Live Cell Monitoring of μ-Opioid Receptor-mediated G-protein Activation Reveals Strong Biological Activity of Close Morphine Biosynthetic Precursors

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G-protein activation by receptors is generally measured using 35S-GTPγS binding assays in cell membranes and cannot be well assessed in intact cells. We have recently developed a fluorescence resonance energy transfer (FRET)-based approach to monitor G-protein activation in living cells. Here we report that this technique can be used to determine structure-activity relationships of receptor agonists in intact cells. We have recently shown that morphine is biosynthesized de novo by mammals via a multistep pathway different from that in plants. However, the pharmacological properties of morphine precursors are poorly understood. Here, we directly monitored μ-opioid receptor (MOR)-mediated G-protein activation in living cells by FRET and validated this method with classical GTPγS binding assays. Receptor binding studies and FRET measurements demonstrated that several (R)-configured morphine precursors such as (R)-reticuline, salutaridine, salutaridinol, thebaine, and codeine were partial MOR agonists. Some closer precursors such as oripavine, codeinone, and morphinine activated G-proteins as strongly as morphine, but with slightly lower potencies. The more distant the precursors were positioned in the pathway with respect to morphine, the less efficient and potent they were at MOR. Comparison of pharmacological properties of close morphine precursors and concentrations in which they occur in animal tissues suggests that they might activate MOR signaling under physiological conditions. Taken together, our data indicate that FRET-based assays of G-protein activation can serve to determine the abilities of compounds to activate G-protein signaling directly and in living cells.

Fluorescence resonance energy transfer (FRET) is a powerful technique for real-time monitoring of biochemical processes in intact living cells. Recently we have described a FRET-based method to directly monitor activation of G-proteins in living cells (1). For the α2A-adrenergic receptor (α2A-AR) this approach has proved useful to differentiate among the agonists, antagonists, inverse and partial agonists of different chemical structures (2–4). This method revealed that partial agonists switch α2A-AR into multiple conformational states, thereby inducing G-protein responses of distinct efficacies and kinetics (2). However, this method has not been used to analyze pharmacological properties of the ligands of other G i-coupled receptors. Here, we perform an extensive validation of this FRET-based approach and analyze a series of newly discovered endogenous μ-opioid receptor (MOR) ligands.

Morphine plays an important role in the therapy of acute and chronic pain. Exogenously delivered morphine acts in the body by activating different subtypes of opioid receptors, including μ-, κ-, and δ-opioid receptors (5, 6). Most pharmacological effects of morphine appear to be mediated via MOR present on cells of neuronal and immune systems. These receptors are classical seven-transmembrane proteins that couple to the G1 class of heterotrimeric G-proteins, activating a signaling cascade, which is responsible for the antinociceptive effects of morphine (7).

Over the last decades, suggestions have accumulated that morphine present in mammalian tissues and fluids comes not only from an exogenous or dietary origin, but may also be biosynthesized de novo. Recent studies have unequivocally proven the existence of a mammalian morphine biosynthetic pathway, which consists of at least 19 steps and is different in its early steps from that occurring in plants (8, 9). Because the endogenous morphine biosynthesis takes place in mammals, their cells and tissues are constantly exposed to certain amounts of morphine and its precursors (10, 11).

However, only a few reports describe pharmacological properties of the individual intermediates of the morphine biosynthetic route. Both (R)- and (S)-reticulines, for instance, have...
been shown to exhibit hypotensive (12), antiaggregant (13), and central depressant (14) effects. Salutaridine has only been characterized as a partial agonist at γ-aminobutyric acid, type A receptors (15), whereas natural (−) thebaine is poorly antinociceptive in mice (16). In contrast, oripavine and codeinone have almost similar analgesic potential as morphine and cause a withdrawal syndrome, which can be suppressed by morphine and vice versa (17, 18). Morphinone has been described as a toxic morphine metabolite and a MOR agonist (19). Although the action of morphine precursors via MOR might be expected, no investigations have compared their pharmacological properties in one experimental system to answer the question, whether and to which extent these endogenously occurring compounds can activate MOR and its cognate G_{i/0} proteins.

