Role of Phosphorylation Sites in Desensitization of \( \mu \)-Opioid Receptor

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Received February 3, 2015; accepted May 12, 2015

ABSTRACT

Phosphorylation of residues in the C-terminal tail of the \( \mu \)-opioid receptor (MOPr) is thought to be a key step in desensitization and internalization. Phosphorylation of C-terminal S/T residues is required for internalization (Just et al., 2013), but its role in desensitization is unknown. This study examined the influence of C-terminal phosphorylation sites on rapid desensitization of MOPr. Wild-type MOPr, a 3S/T-A mutant (S363A, T370A, S375A) that maintains internalization, 6S/T-A (S369A, T370A, S375A, T376A, T379A, T383A) and 11S/T-A (all C-terminal S/T residues mutated) mutants not internalized by MOPr agonists were stably expressed in AtT20 cells. Perforated patch-clamp recordings of MOPr-mediated activation of G-protein-activated inwardly rectifying potassium channel (K\(r_{3.3}\)) (GiRK) conductance by submaximal concentrations of Met\(^2\)-enkephalin (ME) and somatostatin (SST; coupling to native SST receptor [SSTR]) were used to examine desensitization induced by exposure to ME and morphine for 5 minutes at 37° C. The rates of ME- and morphine-induced desensitization did not correlate with phosphorylation using phosphorylation site-specific antibodies. ME-induced MOPr desensitization and resensitization did not differ from wild-type for 3S/T-A and 6S/T-A but was abolished in 11S/T-A. Morphine-induced desensitization was unaffected in all three mutants, as was heterologous desensitization of SSTR. Morphine-induced desensitization (but not ME) was reduced by protein kinase C inhibition in wild-type MOPr and abolished in the 11S/T-A mutant, as was heterologous desensitization. These findings establish that MOPr desensitization can occur independently of S/T phosphorylation and internalization; however, C-terminal phosphorylation is necessary for some forms of desensitization because mutation of all C-terminal sites (11S/T-A) abolishes desensitization induced by ME.

Introduction

Opioids are effective analgesics, but tolerance and addiction limit their utility (Williams et al., 2013). A range of mechanisms causing tolerance have been proposed (Williams et al., 2013). Agonist-induced desensitization of the \( \mu \)-opioid receptor (MOPr) is widely considered an initial step in the development of opioid tolerance (Williams et al., 2013). Potential mechanisms include phosphorylation of specific MOPr C-terminal S/T residues by G-protein–coupled receptor kinases (GRKs), followed by \( \beta \)-arrestin binding that causes internalization (Just et al., 2013; Williams et al., 2013); however, the relationship between these events and receptor desensitization is uncertain (Canals, 2015). 

Agonist-mediated phosphorylation of MOPr is rapid and prominent at S375 within the C terminus, which may facilitate phosphorylation of other residues, including T370, T379, and T376, respectively (El Kouhen et al., 2001; Schulz et al., 2004; Doll et al., 2011; Lau et al., 2011; Just et al., 2013). Efficiency of phosphorylation of S375 correlates with the propensity of an opioid agonist to induce internalization (Zhang et al., 1998; McPherson et al., 2010). Using quantitative mass spectrometry, Lau et al. (2011) identified two clusters of MOPr residues, 354TSST357 and 375STANT379, that undergo opioid-induced phosphorylation. Phosphorylation site-specific antibodies have identified a similar pattern of C-terminal phosphorylation events (Doll et al., 2011; Just et al., 2013). Different opioids produce both different phosphorylation patterns, with multiple phosphorylation in the region S354 to T379 occurring robustly for agonists that induce internalization versus less phosphorylation of fewer sites for those that do not (Doll et al., 2011; Lau et al., 2011; Just et al., 2013).

Mutation of potential phosphorylation sites has also been used to examine the relationship between phosphorylation and desensitization.
MOPr internalization. Mutation of S375 to alanine slowed internalization, but mutation of S363 and T370 did not (El Kouhen et al., 2001; Schulz et al., 2004), suggesting that S375 is important for β-arrestin recruitment and endocytosis. Mutation of potential S/T phosphorylation sites in the 375–379 region slowed internalization by [D-Ala2-MePhe4-Gly-ol] enkephalin (DAMGO), but mutating the 354–357 region did not. Just et al. (2013) reported that mutation of S363, T370, and S375 to A (3S/T-A) partially inhibited agonist-induced internalization, but additional mutation of T376, T379, and T383 (6S/T-A) abolished internalization, as did mutation of all C-terminal S and T residues (11S/T-A). These findings strongly suggest that multiple residues in the vicinity of S375 must be phosphorylated for efficient internalization, presumably because they are required for β-arrestin recruitment to the MOPr, but the effect of these mutations on desensitization is unknown.

