding. For [3H]diprenorphine saturation binding assay, membranes (20 μg of protein) were incubated with...
different concentrations (0.5–5 nM) of [3H]diprenorphine in buffer containing 50 mM Tris-HCl, pH 7.4, and 5 mM EDTA at 23°C for 1 h. For competitive binding experiments, 0.5 nM [3H]diprenorphine was incubated with 20 μg of membranes in the absence or presence of different concentration of tested compounds at 23°C for 1 h. Incubations were terminated by rapid filtration onto glass fiber filters (Whatman Schleicher and Schuell, Keene, NH). The filters were washed with 10 ml of ice-cold PBS, and the radioactivity was quantified using a liquid scintillation counter.

Data Analysis. Results are expressed as means ± S.D. for at least three experiments, each performed in duplicate. Statistical analysis and curve fits of dose-response curves were performed using Prism (GraphPad Software Inc., San Diego, CA).

Results

Binding Characteristics and Antagonistic Effects of Naltrexone Analogs on MOR, DOR, and KOR. We performed competitive receptor binding assays in membrane expressing MOR, DOR, and KOR to determine the binding affinity of test compounds, using the tracer [3H]diprenorphine, which is a nonselective opioid ligand. Saturation binding assays showed that the $K_i$ values of [3H]diprenorphine were 0.39, 0.44, and 0.27 nM in MOR, DOR, and KOR membranes, respectively. Both 6β-naltrexol and 6β-naltrexamide have highest affinity for MOR, followed by KOR, whereas they are 20 to 30 times less potent for DOR (Table 1). The binding affinity of 6β-naltrexol for MOR and KOR is 2- to 5-fold higher than naloxone and 2-fold less potent than naltrexone, whereas it is 3- to 4-fold less potent than naloxone and naltrexone for DOR (Table 1). The binding affinity of 6β-naltrexamide is 3-fold more potent than naloxone and 4-fold less potent than naltrexone at MOR, whereas it is 2- to 7-fold and 7- to 10-fold less potent than naloxone at KOR and DOR, respectively (Table 1).

To test the antagonistic properties of these compounds, the inhibition of submaximal concentration of DAMGO- (1 μM), DPDPE- (30 nM), or U50,488H- (300 nM) stimulated [35S]GTP$\gamma$S binding was determined in MOR, DOR, and KOR membranes. Naloxone, naltrexone, and analogs dose-dependently inhibited agonist-stimulated [35S]GTP$\gamma$S binding in MOR, DOR, and KOR membranes with similar potency order as observed for binding affinity (Table 1). This result indicates that naloxone, naltrexone, 6β-naltrexol, and 6β-naltrexamide all act as full antagonists at each of the three opioid receptors tested (Wang et al., 2001). On the other hand, the antagonistic potency ($K_i$) of BNTX, ICI 174,864, and nor-BNI is greater than the [3H]diprenorphine binding affinities ($K_i$) (Table 1). This result may be related to their status as full inverse agonists at the respective opioid receptors (see below).

Basal Signaling Activity of MOR and the Effects of Naltrexone Analogs. We have previously reported that pretreatment of MOR with morphine increases the inverse agonist effect of β-chloronaltrexamine (Wang et al., 2000, 2001). To test the effects of DAMGO pretreatment on basal activity and inverse agonist effects, we pretreated MOR with DAMGO for 24 h, followed by removal of the agonist by washing the cells thoroughly. DAMGO pretreatment increased the $E_{max}$ value of DAMGO and decreased the $E_{max}$ value (Fig. 2a, Table 2), indicating receptor desensitization and down-regulation has occurred. In contrast, both DAMGO and morphine pretreatment increased inverse agonist effects of BNTX and shifted the dose-response curve of BNTX to the