Here, we apply G_{i}-FRET technique to monitor activation of MOR in single intact cells and compare pharmacological effects of the key intermediates of the mammalian morphine biosynthetic pathway. We demonstrate that several close morphine precursors, late in the biosynthetic pathway can activate MOR in living cells.

**EXPERIMENTAL PROCEDURES**

Substances—Codeine (Merck, Darmstadt, Germany), morphine (Merck), (R,S)-norlaudanosoline (Sigma), thebaine (Sanofi Synthelabo, Paris, France), and all opioid peptides (Sigma) are commercially available. Oripavine was a gift from Prof. E. Brockmann-Hanssen (UCSF, CA). Codeinone, morphinone, (R)- and (S)-reticuline, salutaridine, salutaridinol, and 7-epi-salutaridinol were synthesized and verified by NMR. Naloxone, naltrexone, and guanosine diphosphate were from Sigma.

Plasmids, Cells, and Transfection—Human MOR cDNA was a kind gift from Prof. S. Schultz. The receptor cDNA sequence was subcloned into pcDNA3 expression vector (Invitrogen) between HindIII and XbaI restriction sites.

HEK293a cells (which have no endogenous expression of G_{ai}-proteins) were grown under standard conditions, seeded onto 57-mm plates and transfected with (all in pcDNA3 vector) 0.7 μg of receptor DNA, 1.5 μg of PTX-insensitive G_{ai} (C351I mutant) labeled with enhanced yellow fluorescent protein (YFP) at position 91/92, 0.5 μg of G_{ai}, and 0.3 μg of G_{i2} C-terminally labeled with enhanced cyan fluorescent protein (CFP) using Effectene transfection reagent (Qiagen). 24 h after transfection the cells were plated onto 24-mm-glass coverslips and incubated for another 24 h.

Receptor Binding Assays—MOR-expressing HEK293a cell membranes (5 μg of protein) were incubated for 2 h at 30 °C in assay buffer (50 mM Tris, 3 mM MgCl_{2}, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4) with 0.1–10 nM [3H]naloxone (Amer-sham Biosciences) as described (20). Nonspecific binding was determined in the presence of 10 μM naltrexone or unlabeled naloxone. The reactions were terminated by vacuum filtration through GF/F glass fiber filters (Millipore, Schwalbach, Germany).

^{35}S-GTPγS Binding—The assays were performed essentially as described by Traynor and Nahorski (21). Briefly, 25 μg of membrane protein in 100 μl of binding buffer (20 mM HEPES, 10 mM MgCl_{2}, and 100 mM NaCl, pH 7.4) containing 3 μM guanosine diphosphate and 100 pm ^{35}S-GTPγS (PerkinElmer Life Sciences) were incubated with different concentrations of morphine or Tyr-D-Ala-Gly-N-Methyl-Phe-Gly-ol (DAMGO). After 1-h incubation at 30 °C the samples were rapidly filtered through GF/F.

**FRET Measurements of G_{i}-Protein Activation**—Coverslips with adherent cells were washed once, maintained in a physiological buffer A (containing 144 mM NaCl, 5.4 mM KCl, 2 mM CaCl_{2}, 1 mM MgCl_{2}, and 20 mM HEPES, pH 7.3) at room temperature, and placed on a Zeiss Axiovert 200 microscope equipped with 100× oil immersion objective, Polychrome IV light source, beam splitter dichroic long path 505 nm, photometric detection system (Till Photonics, Gaefelfingen, Germany), and analog to digital converter (Digidata 1322A; Axon Instruments). FRET was monitored as the emission ratio F_{YFP}/F_{CFP} upon 436 nm excitation (filters YFP 535 ± 15 nm, CFP 480 ± 20 nm). For each measurement, emission values were corrected for bleedthrough, direct YFP excitation, and photobleaching as described (1, 22).

The expression ratio of G_{i}-protein subunits was systematically monitored by comparing YFP (α-subunits) and CFP (βγ-subunits) emissions and kept similar in all experiments. Based on single cell fluorescence, G_{is}-YFP expression was typically limiting for the heterotrimer formation and less than 2 pmol/mg membrane protein. To study agonist-dependent changes in FRET, single cells were continuously superfused with buffer A alone or containing different substances using a computer-assisted solenoid valve-controlled rapid superfusion device (ALA-VM8; ALA Scientific Instruments). Data sampling and analysis were performed using a personal computer software Clampex 8.1 (Axon Instruments), Origin 6.1 (Microcal), and Prism 4.0 (GraphPad).