Rapid MOPr desensitization of MOPr coupling to membrane effectors such as voltage-gated calcium channels (VGCCs) and inwardly rectifying K channels (GIRKs; Kir3.X) precedes internalization, but its relationship to phosphorylation events and arrestin recruitment is unclear (Dang and Christie, 2012; Williams et al., 2013). Early studies of MOPr desensitization (Wang, 2000; Qiu et al., 2003; Schulz et al., 2004) used assays with time courses that significantly overlap with internalization, complicating the interpretation of mechanisms (Williams et al., 2013). More direct measurement of loss of coupling to VGCCs and GIRKs has examined the influence of kinase inhibitors to modulate desensitization. Johnson et al. (2006) used protein kinase C (PKC) inhibition in human embryonic kidney 293 (HEK293) cells, and Bailey et al. (2009) inhibited PKC and GRK2 in locus coeruleus (LC) neurons to suggest that PKC and GRK2 in locus coeruleus (LC) neurons to suggest that PKC and GRK2 are required for efficient internalization, presumably because they are required for β-arrestin recruitment to the MOPr, but the effect of these mutations on desensitization is unknown. Just et al. (2013) reported that mutation of S363, T370, and S375 to A (3S/T-A) partially inhibited agonist-induced internalization, but additional mutation of T376, T379, and T383 (6S/T-A) abolished internalization, as did mutation of all C-terminal S and T residues (11S/T-A). These findings strongly suggest that multiple residues in the vicinity of S375 must be phosphorylated for efficient internalization, presumably because they are required for β-arrestin recruitment to the MOPr, but the effect of these mutations on desensitization is unknown.

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In the present study, we examined desensitization of MOPr activated GIRK (Kir3.X) conductance in AtT20 cells transfected with the phosphosite mutants described previously (Just et al., 2013) using perforated patch-clamp recording, as well as phosphorylation and internalization under the same conditions. Our results show that desensitization by ME is completely abolished in a MOPr mutant having all 11 sites mutated to A (11S/T-A) but was maintained in the other two mutants (3S/T-A and 6S/T-A). By contrast, morphine-induced desensitization persisted in all mutants and was inhibited by a PKC inhibitor, supporting previous findings of distinct mechanisms between the internalizing agonists, ME and DAMGO, and noninternalizing agonists such as morphine (Johnson et al., 2006; Bailey et al., 2009). Consistent with distinct mechanisms, recovery of MOPr function after desensitization was faster after exposure to ME than to morphine. These results indicate that desensitization of MOPrs by high-efficiency, internalizing agonists such as ME requires phosphorylation of multiple residues in the C-terminal region. Morphine engages a desensitization mechanism that is independent of C-terminal S/T phosphorylation but involves a PKC-dependent mechanism.

Materials and Methods

MOPr Mutants Cell Culture. Wild-type MOPr, 3S/T-A (S363A, T370A, S375A), 6S/T-A (S363A, T370A, S375A, T376A, T379A, T383A), and 11S/T-A (T354A, S355A, S356A, T357A, S363A, T370A, S375A, T376A, T379A, T383A, T394A) were all cloned in pcDNA3.1 plasmids with a hemagglutinin (HA) tag as previously described (Just et al., 2013) and were expressed stably in AtT20 cells as previously described (Borgland et al., 2003). For patch-clamp experiments, AtT20 cells were seeded on 35-mm polystyrene culture dishes (Becton Dickinson, and Company, East Rutherford, NJ) in Dulbecco’s modified Eagle’s medium (Gibco/Life Technologies, Melbourne, Australia) containing 4.5 g/l of glucose, penicillin-streptomyacin (100 μl/ml), G418 (50 mg/ml/G418), and 10% fetal bovine serum. Cell cultures were kept in humidified 5% CO2 atmosphere at 37°C. Cells were ready for recording after 24 hours of incubation.

MOPr Binding Density. MOR binding density was determined on intact cells as described by Gomes et al. (2000). Briefly, approximately 2 × 10^5 cells were plated in a 24-well plate coated with poly-l-lysine and incubated overnight. Cells were then rinsed gently twice with 50 mM Tris-Cl (pH 7.4), placed on ice, and incubated with 5 nM [3H]DAMGO (PerkinElmer, Waltham, MA) for 2 hours. Non-specific binding was determined in the presence of unlabeled DAMGO (10 μM). At the end of incubation, plated cells were rinsed three times with 0.5 ml of 50 mM Tris-Cl (pH 7.4) at 4°C. Cells in each well were then digested for 1 hour at room temperature with 100 μl of 1 N NaOH, and 100 μl of 1 N HCl was then added to each well and collected into scintillation vials; bound ligand was determined using a liquid scintillation counter (MicroBeta/PerkinElmer). A parallel set of incubated cells was collected for total protein assay. Clones of wild-type, 3S/T-A, 6S/T-A, and 11S/T-A with similar binding densities were selected for all experiments (4.2, 3.0, 4.4, and 3.5 pmol/mg protein, respectively).

