Opioid Agonists Have Different Efficacy Profiles for G Protein Activation, Rapid Desensitization, and Endocytosis of Mu-opioid Receptors*

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The differential ability of various µ-opioid receptor (MOP) agonists to induce rapid receptor desensitization and endocytosis of MOP could arise simply from differences in their efficacy to activate G proteins or, alternatively, be due to differential capacity for activation of other signaling processes. We used AtT20 cells stably expressing a low density of FLAG-tagged MOP to compare the efficacies of a range of agonists to 1) activate G proteins using inhibition of calcium channel currents (I_{Ca}) as a reporter before and after inactivation of a fraction of receptors by β-chloro-naltrixemamine, 2) produce rapid, homologous desensitization of I_{Ca} inhibition, and 3) internalize receptors. Relative efficiencies determined for G protein coupling were [Tyr-D-Ala-Gly-MePhe-Gly-ol]enkephalin (DAMGO) (1) > methadone (0.96) > morphine (0.58) > pentazocine (0.15). The same rank order of efficacies for rapid desensitization of MOP was observed, but greater concentrations of agonist were required than for G protein activation. By contrast, relative efficacies for promoting endocytosis of MOP were DAMGO (1) > methadone (0.59) >> morphine (0.07) > pentazocine (0.03). These results indicate that the efficacy of opioids to produce activation of G proteins and rapid desensitization is distinct from their capacity to internalize µ-opioid receptors but that, contrary to some previous reports, morphine can produce rapid, homologous desensitization of MOP.

Tolerance to the analgesic and other effects of opioid drugs, such as morphine, undermines their use in long term treatment (1). Although analgesic tolerance is likely to be a complex phenomenon, an understanding of how µ-opioid (MOP) receptors are regulated by opioid agonists will lead to important insights into this phenomenon. MOPs are similar to many other G protein-coupled receptors (GPCR) in that they undergo desensitization within several minutes of stimulation by agonists (2–4), and continued agonist exposure results in removal of receptors from the cell surface (5–7). MOP phosphorylation is increased as a consequence of agonist exposure, leading to a commonly held assumption that receptor phosphorylation is essential for MOP desensitization and internalization (5, 8–10). The mechanism that may be responsible for uncoupling MOP from G protein activation and then promoting receptor sequestration is as follows: phosphorylation of the agonist occupied MOP by a G protein receptor kinase (GRK), subsequent binding of β-arrestins to the phosphorylated receptor, and removal of MOP from the cell surface via a clathrin-dependent process (11–15). The enhanced analgesic potency of morphine and diminished analgesic tolerance in β-arrestin2 knockout mice suggest that the capacity of opioid agonists to cause receptor desensitization and endocytosis is one of the key cellular mechanisms underlying tolerance (16). Phosphorylation of MOP by protein kinase C (PKC) or calmodulin-dependent kinase II can also reduce the capacity of MOP to activate G proteins (8, 17), also contributing to opioid tolerance (18). However, there is no evidence that phosphorylation by either PKC or calmodulin-dependent kinase II contributes to MOP internalization. GPCR internalization is not simply a secondary consequence of receptor activation by agonists but can be affected differentially by different ligands independently of their efficacy of coupling to G proteins, and for some receptors even apparent antagonists can induce receptor internalization (19, 20).

MOP agonists appear to have differential capacities to promote receptor internalization. Etorphine, sufentanil, methadone, and DAMGO produce significant MOP endocytosis, whereas others, most notably morphine, are much less effective (5–7, 11, 13, 21–23). The relationship between the coupling efficiency of MOP agonists to stimulate G proteins and induce receptor internalization is controversial. Some studies have reported that morphine and other agonists such as DAMGO and methadone have similar signaling efficacies but that morphine has a much lower efficacy to induce receptor internalization (11, 22). Other studies suggest that the capacity of MOP expressing a FLAG tag at the N terminus; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; I_{Ca}, voltage-dependent calcium channel current; PBS, phosphate-buffered saline; PKC, protein kinase C; SRIF, somatostatin; FITC, fluorescein isothiocyanate; GTP-γ-S, guanosine 5’-3’-O-(thio)triphosphate; pEC_{50}, agonist potency; pcDNA, plasmid complimentary DNA; LC, locus coeruleus.

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††††††††† The abbreviations used are: MOP, µ-opioid receptor; AtT20MOP, AtT20 neuroblastoma cells stably expressing FLAG epitope-tagged mouse µ-opioid receptor; β-CNA, β-chloro-naltrixemamine; CTAP, d-Phe-Cys-Tyr-d-Trp-Arg-Thr-Pen-Thr-NH₂; DAMGO, [Tyr-d-Ala-Gly-MePhe-Gly-ol]enkephalin; FLAG-MOP, mouse µ-opioid receptor ex-
agonists to induce desensitization or endocytosis is simply a function of their efficacy to induce activation of G proteins (24, 25) and that the partial agonist properties of morphine are the reason for its poor efficacy to induce internalization. The uncertainty concerning differential efficacy of MOP agonists for G protein activation, rapid desensitization, and endocytosis have probably arisen for three main reasons. First, most studies of these processes have used heterologous expression systems that greatly overexpress receptors, thus disrupting quantitative determination of relative signaling efficacy as well as the stoichiometry of MOP/GRK/β-arrestin interactions (11, 26, 27).