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>[3H]Diprenorphine Binding, $K_i$ (nM)</th>
<th>MOR</th>
<th>DOR</th>
<th>KOR</th>
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<tr>
<td>nor-BNI</td>
<td>25 ± 11</td>
<td>11 ± 3</td>
<td>0.2 ± 0.1</td>
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<tr>
<td>ICI 174,864</td>
<td>&gt;10,000</td>
<td>31 ± 12</td>
<td>&gt;10,000</td>
<td></td>
</tr>
<tr>
<td>BNTX</td>
<td>12 ± 1</td>
<td>1 ± 1</td>
<td>48 ± 1</td>
<td></td>
</tr>
<tr>
<td>Naloxone</td>
<td>7 ± 1</td>
<td>8 ± 1</td>
<td>4 ± 1</td>
<td></td>
</tr>
<tr>
<td>Naltrexone</td>
<td>0.5 ± 0.1</td>
<td>7 ± 1</td>
<td>1 ± 1</td>
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<tr>
<td>6β-Naltrexol</td>
<td>1.4 ± 0.1</td>
<td>29 ± 14</td>
<td>2 ± 1</td>
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<td>6β-Naltrexamide</td>
<td>2.3 ± 0.1</td>
<td>61 ± 12</td>
<td>9 ± 1</td>
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<th>Compound</th>
<th>Antagonistic Effects, $K_i'$ (nM)</th>
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<td>nor-BNI</td>
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<tr>
<td>ICI 174,864</td>
<td>N.D.</td>
</tr>
<tr>
<td>BNTX</td>
<td>N.D.</td>
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<tr>
<td>Naloxone</td>
<td>0.5 ± 0.2</td>
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<tr>
<td>Naltrexone</td>
<td>2 ± 1</td>
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<tr>
<td>6β-Naltrexol</td>
<td>4 ± 1</td>
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<tr>
<td>6β-Naltrexamide</td>
<td>76 ± 13</td>
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N.D., not determined.

Fig. 2. Regulation of agonist and inverse agonist effects on MOR after DAMGO and morphine pretreatment in transfected HEK-MOR cells. HEK-MOR cells were pretreatment with 1 μM DAMGO or 10 μM morphine for 24 h, and then cell membranes were prepared for [35S]GTP$\gamma$S binding. a, dose-response curves of DAMGO in control and DAMGO-pretreated membranes. b, dose-response curves of BNTX in control, DAMGO-, or morphine-pretreated membranes. Data are means ± S.D., n = 6.
TABLE 2
Effects of different opioid antagonists on basal [\(^{35}\)S]GTP\(S\) binding in MOR cell membranes with or without agonists pretreatment.
Maximal effects (\(E_{\text{max}}\)) are expressed as percentage of change from basal. Positive values indicate agonistic effects, whereas negative values indicate inverse agonist effects.
Data are means ± S.D., \(n = 4\) to 8.

<table>
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<tr>
<th>Compound</th>
<th>No Pretreatment</th>
<th>Pretreatment</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(EC_{50})</td>
<td>(E_{\text{max}})</td>
<td>(EC_{50})</td>
</tr>
<tr>
<td></td>
<td>nM</td>
<td>% change</td>
<td>nM</td>
</tr>
<tr>
<td>DAMGO</td>
<td>74 ± 13</td>
<td>92 ± 5</td>
<td>164 ± 59*</td>
</tr>
<tr>
<td>BNTX</td>
<td>402 ± 250</td>
<td>-7 ± 2**</td>
<td>31 ± 16*</td>
</tr>
<tr>
<td>Naloxone</td>
<td>2 ± 1</td>
<td>14 ± 1**</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>N.D.</td>
<td>29 ± 6**</td>
<td>N.D.</td>
</tr>
<tr>
<td>6β-Naltrexol</td>
<td>3 ± 2</td>
<td>12 ± 1**</td>
<td>N.D.</td>
</tr>
<tr>
<td>6β-Naltrexol</td>
<td>3 ± 2</td>
<td>22 ± 1**</td>
<td>29 ± 15*</td>
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N.D., not determined.
\* \(P < 0.01\), compared with no pretreatment.
** \(P < 0.01\), compared with zero; ANOVA with Dunnett's post test.

left, with no difference observed between morphine and the receptor-selective agonist DAMGO (Fig. 2b; Table 2). These results demonstrate that morphine and DAMGO pretreatment increases the inverse agonist effects of BNTX, implying increased basal signal activity of MOR, consistent with previous results (Wang et al., 2001), and also sensitized MOR to the inverse agonist effects of BNTX, even though the receptor was partially desensitized to agonist activation. Similar to previous results (Wang et al., 2001), naloxone and naltrexone marginally increased basal [\(^{35}\)S]GTP\(S\) binding in untreated MOR membranes, but decreased it after DAMGO or morphine pretreatment with similar \(EC_{50}\) values (Table 2). This is consistent with previous observations indicating that morphine or DAMGO pretreatment turns naloxone and naltrexone into inverse agonists. In contrast, 6β-naltrexol and 6β-naltrexamide did not decrease basal [\(^{35}\)S]GTP\(S\) binding in even agonist-pretreated MOR membranes (Table 2), consistent with neutral antagonism without protean property as reported previously for 6β-naltrexol (Wang et al., 2001).