MOPr Internalization. MOPr internalization was measured using a similar method to that described in Borgland et al. (2003). Briefly, AtT20 cells stably expressing different mutant HA-tagged MOPr were seeded on 10-mm glass coverslips and incubated for 24 hours to achieve about 50% confluence. Cells were incubated for 30 minutes in serum-free Dulbecco’s modified Eagle’s medium, followed by the addition of ME (30 μM at 37°C). After 30 minutes, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature and then permeabilized using 0.1% Triton X-100. Cells were then immune-stained with 1:300 dilution of anti-HA antibody (Santa Cruz Biotechnology, Dallas, TX) at 1 hour at room temperature, rinsed in PBS, followed by incubation with 1:1000 Alexa Fluor 488–conjugated goat anti-rabbit antibody (Invitrogen) for 1 hour at room temperature. Coverslips were then washed 3 × 10 minutes with PBS and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL) on glass slides. Images were acquired using Olympus (Shinjuku, Japan) FV3000 laser scanning confocal microscope. High-resolution images (optical magnification: 60×; resolution: 1024 × 1024 pixels) were obtained using a 60× oil objective (Olympus UPLANFL N). All the software settings, including laser intensity, photomultiplier tube voltage, and offset, remained constant for the same experiment. Single confocal images that included the nucleus and a large area of cytoplasm were taken from cells chosen at random. The mean of fluorescence intensity (mean of gray value, gray values ranging from 0 to 4096) was quantified from the raw 16-bit images using ImagJ Software (v. 1.44p, National Institutes of Health, Bethesda, MD). For each image, a line was drawn around the outside of the cell, and the number of pixels with intensity above threshold was defined as the total fluorescence (membrane plus cytoplasm). 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 within this line (cytoplasm) was measured. Membrane fluorescence was defined as total minus cytoplasm fluorescence. The percent of internalized receptors was estimated as a ratio of [1 − (membrane fluorescence/total fluorescence)] × 100.

Western Blots. Cells were seeded onto poly-l-lysine–coated 60-mm dishes and grown to 80% confluence. After treatment with either 10 μM morphine or 10 μM ME at 37°C, cells were lysed in detergent buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM diisoumpyrophenylate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of protease
and phosphatase inhibitors, complete mini- and PhosSTOP (Roche Diagnostics, Mannheim, Germany). Glycosylated proteins were partially enriched using wheat germ lectin–agarose beads as described (Koch et al., 2001; Schulz et al., 2004). Proteins were eluted from the beads using SDS-sample buffer for 20 minutes at 45°C. Samples were split and resolved on 7.5% SDS-PAGE gels. After electrophoresis, membranes were incubated with anti-pS356/pT357 (4879), anti-pT376 (3190), anti-pS375 (2490), anti-pT376 (3722), or anti-pT379 (3686) antibodies, followed by detection using an enhanced chemiluminescence detection system (Amersham, Braunschweig, Germany). Blots were stripped and incubated again using the phosphorylation-independent anti-HA antibody to ensure equal loading of the gels.

**Electrophysiology.** For perforated patch recordings, patch pipettes were pulled from borosilicate glass (AM Systems, Everett, WA) having input resistance ranged between 3.5 and 4.5 MΩ. Pipettes were filled with internal solution containing 135 mM potassium gluconate, 3 mM MgCl₂, 5 mM HEPES, and 5 mM EGTA (pH adjusted to 7.4 with potassium hydroxide). The recording electrodes were first front filled with this internal solution and then backfilled with the same solution containing 200 μg/ml amphotericin B (in 0.8% dimethylsulfoxide). Cells were initially superfused with external bath solution containing 140 mM NaCl, 1.8 mM KCl, 1.2 mM MgCl₂, 10 mM HEPES, and 10 mM glucose (pH adjusted to 7.4 with sodium hydroxide). For measuring IᵢGKR, the KCl concentration in the bath was increased to 20 mM KCl (substituted for NaCl) before the start of the measurements and was maintained throughout the experiments. Liquid junction potential was calculated to be +16 mV and was adjusted before the start of every experiment.

Currents were recorded at 37°C in a fully enclosed, temperature-controlled recording chamber using an Axopatch 200B amplifier and pCLAMP 9.2 software and digitized using Digidata 1320 (Axon Instruments/Molecular Devices, Sunnyvale, CA). Currents were sampled at 100 Hz, low-pass filtered at 50 Hz, and recorded on hard disk for later analysis. IᵢGKR was recorded using a 200-millisecond voltage step of −120 mV from a holding potential of −60 mV applied every 2 seconds. Drugs were perfused on to the cells using ValveLink8.2 pressurized pinch valve perfusion system (AutoMate Scientific, Berkeley, CA). In all recorded cells, solution exchange reached steady state within 200 milliseconds (usually within 100 milliseconds), which was confirmed by examination of the current produced at −60 mV by switching from low (3 mM) to high K⁺ (20 mM) solution.