Second, MOP activation, desensitization, and endocytosis have generally been compared between different cellular expression systems under different conditions (22, 24, 25). This is mostly because cell lines do not generally express receptor proteins that can be used as rapid reporters of MOP activation, whereas receptor internalization is difficult to study quantitatively in native cells because of relatively low levels of native receptor expression. Finally, the term “receptor desensitization” has probably been used to describe different phenomena in different types of studies. In biochemical studies of MOP receptor function that measure agonist inhibition of cAMP generation or stimulation of GTP-γ-S binding to G proteins, the basic assays take a minimum of 15 min and often longer. Studies of MOP responses in neurons indicate that fast desensitization occurs with a time constant of about 3 min (4), and substantial internalization is apparent as early as 5–10 min in MOP-expressing cell lines (6, 11), meaning that the biochemical studies are starting from a baseline of an at least partially desensitized and/or internalized receptor. Thus, studies to date have not been able to adequately resolve MOP agonist efficacy for activation of G proteins, rapid desensitization, or internalization under similar experimental conditions.

In the present study, we have constructed a cell line in which the efficacy and potency of a range of opioid agonists has been determined in a homogenous cellular background with low receptor density. We have stably transfected mouse pituitary AtT20 cells with FLAG epitope-tagged MOP (FLAG-MOP) and selected for clones that express low amounts of receptor protein. This allowed for a comparison of the relative abilities of four opioid agonists to activate G proteins, to induce rapid desensitization, and to internalize MOP under similar experimental conditions in a single biological system. Opioid agonist efficacies for receptor activation were determined before and after inactivation of a fraction of FLAG-MOP with an irreversible antagonist, using inhibition of calcium channels (I\textsubscript{Ca}) as a rapid (within several seconds) reporter of release of G protein βγ subunits. The relative ability of each agonist to promote rapid, homologous receptor desensitization was also determined using inhibition of I\textsubscript{Ca} as a reporter. Finally, agonist-induced receptor internalization was determined under the same incubation conditions. The results showed that, when compared with DAMGO and methadone, the relative efficacy of morphine to promote FLAG-MOP internalization is much less than its relative efficacy to activate G proteins or to promote rapid FLAG-MOP desensitization.

**EXPERIMENTAL PROCEDURES**

**AtT20MOP Cell Culture**—Mouse AtT20 neuroblastoma cells were stably transfected with the cDNA encoding the FLAG epitope-tagged mouse μ-opioid receptor using the transfectant LipofectAMINE (Invitrogen). The pcDNA3 FLAG-MOP construct was a kind gift from Dr. Lakshmi Devi (Mt. Sinai School of Medicine, New York, NY). Genetecin (500 μg ml\(^{-1}\)) was added to select for clones expressing FLAG-MOP protein, and subsequently 16 single clones were isolated by diluting cells twice at 1:550 and 1:1000. Each of these single cells was grown to confluence for subsequent determination of FLAG-MOP density. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (both Invitrogen or CSL) and penicillin/streptomycin (50 units/5 μg ml\(^{-1}\)). All experiments were performed on cells within 10 passages of isolation.

**Radioligand Binding**—Membrane fractions from confluent cultures of each of the AtT20MOP clones were prepared as described previously (28). AtT20MOP membranes, at a final protein concentration of ~400 μg ml\(^{-1}\), were incubated with 5 nM [\(^{3}H\)]naloxone (PerkinElmer Life Sciences) in 50 mM Tris-HCl for 90 min at room temperature. The reaction was terminated by rapid filtration using a Brandel 24-cell tissue harvester (Gaithersburg, MD) through Whatman GF/B glass fiber filters that had been pre-soaked for 1 h in 0.5% polyethylenimine. The filters were washed three times with 3 ml of ice-cold Tris-HCl buffer, and bound radioactivity was determined by liquid scintillation counting. Non-specific [\(^{3}H\)]naloxone binding was determined by incubating with unlabeled naloxone. Two clones expressing a moderate amount of [\(^{3}H\)]naloxone binding were selected for saturation analysis of FLAG-MOP density.

For saturation analysis, membrane protein (~200 μg ml\(^{-1}\)) was incubated in 50 mM Tris-HCl solution with a range of [\(^{3}H\)]naloxone concentrations (0–16 μM), and specific binding was determined as described above. Receptor density (B\textsubscript{max}) and affinity (K\textsubscript{D}) were calculated using a one-site binding curve fitted using GraphPad Prism software (version 3). All experiments were performed three times in quadruplicate.

**Electrophysiological Recordings**—Cells were grown in 35-mm plastic tissue culture plates at low density and used up to 3 days after initial plating, providing cells were not in contact. I\textsubscript{Ca} was recorded using the whole-cell configuration of the patch-clamp technique (29) at 32–34 °C. I\textsubscript{Ca} was isolated with an extracellular solution (containing, in mM: cesium methanesulfonate 140, HEPES 10, glucose 10 (pH 7.2), and CsOH (330 ± 5 mmos)) and a pipette solution (containing, in mM: cesium methanesulfonate 130. HEPES 30, BAPTA 10, CaCl\(_2\) 2, MgATP 5, NaGTP 0.2, NaCl 5 (pH 7.3), and CsOH (310 ± 3 mmos)). Electrodes were made with fire-polished borosilicate glass (A-M Systems, Everett, WA) and had an input resistance of about 2 megohms. Recordings were made using an EPC-9 patch-clamp amplifier and Pulse software from Heka Electronics. I\textsubscript{Ca} were sampled at 20 kHz, filtered at 2.83 kHz, and recorded onto a hard disk for later analysis. Cell capacitance was between 8 and 30 picofarads, and series resistance was less than 5 megohms and was compensated for by at least 80% in all experiments. Capacitance transients were compensated for on-line, and the leak current was subtracted using a P/8 protocol.

