The role of phosphorylation sites in desensitization of µ-opioid receptor

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Abbreviations: A, alanine; DAMGO, [D-Ala2-MePhe4-Gly-ol]enkephalin; HEK 293, human embryonic kidney 293; ERK1/2, extracellular signal regulated kinases 1 and 2; GIRK, G protein activated inwardly rectifying potassium channel (Kir3.x); GPCR, G-protein coupled receptor; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; LC, locus coeruleus, ME, Met⁵-enkephalin, MOPr, μ-opioid receptor; PKC, protein kinase C; S, serine; SST, somatostatin; T, threonine; VGCC, voltage-gated calcium channel
ABSTRACT

Phosphorylation of residues in the C-terminal tail of the µ-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, 6 S/T-A (S363A, 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 GIRK (Kir3.X) conductance by submaximal concentrations of Met⁻⁻⁵-Enkephalin (ME) and somatostatin (SST coupling to native SSTR) were used to examine desensitization induced by exposure to ME and morphine for 5 min 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 µ-opioid receptor (MOPr) is widely considered to be an initial step in the development of opioid tolerance (Williams et al., 2013). Potential mechanisms include phosphorylation of specific MOPr C-terminal Serine and Threonine (S/T) residues by G-protein coupled receptor kinases (GRK), followed by β-arrestin binding that causes internalization (Just et al., 2013; Williams et al., 2013). However, the relationship between these events and receptor desensitization is uncertain.

Agonist mediated phosphorylation of MOPr is rapid and prominent at S375 within the C-terminal, 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, $^{354}$TSST$^{357}$ and $^{375}$STANT$^{379}$ 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 MOPr internalization. Mutation of S375 to Alanine (A) slowed internalization but mutation of S363 and T370 did not (El Kouhen et al., 2001; Schulz et al., 2004), suggesting S375 is important for β-arrestin recruitment and endocytosis. Mutation of potential S/T phosphorylation sites in the 375-379 region slowed internalization by 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 effects 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 interpretation of mechanisms (Williams et al., 2013). More direct measurement of loss of coupling to VGCCs and GIRKs have examined the influence of kinase inhibitors to modulate desensitization. Johnson et al., (2006) used protein kinase C
(PKC) inhibition in HEK293 cells and Bailey et al., (2009) inhibited PKC and GRK2 in locus coeruleus (LC) neurons to suggest that desensitization by DAMGO required GRK2 but morphine required PKC. By contrast, Dang et al., (2009) suggested that inhibition of both β-arrestin-dependent and ERK-dependent mechanisms drive desensitization by ME.

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 non-internalizing 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 morphine. These results indicate that desensitization of MOPr by high efficacy, 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), 6 S/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 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 (Beckton, Dickinson Biosciences) in Dulbecco modified Eagle medium (Gibco, Life Technologies, Australia) containing 4.5 g/L glucose, penicillin-streptomycin (100µl/ml.), G418 (50mg/ml.) (Gibco, Invitrogen) and 10% FBS. Cell cultures were kept in humidified 5% CO₂ atmosphere at 37°C. Cells were ready for recording after 24 hours incubation.