**Data Analyses.** All data are shown as the mean ± S.E.M. and analyzed using GraphPad Prism v 5. All data points are plotted as pure GIRK conductance G (nS) using the following calculation: \[ I_{GKR} \, (−60 \, mV) - I_{KCl} \, (−120 \, mV)/60 \, mV. \] The rate of μ-opioid receptor desensitization and its recovery was calculated as a percentage by using the following formula: \[ \text{post GME/pre GME} \times 100. \] Where, pre and post GME are values of GIRK conductance averaged for 4 to 5 points during the maximal response (colored points in figures) in response to ME (red). Agonist-induced GIRK conductance increases were calculated at 2-second intervals using the voltage-step protocol shown in Fig. 2A (blue trace), and the chord conductance G was plotted during application of agonists as shown in Fig. 2B. Conductance increases (Fig. 2B, red circles) were normalized to the response produced by a supramaximal concentration of somatostatin (1 μM) acting on the somatostatin receptor (SSTR) (Borgland et al., 2003) in the same cell, and concentration-response curves were determined as shown in Fig. 2C, which shows that morphine acts as a partial agonist compared with ME and SST. As widely reported (Williams et al., 2001), MOPr and other Gᵢ-coupled G-protein–coupled receptors usually converge on a single population of GIRK channels in a single cell. We confirmed that this was the case for MOPr and natively expressed SSTR receptors. SSTR types 1–4 are natively expressed in AtT20 cells (Patel et al., 1994; Atwood et al., 2011). A supramaximal concentration of SST (1 μM) was applied to MOPr transfected cells for 1 minute, followed by coapplication of a supramaximal concentration of ME (10 μM) as shown in Fig. 2D. No additional response to ME was observed when coapplied with SST (n = 5), showing that both receptors (SSTR2 and MOPr) activate a common population of GIRK channels.

**Results**

**MOPr Function in Different MOPr Mutants.** Figure 1 shows the amino acid sequence of C-terminal tails of different mutants of MOPr that were stably transfected into AtT20 cells.

Figure 2A shows the voltage-clamp recording protocol used to study the receptor function in wild-type MOPr and different mutants in elevated external K⁺ (20 mM). The ramp IV trace shows the basal conductance (gray) and increased conductance in response to ME (red). Agonist-induced GIRK conductance increases were calculated at 2-second intervals using the voltage-step protocol shown in Fig. 2A (blue trace), and the chord conductance G was plotted during application of agonists as shown in Fig. 2B. Conductance increases (Fig. 2B, red circles) were normalized to the response produced by a supramaximal concentration of somatostatin (1 μM) acting on the somatostatin receptor (SSTR) (Borgland et al., 2003) in the same cell, and concentration-response curves were determined as shown in Fig. 2C, which shows that morphine acts as a partial agonist compared with ME and SST. As widely reported (Williams et al., 2001), MOPr and other Gᵢ-coupled G-protein–coupled receptors usually converge on a single population of GIRK channels in a single cell. We confirmed that this was the case for MOPr and natively expressed SSTR receptors. SSTR types 1–4 are natively expressed in AtT20 cells (Patel et al., 1994; Atwood et al., 2011). A supramaximal concentration of SST (1 μM) was applied to MOPr transfected cells for 1 minute, followed by coapplication of a supramaximal concentration of ME (10 μM) as shown in Fig. 2D. No additional response to ME was observed when coapplied with SST (n = 5), showing that both receptors (SSTR2 and MOPr) activate a common population of GIRK channels.
MOPr Desensitization by ME Requires Multiple Phosphorylation Sites. Figure 3, A and B, shows representative Western blots and densitometry of the time course of MOPr phosphorylation induced by ME in AtT20 cells. Results were similar to our previous results in HEK293 cells (Doll et al., 2011; Just et al., 2013). ME-induced maximal receptor phosphorylation of S375 within 20 seconds, with maximal phosphorylation of T370 and T379 occurring within 1 minute. S356/T357 and T376 were not maximally phosphorylated until exposed to ME for 20–30 minutes. It should be noted that the slow phosphorylation of S356/T357 could be due, in part, to our finding that the phosphosite antibody (4879) recognizes the epitope only when both sites are phosphorylated (data not shown).

The time course of desensitization during exposure to ME (10 μM) for 6 minutes did not correlate with the time course of MOPr phosphorylation (Fig. 3, C and D). A very rapid component of desensitization of the GIRK conductance was observed during exposure to supramaximal concentrations of ME and reached a plateau within 6 seconds (34 ± 3% decline in the 6 seconds after peak response, n = 11; Fig. 3C). The very rapid component of desensitization was not related to S/T phosphorylation because it persisted in all mutants exposed to ME (see as follows). It resembles the rapid nonenzymatic direct regulation of GIRK by GRK2 reported by Raveh et al. (2010). Figure 3C shows a representative example of the time course of desensitization of MOPr coupling to GIRK. Excluding this initial 6-second component, there was a modest decline in the response to the supramaximal concentration of ME (10 μM) during the 6-minute exposure (20 ± 3%, n = 10), but this measure of desensitization is very insensitive for high intrinsic efficacy agonists such as ME, particularly when there is considerable receptor reserve (Connor et al., 2004). Exposure to a submaximal or “probe” concentration of agonist is the most reliable approach to determine desensitization (Connor et al., 2004). MOPr desensitization was therefore measured by brief exposure to a submaximal probe concentration of ME (Fig. 2C; 10 nM, 20 seconds) during and after exposure to a supramaximal concentration of ME (10 μM, 6 minutes). A 20-second exposure was considered sufficient to wash out ME (10 μM) and achieve a final concentration of ME of 10 nM because we have independently determined the off rate (τ_{off}) of ME and morphine by rapid switching to 10 μM naloxone to be approximately 4 seconds for ME and 3 seconds for morphine (data not shown), similar to the τ_{off} of ~3 and 2 seconds, respectively, reported by Williams (2014) using caged naloxone in brain slices. Brief (20 second) preexposure to a near-maximal concentration of SST (100 nM) was used to normalize MOPr responses due to variation in GIRK expression from cell to cell.