Cells were voltage-clamped at −80 mV, and peak inward current occurred at test steps to +20 mV (data not shown). I\textsubscript{Ca} was evoked by steps to +20 mV every 20 s and monitored for current stability before drugs were applied. Cells were not used if the current increased or decreased by more than 2% in the first 90 s. Drugs were applied via a series of flow pipes (200 μm diameter) positioned above the cells, and complete exchange of drug solutions was achieved within 5 s by switching pairs with a motorized pipette manipulator. Agonist-induced I\textsubscript{Ca} was quantified by determining the current in the presence and absence of drug, at the time of the peak inward current before drug application.

**Internalization of FLAG-MOP**—For immunohistochemical experiments, AtT20MOP cells were seeded on 10-mm glass coverslips, and experiments performed the next day. Cells were washed twice in phosphate-buffered saline (PBS) and incubated with the appropriate concentration of opioid in serum-free Dulbecco’s modified Eagle’s medium with 0.5% bovine serum albumin and 1% normal horse serum. Cells were incubated in monoclonal M2-anti-FLAG primary antibody (10 μg ml\(^{-1}\), Sigma) overnight at 4 °C. The cells were then washed twice with PBS and permeabilized with methanol for 20 min on ice. Coverslips were washed twice at 10-min intervals with PBS, then incubated for 20 min at room temperature in permeabilization/blocking solution comprising PBS, 0.01% saponin, and 1% normal horse serum. Cells were blocked in monoclonal A-anti-mouse secondary antibodies (1:1000, Vector Laboratories) at room temperature for 2 h. After extensive washing, cells were incubated with streptavidin coupled to fluorescein isothiocyanate (FITC, 1:50, Amersham Biosciences) for 90 min at room temperature, washed twice with PBS, and mounted with buffered glycerol (pH 8.6) on glass slides. Images were acquired with a Nikon Diaphot 200 microscope with a Bio-Rad MRC1024 confocal scanning laser system using a 488-nm excitation filter and 522- to 535-nm emission filter. Single confocal images (nominal optical section thickness, 0.5 μm) that included the nucleus and a large area of cytoplasm were
taken from cells chosen at random. Images of 512 × 512 pixels were collected using a 100× objective (numerical aperture 1.4) and processed using Confocal Assistant and Corel Draw software. Subcellular distribution of FLAG-MOP was quantified using National Institutes of Health Image software (Scion Analysis, available at rsb.info.nih.gov/nih-image) as described previously (30). The nucleus did not contain FLAG-MOP receptor protein, and the background fluorescence of the nucleus was used as the threshold fluorescence. For each image, a line was drawn around the outside of the cell and the total cell fluorescence (plasma membrane plus cytoplasm) was measured as the number of pixels with intensity above threshold. A second line was drawn inside the cell membrane, 0.5 μm from the first line, and the number of pixels with intensity above threshold in the cytoplasm only was measured. The percentage of total fluorescence in the cytoplasm was calculated by dividing the immunoreactivity in the cytoplasm only by total immunoreactivity. Each point represents three separate experiments with en- docytosis quantified for 10 cells per point per experiment.

Analysis of Concentration Response Data—Nonlinear regression fits of the data were made using the NLR routine in the SPSS statistical package. Control data obtained for each agonist were fit with a three parameter logistic equation, as in shown Equation 1.

\[
\text{Response} = \frac{E_{\text{max}}}{1 + e^{\text{log} \left( \frac{\text{EC}_{50}}{\text{K}_{A}} \right)}} \times 100 + E_{\text{max}} \quad (\text{Eq. 1})
\]

The null model for receptor occlusion was used to estimate the equilibrium dissociation constant (\(K_A\)) of the full agonist DAMGO (31–33). In this model, the occluded curve obtained for DAMGO after receptor inactivation with β-CNA is represented as a right-shifted version of the control curve. If [A'] in the occluded curve is equally effective to [A] in the control curve, then [A'] divided by the dose shift can be used to calculate [A] according to the equation,

\[
[A] = [A'] \left( \frac{1}{q} + \frac{1 - q}{q} \times \frac{1}{K_A} \times [A'] \right) \quad (\text{Eq. 2})
\]

where \(q\) is the fraction of functional receptors remaining after inactivation, and \(K_A\) is the agonist dissociation constant. In exponential form, Equation 2 is equivalent to ln([A']) = ln([A]) − ln(shift), which can be substituted for [A] in Equation 1 resulting in Equation 3.

\[
\text{Response} = \frac{E_{\text{max}}}{1 + e^{\text{log} \left( \frac{\text{ln}[A'] - \text{ln}(\frac{r_A \text{EC}_{50}}{\text{K}_A}) - \text{ln}(\frac{r_A}{\text{K}_A})}{} \right)}} + E_{\text{max}} \quad (\text{Eq. 3})
\]

Simultaneous fits of Equations 1 and 3, respectively, to control and occluded DAMGO curves, provides estimates of \(q\) and \(K_A\). A null model was also used to estimate the equilibrium dissociation constant of the partial agonists (\(K_A\)) and their relative efficacy to DAMGO (33). The relationship between equally effective concentrations of a partial agonist and DAMGO can be described by,

\[
[A] = [A_{\text{max}}] \left( \frac{r_A \text{K}_{\text{EC}_{50}}}{\text{K}_A} + \frac{1}{\text{K}_A} \times [A_{\text{max}}] \right) \quad (\text{Eq. 4})
\]

where \(r_A\) is the relative efficacy (\(n_{\text{H}1}\text{halcane}/n_{\text{H}2}\text{halcane}) Substituting for [A] in Equation 1 results in Equation 5.

