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Morphine induces terminal μ-opioid receptor desensitization by sustained phosphorylation of serine-375

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Morphine is a poor inducer of μ-opioid receptor (MOR) internalization, but a potent inducer of cellular tolerance. Here we show that, in contrast to full agonists such as [D-Ala^2-MePhe^4-Gly-ol]enkephalin (DAMGO), morphine stimulated a selective phosphorylation of the carboxy-terminal residue 375 (Ser^{375}). Ser^{375} phosphorylation was sufficient and required for morphine-induced desensitization of MOR. In the presence of full agonists, morphine revealed partial agonistic properties and potently inhibited MOR phosphorylation and internalization. Upon removal of the drug, DAMGO-desensitized receptors were rapidly dephosphorylated. In contrast, morphine-desensitized receptors remained at the plasma membrane in a Ser^{375}-phosphorylated state for prolonged periods. Thus, morphine promotes terminal MOR desensitization by inducing a persistent modification of Ser^{375}.

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Introduction

Morphine is a powerful pain reliever; however, the utility of morphine for the treatment of chronic pain is hindered by the development of tolerance (Nestler, 1996; Nestler and Aghajanian, 1997; Koob *et al*, 1998). Morphine tolerance occurs on continued use of the drug such that the amount of drug required to elicit pain relief must be increased to compensate for diminished responsiveness (Nestler, 1996; Nestler and Aghajanian, 1997; Koob *et al*, 1998). In many systems, decreased responsiveness to agonists has been correlated with desensitization and internalization of G protein-coupled receptors (Pitcher *et al*, 1998). However, morphine profoundly differs from other opioids such as [D-Ala^2-MePhe^4-Gly-ol]enkephalin (DAMGO) in that it activates the μ-opioid receptor (MOR, MOP) without causing its rapid internalization (Keith *et al*, 1996). The differential regulation of receptor endocytosis by distinct opioid agonists appears to be related to their ability to promote G protein-coupled receptor kinase (GRK)-dependent phosphorylation of MOR (Zhang *et al*, 1998). Whereas DAMGO elicits robust phosphorylation and internalization of MOR, morphine induces little MOR phosphorylation (Zhang *et al*, 1998; Koch *et al*, 2001). Upon overexpression of GRK2, morphine gains the capacity to induce MOR phosphorylation and internalization (Zhang *et al*, 1998).

Previously, we have reported that prolonged exposure to both DAMGO and morphine promotes desensitization of MOR signaling in transfected cells (Koch *et al*, 1998). Upon removal of the drug, DAMGO-desensitized receptors regained functional activity within minutes. In contrast, morphine-desensitized receptors failed to resensitize (Koch *et al*, 2001). Based on these results, we proposed that regulated endocytosis of MOR is required for its resensitization, and that the inability of morphine to cause substantial receptor endocytosis and recycling may facilitate the development of tolerance upon chronic drug exposure (Koch *et al*, 1998, 2001).

Recent evidence suggests that DAMGO-induced internalization of MOR is regulated by phosphorylation of several Ser/Thr residues including serine-363 (Ser^{363}), Thr^{370} and Ser^{375} of the COOH-terminal tail (El Kouhen *et al*, 2001). Here, we show that exposure of MOR to morphine selectively induces the phosphorylation of Ser^{375} (corresponds to Ser^{377} of human MOR). In contrast to DAMGO, morphine-induced phosphorylation of Ser^{375} is sustained for prolonged periods and, hence, promotes persistent desensitization of MOR.

Results

First, we assessed whole-cell receptor phosphorylation in MOR-expressing HEK 293 cells in response to both DAMGO and morphine. DAMGO induced a rapid and robust phosphorylation of MOR (~4-fold over basal). In contrast, the extent of morphine-induced phosphorylation of MOR reached only $32 \pm 6\%$ ($n = 3$) of DAMGO-induced phosphorylation (Figure 1). Mutation of Ser^{375} to Ala (S375A) reduced DAMGO-induced phosphorylation to $43 \pm 4\%$ ($n = 3$) of wild-type MOR and completely diminished morphine-induced phosphorylation ($6 \pm 4\%$, $n = 3$; Figure 1). These results indicate that Ser^{375} is a primary phosphoacceptor site for the DAMGO-activated MOR. However, the residual DAMGO-induced phosphorylation of the S375A mutant suggests that the DAMGO-activated MOR acquires a conformation, which also allows phosphorylation of several other Ser/Thr residues in addition to Ser^{375}. In contrast, the morphine-activated MOR undergoes selective phosphorylation of Ser^{375}.

We next examined agonist-dependent Ser^{375} phosphorylation using an antibody that selectively recognizes the Ser^{375}-phosphorylated form of MOR (see Supplementary data Figure 1). We found that Ser^{375} phosphorylation was at or near the

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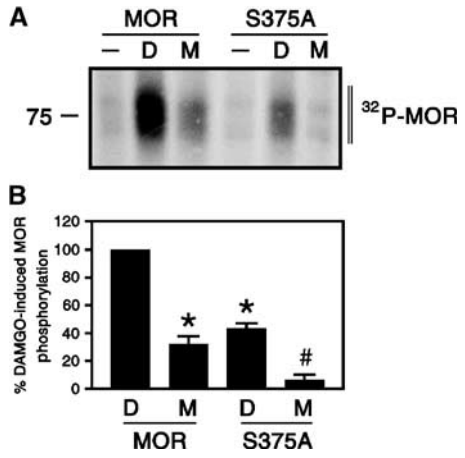