**MOPr binding density.** MOR binding density was determined on intact cells as described by Gomes *et al.* (2000). Briefly, approximately $2 \times 10^5$ 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 5nM [$^3$H] DAMGO (PerkinElmer, Waltham, MA) for 2 h. Nonspecific binding was determined in the presence of unlabeled DAMGO (10 µM). At the end of the incubation, plated cells were rinsed three times with 0.5mL 50 mM Tris-Cl (pH 7.4) at 4 °C. Cells in each well were then digested for 1 hr at room temperature with 100 µL of 1N NaOH. 100 µL 1N HCl was then added to each well and collected into scintillation vials and bound ligand was determined using a liquid scintillation counter (MicroBeta, Perkin Elmer, Waltham MA, USA). A parallel set of incubated cells was collected for total protein
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 ~50% confluence. Cells were incubated for 30 min in serum-free Dulbecco’s modified Eagle’s medium followed by addition of ME (30 μM at 37°C). After 30 min, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 min at room temperature and then permeabilized using 0.1% Triton X-100. Cells then were immune-stained with 1:300 dilution of anti-HA antibody (Santa Cruz, 1 hour at room temperature), rinsed in PBS followed by incubation with 1:1000 Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen, 1 hour at room temperature). Coverslips were then washed 3 × 10 minute with PBS and mounted with Fluormount-G (SouthernBiotech) on glass slides. Images were acquired using Olympus FV300 laser scanning confocal microscope. High-resolution images (optical magnification: 60X; resolution: 1024 × 1024 pixels) were obtained using a 60X oil objective (UPFL). 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 ImageJ (v 1.44p, Wayne Rasband, National Institutes of Health, USA) software. 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 percentage of internalized receptors was estimated as a ratio of \[1 - \frac{\text{membrane fluorescence}}{\text{total fluorescence}}\] × 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 disodiumpyrophosphate, 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 lectinagarose beads as described (Schulz *et al.*, 2004, Koch *et al.*, 2001). Proteins were eluted from the beads using SDS-sample buffer for 20 min at 45°C. Samples were split, resolved on 7.5% SDS-polyacrylamide gels, and after electroblotting, membranes were incubated with either anti-pS356/pT357 {4879}, anti-pT370 {3196}, antipS375 {2493}, 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, USA) having input resistance ranged between 3.5-4.5 MΩ.
Pipettes were filled with internal solution containing 135 mM potassium gluconate, 3 mM MgCl₂, 5 mM HEPES, 5 mM EGTA (pH adjusted to 7.4 with KOH). 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 [DMSO]). Cells were initially superfused with external bath solution containing 140 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 10mM HEPES and 10mM glucose (pH adjusted to 7.4 with NaOH). For measuring \( I_{\text{GIRK}} \) 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, USA). Currents were sampled at 100 Hz, low pass filtered at 50 Hz and recorded on hard disk for later analysis. \( I_{\text{GIRK}} \) was recorded using a 200-ms voltage step of -120 mV from a holding potential of -60 mV applied every 2 s. Drugs were perfused on to the cells using ValveLink8.2 pressurized pinch valve perfusion system (AutoMate Scientific, USA). In all recorded cells, solution exchange reached steady state within 200 ms (usually within 100 ms), 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 ± SEM and analysed using GraphPad Prism v. 5. All data points are plotted as pure GIRK conductance $G$ (nS) using the following calculation: $[I_{GIRK}(-60 \text{ mV}) - I_{GIRK}(-120 \text{ mV})] / 60 \text{ mV}$. The rate of $\mu$-opioid receptor desensitization and its recovery was calculated as a percentage by using the following formula: $(\text{Post G}_{\text{ME}} / \text{Pre G}_{\text{ME}}) \times 100$. Where, “Pre” and “Post G$_{\text{ME}}$” are values of GIRK conductance averaged for 4-5 points during the maximal response (coloured points in figures), in response to probe ME (10nM). Comparisons of desensitization for different mutants were analysed using the Kruskal Wallis test. Comparison for heterologous desensitization at SST receptors was analysed using one way ANOVA followed by Dunnett’s multiple comparison test. The desensitization and recovery for different $\mu$-opioid receptor mutants were analysed using a two-factor ANOVA followed by Bonferroni post-hoc tests.

**Drugs and chemicals.** Somatostatin, ME and protein kinase C blocker Calphostin C and amphotericin B were obtained from Sigma-Aldrich (Australia), Staurosporine from Tocris Biosciences, morphine HCl was from GlaxoSmithKline (Boronia, Victoria, Australia).
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. Receptor internalization was studied in all MOPr mutants using a full agonist (30 µM ME) at 37°C and was consistent with our previously reported results in HEK293 cells (Just et al., 2013). The images in Figure 1 show that incubation of MOPr and the 3S/T-A mutants with ME (30 µM) for 30 min produced internalization, as previously reported (Just et al., 2013; 65 ± 2 % internalized in wild type MOPr and 67 ± 4 % in 3S/T-A, n = 7 for each). As previously reported (Just et al., 2013), incubation of 6S/T-A and 11S/T-A with ME (30 µM) did not produce significant internalization (% internalization < 5 % in 6S/T-A and 11S/T-A, n = 7 for each).