\[
\text{Response} = \frac{E_{\text{max}}}{1 + e^{\text{log} \left( \frac{\text{ln}[A_{\text{max}}] - \text{ln}(\frac{r_A \text{EC}_{50}}{\text{K}_A}) - \text{ln}(\frac{r_A}{\text{K}_A})}{} \right)}} + E_{\text{max}} \quad (\text{Eq. 5})
\]

Simultaneous fits of Equations 1, 3, and 4, respectively, to control and occluded DAMGO curves and the partial agonist curve provides estimates of \(K_A\) and \(r_A\). The estimates of \(K_A\) and \(K_B\) obtained from the full and partial agonist null models were used to estimate approximate relative efficacies from receptor desensitization and internalization data (34, 35). By estimating the receptor reserve as the quotient \(K_B/\text{EC}_{50}\), the midpoint shift ratio can be used to calculate relative efficacy using Equation 6.

\[
r_A = \frac{\left(\frac{E_{\text{max}}}{\text{EC}_{50})/K_A}{[E_{\text{max}}]/[\text{EC}_{50})/K_B} \right)}{\left(\frac{K_A}{[\text{EC}_{50})/K_B}\right)} \quad (\text{Eq. 6})
\]

All data are expressed as mean ± S.E. unless otherwise indicated. Where noted, significant differences between means were tested, using a paired or unpaired two-tailed Student’s t test.

Drugs and Chemicals—DAMGO and CTAP were from Auspep. Morphine HCl was from Glaxo. ω-Agatoxin IVA was from the Peptide Institute. b-chloralnaltrexone was from Sigma. Pentazocine was from Winthrop, and methadone was from Burroughs Wellcome Sydney. Buffer salts were from BDH or Sigma; all other reagents were from Sigma.

RESULTS

Expression of FLAG-MOP in Mouse Pituitary AtT20 Cells—Mouse pituitary AtT20 wild-type cells do not express endogenous opioid receptors (36). From 16 clones stably transfected with a construct coding for a FLAG epitope-tagged mouse MOP, we selected a line that expressed 260 ± 30 fmol of \([1^3]\text{H}1\text{nalone binding sites per milligram of membrane protein. This represents} \sim 31000 \text{ receptors per cell. The K}_I^A \text{ for} \ [1^3]\text{H}1\text{nalone was 4} \pm 0.5 \text{ nm.}

Opioids Inhibit I_{Ca} in AtT20 MOP Cells with Different Relative Intrinsic Efficacies—Wild-type AtT20 cells predominately express a native P/Q-type \(I_{Ca}\) (\(C_{AV2.1}, 37\). \(I_{Ca}\) in AtT20 MOP cells was elicited by a step from a holding potential of −80 mV to a test potential of +20 mV. Application of the P/Q-type \(I_{Ca}\) blocker, ω-agatoxin IVA (500 nM), to AtT20 MOP cells inhibited \(I_{Ca}\) by 88 ± 1%; the remaining \(I_{Ca}\) was inhibited by the nonselective \(I_{Ca}\) blocker, Cd²⁺ (30 μM, n = 7). The opioid agonists DAMGO, methadone, morphine, and pentazocine each inhibited \(I_{Ca}\) in AtT20 MOP cells (Fig. 1). The inhibition of \(I_{Ca}\) by DAMGO (1 μM) was prevented by superfusion of the selective MOP antagonist CTAP (1 μM, n = 4). DAMGO did not inhibit \(I_{Ca}\) in untransfected AtT20 cells (not shown).

Concentration-effect relationships for each agonist were obtained by superfusing one or more concentrations of drug onto individual AtT20MOP cells. These curves are shown in Fig. 2A. The estimated maximal inhibition (\(E_{\text{max}}\)) and \(\text{EC}_{50}\) for DAMGO and methadone inhibition of \(I_{Ca}\) were similar; however, morphine and pentazocine inhibited \(I_{Ca}\) with a lower estimated \(E_{\text{max}}\) and higher \(\text{EC}_{50}\), indicating they may be less potent partial agonists when compared with DAMGO or methadone (Table 1). To quantify the relative efficacy of the opioid agonists to inhibit \(I_{Ca}\), we re-examined inhibition of \(I_{Ca}\) under conditions where full receptor occupancy did not produce a maximal response, a situation where relative intrinsic efficacy (\(r_A\)) correlates well with the relative maxima. This condition of reduced maximal effectiveness was achieved by reducing the number of functional FLAG-MOP with the opioid receptor alkylation agent β-CNA (100 nM for 20 min at 37 °C). After β-CNA treatment, the inhibition of \(I_{Ca}\) by a high concentration of DAMGO (30 μM) was reduced by 57%. The concentration-effect curves for each agonist obtained after preincubation with β-CNA are shown in Fig. 2B.
We analyzed the DAMGO concentration-effect curves for inhibition of \( I_{\text{ca}} \) in control conditions and following \( \beta\)-CNA treatment using a null model to estimate the ligand \( K_A \) and the fraction of functional receptors remaining after \( \beta\)-CNA inactivation. The \( K_A \) for DAMGO was 130 nM (pK\(_A\) = 6.89), and the fraction of receptors remaining after \( \beta\)-CNA treatment was 0.32 (g). Using these values, a further three-curve analysis with a null model provided estimates of the dissociation constants \( (K_D) \) and relative intrinsic efficacy \( (r_e) \) values for methadone, morphine, and pentazocine (Fig. 2, C–E, and Table II). The relative efficacy values \( (\frac{r_e}{r_e^{\text{DAMGO}}}) \) for inhibition of \( I_{\text{ca}} \) obtained with the null method were similar to those obtained with the method using the dissociation constants to estimate the midpoint shift ratio (Equation 6 and Table II). The rank order of efficacy was DAMGO > methadone > morphine > pentazocine.