Figure 1 Agonist-selective phosphorylation of MOR and its S375A mutant. HEK 293 cells expressing HA-tagged MOR or HA-tagged S375A receptors were exposed to 10 μM DAMGO or 10 μM morphine for 30 min, and whole-cell receptor phosphorylation was determined as described under 'Materials and methods'. (**A**) Representative autoradiographs from one of three independent experiments are shown. (**B**) Mean ± s.e. of three independent experiments quantified by phosphorimager analysis. Data were normalized to DAMGO-induced phosphorylation of MOR. Results were analyzed by two-tailed Student's paired *t*-test (**P*<0.05 versus DAMGO-induced phosphorylation of MOR; #*P*<0.05 versus morphine-induced phosphorylation of MOR). Note, (I) morphine-induced phosphorylation of MOR was significantly less than DAMGO-induced phosphorylation, (II) mutation of Ser^{375} to Ala (*S375A*) strongly reduced DAMGO-induced phosphorylation and completely diminished morphine-induced phosphorylation. The positions of molecular mass markers are indicated on the left (in kDa).

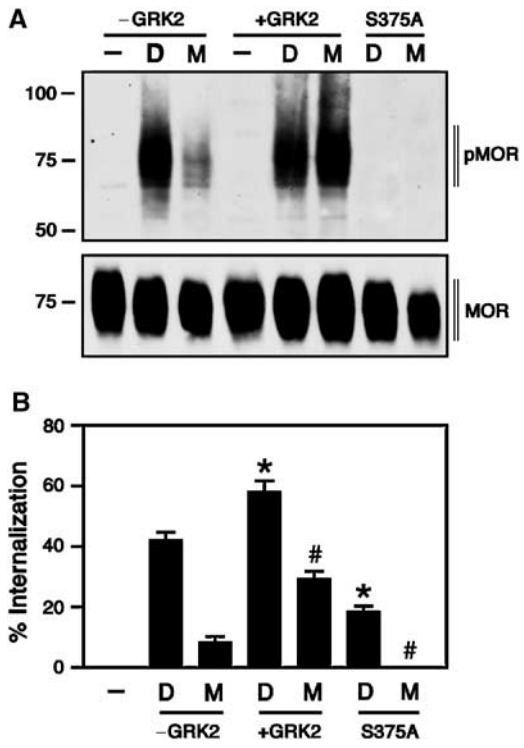

Figure 2 Relationship between Ser^{375} phosphorylation and internalization of MOR. (**A**) HEK 293 cells expressing the wild-type MOR or the S375A mutant were transiently transfected with GRK2 (+*GRK2*) or empty vector (−*GRK2*). After 2 days, cells were either not treated (−) or treated with 10 μM DAMGO (D) or 10 μM morphine (M) for 30 min, lysed and immunoblotted with an antibody specific for the Ser^{375}-phosphorylated MOR (pMOR, upper panel). Expression of HA-tagged MOR and HA-tagged S375A receptors was confirmed by immunoblotting with an anti-HA antibody (MOR, lower panel). Note, (I) Ser^{375} phosphorylation was not detectable in untreated cells, (II) the morphine-induced Ser^{375} phosphorylation was ∼35% of DAMGO-induced phosphorylation, (III) overexpression of GRK2 strongly enhanced the morphine-induced Ser^{375} phosphorylation and (IV) phospho-Ser^{375} immunoreactivity was not detectable in cells expressing the S375A mutant. The positions of molecular mass markers are indicated on the left (in kDa). Three additional experiments gave similar results. (**B**) HEK 293 cells expressing the wild-type MOR or the S375A mutant were transiently transfected with GRK2 (+*GRK2*) or empty vector (−*GRK2*). After 2 days, cells were either not treated (−) or treated with 10 μM DAMGO (D) or 10 μM morphine (M) for 30 min. Cell surface receptors were labeled with rabbit anti-HA antibodies, followed by a peroxidase-conjugated secondary antibody. Receptor sequestration, quantified as the percent loss of cell-surface receptors in agonist-treated cells, was measured by ELISA. Data are presented as mean ± s.e. of five independent experiments performed in quadruplicate. The results were analyzed by two-tailed Student's paired *t*-test (**P*<0.05 versus DAMGO (−GRK2); #*P*<0.05 versus morphine (−GRK2)). Note, (I) in contrast to DAMGO, morphine failed to cause substantial receptor endocytosis, (II) overexpression of GRK2 strongly enhanced the level of morphine-induced internalization and (III) mutation of Ser^{375} to Ala (S375A) strongly reduced agonist-driven MOR endocytosis.