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 I/V trace shows the basal conductance (grey) and increased conductance in response to ME (red). Agonist-induced GIRK conductance increases were calculated at 2 s intervals using the voltage step protocol shown in Figure 2A (blue trace), the chord conductance ‘G’ was plotted during application of agonists as shown in Figure 2B. Conductance increases (Figure 2B, red circles) were normalized to the response produced by a supramaximal concentration of somatostatin (SST, 1 µM) acting on SSTR (Borgland et al., 2003) in the same cell and concentration-response curves determined as shown in Figure 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 G protein-coupled GPCRs 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 SST receptors. SSTR types1-4 are natively expressed in AtT20 cells (Patel et al., 1994; Atwood et al., 2011). A supramaximal concentration of SST (1µM) was applied to MOPr transfected cells for 1 min followed by co-application of a supramaximal concentration of ME (10µM) as shown in figure 2D. No additional response to ME was observed when co-applied 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.** Figures 3A and 3B show 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 s, with maximal phosphorylation of T370 and T379 occurring within to 1 min. S356/T357 and T376 were not maximally phosphorylated until exposed to ME for 20-30 min. 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 min did not correlate with the time course of MOPr phosphorylation (Figures 3C and 3D). A very rapid component of desensitization of the GIRK conductance was observed during exposure to supramaximal concentrations of ME which reached a plateau within 6 s (34 ± 3 % decline in the 6s after peak response, n = 11; Figure 3C). The very rapid component of desensitization was not related to S/T
phosphorylation because it persisted in all mutants exposed to ME (see below). It resembles the rapid non-enzymic 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 s component, there was a modest decline in the response to the supramaximal concentration of ME (10 μM) during the 6 min 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 (Figure 2C; 10 nM, 20 s) during and after exposure to a supramaximal concentration of ME (10 μM, 6 min). A 20s exposure was considered sufficient to washout 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 4s for ME and 3 s for morphine (data not shown), similar to the τ_{off} of ~3 and 2 s, respectively reported by Williams (2014) using caged naloxone in brain slices. Brief (20 s) pre-exposure 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 Figure 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 min time
point (not shown in Figure 3D), the time constant for desensitization was ~ 2.6 min. The probe procedure during application of ME (10 μM) might have slowed the rate of desensitization but did not greatly affect the magnitude because desensitization measured 1 min after washout of 10 μM ME did not differ when the probe was omitted during desensitization (8.3 ± 0.4 %, n = 5 in presence of probe and 9.6 ± 1.6 %, n = 8 in its absence, P = 0.59, unpaired t-test).

Figures 4A and 4B show representative Western blots and densitometry of the time course of MOPr phosphorylation induced by morphine in AtT20 cells. As previously described in HEK 293 cells (Doll et al., 2011; Just et al., 2013), morphine produced weak phosphorylation of S375 that peaked after 10 min and very weak phosphorylation of pT370 and pT376 that was slow, reaching maxima after 10 min. 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 min was similar to that produced by ME (Figures 4C and 4D). In contrast to ME, the rapid component of GIRK desensitization during the first 6 s exposure to morphine was significantly smaller than that produced by ME (9.9 ± 0.9 % n = 14, P < 0.0001 versus ME, Figure 4C). Excluding the first 6s 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 min exposure (Figure 4C), as expected for a partial agonist during desensitization (Connor et al., 2004). Desensitization measured 1 min after washout of morphine (10 μM) was similar to that produced by ME (11 ± 1 %, n = 5 in 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 min time point (not shown in Figure 4 D) the time constant, for desensitization was 2.0 min.

**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. Figures 5A and B show representative examples of time course of ME-induced desensitization and recovery in wild type MOPr and the 11S/T-A mutant. As introduced above, an initial very rapid GIRK desensitization was observed that reached a plateau in 2-6 s 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 6 s 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 Figure 3C but no decline was observed in the 11S/T-A mutant (Figure 5B). The probe concentration of ME (10 nM) applied 1 min after washing ME in wild type MOPr transfected cells showed similar desensitization to that shown in Figure 3B. Desensitization was not affected 3S/T-A and 6S/T-A mutants but was abolished in the 11S/T-A mutant (Figure 5C).