**Desensitization of MOP Agonist Inhibition of \( I_{\text{ca}} \)**—Prolonged application of MOP agonists to AtT20MOP cells often resulted in a rebound “facilitation” of \( I_{\text{ca}} \). The precise mechanism underling this in AtT20MOP is known, although in other neuronal cells it has been suggested to be due to removal of G protein \( \beta\gamma \) subunits constitutively associated with the channels before agonist application (38–40), because the degree of agonist-induced facilitation can be enhanced by intracellular manipulations that alter the level of constitutive G protein activation (39). The \( I_{\text{ca}} \) rebound confined assessment of desensitization, so before opioid agonist superfusion G proteins were “reset” by superfusion of somatostatin (SRIF) (100 nM, 60 s), which inhibits \( I_{\text{ca}} \) via native somatostatin receptors in the AtT20 cells (41). Rapid desensitization of the opioid inhibition of \( I_{\text{ca}} \) induced by morphine is illustrated in Fig. 3A. Shown is a progressive reduction in the effect of 300 nM morphine when interchanged at regular intervals during an extended application of 10 \( \mu \)M morphine. The high concentration of agonist was rapidly washed off prior to superfusion with the low concentration via rapid switch of the solution flow pipes. Data obtained using this protocol with DAMGO (100 nM probe/10 \( \mu \)M test), methadone (100 nM/10 \( \mu \)M), or morphine (300 nM/10 \( \mu \)M) are summarized in Fig. 3B. In each case desensitization appeared to reach a steady-state level. When the data were fit with a single exponential, the desensitization of inhibition of \( I_{\text{ca}} \) by DAMGO proceeded with a time constant of 0.48 min to a maximum of 58 ± 1.3% (n = 6). The values for methadone were 0.57 min and 53 ± 0.2%, respectively, and for morphine 0.62 min and 46 ± 0.6%. The amount of desensitization produced by pentazocine was too little to quantify. The desensitization induced by opioid agonists appeared to be homologous, because inhibition of \( I_{\text{ca}} \) by SRIF (10 nM) was not affected by a treatment with DAMGO (10 \( \mu \)M for 3 min) that maximally desensitized DAMGO inhibition of \( I_{\text{ca}} \) (n = 6, Fig. 3C).

The relative ability of opioid agonists to induce rapid receptor desensitization was assessed by measuring reduction of \( I_{\text{ca}} \) inhibition by test applications of 100 nM DAMGO. A submaximally effective test concentration of DAMGO was used, because submaximal responses will be reduced by small amounts of receptor desensitization, even if there is no change in maximal response in a situation where maximal inhibition of \( I_{\text{ca}} \) can occur at fractional receptor occupancy. We chose a 3-min application of desensitizing agonist, because the time courses defined above indicated that rapid desensitization is complete at this time. Initially, rapid desensitization was induced in whole cell configuration, as described above, and in these experiments DAMGO had an \( EC_{50} \) of 0.9 \( \mu \)M (pEC\(_{50}\) = 6.05 ± 0.1) to induce desensitization of inhibition of \( I_{\text{ca}} \) by 100 nM DAMGO. We were concerned that prolonged whole cell recordings may dialyze intracellular components that influence MOP desensitization, so we redetermined the DAMGO concentration effect relationship with the DAMGO being applied to the cell during a cell attached recording. In these experiments, the cell membrane under the pipette was held at the holding potential (~80 mV), but there was no pipette or electrical access during

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**Table I**

<table>
<thead>
<tr>
<th>Inhibition of ( I_{\text{ca}} )</th>
<th>Inhibition of ( I_{\text{ca}} ) after ( \beta)-CNA</th>
<th>Acute MOP desensitization</th>
<th>FLAG-MOP internalization</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>( p\text{EC}_{50} ) Max</td>
<td>( p\text{EC}_{50} ) Max</td>
<td>( p\text{EC}_{50} ) Max</td>
</tr>
<tr>
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</tr>
<tr>
<td>Pentazocine</td>
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* ND, too little desensitization to determine potency.
Distinct Agonist Efficacy Profiles for Regulation of MOP

Concentration response data for opioid agonist inhibition of $I_{Ca}$ was obtained before and after alkylation of MOP by β-CNA (100 nM, 20 min), opioid potency for desensitizing the inhibition of $I_{Ca}$ by 100 nM DAMGO was measured after a 3-min application of each agonist, and agonist-induced internalization of FLAG-MOP fluorescence was determined after a 30-min incubation in each agonist. The relative efficacy ($r$) of each agonist compared to DAMGO was determined either by simultaneous fitting of the concentration response data to Equations 1, 2, and 4 (null method) or by using the midpoint shift ratio (Equation 6).

### Table II

<table>
<thead>
<tr>
<th></th>
<th>DAMGO $= 1$</th>
<th>Methadone</th>
<th>Morphine</th>
<th>Pentazocine</th>
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* ND, not determined.