The extent of desensitization was calculated each minute as plotted in Fig. 3D. After washout of ME (10 μM), the response to the probe concentration was 8.3 ± 0.4% (n = 5) of the response before desensitization. When fitted to a single exponential function by including the 7-minute time point (not shown in Fig. 3D), the time constant for desensitization was ~2.6 minutes. The probe procedure during application of ME (10 μM) might have slowed the rate of desensitization, but it did not greatly affect the magnitude because desensitization measured 1 minute after washout of 10 μM ME did not differ when the probe was omitted during desensitization (8.3 ± 0.4%, n = 5 in the presence of probe and 9.6 ± 1.6%, n = 8 in its absence; P = 0.59, unpaired t test).

Figure 4, A and B, shows representative Western blots and densitometry of the time course of MOPr phosphorylation induced by morphine in AtT20 cells. As previously described in HEK293 cells (Doll et al., 2011; Just et al., 2013), morphine produced weak phosphorylation of S375 that peaked after 10 minutes and very weak phosphorylation of pT370 and pT376 that was slow, reaching maxima after 10 minutes. No phosphorylation of S356/T357 or T379 could be detected during exposure to morphine. In contrast to the slow, weak phosphorylation, the time course and extent of morphine-induced MOPr desensitization during exposure to a supramaximal concentration of morphine (10 μM) for 5–6 minutes were similar to those
produced by ME (Fig. 4, C and D). In contrast to ME, the rapid component of GIRK desensitization during the first 6-second exposure to morphine was significantly smaller than that produced by ME (9.9 ± 0.9%, n = 14; P < 0.0001 versus ME; Fig. 4C). Excluding the first 6 seconds of exposure to morphine, the response to the supramaximal concentration of morphine declined by 40 ± 3% (n = 6, P < 0.001, paired t test) during the 6-minute exposure (Fig. 4C), as expected for a partial agonist during desensitization (Connor et al., 2004). Desensitization measured 1 minute after washout of morphine (10 μM) was similar to that produced by ME (11 ± 1%, n = 5 in the presence of probe and 9 ± 1%, n = 5 in its absence; P = 0.6). When exposure to the probe concentration of ME (10 nM) was fitted to a single exponential function by including the 7-minute time point (not shown in Fig. 4D), the time constant for desensitization was 2.0 minutes.

**Mutation of All C-Terminal Sites (11S/T-A) but Not 3S/T-A or 6S/T-A Abolishes Desensitization by Met-Enkephalin.** ME-induced desensitization and the rate of recovery from desensitization were measured for wild-type MOPr and the three mutants. Figure 5, A and B, shows representative examples of time course of ME-induced desensitization and recovery in wild-type MOPr and the 11S/T-A mutant. As introduced in the preceding, an initial very rapid GIRK desensitization was observed that reached a plateau in 2 to 6 seconds in wild-type MOPr and all mutants (33 ± 3% decline in wild-type MOPr, 33 ± 5% in 3S/T-A, 37 ± 4% in 6S/T-A and 26 ± 4% in 11S/T-A within 60-second exposure to ME, n = 5 for each). Subsequent desensitization of wild-type MOPr during application of ME (10 μM) was similar to that shown in Fig. 3C, but no decline was observed in the 11S/T-A mutant (Fig. 5B). The probe concentration of ME (10 nM) applied 1 minute after washing ME in wild-type MOPr transfected cells showed similar desensitization to that shown in Fig. 3B. Desensitization was not affected 3S/T-A and 6S/T-A mutants but was abolished in the 11S/T-A mutant (Fig. 5C).

The probe concentration of ME was applied successively 1, 4, 6, 8, and 10 minutes after washout of ME (10 μM) to determine the rate of recovery from desensitization (Fig. 5). Figure 5, A and D, shows recovery of MOPr after successive wash to original response to 10 nM ME. Recovery from desensitization occurred with a similar rate for MOPr, 3S/T-A and 6S/T-A mutants and was complete within 10 minutes.

**Desensitization by Morphine Is Not Disrupted by Mutation of S/T Phosphorylation Sites.** As shown in Fig. 6, MOPr desensitization by morphine was unaffected by 3S/T-A, 6S/T-A, or 11S/T-A mutations (i.e., mutation of all S/T phosphorylation sites in the C-terminal region of MOPr had
The desensitization during application of morphine (10 μM) was similar to that shown in Fig. 4C for wild-type MOPr, 3S/T-A, 6S/T-A, and 11S/T-A (34 ± 5%, 36 ± 1%, 30 ± 3% and 31 ± 2% reduction, respectively, n = 4 for each, calculated excluding initial 6 seconds). The response to the probe concentration of ME (10 nM) 1 minute after washout was also unaffected by any of the mutations (Fig. 6C). The rate of recovery of MOPr function after desensitization with morphine (10 μM), for all wild-type MOPr and all mutants was more prolonged than the recovery from ME-induced desensitization; it was also unaffected by any of the mutations (Fig. 6, A, B, and D; n = 5 for each mutant). These results clearly establish that C-terminal S/T phosphorylation is not required for desensitization by morphine.