**RESULTS**

Real-time Monitoring of MOR-induced G-protein Activation—To study the pharmacological effects of different ligands at MOR, we decided to develop a real-time monitoring system to measure MOR-mediated G_{i/-protein} activation. To do so, we monitored FRET between fluorescently labeled subunits of heterotrimeric G_{i/-protein} (Fig. 1A). The α_{i/-subunit} was labeled with YFP in position 91/92, whereas the γ_{i/-subunit} of a β_{i}γ_{i-} complex was C-terminally labeled with CFP (1). In intact living HEK293 cells expressing human MOR, addition of micromolar morphine concentrations resulted in a fast decrease of FRET with a half-life of ∼1.4 s (Fig. 1B) comparable with the kinetic rates measured for G_{i}-activation by α_{2A}AR (1, 2). The decrease in FRET between CFP and YFP fused to G_{i2} and α_{i/-subunits} of the trimeric G_{i/-protein} complex was reversible upon withdrawal of morphine (Fig. 1B), reflecting ligand-dependent conformational rearrangements in the G_{i/-protein} heterotrimeric complex (1, 4, 23).

To confirm the specificity of the FRET signal for MOR activation, we performed three different experiments. First, we stimulated HEK293a cells not transfected with MOR with morphine or its precursors and were not able to see any signal (data not shown). Next, we treated morphine-stimulated MOR-expressing cells with a specific receptor antagonist naloxone, which completely blocked the effect of morphine, whereas naloxone given alone did not produce any change in FRET (Fig. 1C). Finally, treatment of MOR-expressing cells with non-opi-
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![Diagram](image)

**Figure 1. Monitoring MOR activation in living cells by FRET between Gi-protein subunits.** A, to perform real-time measurements of MOR-induced Gi-protein activation we co-expressed MOR with fluorescently tagged G$_{\alpha}$-subunits. B, addition of morphine (10 µM) to HEK293a cells expressing human MOR and fluorescently labeled G$_{\alpha}$-subunits leads to a rapid reversible decrease in FRET (measured as ratio $F_{\text{YFP/F645}}$) characterized by decrease in YFP ($F_{\text{YFP}}$) and concomitant increase in CFP ($F_{\text{CFP}}$) fluorescence reflecting Gi-protein rearrangement during receptor-induced activation. C, morphine-induced change in FRET is specific for MOR activation and can be fully blocked by MOR antagonist naloxone (10 µM). D, kinetics of the FRET signal induced by application of 10 µM morphine (Mo) with subsequent washout of the ligand (wash) or wash-in of 10 µM naloxone (Mo + Nal). $t_{1/2}$ values were calculated as described (1, 2) and presented as mean ± S.E. ($n = 7$). E, effect of the partial MOR agonist buprenorphine (10 µM) on the FRET signal induced by 10 µM morphine. Naloxone (10 µM) was used to block MOR activity. Representative experiment ($n = 5$).

Opioid ligands such as clonidine or norepinephrine did not affect the FRET signal (supplemental Fig. 1A). We further analyzed the kinetics of the onset and offset of morphine-induced FRET signals and found that MOR inhibition with naloxone led to a much faster inactivation of Gi-proteins than monitored upon washout of morphine (Fig. 1D). To test, whether our experimental system can distinguish the effects of partial agonists, we used a well known MOR partial agonist buprenorphine, which could partially block the effect of morphine on Gi-protein activation and showed ~50% change in FRET as compared with morphine (Fig. 1E).

**Validation of the FRET Method for MOR-induced G-protein Activation**—Next, we sought to validate our FRET-based approach by testing how accurately it can report G-protein activation. For this, we performed a side-by-side comparison of the FRET results with the data obtained from a classical $[^{35}\text{S}]$GTPyS binding assay. Both methods were used to measure concentration-response dependencies for the full MOR agonist DAMGO and the strong partial agonist morphine (Fig. 2A). The two experimental approaches yielded curves with almost identical EC$_{50}$ values, which were in good agreement with previously reported values for MOR (20, 24). Similarly, we have previously demonstrated that concentration-response curves for $\alpha_{2a}$AR-mediated Gi-protein-regulated inwardly rectifying potassium channel activation were superimposable with measurements of Gi-protein activation by FRET (1).