### Fig. 3

Opioid agonists produce rapid, homologous desensitization of the inhibition of $I_{Ca}$ in AtT20MOP cells with a similar efficacy to opioid agonist inhibition of $I_{Ca}$. A, time plot of a representative experiment used to determine the time course of fast desensitization. SRIF was applied at the beginning of the experiment to reset G proteins, then the degree of $I_{Ca}$ inhibition by probe concentration of morphine was determined. The inhibition of $I_{Ca}$ by the probe concentration was retested during a continuous application of a high concentration of the inhibition of $I_{Ca}$ by 100 nM DAMGO was measured after a 3-min application of each agonist, and agonist-induced internalization of FLAG-MOP fluorescence was determined after a 30-min incubation in each agonist. The relative efficacy ($r$) of each agonist compared to DAMGO was determined either by simultaneous fitting of the concentration response data to Equations 1, 2, and 4 (null method) or by using the midpoint shift ratio (Equation 6).

**Relative Efficacies of Opioids for Receptor Activation, Desensitization, and Endocytosis**—The capacities of the opioid agonists to inhibit $I_{Ca}$ and internalize FAST-MOP are shown graphically in Fig. 5. The experimentally derived responses have been plotted as a function of receptor occupancy, which was calculated by dividing agonist concentration by the agonist $K_a$, derived from the receptor inactivation experiments described above. The maximum effect for each agonist has been normalized to the maximum effect obtained with DAMGO. For comparison, the theoretical fraction of agonist bound to recep-
tors is included as a dotted line (42).

Fig. 5A shows the response curves obtained for G protein signaling, as measured by inhibition of $I_{Ca}$. These data show that the signaling of DAMGO and methadone are quite similar but the lower efficacy of morphine can be seen by reduced maximum response. Even though $I_{Ca}$ is inhibited by activated G protein $\beta\gamma$ subunits, making the coupling between receptor and channel quite direct, some response amplification by the G protein step is apparent from the leftward separation of the response curves from the occupancy curve for DAMGO, methadone, and morphine. In contrast, pentazocine is a weaker agonist and the signal never exceeds receptor occupancy. Fig. 5B shows the response curves obtained for rapid desensitization...
tion as measured by loss of the Ica inhibition. These data show that high levels of receptor occupancy are required to induce receptor desensitization relative to G protein signaling. Nevertheless, the relative efficacies of the agonists to cause desensitization are similar to that for activating G protein signaling. In contrast, as illustrated in Fig. 5C, the relative agonist efficiencies for receptor internalization are different. The agonist-response curves for DAMGO and methadone are quite close to the theoretical receptor occupancy curves, whereas the morphine agonist-response curve is close to the curve obtained for pentazocine. These data show that the weak ability of morphine to induce FLAG-MOP receptor internalization does not correlate with its ability to induce a G protein signal or stimulate rapid FLAG-MOP desensitization in AtT20 cells.

**DISCUSSION**

We have used a single biological system to compare the abilities of four opioid agonists of different strengths to couple to three important components of the MOP signaling cycle, namely receptor activation, rapid receptor desensitization, and receptor internalization. The moderate levels of FLAG-MOP expression in the AtT20MOP cell line we chose to examine, coupled with the use of receptor inactivation via alkylation, ensured that the determination of relative agonist efficacies were not confounded by the presence of spare receptors. The use of FLAG-MOP inhibition of Ica, a rapid reporter of G protein activation, enabled resolution of receptor activation, desensitization, and endocytosis in as similar conditions as possible, i.e. in intact cells at 35–37 °C. We found that there were consistent differences in the potencies of MOP agonists to mediate G protein activation, fast receptor desensitization, and receptor internalization. There were two major findings of this study. First, the efficacies of DAMGO, methadone, morphine, and pentazocine for inducing rapid FLAG-MOP desensitization appear to be highly correlated with their efficacies for activation of FLAG-MOP G protein-coupled signaling. Second, we found that morphine displayed greatly reduced ability to internalize FLAG-MOP, which was not correlated well with its efficacy to activate or desensitize these receptors.

**Our Model Cells**—The AtT20MOP cell line we used in this study expressed about 260 fmol of receptor mg−1 protein, which is a relatively low MOP density when compared with that reported in many other expression systems (5–6, 26, 43). Most of the FLAG-MOP receptors appeared to correctly localize to the cell surface (e.g. Fig. 4). A previously described AtT20 cell line, expressing rat MOP (44), expressed about 2.5 times as many receptors as the cell line we used. A major advantage of the relatively low receptor density in our AtT20MOP cells was that the partial agonist effects of morphine and pentazocine could be readily detected, and the efficacy of DAMGO and methadone could be significantly reduced through mild receptor depletion via alkylation with β-CNA. A second major advantage of AtT20MOP cells is that they express an excellent reporter system for G protein activation, an apparently homogenous population of P/Q-type Ica, the only opioid-inhibitable Ica in our cells (data not shown). Opioid-mediated inhibition of Ica provides a measurement of G protein activation that reaches steady state within several seconds and is readily reversible (45–46). G/Ica-coupled GPCRs inhibit P/Q Ica largely via G protein βγ subunit release and subsequent direct interaction with the channel (47–49), a process not confounded by a large amplification due to activation of second messenger systems. Finally, because the MOP expressed an FLAG epitope tag, we could quantify receptor internalization under very similar conditions to those used to determine agonist signaling efficacy.