As a critical test of basal MOR activity, neutral antagonists are expected to inhibit the effects of inverse agonist. Indeed, the inverse agonist effect of BNTX was inhibited by naloxone and naltrexone analogs in untreated MOR membranes, and the inverse agonist effect of naloxone in DAMGO-pretreated MOR membranes was also inhibited by the naltrexone analogs (Fig. 3, a and b). These results indicate that 6β-naltrexol and 6β-naltrexamide are neutral antagonists regardless of agonist pretreatment. Although part of these results have been reported previously (except for 6β-naltrexamide), they are essential in this report for direct comparison with subsequent results obtained under identical conditions with DOR and KOR.

Fig. 3. A, inhibition of inverse agonist effects of 1 \(\mu\)M BNTX by naloxone (Nal), 6β-naltrexol (6β-nal), and 6β-naltrexolamide (6β-NXM) (all 10 \(\mu\)M) in untreated HEK-MOR cell membranes. B, inhibition of inverse agonist effects of naloxone in DAMGO-pretreated HEK-MOR membranes by 6β-naltrexol and 6β-naltrexolamide. Data are means ± S.D., \(n = 6\), * \(P < 0.05\) and **, \(P < 0.01\), compared with control (C).

Fig. 4. Regulation of agonist and inverse agonist effects on DOR after DPDPE and morphine pretreatment in transfected HEK-DOR cells. HEK-DOR cells were pretreatment with 1 \(\mu\)M DPDPE or 50 \(\mu\)M morphine for 24 h, and then cell membranes were prepared for [\(^{35}\)S]GTP\(S\) binding. A, dose-response curves of DPDPE in control, DPDPE-, or morphine-pretreated membranes. B, dose-response curves of ICI 174,864 in control, DPDPE-, or morphine-pretreated membranes. Data are means ± S.D., \(n = 6\), * \(P < 0.05\) and **, \(P < 0.01\), compared with control (C).
Basal Signaling Activity of DOR and the Effects of Naltrexone Analogs. Pretreatment of DOR with DPDPDE has been shown to decrease the inverse agonist effect of ICI 174,864 while turning naloxone into an inverse agonist (Liu and Prather, 2002). To test whether DPDPDE and morphine exposure regulates DOR basal activity differently, we pretreated HEK-DOR cells with DPDPDE or morphine for 24 h, followed by washout of the agonists. Agonist pretreatment increased the EC$_{50}$ value and decreased the $E_{\text{max}}$ value for DPDPDE (Fig. 4a; Table 3), indicating receptor desensitization and down-regulation. Different from MOR, and consistent with previous results, pre-exposure of DOR to DPDPDE decreased inverse agonist effects of ICI 174,864 without changing the EC$_{50}$ value (Fig. 4b; Table 3). In contrast, pretreatment of DOR with morphine shifted the dose-response curve for ICI 174,864 to the left without affecting $E_{\text{max}}$ (Fig. 4b; Table 3). These results show that DPDPDE and morphine regulate basal DOR activity differently. Similar to MOR, in untreated DOR membranes, naltrexone and its analogs showed small increase (8–41%) in [35S]GTP$\gamma$S binding in KOR membranes. As shown in Fig. 6, nor-BNI and GNTI dose-dependently decreased basal [35S]GTP$\gamma$S binding, indicating conversion into an inverse agonist as reported previously (Liu and Prather, 2002). Likewise, morphine pretreatment also converted naloxone into inverse agonist (Table 3). However, in contrast to the findings with MOR, naltrexone did not turn into inverse agonist at DOR after either DPDPDE or morphine pretreatment (Table 3). On the other hand, 6β-naltrexol and 6β-naltrexamide remained neutral after both agonists pretreatment (Table 3); therefore, they are neutral antagonists regardless of agonist pretreatment in DOR, as shown for MOR. The inverse agonist effect of ICI 174,864 in untreated DOR membranes was inhibited by all four antagonists, and the inverse agonist effect of naloxone in DPDPDE-pretreated membranes was inhibited by naltrexone and analogs (Fig. 5). These results for the first time distinguish the effects of naloxone from those of naltrexone.