**PKC Inhibition Reduces Morphine-Induced but Not Met-Enkephalin-Induced Desensitization.** To determine whether PKC has a role in MOPr desensitization induced by ME and morphine, the effects of several membrane-permeable PKC inhibitors were examined. Commonly used, specific PKC inhibitors reduce EPSP amplitudes, and inhibit ME-induced desensitization. The results are depicted in Fig. 4, and in the following results.

**Fig. 4.** Time course of phosphorylation and MOPr desensitization induced by morphine. (A) Representative Western blots of phosphorylation at specific C-terminal sites on MOPr induced by morphine (10 μM). (B) Time course of densitometry of data in panel for the period of desensitization (A) expressed as percentage of the maximal density. (C) Example of GIRK conductance changes during application of SST (blue) and a submaximal probe concentration of ME (10 nM red) to determine the extent of desensitization applied to a MOPr wild-type cell before, during, and after 6-minute exposure to a supramaximal concentration of morphine (10 μM, brown). (D) The red circles were averaged and for the group data. (D) Rate of desensitization expressed as percentage of the amplitude of the presensitization probe exposure to ME (10 nM).

**Fig. 5.** Desensitization induced by ME in different MOPr mutants. (A) ME-induced desensitization in wild-type MOPr probed (ME 10 nM, red) as shown in Fig. 3 without testing during exposure to supramaximal ME (10 μM, orange) and probed before and after desensitization with SST (100 nM, blue) (B) Example of ME-induced desensitization in 11S/T-A. (C) Comparison of GIRK conductance to MOPr probe (10 nM) 1 minute after desensitization as a percent of response 1 minute before desensitization by ME (10 μM, 5 minutes) in wild-type and different mutants (n = 6 cells per point). (D) Rate of recovery from desensitization (resensitization) in wild-type and different mutants (n = 6 cells per curve). ***P < 0.001; *P < 0.05. Two-way analysis of variance, Bonferroni post-tests 11S/T-A versus MOPr; no other between-mutant differences were seen.
inhibitors such GF109203X [3-(N-[dimethylamino]propyl-3-indolyl)-4-(3-indolyl) maleimide] (1 μM) and chelerythrine (1 μM) were tested initially but, as reported previously by Johnson et al. (2006), both these directly inhibited opioid activated GIRK currents in AtT20 cells. Continuous application of calphostin C (30 nM, activated by exposure to visible light for at least 20 minutes) throughout the experiments disrupted agonist-activated GIRK currents in some cells, but a 10-minute incubation followed by washout did not affect basal GIRK currents. Preincubation with calphostin C (30 nM) reduced the morphine-induced desensitization in wild-type MOPr cells (56 ± 2% of prepulse) completely blocked desensitization in 11S/T-A mutant (Fig. 7, A, C, and E), and remained constant even after application of morphine (10 μM) for 10 minutes (data not shown). A more prolonged preincubation (30 minutes) with a higher concentration of calphostin C (1 μM) did not produce greater inhibition of morphine-induced desensitization in wild-type MOPr cells (56 ± 2% of prepulse-desensitization probe), completely blocked desensitization in 11S/T-A mutant (Fig. 7, A and B; 64 ± 4% of prepulse, n = 5 in MOPr versus 61 ± 4%, n = 5 in 11S/T-A; P = 0.52). Calphostin C (30 nM, did not affect heterologous desensitization induced by ME or morphine in wild-type MOPr (68 ± 5% of prepulse, n = 3 for ME and 65 ± 5%, n = 4 for morphine). However, as for morphine-induced desensitization of MOPr, calphostin C nearly abolished morphine-induced heterologous desensitization in the 11S/T-A mutant (96 ± 3% of prepulse, n = 4; P = 0.19 versus prepulse) but did not affect ME-induced heterologous desensitization (65 ± 5% of prepulse, n = 3).