The probe concentration of ME was applied successively 1, 4, 6, 8 and 10 min after washout of ME (10 μM) to determine the rate of recovery from desensitization (Figure 5). Figure 5A and D show recovery of MOPr after successive wash to original response to 10nM ME. Recovery from desensitization occurred with a similar rate for MOPr, 3S/T-A and 6S/T-A mutants and was complete within 10 min.
Desensitization by morphine is not disrupted by mutation of S/T phosphorylation sites. As shown in Figure 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 no effect on desensitization by morphine. The desensitization during application of morphine (10 μM) was similar to that shown in Figure 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 s). The response to the probe concentration of ME (10 nM) 1 min after washout was also unaffected by any of the mutations (Figure 6C). The rate of recovery of MOPr function after desensitization with morphine (10µM), for all wild type MOPr and all mutants was more prolonged than recovery from ME-induced desensitization and was also unaffected by any of the mutations (Figure 6A, 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. In order to determine if 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 such GF109203X (1µM) and chelerythrin (1µM) were tested initially but as reported previously by Johnson et al. (2006), both of 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 min) throughout experiments disrupted agonist activated
GIRK currents in some cells but a 10 min incubation followed by washout did not affect basal GIRK currents. Preincubation with calphostin C (30nM) reduced the morphine-induced desensitization in wild-type MOPr cells (56 ± 2% of pre-desensitization probe) and completely blocked desensitization in 11S/T-A mutant (Figure 7A, C and E), and remained constant even after application of morphine (10 μM) for 10 min. (Data not shown). A more prolonged pre-incubation (30 min) 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 pre-desensitization probe, n = 4). As shown in Figure 7E, the blunted desensitization produced by morphine after pre-incubation in calphostin C showed little or no recovery over the ensuing 10 min. By contrast, calphostin C had no effect on ME-induced desensitization or recovery in wild type MOPr or 11S/T-A (Figure 7 B, D and F). Staurosporine (1 μM), a broad range protein kinase inhibitor, including PKC, produced similar inhibition of morphine-induced desensitization in wild type MOPr cells (68 ± 5%, n = 4).

Met-enkephalin and morphine induce heterologous desensitization at SSTR. In addition to homologous desensitization, heterologous desensitization of SSTRs and α2-adrenergic receptors has been previously reported following stimulation of MOPr in LC neurons (Dang et al., 2012; Llorente et al., 2012). In the present study heterologous desensitization of native SSTR2 responses was observed after 5 min exposure to morphine or ME (10 μM). Figure 8 A shows that the response to a near maximal concentration of SST (100 nM) the applied at a 6.5 min interval produces no decline in GIRK activation. Figure 8B shows that 5 min exposure to morphine (10 μM) (B) produces heterologous desensitization of the response to subsequent
application of SST. Figure 8C shows that similar heterologous desensitization of responses to SST was observed following exposure to desensitizing concentrations of either morphine or ME, regardless of whether a submaximal or supramaximal concentration of STT (1 μM) was used to probe heterologous desensitization.

As shown in Figure 8D (see examples in Figure 5A and B), heterologous desensitization induced by ME 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 (Figure 6A and B; 64 ± 4% of pre-pulse, n = 5 in MOPr versus 61 ± 4%, n = 5 in 11 S/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 pre-pulse, 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 pre-pulse, n = 4, P = 0.19 versus pre-pulse) but did not affect ME-induced heterologous desensitization (65 ± 5% of pre-pulse, 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 considerably slower than the time to maximal phosphorylation of S375, T370 and T379, and much faster than phosphorylation of S356/T357 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 produced by ME even though the 6S/T-A mutation abolished internalization and, presumably, arrestin recruitment. Finally, as previously reported (Johnson et al., 2006; Bailey et al., 2009), the mechanism of morphine-induced desensitization differed from ME. It was mediated at least partly by PKC but did not require the known PKC phosphorylation sites in MOPr (S363, T370; Feng et al., 2011; Illing et al., 2014; Mann et al., 2015).