Our FRET approach has also allowed to distinguish between full agonists and partial agonists at MOR. Morphine as a partial agonist produced significantly smaller effects than the full synthetic agonist DAMGO (Fig. 2. A and B). In line with this observation, naturally occurring opioid peptides such as Met-enkephalin and $\alpha$-endorphin also stimulated the Gi-protein activity to a higher extent than morphine (Fig. 2. C and D). Among the endogenous opioid peptides, only $\beta$-endorphin at high concentration demonstrated about the same amplitude as morphine (Fig. 2E). The extent of MOR activation by natural opioid peptides was very similar to the biochemical data from GTPyS binding assays reported previously (25). Washout of these opioid peptides did not rapidly deactivate G-proteins consistent with their high affinity for the receptors. We analyzed the kinetics of G-protein inactivation after $\beta$-endorphin treatment upon washout of the peptide and receptor inhibition by naloxone (Fig. 2, F and G). Interestingly, the FRET signals showed very slow offset kinetics even upon naloxone treatment, suggesting a high affinity of $\beta$-endorphin at MOR. Taken together, the FRET measurements performed with morphine and opioid peptides and side-by-side comparison with $[^{35}\text{S}]$GTPyS data confirmed that our FRET-based approach represented a highly sensitive and specific method to monitor the ligand-induced MOR activation in living cells and could be used to analyze the pharmacological properties of different full and partial agonists at the receptor.

**Analysis of Morphine Precursors and Their Efficacy at MOR**—Using Gi-protein activation as a measure of ligand efficacy at the receptor level we studied pharmacological effects of several intermediates of the mammalian morphine biosynthetic pathway. Starting from the very early precursors of morphine such as norlaudanosoline (tetrahydropapaveroline) and reticulines, we first stimulated the cells with saturating concentrations of a precursor (10 or 100 µM) and subsequently applied 10 µM morphine to determine the efficacy of different compounds at MOR signal (Fig. 3). (R,S)-Norlaudanosoline and (S)-reticuline at concentrations up to 100 µM did not produce any significant activation of Gi-proteins. In contrast, (R)-reticuline, a precursor with a (R)-configuration of the C-1 atom, typical for morphine and other morphinans (C-9 in morphine), demonstrated already a small FRET signal with amplitude of ~16% of the maximal morphine response. Closure of the C-12–C-13 chemical bond in salutaridine dramatically increased the ability to activate MOR signaling (~54% of morphine).

Salutaridinol, an intermediate in the two-step enzymatic conversion of salutaridine to thebaine, had a lower efficacy than salutaridine, which was close to that of (R)-reticuline, suggesting that the presence of a hydroxyl at the C-7 position of salutaridinol (not typical for other morphinans) reduced the ability to activate the receptor. In line with this observation, 7-episalutaridinol, which does not participate in mammalian mor-
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Comparison of the Potencies of Different MOR Ligands—To understand whether some morphine precursors can activate MOR signaling under physiological conditions and in concentrations in which they occur in mammals, we recorded concentration-response dependencies for the compounds, which significantly activated the receptor. Analysis of the EC50 values, i.e. the concentrations at which a half-maximal activation of G\textsubscript{i} was achieved, for each individual substance revealed that the potencies of the compounds were inversely proportional to the amplitude of the FRET signal (Fig. 4, Table 1). For two distant morphine precursors, (R)-reticuline and salutaridinol, concentration-response dependencies could not be measured because of their small signals. Other morphinans, which activated MOR less than morphine, had potencies in the following order: thebaine ≡ codeine > salutaridinol. Codeine, a much less potent partial agonist than morphine, demonstrated almost 1000-fold higher EC50 values, suggesting that extremely high concentrations of this alkaloid were necessary to achieve an effect comparable with that of morphine (Fig. 4, Table 1).