**Discussion**

This study has identified multiple mechanisms of MOPr desensitization. Agonist-induced S/T phosphorylation in the MOPr C-terminal does not directly cause desensitization. Desensitization rates by either ME or morphine were similar to those reported previously in a range of cell types (Dang and Christie, 2012) and for coupling to VGCC currents in AtT20 cells (Borgland et al., 2003), but they were considerably slower than the time to maximal phosphorylation of S375, T370, and T376. A likely explanation is that phosphorylation more slowly recruits interacting proteins such as arrestins (e.g., McPherson et al., 2010) that inactivate the receptor; however, progressive mutation of most phosphorylation sites (3S/T-A and 6S/T-A) had no effect on desensitization. Agonist-induced S/T phosphorylation in the C-terminal does not directly cause desensitization. Desensitization induced by morphine was unaffected in 3S/T-A, 6S/T-A, and 11S/T-A mutants. Similarly, heterologous desensitization induced by morphine was unaffected in the 11S/T-A mutant (Fig. 6, A and B; 64 ± 4% of prepulse, n = 5 in MOPr versus 61 ± 4%, n = 5 in 11S/T-A; P = 0.52). Calphostin C (30 nM), did not affect heterologous desensitization induced by ME or morphine in wild-type MOPr (68 ± 5% of prepulse, n = 3 for ME and 65 ± 5%, n = 4 for morphine). However, as for morphine-induced desensitization of MOPr, calphostin C nearly abolished morphine-induced heterologous desensitization in the 11S/T-A mutant (96 ± 3% of prepulse, n = 4; P = 0.19 versus prepulse) but did not affect ME-induced heterologous desensitization (65 ± 5% of prepulse, n = 3).

**Fig. 6.** Desensitization induced by morphine in different MOPr mutants. (A) Morphine-induced desensitization (mrp) in wild-type MOPr probed (ME 10 nM, red) as shown in Fig. 3 without testing during exposure to supramaximal morphine (10 μM, brown) and probed before and after desensitization with SST (100 nM, blue) (B). Example of morphine-induced desensitization in 11S/T-A. (C) Comparison of GIRK conductance in response to ME probe (10 nM) 1 minute after desensitization as a percent of the response 1 minute before desensitization by morphine (10 μM, 5 minutes) in wild-type and different mutants (n = 6 cells per point). (D) Rate of recovery from desensitization (resensitization) in wild-type and different mutants (n = 6 cells per curve). Two-way analysis of variance, Bonferroni post-tests found no differences between mutants.
Desensitization by ME and morphine were both unaffected in 3S/T-A that removes S375, which is efficiently phosphorylated by GRK2/3, as well as two proximal residues, T370, that is quite efficiently phosphorylated (Doll et al., 2011), and S363 which is constitutively phosphorylated and a substrate for PKC-phosphorylation (Illing et al., 2014; Mann et al., 2015). This finding suggests other S/T residues may be phosphorylated to contribute to desensitization and internalization. Mutation of all S/T residues in close proximity to S375 (6S/T-A) completely abolished internalization (also Just et al., 2013) but did not affect desensitization induced by ME or morphine. This finding suggests that desensitization can occur independently of phosphorylation of most residues as well as recruitment of arrestins, which is consistent with findings in sensory (Walwyn et al., 2007) and LC neurons (Dang et al., 2009, 2011; Quillinan et al., 2011) that disruption of β-arrestin-2, GRK2 does not impair MOPr desensitization by ME or DAMGO. It remains possible that both arrestin-mediated and arrestin-independent mechanisms contribute in a parallel manner, which maintains desensitization in the 6S/T-A mutant because desensitization by ME in LC neurons from β-arrestin-2 knockout, but not the wild type, was blocked by ERK1/2 inhibitors (Dang et al., 2009).

Based on our findings in HEK293 cells (Just et al., 2013), the 3S/T-A mutant was expected to produce partial inhibition of ME-induced internalization. The reason for robust ME-induced internalization in the present study is uncertain but might be due to differing patterns of expression of regulatory proteins such as GRKs and arrestins between HEK293 and AtT20 cells (Atwood et al., 2011).

The finding that desensitization by ME was abolished only when all C-terminal S/T residues were mutated suggests that...
phosphorylation of sites additional to those required for arrestin recruitment (lost in the 6S/T-A mutant) are necessary (Birdsong et al., 2015; Canals, 2015). T394 might not be crucial because Lau et al. (2011) were unable to detect agonist-induced phosphorylation of this site. The mechanism underlying the influence of the 354TSST357 region remains uncertain and should be explored in future studies. It could involve facilitation of recruitment of any of the many proteins that have been shown to interact directly with MOPr (Georgoussi et al., 2012).

We also identified a very rapid component of desensitization that is likely to reflect regulation of GIRK by GRK2 recruited to the receptor-channel complex. GRK2 has been shown to nonenzymatically disrupt the PIP2-Gbg interaction needed for GIRK channel opening (Raveh et al., 2010). Supporting evidence includes the initial rapid desensitization includes its rapid time course (<6 seconds), similar to findings of Raveh et al. (2010), which is consistent with rapid recruitment of GRK2 inferred from full phosphorylation of S375 by GRK2 in <20 seconds. Second, ME produced a robust effect but morphine, which does not effectively recruit GRK2 (Doll et al., 2012), produced little very rapid desensitization. Finally, consistent with the mechanism described by Raveh et al. (2010), this component was not attenuated in any of the mutants, including 11S/T-A. Morphine recruits GRK5 in HEK293 cells to mediate phosphorylation of S375 (Doll et al., 2012), but this is unlikely to account for either the slow morphine-induced phosphorylation of MOPr or modest very rapid desensitization because expression of mRNA for GRK5 is undetectable in AtT20 cells (Atwood et al., 2011).