detection limit in the basal state (Figure 2A). Exposure of MOR-expressing HEK 293 cells to 10 μM DAMGO for 30 min induced a robust phosphorylation of Ser^{375}. Exposure to 10 μM morphine for 30 min also induced a clearly detectable Ser^{375} phosphorylation, however, to a much lesser extent ($35 \pm 8\%$, $n = 6$) than that observed with DAMGO (Figure 2A). Quantitative analysis of receptor internalization revealed that DAMGO-activated MOR receptors were efficiently removed from the cell surface, whereas morphine failed to cause substantial receptor endocytosis (Figure 2B). Overexpression of GRK2 strongly enhanced the level of morphine-induced Ser^{375} phosphorylation and internalization of MOR (Figure 2A and B, see Supplementary data Figure 2). Mutation of Ser^{375} to Ala (S375A) completely diminished detectable phospho-Ser^{375} immunoreactivity and strongly reduced agonist-driven receptor endocytosis (Figures 2A, B and 3). Confocal microscopy revealed that Ser^{375}-phosphorylated MOR receptors were detectable at the plasma membrane within 2 min of DAMGO treatment (see Supplementary data Figure 3). In cells treated for 30 min with DAMGO, the majority of Ser^{375}-phosphorylated MOR receptors were confined to clusters of intracellular vesicles presumably representing the perinuclear recycling compartment (Figure 3, see Supplementary data Figure 3). In contrast, in cells treated for 30 min with morphine, the majority of Ser^{375}-phosphorylated MOR receptors were seen as punctate staining at the plasma membrane (Figure 3). These results provide direct evidence for agonist-induced phosphorylation of Ser^{375} within the carboxyl-terminal portion of the receptor, and suggest a direct relationship between Ser^{375} phosphorylation and internalization of MOR. Morphine appears to restrain the receptor in a conformation that is a poor substrate for GRK2-mediated phosphorylation of Ser^{375}.

We then examined the dose–response relationship of various opioids to induce Ser^{375} phosphorylation and internalization of MOR (see Supplementary data Figure 4). The full agonists DAMGO, sufentanil and etorphine were

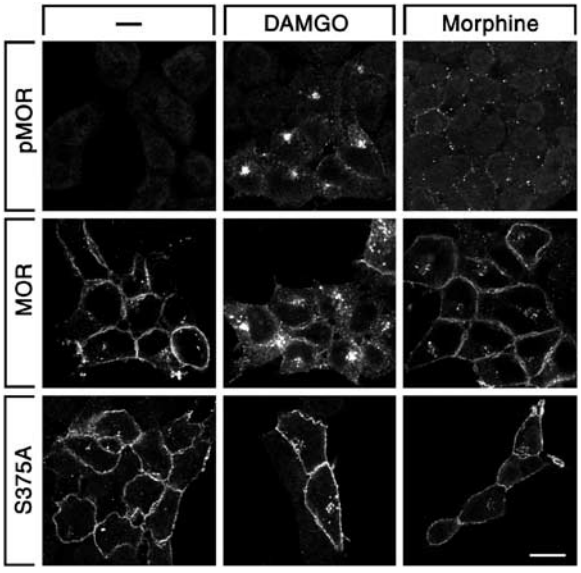

Figure 3 Agonist-selective internalization of Ser^{375}-phosphorylated MOR receptors. HEK 293 cells expressing MOR or S375A were either not exposed (−) or exposed to 10 μM DAMGO or 10 μM morphine for 30 min, immunofluorescently labeled with anti-phospho-Ser^{375} (pMOR, upper panel) or anti-HA (MOR, middle and lower panels) antibodies and examined under a confocal microscope. Note, (I) in DAMGO-treated cells, the majority of Ser^{375}-phosphorylated MOR receptors were confined to perinuclear clusters of vesicles, whereas, in morphine-treated cells, the majority of Ser^{375}-phosphorylated MOR receptors was seen as punctate staining at the plasma membrane, (II) mutation of Ser^{375} to Ala (S375A) strongly reduced agonist-driven MOR endocytosis. Representative images from one of four independent experiments performed in duplicate are shown. Scale bar, 20 μm.