**Opioid Agonist Efficacy of G Protein Activation**—The rank order of efficacy to inhibit Ica was determined by using the opioid receptor alkylling agent β-CNA to inactivate a fraction of FLAG-MOP receptors and subsequently fitting the data to a null model. This type of operational measurement is derived from classic receptor theory and the methods originally developed by Furchgott (50), and attempts to cancel the effects of efficacy so that the base affinity of the agonist for the inactive receptor can be measured. This empirical approach does not address the molecular mechanisms that lead to efficacy and is based on assumptions that make no theoretical provision for more recently identified features of G protein receptor signaling such as multiple receptor-active states or promiscuous coupling of single receptors to multiple G proteins. However, provided these constraints are recognized, the principles of null methods do appear to be valid for providing an index of the relationship between agonist concentration and the initial stimulus that the receptor provides to initiate signaling (34, 50–53).

In the present study the relative efficacy of a range of agonists to activate FLAG-MOR was determined in conditions not confounded by rapid desensitization. The rank order of DAMGO > methadone > morphine > pentazocine is consistent with that determined in studies with native MOP on rat locus coeruleus neurons or thalamic membranes, in guinea pig ileum or human SH-SY5Y neuroblastoma cells (43, 54–56), as well as efficacies determined using quantitative methods in cells heterologously expressing MOP (43, 57). One study in Xenopus oocytes expressing MOP reported a relatively lower efficacy for methadone than morphine in the activation of recombinant GIRK1 (9), as did a study in HEK293 cells expressing recombinant GIRK1/4 and FLAG-MOR receptors (22). In both studies multiple agonists were applied to each cell in the apparent absence of controls for fast desensitization, and in the latter study only a single concentration of each agonist was used, making absolute determination of efficacy problematic.

**Opioid Agonist Efficacy of Fast Desensitization**—DAMGO, morphine, and methadone all induced rapid, homologous FLAG-MOP desensitization, with a similar rank order of efficacy as for acute inhibition of Ica. The potency of all agonists to desensitize FLAG-MOP was at least 10-fold lower than for G protein activation. These results suggest that the capacity of an agonist to produce rapid desensitization might be related to its efficacy for activation of G proteins, although the efficiency of fast desensitization is much lower than receptor activation, as has generally been indicated in previous studies (24, 25, 58). The desensitization of FLAG-MOP inhibition of Ica appeared to be homologous, because inhibition of Ica by moderate concentrations of SRIF was not affected by treatment with concentrations of DAMGO that produced maximum MOP desensitization. The results of the present study are similar to studies of the desensitization of δ-opioid receptor-mediated inhibition of Ica in the NG108-15 neuroblastoma cell line, where desensitization was reported to be rapid and homologous (59, 60). The potency for δ-opioid receptor desensitization was also at least one order of magnitude less than that for acute inhibition of Ica (60). The recovery of δ-opioid receptors from desensitization required receptor dephosphorylation, and because inhibitors of protein kinase A or PKC did not block desensitization, receptor phosphorylation by a GRK was suggested to mediate the desensitization (60). Interestingly, previous reports of rapid desensitization of MOP inhibition of Ica have only identified heterologous desensitization (61, 62), with the cross-desensitization suggested to occur at the level of the channel rather than the receptors (62). The reasons for the differences in the nature of desensitization between this study and those in sensory neurons (61, 62) are not known but may arise because of the different types of Ica in the cells, with sensory neurons expressing predominantly N-type Ica (e.g. Ref. 62). Interestingly, MOP
inhibition of the P/Q-type $I_{\text{Ca}}$, in sensory neurons was not susceptible to heterologous desensitization with high concentrations of DAMGO, similar to our observations with the P/Q-type $I_{\text{Ca}}$ in AtT20 cells (62).

MOP agonists activate native GIRK channels in LC neurons, and desensitization of this response proceeds with a time constant of about 3 min if high concentrations of the efficacious agonist met-enkephalin are used (2, 4). Most studies have found this desensitization to be largely homologous (Refs. 2–4, 63, but see Ref. 58). Because it is not possible to rapidly equilibrate and remove drugs from brain slices, the determination of efficacy for rapid desensitization in the LC has been somewhat problematic, but the degree of MOP activation and subsequent desensitization are correlated. Methadone is less efficacious than met-enkephalin and more efficacious than morphine (25, 58), consistent with the findings of the present study. Morphine induces no measurable macroscopic desensitization of GIRK activation in LC neurons, but it has not been possible to examine morphine- or methadone-induced desensitization with lower concentrations of agonist because of the slow equilibration time of these agonists in brain slices (25, 58).

Studies of desensitization of MOP activation of GIRK channels in Xenopus laevis oocytes have also reported significant reductions in opioid agonist responses with prolonged incubations (8, 24, 64, 65). In studies where MOP desensitization is promoted by coexpressed GRK and β-arrestin, the desensitization to high efficacy agonists such as DAMGO proceeds with a similar time course to that in the LC. Also, it was suggested that agonist efficacy for promoting desensitization was directly related to efficacy for activating GIRK (24), although, as in the LC, no rapid desensitization was apparent with morphine. We do not know what process or processes mediate rapid FLAG-MOP receptor desensitization in AtT20 cells, but it is clear from the present study that morphine can produce rapid MOP desensitization with a similar time course and to a similar maximal extent as DAMGO, which is in contrast to the studies in oocytes. The differences between this study and those in oocytes may result partly from the experiments in oocytes being performed at room temperature rather than at 35 °C, but probably mostly reflects very different cellular environments with their distinct ensembles of protein kinases and phosphatases (10).