The analysis of the EC50 values for strong partial agonists having the same amplitude as morphine was intriguing to compare their activating potential for MOR signaling systems. Both oripavine and codeinone, intermediates of two parallel branches of the biosynthetic conversion of thebaine to morphine in mammals, had high nanomolar EC50 values (200–300 nM, ~40-fold less potent than morphine). Morphinone, however, was significantly more potent and only 4–5-fold less efficacious than morphine (Fig. 4).

To compare the potencies of the compounds at G\textsubscript{i}-protein activation with their affinities at MOR, we performed receptor binding experiments to determine K\textsubscript{i} values for different morphine precursors. The cells used in this study expressed 2.4 ± 0.6 pmol MOR per mg membrane protein (n = 6, mean ± S.E.), which corresponds to ~40 amol (240,800) receptors per cell. K\textsubscript{i} values obtained from competitive binding displacement experiments were in general quite close to the EC50 values measured by the FRET assay (Table 1), suggesting a moderate amount of spare receptors. This comparison further suggests that our G\textsubscript{i}-FRET assay faithfully reports G\textsubscript{i}-protein activation and can be used to investigate the pharmacological properties of compounds acting at G-protein coupled receptors.

DISCUSSION

In this study we present a FRET-based approach to monitor MOR-mediated G\textsubscript{i}-protein activation in intact living cells and
use this method for pharmacological characterization of different receptor ligands, including major intermediates of morphine biosynthetic pathway.

Previously, such a FRET method has been used to study effects of different \( \alpha_2 \)-AR ligands and to enlighten the mechanism of partial agonism at this receptor (1, 2, 4).

FIGURE 3. Different morphine precursors activate Gi-proteins with distinct efficacies. Representative FRET-ratio traces (\( n = 3–6 \)) for each substance are shown together with its chemical structure and position in the biosynthetic pathway (indicated by arrows). HEK293a cells expressing human MOR and fluorescently tagged Gi-subunits were first stimulated with saturating concentrations of a precursor (for norlaudanosoline, reticulines, salutaridine, salutaridinol, thebaine, and codeine 100 \( \mu \)M were used; oripavine, codeinone, and morphinone were applied at 10 \( \mu \)M) and subsequently with 10 \( \mu \)M morphine to determine a relative efficacy for each compound. The changes in FRET reflecting MOR-dependent Gi-protein activation (\( \Delta F_{\text{YFP/CFP}} \)) are normalized to the maximal response induced by morphine. Relative efficacies for all compounds are summarized in Table 1.

FIGURE 4. Comparison of relative efficacies and potencies of major morphine biosynthetic precursors. Concentration-response dependencies for morphine and its precursors were derived from Gi-FRET experiments. The changes in FRET-ratio were measured at different concentrations of morphine and precursors and normalized to the maximal signal evoked by 10 \( \mu \)M morphine. The data are from 4–6 independent experiments, mean \( \pm \) S.D. EC\text{50} values are presented in Table 1.

TABLE 1
Binding properties, relative efficacies, and potencies of morphine biosynthetic precursors at \( \mu \)-opioid receptor (MOR)

| Substance          | \( G_i \) activation by FRET | MOR binding assays \( K_i \) |
|--------------------|-------------------------------|-----------------------------|
|                    | % morphine \( E_{\text{max}} \) | \( \mu \)M | EC\text{50} \( \mu \)M |
| Morphine           | 100 0.006 ± 0.002              | 0.004 ± 0.001 |
| Morphinone         | 100 0.028 ± 0.006              | 0.146 ± 0.017 |
| Oripavine          | 100 0.277 ± 0.029              | 0.286 ± 0.064 |
| Codeinone          | 100 0.294 ± 0.041              | 0.459 ± 0.111 |
| Codeine            | 81.3 ± 3.7 6.1 ± 2.4           | 6.3 ± 2.1 |
| Thebaine           | 76.3 ± 3.2 5.6 ± 1.4           | 7.4 ± 4.9 |
| Salutaridine       | 54.4 ± 3.4 32.5 ± 7.8          | 34.6 ± 7.7 |
| Salutaridinol      | 17.7 ± 1.5 ND                  | ND |
| (R)-Reticuline     | 15.9 ± 2.4 ND                  | ND |
In the present study, we compare the effects of endogenously occurring morphine biosynthetic precursors and their efficacies at MOR, which represent the main opioid receptor subtype accounting for morphine-induced analgesia (7, 27, 28). Recently, using α2AR we have demonstrated that the degree of Gi-protein activation measured in the FRET system had a linear correlation with the extent of receptor activation by partial agonists, suggesting that the Gi-sensor could be used to compare the efficacy of partial agonists of Gi-coupled receptors (2). This sensor was able to detect minor conformational changes in the receptors driven by weak partial agonists with a high sensitivity.