Basal Signaling Activity of KOR and the Effects of Naltrexone Analogs. Different from MOR and DOR, basal activity for KOR had not been fully demonstrated, although one KOR antagonist was found to have inverse agonist effect on KOR in a ligand screen (Becker et al., 1999). To test whether KOR displays basal activity, we tested the effects of KOR antagonists nor-BNI and GNTI on basal [35S]GTP$\gamma$S binding in KOR membranes. As shown in Fig. 6, nor-BNI and GNTI dose-dependently decreased basal [35S]GTP$\gamma$S binding with EC$_{50}$ values in the femtomolar range and an $E_{\text{max}}$ value of $\sim$10% (Table 4). These results support the existence of basal activity of KOR and indicate that nor-BNI and GNTI are inverse agonists at KOR.

To test whether agonist pretreatment changes basal activity and/or inverse agonist effects in KOR, we pretreated HEK-KOR with the KOR-selective agonist U-69593, followed by washout. Pretreatment of KOR with 1 μM U-69593 shifted the dose-response curve of U-69593 to the right and decreased $E_{\text{max}}$ (Fig. 7a; Table 4), indicating KOR desensitization and down-regulation. As observed for MOR, U-69593 pretreatment of KOR increased the inverse agonist effects of nor-BNI and GNTI, and additionally, decreased their EC$_{50}$ value 3- to 4-fold (Fig. 7b; Table 4). Similar results were obtained by pretreatment with another KOR-selective ago-

### Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>1 μM DPDPDE</th>
<th>10 μM Morphine</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$</td>
<td>E$_{\text{max}}$</td>
<td>% change</td>
</tr>
<tr>
<td></td>
<td>nM</td>
<td>nM</td>
<td>nM</td>
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<td>DPDPDE</td>
<td>0.7 ± 0.1</td>
<td>88 ± 2</td>
<td>88 ± 2</td>
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<td>ICI 174,864</td>
<td>64 ± 1</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
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<td>Naloxone</td>
<td>0.1 ± 0.2</td>
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<td>6β-Naltrexol</td>
<td>1.0 ± 0.2</td>
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<td>6β-Naltrexamide</td>
<td>8 ± 1</td>
<td>21 ± 1</td>
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</table>

Data are means ± S.D., n = 4 to 8.

P$_{<0.05}$, compared with no pretreatment; ANOVA with Dunnett's post test.

* P$_{<0.01}$, compared with zero; ANOVA with Dunnett post test.

N.D. = not determined.
agonist effect of naloxone in morphine-pretreated KOR membranes (Fig. 8). Likewise, 6β-naltrexol inhibited inverse agonist effect of 6β-naltrexol in U-69593-pretreated membranes (Fig. 8).

**Discussion**

In this study, we have found that each of the three opioid receptors displayed basal activity in transfected HEK cell membranes, which was modulated by agonist pretreatment. However, the regulation of basal signaling differs in some detail for MOR, DOR, and KOR, and with pretreatment by different agonists (Table 5). These results suggest that basal receptor activity can have physiological significance, and moreover, that it is subject to regulation as a result of receptor stimulation.

In contrast to agonist-induced receptor desensitization, our results indicate that agonist-pretreatment increases basal activity and/or sensitizes inverse agonist effects at MOR, DOR, and KOR, based on the following observations (Table 5). 1) For MOR, both potency and efficacy of BNTX increased, and naloxone and naltrexone turned into inverse agonist after agonist pretreatment. 2) For KOR, agonist pretreatment increased efficacy and potency of nor-BNI and GNTI (with the exception of morphine, which did not affect potency). In addition, naloxone and 6β-naltrexol turned into inverse agonist after agonist pretreatment. 3) For DOR, although we observed a decrease in efficacy of ICI 174,864 after DPDPE pretreatment (possibly a result of partial receptor down-regulation), the potency of ICI 174,864 was increased after morphine pretreatment, indicating sensitization to inverse agonism after morphine pretreatment. Moreover, both morphine and DPDPE pretreatments turned naloxone into an inverse agonist.