Opioid Agonist Efficacy for Receptor Internalization—The relative efficacy and potency for internalization of FLAG-MOP in this study is consistent with previously published reports (11, 22, 23). Both DAMGO and methadone induced a rapid and significant internalization of FLAG-MOP, whereas morphine and pentazocine produced a very modest loss of cell surface receptor. The potencies of opioids to induce endocytosis were approximately one order of magnitude lower than for G protein-mediated inhibition of $I_{\text{Ca}}$ but were more potent than for induction of rapid desensitization. This suggests that FLAG-MOP coupling to endocytosis was more efficacious than for desensitization in AtT20-MOP cells. With the exception of morphine, the relative efficacies of opioids to induce endocytosis were well correlated with their efficacies for receptor activation. However, the efficacy of morphine for endocytosis was much lower than expected from its relative efficacy to produce either receptor activation or rapid desensitization.

The failure of morphine to induce significant MOP endocytosis has been explained by morphine being unable to stabilize the same conformations of MOP as agonists that promote internalization such as DAMGO or etorphine (11–13, 66). This results in morphine being unable to elicit a “threshold activation state” that puts the MOP in a specific conformation that is favorable for entering the endocytotic pathway (67). Implicit in these theories is the idea that the active conformation for receptor signaling is different to that for recruitment of proteins involved in receptor internalization. The efficacy to activate G proteins and stimulate receptor internalization is well correlated for some GPCR (e.g. 42, 68), but for other receptors a clear separation between the two processes is evident (69). In extreme cases, the phosphorylation and internalization of angiotensin AT1 or cholecystokinin A receptors can be stimulated by ligands that do not activate signaling through the receptor (19, 20). Mutant GPCRs have also been constructed in which agonist-induced G protein signaling is abolished while processes leading to receptor internalization are preserved (e.g. 70, 71). These findings strongly suggest that G protein activation and recruitment of receptor regulatory proteins can be mediated by distinct conformations of GPCRs, and these conformational states are not necessarily stabilized by all ligands.

It is generally accepted that MOP desensitization and internalization are sequential processes that involve receptor phosphorylation, recruitment of β-arrestin, and subsequent removal of the phosphorylated receptor from the cell surface via an endocytotic pathway. It has not been established at which point during this process receptors become uncoupled from their cognate G proteins or which of these events many correspond to the desensitization observed in the present study. Both phosphorylation of GPCR by GRKs (e.g. 72, 73) and binding of β-arrestins to GPCR have been shown to inhibit GPCR signaling to G proteins (14, 15). Internalization of GPCR does not necessarily abolish MOP signaling (74, 75), but the close proximity of $I_{\text{Ca}}$-coupled GPCR to the channels makes it likely that once MOP are internalized they can no longer inhibit $I_{\text{Ca}}$ (45). It should be noted that we have not established whether the desensitization we observed in this study is related to MOP interactions with the GRK/β-arrestin/internalization pathway. Desensitization of GPCR signaling can proceed by many pathways distinct from GRK-mediated phosphorylation or β-arrestin binding (e.g. Refs. 8, 17, and 76) and the fact that morphine promotes desensitization but not internalization could be interpreted as evidence for MOP desensitization of inhibition of $I_{\text{Ca}}$ occurring via a mechanism unrelated to the internalization pathway. Agonist-stimulated endocytosis and recycling of MOP is thought to be regulated by phosphorylation of residues in the C-terminal region of the receptor by GRKs (12, 13, 77), whereas acute desensitization of MOP might involve phosphorylation of a residue in the second intracellular loop (63). A differential ability to induce phosphorylation of these residues could provide a structural basis for the apparent separation between the efficacy for desensitization and internalization evident with morphine. Intriguingly, several putative C-terminal splice variants of the mouse MOP undergo internalization in response to morphine exposure (77, 78), and these variants are also more heavily phosphorylated in response to morphine binding (77). A number of residues of MOP can be phosphorylated in response to opioid agonist exposure (79), although it is not known whether different agonists promote differential phosphorylation of specific sites. Because morphine can induce rapid and significant MOP phosphorylation (Refs. 5, 8, 9, 77, but see Ref. 13), it is tempting to speculate that morphine can induce phosphorylation of residues involved in rapid desensitization but that it does not very effectively stabilize the conformation of MOP necessary for the subsequent phosphorylation of residues important for β-arrestin binding and endocytosis.

We have provided evidence that morphine couples to G proteins, promotes acute desensitization, and causes receptor endocytosis with different relative efficacies to some other MOP agonists. This is the first study to examine these parameters in
Distinct Agonist Efficacy Profiles for Regulation of MOP

the same cells under similar conditions. Although it is clear that morphine has a much lower efficacy than expected for promoting MOP internalization, our results are in contrast to assertions from previous studies that claim morphine is incapable of producing fast MOP desensitization (22). Morphine can recruit mechanisms to rapidly limit its own signaling through MOP, and it does so with a similar relative efficacy to activating other G proteins. Our data suggest that it may be post-desensitization mechanisms that are not recruited efficiently by morphine, and it will be of great interest to discover which of the many putative agonist-induced regulatory events (e.g., Ref. 80) are differentially stimulated by morphine and other opioid ligands.

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Opioid Agonists Have Different Efficacy Profiles for G Protein Activation, Rapid Desensitization, and Endocytosis of Mu-opioid Receptors
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