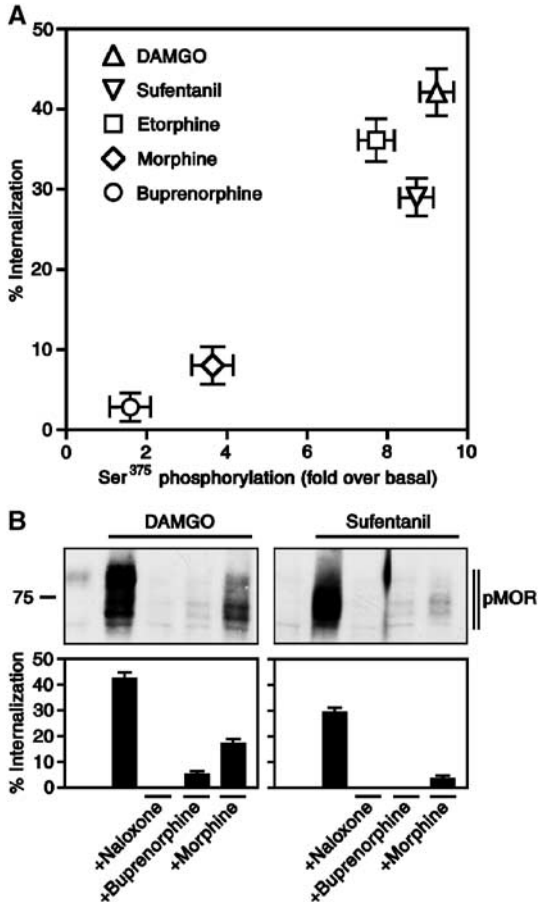

Figure 4 Partial agonistic properties of morphine. (**A**) HEK 293 cells expressing MOR were treated with saturating concentrations of DAMGO (10 μM), sufentanil (0.1 μM), etorphine (0.1 μM), morphine (10 μM) and buprenorphine (1 μM) for 30 min. For quantitative determination of receptor phosphorylation, cells were lysed and immunoblotted with an antibody specific for the Ser^{375}-phosphorylated MOR. The levels of Ser^{375}-phosphorylated MOR were quantified by densitometric analysis. Data were normalized to total MOR and expressed as the fold Ser^{375} phosphorylation over basal in untreated cells. Values represent means ± s.e. of three to five independent experiments. For quantitative determination of receptor endocytosis, cell surface receptors were labeled with anti-HA antibodies, followed by a peroxidase-conjugated secondary antibody. Receptor sequestration, quantified as the percent loss of cell-surface receptors in agonist-treated cells, was measured by ELISA. Data are presented as mean ± s.e. of three to five independent experiments performed in quadruplicate. Data for Ser^{375} phosphorylation and internalization were analyzed for correlation by the two-tailed Pearson test ($r^2 = 0.9057$; $P < 0.05$). Note, morphine was much less potent than the full agonists DAMGO, sufentanil and etorphine, but more potent than the partial agonist buprenorphine. (**B**) HEK 293 cells expressing MOR were treated with 10 μM DAMGO (left panel) or 0.1 μM sufentanil (right panel) in the presence or absence of 1 μM naloxone, 1 μM buprenorphine or 10 μM morphine for 30 min. For determination of receptor phosphorylation, cells were lysed and immunoblotted with an antibody specific for the Ser^{375}-phosphorylated MOR (pMOR). The position of molecular mass marker is indicated on the left (in kDa). Three additional experiments gave similar results. For determination of receptor endocytosis, cell surface receptors were labeled with anti-HA antibodies followed by a peroxidase-conjugated secondary antibody. Receptor sequestration, quantified as the percent loss of cell-surface receptors in agonist-treated cells, was measured by ELISA. Data are presented as mean ± s.e. of three independent experiments performed in quadruplicate. Note, morphine behaved like the partial agonist buprenorphine and potently inhibited both Ser^{375} phosphorylation and internalization of DAMGO- and sufentanil-activated MOR.

potent inducers of both Ser^{375} phosphorylation and internalization (Figure 4A). Although morphine was more effective than the partial agonist buprenorphine, it induced Ser^{375} phosphorylation and internalization to a much lesser extent than full agonists (Figure 4A). In the presence of DAMGO or sufentanil, morphine behaved like the partial agonist buprenorphine and potently inhibited both Ser^{375} phosphorylation and internalization of MOR (Figure 4B). We also examined Ser^{375} phosphorylation and internalization after coapplication of morphine with low concentrations of DAMGO. However, none of the combinations of both drugs was able to increase the levels of Ser^{375} phosphorylation and internalization above those observed with morphine alone (see Supplementary data Figure 5). These results indicate that morphine exhibits clear partial agonistic properties.

Recent studies suggest that, when compared with DAMGO, the relative efficacy of morphine to promote rapid desensitization and internalization of MOR is much less than its relative efficacy to activate G proteins (Yu *et al*, 1997; Alvarez *et al*, 2002; Borgland *et al*, 2003). We then examined the time course of Ser^{375} phosphorylation after exposure of MOR-expressing cells to either DAMGO or morphine. DAMGO stimulated a robust phosphorylation of Ser^{375} within 2 min, which remained at high levels throughout the 30-min treatment period (Figure 5, left panel). In contrast, morphine-induced Ser^{375} phosphorylation was first detectable after 5 min and increased steadily throughout the 30-min treatment period (Figure 5, left panel). Thus, the delayed onset of

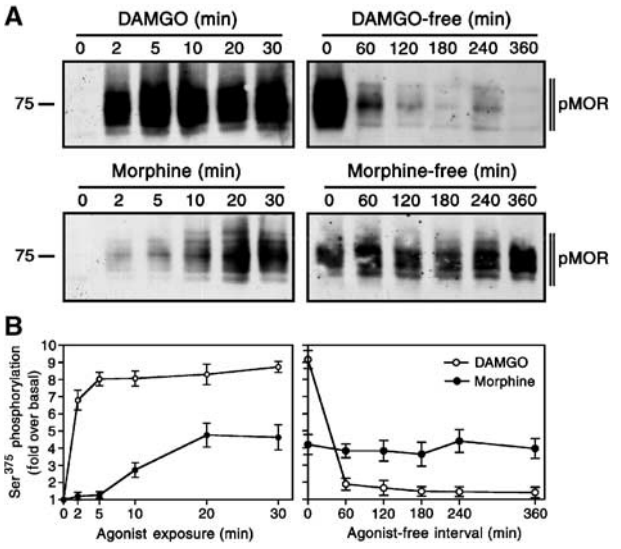

Figure 5 Time course of agonist-induced Ser^{375} phosphorylation and dephosphorylation. Left panel, HEK 293 cells expressing MOR were treated with 10 μM DAMGO or 10 μM morphine for either 0, 2, 5, 10, 20 or 30 min. Right panel, HEK 293 cells expressing MOR were treated with 10 μM DAMGO or 10 μM morphine for 30 min. Cells were extensively washed, incubated in the absence of agonist for either 0, 60, 120, 180, 240 or 360 min, lysed and immunoblotted with an antibody specific for the Ser^{375}-phosphorylated MOR (pMOR). (**A**) Representative immunoblots from one of three independent experiments are shown. (**B**) Mean ± s.e. of three independent experiments quantified by densitometric analysis. Data were expressed as the fold Ser^{375} phosphorylation over basal in untreated cells. The position of molecular mass markers is indicated on the left (in kDa).

morphine-induced Ser^{375} phosphorylation may provide a potential explanation for its relatively poor efficacy to induce rapid desensitization of MOR signaling.