It seems paradoxical that basal activity and inverse agonist properties of antagonists tend to increase after agonist pretreatment, whereas agonist-stimulation is desensitized. One possibility is the unmasking of basal receptor activity, for example by shedding calmodulin from binding sites in the i3 loop involved in G protein coupling (Wang et al., 1999). Increased constitutive state of receptor probably is associated with a change in receptor conformation after agonist pretreatment. This is supported by opioid receptor mutants that have enhanced constitutive activity (Brillet et al., 2003). The different pharmacological behavior of opioid antagonists may be caused by different affinities for various receptor conformations they act on. Previous studies have shown that pretreatment with the inverse DOR agonist (IC1 174,864) produces new receptor sites with 1000-fold higher affinity for

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**Fig. 6.** Dose-response curves of nor-BNI and GNTI on basal [35S]GTPγS binding in HEK-KOR membranes. Data are means ± S.D., n = 5.
naloxone at DOR (Pineyro et al., 2005). Alternatively, basal signaling could proceed via distinct signaling pathways that are activated while agonist-stimulated pathways are downregulated. Switching of signaling pathways has been reported between different ligands and after pretreatment (Chakrabarti et al., 2001; Dupre et al., 2004). In either case, agonist pretreatment alters the opioid receptor system, modulating both agonist-stimulated and basal signaling.

Our current results are consistent with our previous reports (Wang et al., 2000, 2001, 2004) as well as a report by Liu and Prather (2001), showing that the effect of the inverse MOR agonist β-chloronaltrexamine was increased after morphine pretreatment. Our results did not show differences in inverse agonist effects between morphine and DAMGO pretreatment, inconsistent with a previous report showing DAMGO pretreatment increased inverse agonist effects more strongly than morphine (Liu and Prather, 2001). The discrepancy may be caused by the different cell culture (GH3 cell in the previous study) or by different DAMGO concentrations used for pretreatment. In this study, we have chosen concentration of agonists based on their ability to produce similar response (1 μM for DAMGO and 10 μM for morphine), whereas the previous report used the same dose (10 μM for both). Because DAMGO is known to be more efficacious than morphine, we would expect it to have greater effects on receptor regulation at equal doses. However, whereas agonist-stimulated responses are more strongly affected by DAMGO than morphine, this was not the case for the regulation of basal activity in this case. We propose that different mechanisms play a role in these two processes, an interesting possibility, because it then might be feasible to develop ligands that have differential effects and improved in vivo pharmacology. For DOR, our results are consistent with those of others (Liu and Prather, 2002), showing the effect of ICI 174,864 decreased after DPDPE pretreatment, whereas naloxone turned into an inverse agonist. The decrease in the $E_{\text{max}}$ value of ICI 174,864 might have been caused by substantial receptor internalization and down-regulation after DPDPE pretreatment, which does not occur after morphine pretreatment. It is also possible that internalized receptor may not be available to the hydrophilic peptide ligand ICI 174,864. This requires further study using a nonpeptide inverse agonist, such as RTI compounds (Zaki et al., 2001). The emergence of inverse agonist properties of naloxone after DPDPE indicates that the receptor is more sensitive to the antagonist, as observed with MOR. The increased potency of ICI 174,864 after morphine pretreatment also supports a process of sensitization of inverse agonism after morphine pretreatment of DOR, similar to that of MOR (calmodulin is likely to bind to DOR as well, having an identical i3 loop).

In interpreting the results of this report, one needs to consider that transfected cell lines and membrane preparations may not reflect the true pharmacological properties encountered in vivo. Nevertheless, we have taken the results from such in vitro studies, and similar data obtained with
whether antagonists with neutral or inverse agonist properties in vivo. Specifically, antagonists found to be neutral even after agonist pretreatment of MOR were subsequently shown to cause significantly less withdrawal in morphine-dependent mice (Bilsky et al., 1996; Wang et al., 2001, 2004; Raehal et al., 2005). No such in vitro-in vivo correlations exist for DOR and KOR, but the present in vitro data can form a foundation for testing different opioid antagonist properties in vivo. One further needs to consider that the cell lines are expressing high levels of opioid receptors, and the membrane incubations are done under conditions that facilitate measurements of basal activity. Under these conditions, even antagonists considered devoid of agonist activity did show some stimulation of G protein coupling, as has been observed previously (Wang et al., 2001, 2004). Last, the magnitude of the measured inverse effects is typically less than agonist-stimulated effects. For the latter, one chooses conditions that minimize basal GTP binding by adding high GDP concentrations, whereas one lowers GDP levels to observe basal coupling. Yet, this increases background noise and hence reduces the percentage of decrease that can be observed for inverse agonists. Our in vivo data indicate that the basal signaling levels and inverse effects observed for MOR are relevant to measured antagonist effects in morphine-dependent mice (Wang et al., 2004). Similar relationships may hold for DOR and KOR. It will be of interest to determine how these varying antagonist properties translate into differential pharmacological properties in vivo, in particular in eliciting withdrawal, both centrally and in the peripheral nervous system.