None of the S/T mutations affected morphine-induced desensitization in the absence of PKC inhibition. Previous studies in HEK293 cells and LC neurons have established that inhibition of PKC (Johnson et al., 2006) or knockout of PKCα (Bailey et al., 2009) inhibits morphine-induced desensitization. Consistent with this, a selective (calphostin C) and nonselective (staurosporine) PKC inhibitor reduced morphine-induced desensitization. By contrast, desensitization by ME was unaffected by PKC inhibition, consistent with evidence that morphine, but not DAMGO, increases PKC activity (Chu et al., 2010; Qiu et al., 2014).

The mechanisms underlying PKC involvement are uncertain. The present results suggest that PKC-mediated phosphorylation of S363 and T370 (Doll et al., 2011; Feng et al., 2011; Illing et al., 2014; Mann et al., 2015) is not necessary because morphine-induced desensitization persisted when all S/T sites were mutated. Chu et al. (2010) reported that morphine-induced, PKC-mediated desensitization is mediated partly by phosphorylation of Ga12 subunits, which should be independent of C-terminal phosphorylation. It is possible that PKC-dependent phosphorylation of residues in the intracellular loops (Williams et al., 2013) or in other interacting proteins (Georgoussi et al., 2012) contribute to morphine-induced desensitization. Complex PKC-dependent mechanisms must contribute because calphostin C inhibited by approximately 50% in wild-type MOPr but completely abolished it in 11S/T-A. Moreover, only the component that recovers within 10 minutes was blocked by PKC inhibition in wild-type MOPr, whereas the nonreversing component persisted. This result suggests that the calphostin C–insensitive component of morphine-induced desensitization in wild-type MOPr depends on phosphorylation sites within the C terminus. Constitutive phosphorylation of S363 is probably not involved because this...
would be expected to be blocked by inhibition of PKC in wild-type MOPr (Illing et al., 2014), but other PKC-dependent mechanisms that interact with C-terminal phosphorylation sites might be involved. Whatever PKC-dependent mechanisms are responsible, they must act in parallel rather than additively with other morphine-induced desensitization mechanisms because the 11S/T-A mutation had no effect in the absence of PKC-inhibition. Similarly, parallel mechanisms have been reported in LC neurons (Dang et al., 2009).

The significance of PKC-dependent, morphine-induced desensitization in AtT20 cells (this study) and HEK293 cells (Johnson et al., 2006) for neurons remains uncertain because little morphine-induced desensitization was observed in LC neurons (Dang and Christie, 2012). It remains possible, however, that morphine induces significant desensitization in other neurons; for example, Walwyn et al. (2006) reported similar levels of rapid desensitization by DAMGO and morphine in cultured sensory neurons.

The present results suggest that stimulation of MOPr induces heterologous desensitization of SSTRs, albeit modest. PKC-dependent and -independent mechanisms appear to be involved. Morphine and ME induced similar heterologous desensitization, but the mechanisms are not identical. Phosphorylation of C-terminal S/T residues is not required because desensitization was unaffected for either MOPr agonist in the 11S/T-A mutant; however, inhibition of PKC blocked heterologous desensitization induced by morphine in 11S/T-A, but not wild-type MOPr, which resembles the complete block of morphine-induced MOPr desensitization only in the 11S/T-A mutant and suggests that C-terminal S/T residues may contribute to PKC-dependent mechanisms involved in desensitization of both MOPr and SSTRs. Chu et al. (2010) reported that morphine acting on MOPr stimulates PKCζ to phosphorylate Gβγ, resulting in heterologous desensitization. In contrast to morphine, ME-induced heterologous desensitization was unaffected in both wild-type MOPr and 11S/T-A, similar to Chu et al. (2010). In LC neurons, ME-induced heterologous desensitization of α2-adrenergic receptors was blocked by β-arrestin-2-dependent impairment of μ-opioid receptor resensitization. J Neurosci 31:7122–7130.

In conclusion, the present study has established that C-terminal S/T phosphorylation sites are differentially involved in desensitization of MOPr induced by morphine and ME. ME-induced desensitization does not require phosphorylation of sites in the vicinity of Ser375 that are required for internalization (65/S-T-A) but is blocked if all S/T residues are mutated. By contrast, morphine-induced desensitization is partly PKC-dependent and persists when all S/T residues are mutated. Mechanisms involved in PKC-mediated MOPr desensitization by morphine appear to require an interaction of PKC residues in the 354-TSTTS357, S363, T370, or perhaps T394 region. Mutation of all C-terminal S/T residues does not simply transform the interaction of ME to a morphine-like behavior that recruits PKC, as previously suggested (Zheng et al., 2011), because ME-induced desensitization was insensitive to PKC-inhibition in both the wild-type and 11S/T-A mutants. These findings suggest that differential regulation of MOPr by morphine and strongly internalizing agonists involves multiple mechanisms in addition to differential ability of the agonist occupied receptor to recruit arrestins.

**Authorship Contributions**

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*Conducted experiments:* Yousuf, Miess, Sianati, Du.

*Performed data analysis:* Yousuf, Miess, Sianati, Du.

*Wrote or contributed to the writing of the manuscript:* Yousuf, Miess, Sianati, Du, Schulz, Christie.

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