The functional recovery of desensitized MORs involves dephosphorylation and recycling of internalized receptors to the plasma membrane (Koch *et al*, 1998). Upon removal of the drug, the majority of DAMGO-desensitized receptors was dephosphorylated within 60 min (Figure 5, right panel). Such a rapid dephosphorylation was also observed in sufentanil-treated cells (not shown). In contrast, morphine-desensitized receptors remained in a Ser^{375}-phosphorylated state for at least 360 min in the absence of agonist (Figure 5, right panel). These results indicate that morphine-desensitized MOR receptors resided at the plasma membrane for prolonged periods and were not able to enter the recycling pathway.

We next examined the role of Ser^{375} phosphorylation in agonist-induced desensitization of MOR signaling to both adenylate cyclase and extracellular signal-regulated kinases 1 and 2 (ERK1/2). Prolonged exposure of cells expressing either MOR or its S375A mutant to DAMGO induced a time-dependent desensitization of opioid-induced adenylate cyclase inhibition and ERK1/2 activation (Figure 6A, C and E). The desensitization of mitogenic signaling was homologous because the lysophosphatidic acid (LPA) receptor, which is endogenously expressed in these cells, was still able to activate ERK1/2 after 240 min DAMGO exposure (Figure 6C and E). Prolonged exposure of MOR-expressing cells to morphine also induced a clearly detectable time-dependent desensitization of opioid-induced adenylate cyclase inhibition and ERK1/2 activation (Figure 6B, D and F). In contrast, the S375A mutant did not show any detectable morphine-induced desensitization of adenylate cyclase inhibition or ERK1/2 signaling during the 240-min treatment period (Figure 6B, D and F). These findings indicate that Ser^{375} phosphorylation is both sufficient and required for morphine-induced MOR desensitization. In addition, upon removal of the drug, DAMGO-desensitized receptors regained their ability to inhibit adenylate cyclase within 60 min (Figure 7). In contrast, morphine-desensitized receptors failed to resensitize during this time period, indicating that morphine promotes a persistent desensitization of MOR signaling (Figure 7).

To ensure that Ser^{375} phosphorylation of MOR was not an artifact of the HEK 293 cell model, we examined whether this regulation also occurred in cultured neurons. Cortical neuron cultures were prepared from embryonic rats, transfected with MOR and allowed to mature for 1 week. Similar to that observed in HEK 293 cells, Ser^{375}-phosphorylated MOR receptors were detectable at the plasma membrane within 2 min of DAMGO treatment (Figure 8). In neurons treated for 30 min with DAMGO, nearly all detectable Ser^{375}-phosphorylated MOR receptors were confined to intracellular vesicles (Figure 8). In contrast, in neurons treated for 30 min with morphine, the majority of Ser^{375}-phosphorylated MOR receptors were seen as punctate staining at the plasma membrane (Figure 8). These results strongly indicate that agonist-dependent Ser^{375} phosphorylation also occurs in intact cortical neurons, and that the trafficking of Ser^{375}-phosphorylated MOR receptors is also regulated in an agonist-selective manner in these neurons.

Discussion

At present, divergent views exist concerning the extent to which either opioid receptor desensitization or adenylate cyclase superactivation contribute to the development of tolerance to morphine. The receptor activity versus endocytosis (RAVE) model proposes that morphine may induce adenylate cyclase superactivation to a greater extent than other opioids, which in turn exacerbates the development of tolerance (Whistler *et al*, 1999; Finn and Whistler, 2001). In contrast, studies using mice lacking β-arrestin-2 show that opioid receptor desensitization directly contributes to tolerance and that tolerance and adenylyl cyclase superactivation are two dissociable phenomena (Bohn *et al*, 1999, 2000). Here, we propose that morphine is unique in that it promotes terminal opioid receptor desensitization by inducing a sustained phosphorylation of Ser^{375}. Morphine-desensitized receptors remain at the plasma membrane in a Ser^{375}-phosphorylated state for prolonged periods and are not able to enter the recycling pathway. This hypothesis is compatible with several hallmarks of opioid tolerance. First, morphine fails to promote significant downregulation of MOR even under conditions that induce profound cellular tolerance (Lenoir *et al*, 1984; Simantov *et al*, 1984). Second, when administered chronically at equieffective analgesic doses, etorphine and methadone, which are potent inducers of MOR internalization and recycling, produce less tolerance than morphine (Rezvani *et al*, 1983; Duttaroy and Yoburn, 1995; Mercadante *et al*, 1998). Third, upon withdrawal from morphine, reversal of the tolerant state requires days to