This study also demonstrates qualitative differences between naloxone and naltrexone, both thought to represent prototypical opioid antagonists. Conversion of these two antagonists into inverse agonists at MOR is thought to underlie at least in part their potent ability to precipitate withdrawal in an opioid-dependent state (Wang et al., 2004). However, only naloxone converted into an inverse agonist at DOR and KOR, whereas naltrexone did not. Upon titrating naloxone and naltrexone dose-response curves in measuring various withdrawal effects, clear differences emerge at higher dose levels (E. J. Bilsky, unpublished data). Our results point toward developing safer and more effective opioid antagonists targeting a variety of clinical needs, including long-term treatment of addiction, and opioid-induced gastrointestinal dysfunction.

The opioid antagonist naltrexone has been used to treat opioid overdose, opioid addiction (Gonzalez et al., 2004), and addictions to other drugs of abuse, such as alcohol (Davidson et al., 1999; Chick et al., 2000). Aversive effects of naltrexone, which is similar to opioid withdrawal and occurs even in patients without pre-exposure to opioids (Hollister et al., 1981), limit its widespread use. Neutral opioid antagonists, such as 6β-naltrexol, hold promise for causing less aversive effects in opioid-dependent subjects. As a metabolite of naltrexone in humans, but not in rodents, 6β-naltrexol has been suggested to contribute to the long-term duration of naltrexone action in human (Cone et al., 1974; Verebey et al., 1976). Moreover, serum 6β-naltrexol levels are related to alcohol responses in heavy drinkers after naltrexone administration (McCaul et al., 2000), and 6β-naltrexol also reduces alcohol consumption in rats (Rukstalis et al., 2000; Stromberg et al., 2002). Although MOR is the main target receptor in narcotic analgesia and dependence, DOR and KOR also contribute to these processes, either through heterodimerization with MOR or through presynaptic/postsynaptic regulation of MOR (Narita et al., 2001; Khotib et al., 2004; Wang et al., 2005). Whether antagonists with neutral or inverse agonist prop-

### Table 5

Regulation of basal activity and changes in ligand pharmacological properties in MOR, DOR, and KOR after morphine and receptor-selective agonist pretreatment

BNTX, ICI 174,864, and nor-BNI were used as prototype inverse agonists for MOR, DOR, and KOR, respectively. Naloxone, naltrexone, 6β-naltrexol, and 6β-naltrexamide are neutral antagonist or partial agonist in untreated opioid receptors.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Pretreatment</th>
<th>Effect of Inverse Agonist</th>
<th>Pharmacological Properties of Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR</td>
<td>DAMGO</td>
<td>↑</td>
<td>Inverse</td>
</tr>
<tr>
<td></td>
<td>Morphone</td>
<td>↑</td>
<td>Inverse</td>
</tr>
<tr>
<td>DOR</td>
<td>DPDPE</td>
<td>→</td>
<td>Neutral</td>
</tr>
<tr>
<td>KOR</td>
<td>U69593</td>
<td>↑</td>
<td>Inverse</td>
</tr>
</tbody>
</table>

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**Fig. 8.** a, inhibition of inverse agonist effects of 10 nM nor-BNI by Nal, NTX, 6β-nal, and 6β-NXM (all at 10 μM) in untreated HEK-KOR cell membranes. b, inhibition of inverse agonist effects of naloxone in morphine-pretreated HEK-KOR membranes by 6β-naltrexol and inhibition of inverse agonist effects of 6β-naltrexol in U-69593-pretreated HEK-KOR membranes by 6β-naltrexamide. Data are means ± S.D. *P < 0.05 and **P < 0.01, compared with control (C).
Acknowledgments

Morphine, naloxone, naltrexone, 6J-opioid agonists and antagonists in vivo requires further investigation.

References


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