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 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 suggests that desensitization can occur independently of phosphorylation of most residues as well as recruitment of arrestins. This is consistent with findings in sensory (Walwyn et al., 2007) and LC neurons (Dang et al., 2009; Dang et al., 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 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. 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 non-enzymically disrupt the PIP2-Gβγ interaction needed for GIRK channel opening (Raveh et al., 2010). Supporting evidence includes the initial rapid desensitization includes its rapid time course (<6 s) similar to Raveh et al. (2010), which is consistent with rapid recruitment of GRK inferred from full phosphorylation of S375 by GRK2 in < 20s. Secondly, ME produced a robust effect but morphine, which does not effectively recruit GRK2 (Doll et al., 2012), produced very 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 PKCa (Bailey et al., 2009) inhibits morphine-induced desensitization. Consistent with this, a selective (calphostin C) and non-selective (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 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 Gai2 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 min was blocked by PKC inhibition in wild type MOPr while the non-reversing component persisted. This suggests that the calphostin-C-insensitive component of morphine-induced desensitization in wild type MOPr depends on phosphorylation sites within the C-terminal. 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 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 very little morphine-induced desensitization was observed in LC neurons (see Dang and Christie, 2012). However, it remains possible that morphine induces significant desensitization in other neurons, e.g., 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. This resembles the complete block of morphine-induced MOPr desensitization only in the 11 S/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 PKCe to phosphorylate Gai 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 in β-arrestin knockout mice via an ERK1/2-dependent mechanism (Dang et al., 2012) but not blocked by inhibition of PKC (Llorente et al., 2012). This PKC-independent mechanism is unlikely to account for ME-induced heterologous desensitization in the 11S/T-A mutant, which presumably cannot recruit arrestin to MOPr, so the mechanism remains unknown.

In conclusion, the present study has established that C-terminal S/T phosphorylation sites are differentially involved desensitization of MOPr induced by morphine and ME. ME-induced desensitization does not require phosphorylation of sites in the vicinity of S375 that are required for internalization (6S/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 \[^{354}TSST^{357}\], 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 behaviour 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.

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Authorship Contributions.

*Participated in research design*: Yousuf, Miess, Sianati, Du, Schulz, and Christie.

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

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

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


Footnotes:

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Figure Legends:

Figure 1. Mutation of multiple C-terminal S/T residues blocks MOPr internalization. Amino acid sequences of C-terminal tail of wild type MOPr, 11S/T-A, 6S/T-A and 3S/T-A). Confocal images on left of each sequence show the internalization profile of wild type MOPr and the different mutants in response to incubation in ME (30µM) at 37°C for 30 min.

Figure 2. Opioids and SST activate a common population of GIRK channels in AtT20 cells transfected with MOPr. (A) Voltage-clamp step protocol used in perforated patch-clamp recording showing a single sweep of 2 s at a holding potential of -60 mV and stepping to -120mV for 200 ms (blue) along with evoked current (lower left) in the absence (black) and presence (red) of ME (10 µM). Top right shows voltage-ramp (-120 to -50 mV, 0.2 s) current trace in the absence (grey) and presence of ME (10 µM, red). The slope of the broken line represents the chord conductance. (B) Time course of GIRK response to increasing concentrations of ME in wild type MOPr expressing AtT20 cells. (C) Concentration-response curve of morphine (brown), ME (red) and SST (blue) in wild type cells (n = 6 per point). (D) Time course of GIRK response to SST (1µM, blue bar) then co-application of SST and ME (10µM, red bar).

Figure 3: Time course of phosphorylation and MOPr desensitization induced by ME (10 µM) in MOPr expressing AtT20 cells. (A) representative Western blots of phosphorylation at specific C-terminal sites on MOPr induced by ME. (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 (10nM red) to determine extent of desensitization applied to a MOPr wild type AtT20 cell before, during and after 6 min exposure to a supramaximal concentration of ME (10µM, orange). The red circles were averaged and for the group data in D. (D) The rate of desensitization expressed as percentage of the amplitude of the pre-desensitization probe exposure to ME (10 nM).

**Figure 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 (10nM red) to determine extent of desensitization applied to a MOPr wild type cell before, during and after 6 min exposure to a supramaximal concentration of morphine (10µM, brown). The red circles were averaged and for the group data in D. (D) The rate of desensitization expressed as percentage of the amplitude of the pre-desensitization probe exposure to ME (10 nM).

**Figure 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 Figure 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 in response to ME probe (10nM) 1 min after desensitization as a percentage of the response 1 min before desensitization by ME (10 μM, 5 min) 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 and *P < 0.05 (Two-way ANOVA, Bonferroni post-tests 11S/T-A versus MOPr, no other between mutant differences were seen).

**Figure 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 Figure 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 (10nM) 1 min after desensitization as a percentage of the response 1 min before desensitization by morphine (10 μM, 5 min) 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 ANOVA, Bonferroni post-tests found no between mutant differences).