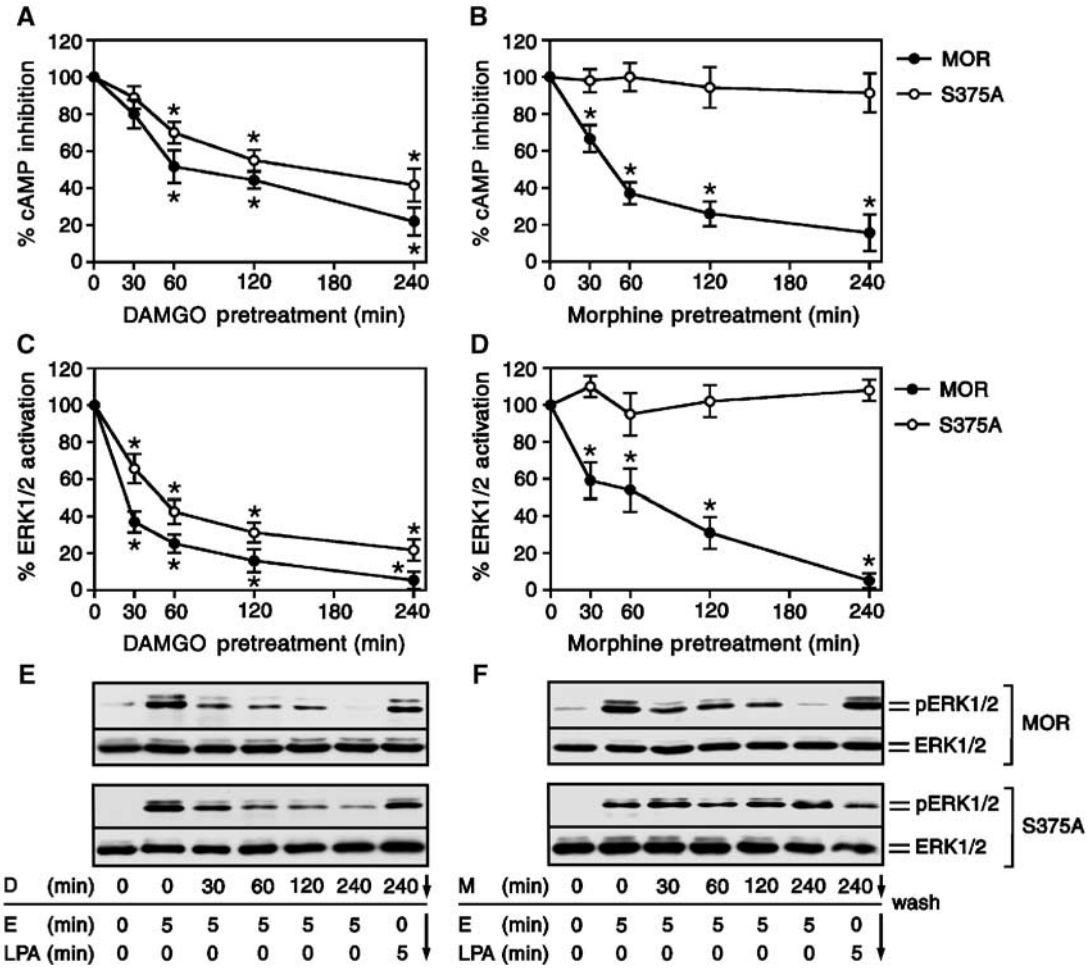

Figure 6 Role of Ser^{375} phosphorylation in morphine-induced desensitization of MOR. (**A, B**) HEK 293 cells expressing the wild-type MOR or the S375A mutant were treated with 10 μM DAMGO or 10 μM morphine for 0, 30, 60, 120 and 240 min. After washing, the cells were treated with either 25 μM forskolin or 25 μM forskolin plus 0.1 μM etonitazene, and cAMP levels were determined as described under 'Materials and methods'. The maximum etonitazene-induced inhibition of forskolin-induced cAMP accumulation (for MOR-expressing cells 87±6%; for S375A-expressing cells 75±8%) without agonist preincubation has been defined as 100%. The values represent means±s.e. from three separate measurements performed in duplicate. * indicates a significant difference ($P<0.05$) between cells preincubated with DAMGO or morphine and cells that had not been preincubated (two-tailed Student's paired *t*-test). (**C–F**) HEK 293 cells expressing the wild-type MOR or the S375A mutant were treated with 10 μM DAMGO (D) or 10 μM morphine (M) for 0, 30, 60, 120 and 240 min. Cells were extensively washed and then exposed to 0.1 μM etonitazene (E) or 1 μM LPA for 5 min. Cells were lysed, equal amounts of protein were resolved by SDS–PAGE and levels of total ERK1/2 and phosphorylated ERK1/2 were determined by immunoblotting. (C, D) Results were quantified by densitometric analysis and normalized to total ERK1/2. For MOR and S375A, the maximum etorphine-stimulated ERK1/2 activation in cells that had not been preincubated was defined as 100%. Values represent means±s.e. of three independent experiments performed in duplicate. * indicates a significant difference ($P<0.05$) between cells preincubated with DAMGO or morphine and cells that had not been preincubated (two-tailed Student's paired *t*-test). (E, F) representative immunoblots for the wild type MOR (upper panel) and the S375A (lower panel) mutant. The positions of phospho-ERK1/2 (*pERK1/2*) and total ERK1/2 (*ERK1/2*) are indicated on the right. Note, exchange of Ser^{375} to Ala completely diminished morphine-induced desensitization of opioid-induced MOR signaling.

weeks (Grecksch *et al*, 1974). Such a considerably long period of time would also be expected for new receptor synthesis to occur.

We show that morphine induces Ser^{375} phosphorylation in cultured cortical neurons, and that Ser^{375}-phosphorylated MOR receptors remain at the plasma membrane in these neurons. Previous studies have reported that morphine does not produce detectable internalization of MOR in neural tissues except for dendrites of nucleus accumbens neurons (Haberstock-Debic *et al*, 2003). We also show that overexpression of GRK2 converts morphine into a potent inducer of Ser^{375} phosphorylation and internalization, suggesting that the cellular complement of GRK2 in many central neurons is not sufficient to drive morphine-induced internalization of MOR.