The amplitude of Gi-activation by morphine precursors depends on the structure of each individual compound (Fig. 3). Early morphine biosynthetic precursors without the C-12–C-13 bond (norlaudanosoline, reticulines) were not able to activate the receptor, suggesting that the mophinan skeleton was important to induce a conformational change in the opioid receptor. Stereochemically, the configuration of C-1 atom of the tetrahydrobenzylisoquinoline precursors (corresponds to C-9 in morphine) seems to play an important role in the receptor activation because the change of configuration from (S) to (R) in reticuline induces an effect at MOR. Further steps of the biosynthesis increase the efficacy of the precursors, forming an optimal ligand structure. Using radioligand binding assays, we determined K_i values for morphine precursors at MOR, which were quite close to the EC_{50} values for G-protein activation measured in living cells by FRET (Table 1). This suggests a moderate amount of spare receptors in the cells used in our study. In accordance with the FRET data, receptor binding studies showed a correlation between binding affinities and efficacies of the ligands. Codeine and more distant precursors such as thebaine and salutaridin exhibited relatively low affinities in micromolar range.

One of the goals of the current study was to investigate whether the morphine biosynthetic pathway and its individual components might activate MORs endogenously present in a mammalian organism. Because de novo formation of morphine via the described route (Fig. 3) has been unequivocally proven (8, 9), the major analgesic alkaloid and its biosynthetic precursors are present in fluids and tissues of the body. The amount of morphine isolated from human cell lines indicates that they may be exposed to 10–20 nM morphine concentrations under physiological conditions (9). Morphine has been detected in human polymorphonuclear leukocytes by radioimmunoassay, which reported ~12 pg of morphine/million cells (29), corresponding to ~80 nM. According to the potency at MOR, such concentrations are capable of activating at least 50% of the Gi-proteins in cells expressing sufficient amounts of receptors (Fig. 4). We assume that under certain circumstances, e.g. inflammation, morphine concentrations in the blood might reach higher nanomolar levels (29) and cause analgesia or other effects via activated opioid receptors. Codeine, however, has no significant analgesic effects unless O-demethylated to morphine (30), suggesting that its strong partiality and low potency (half-maximal Gi-protein activation at only ~10 μM) are not sufficient to produce a strong biological effect unless it is demethylated to morphine (Fig. 4). (--)Thebaine, which has a slightly lower effi-
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cacy than codeine and also a micromolar EC_{50} value, is only poorly antinociceptive in mice injected with up to 30 mg/kg (16). Codeine and thebaine have been isolated from ovine brain, where they are present at 7 and 10 pg/g fresh weight, respectively (11), giving a concentration on the order of 100 nm. These amounts of thebaine and codeine according to our results (Fig. 4) are indeed not able to produce significant effects at MOR. We assume that endogenously occurring morphine precursors, which have lower efficacies and potencies than codeine do not play a significant role in MOR signaling because their concentrations in vivo would not reach high enough values to produce receptor activation.

In contrast, some closer morphine progenitors such as codeinone, oripavine, and morphinone demonstrate much higher efficacies and potencies and might, therefore, exhibit strong partial agonist effects at MOR in high nanomolar concentrations. In support of this hypothesis there are reports on a strong analgesic potential of oripavine and codeinones, which also can induce a withdrawal syndrome typical for morphine (17, 18). However, to date there are no reports available, which give an idea about endogenously occurring concentrations of these precursor-morphinans in animal tissues. This issue deserves further investigation. Because some of the partial agonist oripavine drugs such as buprenorphine are beneficial in the treatment of morphine abuse (31), the possibility of targeted regulation of the endogenous morphine biosynthetic pathway toward the accumulation of certain intermediates, like it has been already achieved in plants (32), might be promising to open up new therapeutic approaches.

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