**Figure 7.** The protein kinase C inhibitor, calphostin-C, inhibits desensitization induced by morphine but not ME. (A, B) Time course showing the effect of calphostin-C (30nM, green) on morphine and ME induced desensitization, respectively, in wild-type MOPr. (C, D) Time course showing the effect of calphostin-C (30nM, green) on morphine and ME induced desensitization,
respectively, in the 11 S/T-A mutant MOPr. Other colour codes are as in Figures 5 and 6. (E) The extent of desensitization and rate of recovery of response to the probe concentration of ME (10 nM) after desensitization with 10µM morphine in the presence of calphostin C in wild type MOPr (white circles) and 11S/T-A (purple circles, n = 4 per curve). Desensitization and recovery data for wild-type MOPr and 11 S/T-A from Figure 6D are re-plotted as shaded symbols with broken lines for comparison. (F) The extent of receptor desensitization and rate of recovery of response to the probe concentration of ME (10 nM) after desensitization with 10µM ME in the presence of calphostin C in wild type MOPr (white circles) and 11S/T-A (purple circles, n = 4 per curve). Desensitization and recovery data for ME in wild-type MOPr and 11 S/T-A from Figure 5D are re-plotted as shaded symbols with broken lines for comparison. Significant differences between wild type MOPr and 11S/T-A were seen at ***P < 0.001 (Two-way ANOVA, Bonferroni post-tests) (n =4).

**Figure 8.** Heterologous desensitization at the somatostatin receptor (SSTR) induced by morphine and ME. (A) Time course showing 20 s application of 100nM SST (blue) before and after 5 min of control solution (20 mM K+) and (B) 10µM morphine. (C) Response to SST (100nM and 1µM) as percentage of the same concentration applied before desensitization after 5 min exposure to control solution (blue), morphine (10 µM, brown) or ME (10µM, red) (n = 4-5 per group). (D) The effect of ME (10 µM)-exposure on heterologous desensitization of SSTR in MOPr wild type cells all mutants. ***P < 0.001 (Two way repeated measures ANOVA, post-versus pre-pulse response to SST, 100 nM) (n = 4-5 per group).
Figure 1

MOPr

FCIPSTSSTEIQQNARIKQHRHSTANTVDRTHQLENLEAALP

MOPr 11 S/T-A

FCIPAAAAIEQQNAARIRQNAREHAAANAVDRAHQNLENLEAALP

MOPr 6 S/T-A

FCIPSTSSTEIQQNARIKQHRHSTANTVDRTHQLENLEAALP

MOPr 3 S/T-A

FCIPSTSSTEIQQNARIKQHRHSTANTVDRTHQLENLEAALP
Figure 2

A

0.2 sec

-60 mV

-120 mV

pA

0

-100

-200

-300

20 K^+

20 K^+ + ME

G

B

1 nM 3 nM 10 nM 30 nM 100 nM 300 nM

GIRK

0.2 nS

1 min

C

D

SST

SST + ME

GIRK (GME/GSST)

MP

ME

Log (Agonist)

0.0

0.5

1.0

1.5

-10

-9

-8

-7

-6

-5
Figure 3
Figure 4

A) Morphine

B) % Phosphorylation over time

C) GIRK activation

D) G_{MOP} (% of pre-desens) over time
Figure 6

A

MOPr

GIRK

0.2 nS

2 min

ST ME ME ME ME ME ME ME

10 mM mprp ME ST ME ME ME ME ME ME

B

11S/T-A

GIRK

0.2 nS

2 min

ST ME ME ME ME ME ME ME

10 mM mprp ME ST ME ME ME ME ME ME

C

G_{ME} (% of pre-desens)

100

50

0

MOPr 11S/T-A 6S/T-A 3S/T-A

D

G_{ME} (% of pre-desens)

100

80

60

40

20

0

2 4 6 8 10 12 14

Time (min.)

MOPr 3S/T-A 6S/T-A 11S/T-A
Figure 7

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Figure 8

A

GIRK

0.2 nS
1 min

0.1 µM ST
Control
0.1 µM ST

B

GIRK

0.3 nS
1 min

0.1 µM ST
10 µM morphine
0.1 µM ST

C

G_{st} (% of pre pulse)

100

50

0

***

morphine
Met-Enkeph
ST (100nM)
ST (1 µM)

-
-
+
-

+
-
+
-

+ 
-
+
-

-
-
+
-

D

G_{st} (% of pre pulse)

100

50

0

***

MOPr
16S/T-A
6S/T-A
30S/T-A

- 
- 
- 
- 

+
-
+
-

+ 
-
+
-

- 
-
+
- 

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