It has recently been reported that subanalgesic doses of DAMGO can prevent morphine tolerance by facilitating the internalization of morphine-activated MOR receptor complexes (He *et al*, 2002). Although this is an intriguing finding, we and others (Bailey *et al*, 2003) failed to provide evidence for synergistic effects of DAMGO and morphine. In contrast, we defined morphine as partial agonist by its ability to inhibit DAMGO- and sufentanil-induced phosphorylation and internalization of MOR. Thus, the mechanistic basis by which DAMGO and morphine may interact to prevent the development of morphine tolerance remains unknown.

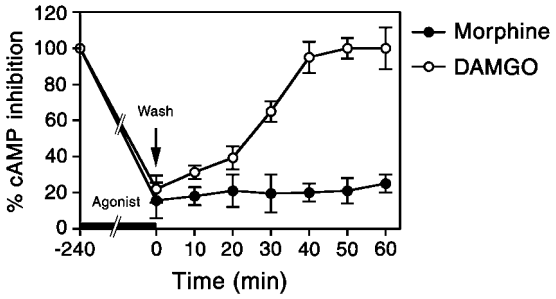

Figure 7 Differential resensitization of DAMGO- and morphine-desensitized MOR receptors. MOR-expressing HEK 293 cells were treated with 10 μM DAMGO or 10 μM morphine for 240 min. Cells were extensively washed and incubated in the absence of agonist for either 0, 10, 20, 30, 40, 50 or 60 min. Cells were then treated with either 25 μM forskolin or 25 μM forskolin plus 0.1 μM etonitazene, and cAMP levels were determined as described under 'Materials and methods'. The maximum etonitazene-induced inhibition of forskolin-induced cAMP accumulation without agonist preincubation has been defined as 100%. The values represent means ± s.e. from three separate measurements performed in duplicate.

In conclusion, we provide evidence for the partial agonistic properties of morphine. As a partial agonist, morphine activates the receptor and restrains it in a conformation that is a poor substrate for GRK2-mediated phosphorylation. Morphine induces selectively the phosphorylation of carboxy-terminal Ser^{375}, which is sufficient to drive receptor desensitization but not sufficient to drive receptor endocytosis. Hence, morphine-desensitized receptors remain at the plasma membrane in a Ser^{375}-phosphorylated state for prolonged periods and are not able to enter the recycling pathway. We propose that sustained Ser^{375} phosphorylation induces persistent desensitization of MOR, which in turn facilitates the development of cellular tolerance.

Materials and methods

Cell lines

HEK 293 cells stably expressing the HA-tagged MOR (B_{max} 778 ± 6 fmol/mg of membrane protein; K_D 1.55 ± 0.3 nM) or the HA-tagged S375A mutant (B_{max} 624 ± 18 fmol/mg of membrane protein; K_D 1.32 ± 0.22 nM) were generated and characterized using radioligand binding assays, Western blot analysis and immunocytochemistry as described elsewhere (Koch *et al*, 2001; Pfeiffer *et al*, 2002). Transient transfections were carried out using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany).

Whole-cell phosphorylation assays

Phosphorylation studies were carried out as described (Koch *et al*, 2001; Pfeiffer *et al*, 2002). After agonist incubation, [^{32}P]orthophosphate-labeled cells were scraped into radioimmune precipitation assay buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin and 10 μg/ml bacitracin) and solubilized. Immunoprecipitations were carried out using 50 μl of HA affinity beads (Covance, Berkeley, CA). Immunocomplexes were eluted from the beads using SDS-sample buffer for 20 min at 56°C. Samples were size-separated by 8% SDS gels followed by autoradiography. The extent of receptor phosphorylation was quantitated using a Fuji PhosphorImaging system and BAS 1000 software. Loading of equal amounts of receptor proteins in each lane was confirmed by Western blot analysis.

Drug treatment

In dose–response studies, cells were treated with 1, 10 or 100 μM DAMGO; 1, 10 or 100 μM morphine; 0.01, 0.1 or 1 μM sufentanil; 0.01, 0.1 or 1 μM etorphine and 0.1, 1 or 10 μM buprenorphine for 30 min. Based on their reported relative binding affinities (Lee *et al*, 1999), the various compounds are expected to reach similar saturating concentrations under these conditions. In time-course studies, cells were exposed to either 10 μM DAMGO, 10 μM morphine or 0.1 μM sufentanil for 0, 2, 5, 10, 20 or 30 min, washed three times with PBS and incubated in culture medium without agonist for 0, 60, 120, 180, 240 or 360 min.

Western blotting

Cells were lysed in radioimmune precipitation assay buffer, and glycosylated proteins were partially enriched using wheat-germ-lectin agarose beads (Amersham, Braunschweig, Germany) as described (Koch *et al*, 2001). Proteins were eluted from the beads using SDS-sample buffer for 20 min at 60°C, and then resolved on 8% SDS–polyacrylamide gels. After electroblotting, the membranes were incubated with anti-phospho-Ser^{375} antibody (1:1000; Cell Signaling Technology, Beverly, MA) or rabbit anti-HA antibody (0.5 μg/ml; Gramsch Laboratories, Schwabhausen, Germany), followed by detection using an enhanced chemiluminescence detection system (Amersham).

Immunocytochemistry

Cells were grown onto poly-L-lysine-coated coverslips overnight. After agonist exposure, cells were fixed, permeabilized and incubated with anti-phospho-Ser^{375} antibody (1:200; Cell Signaling Technology) or rat anti-HA antibody (1:1000; Roche, Mannheim, Germany) for 16 h at room temperature, followed by detection using a cyanine 3.18-conjugated secondary antibody (1:200; Jackson ImmunoResearch, West Grove, PA). Cells were then dehydrated, permanently mounted and examined using a Leica TCS-NT confocal microscope.

Internalization assays

Cells were seeded onto 24-well plates. On the next day, cells were preincubated with 1 μg anti-HA antibody for 2 h in OPTIMEM 1 (Invitrogen) at 4°C. Cells were then exposed to agonist, fixed and developed with peroxidase-conjugated secondary antibody as described (Pfeiffer *et al*, 2002).

cAMP assays

Cells were seeded onto 12-well dishes. On the next day, cells were treated with 10 μM DAMGO or 10 μM morphine for 0, 30, 60, 120 or 240 min, washed three times in PBS and then incubated in medium containing 25 μM forskolin or 25 μM forskolin plus 0.1 μM etonitazene for 15 min at 37°C. Incubation was terminated by removal of the culture medium and subsequent addition of 1 ml of ice-cold HCl/ethanol (1 volume of 1 N HCl with 100 volumes of ethanol). After centrifugation, the supernatant was evaporated, the residue was dissolved in TE buffer (50 mM Tris–EDTA, pH 7.5) and the cAMP content was determined using a commercially available radioimmunoassay kit (Amersham).

ERK assays

Cells were seeded onto 24-well dishes, grown in DMEM medium containing 0.5% FCS overnight. Cells were then treated with 10 μM DAMGO or 10 μM morphine for 0, 30, 60, 120 or 240 min, washed three times in PBS and then exposed to 0.1 μM etonitazene or 10 μM LPA for 5 min at 37°C. Incubation was terminated by removal of the culture medium, and subsequent addition of 150 μl boiling SDS-sample buffer. The samples were assayed by Western blotting as described previously (Pfeiffer *et al*, 2002).

Neuronal cultures

Neurons were prepared from cerebral cortices of embryonic Sprague–Dawley rats at embryonic day 18 as described (Voigt *et al*, 2001). Cells were transfected with MOR using a Nucleofector (Amaxa, Köln, Germany), and then cultivated on poly-D-lysine-coated coverslips in glial-conditioned medium. After 1 week, neurons were treated with 10 μM DAMGO or 10 μM morphine for 0, 2 or 30 min. Cells were were fixed, permeabilized and incubated with anti-phospho-Ser^{375} antibody (1:200; Cell Signaling Technol-

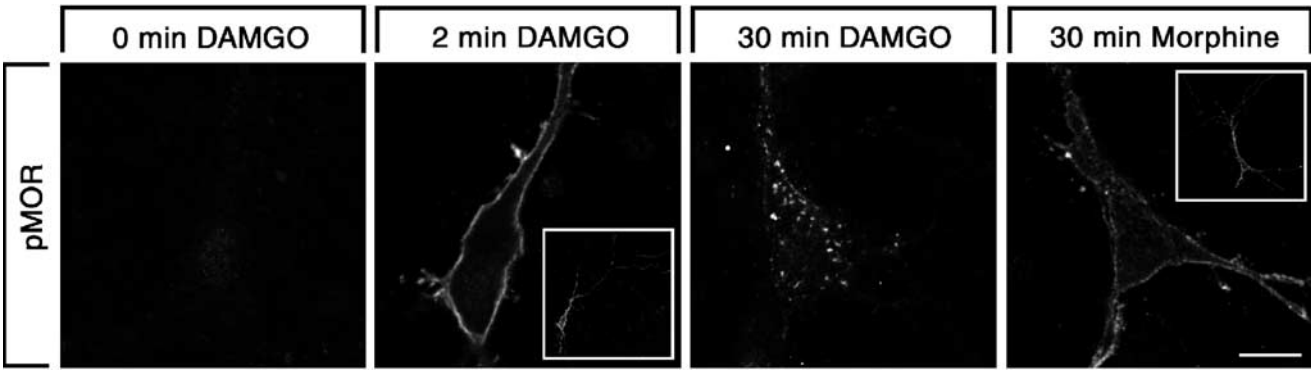

Figure 8 Agonist-induced Ser^{375} phosphorylation of MOR in cultured neurons. Cortical neuron cultures were prepared from embryonic rats at embryonic day 18, transfected with MOR and allowed to mature for 1 week. Neurons were treated with 10 μM DAMGO or 10 μM morphine for 0, 2 or 30 min. Cells were then immunofluorescently labeled with anti-phospho-Ser^{375} antibodies and examined under a confocal microscope. Note, (I) in DAMGO-treated cells, the majority of Ser^{375}-phosphorylated MOR receptors were confined to perinuclear clusters of vesicles after 30 min, whereas, in morphine-treated cells, the majority of Ser^{375}-phosphorylated MOR receptors resided at the plasma membrane. Representative images from one of two independent experiments performed in duplicate are shown. Scale bar, 20 μm.

ogy) for 16 h at room temperature, followed by detection using a cyanine 3.18-conjugated secondary antibody (1:200; Jackson ImmunoResearch). Cells were then dehydrated, permanently mounted and examined using a Leica TCS-NT confocal microscope.

Data analysis

NIH Image 1.62 software was used to densitize and quantify phospho-Ser^{375} and phospho-ERK1/2 levels. Statistical analysis was carried out with the Student's *t*-test and the Pearson test using GraphPad Prism 3.0 software. *P*-values <0.05 were considered as statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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