Phosphorylation of the μ-opioid receptor at tyrosine 166 (Y3.51) in the DRY motif reduces agonist efficacy

Cecilea C. Clayton, Michael R. Bruchas, Michael L. Lee and Charles Chavkin

Department of Pharmacology, University of Washington, Seattle, WA
Running Title: MOR-Y166 phosphorylation reduces agonist efficacy

Corresponding Author:
Dr. Charles Chavkin
Department of Pharmacology, Box 357280
University of Washington School of Medicine
Seattle, WA 98195-7280
206 543 4266 (v) 206 685 3822 (fax)
cchavkin@u.washington.edu

Text Pages: 24
Tables: 1
Figures: 7
Supplemental figure: 1
References: 36 (out of 40 max)
Abstract: 249 (out of 250 words max)
Introduction: 643 (out of 750 words max)
Discussion: 1,484 (out of 1,500 words max)

Abbreviations: MOR, μ-opioid receptor; DRY aspartate-arginine-tyrosine; GPCR, G-protein coupled receptor; HEK293 cells, human embryonic kidney 293 cells; GFP, green fluorescent protein; H2O2, hydrogen peroxide; DAMGO, [D-Ala2,methyl-Phe4,Gly5-ol]enkephalin; EGF, epidermal growth factor; GIRK or Kir3, G-protein-gated inwardly rectifying potassium channel; MAP kinase, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; PKC, protein kinase C; GRK 2, G-protein receptor kinase 2; RTK, receptor tyrosine kinase; EGFR, epidermal growth factor receptor.
Abstract

The effects of phosphorylation of the tyrosine residue in the highly conserved DRY motif expressed in the putative 2nd cytoplasmic loop of the mu opioid receptor were assessed following expression in HEK293 cells. Tyrosine kinase activation by Epidermal Growth Factor (EGF) or hydrogen peroxide treatment effectively increased phosphorylation of the tyrosine-166 in the mu opioid receptor (MOR-Y166p) as measured by a novel phospho-selective antibody. Surprisingly, the increase in MOR-Y166p-immunoreactivity (ir) required co-activation by the opioid agonist, D-Ala²,methyl-Phe⁴,Gly⁵-ol[enkephalin (DAMGO) as demonstrated by both Western blot imaging of membrane proteins and confocal microscopy of transfected cells; MOR-Y166p-ir did not significantly increase after either DAMGO, EGF or H₂O₂ treatment alone. The increase in MOR-Y166p-ir was blocked by pretreatment with the opioid antagonist naloxone or the Src kinase inhibitor PP2. Consistent with these data, mutation of the tyrosine-166 to phenylalanine blocked the increased immunoreactivity, and untransfected HEK293 cells did not increase MOR-Y166p-ir following treatment. DAMGO increased [³⁵S]GTPγS binding to membranes from cells expressing wild type MOR or MOR-Y166F receptors in a dose-dependent manner. Pretreatment of the wild type MOR expressing cells with the combination of DAMGO and EGF completely blocked subsequent DAMGO stimulation of [³⁵S]GTPγS binding membranes; whereas [³⁵S]GTPγS binding to membranes from cells expressing mutated MOR(166F) was only partially inhibited. These results suggest that G protein activation as measured by [³⁵S]GTPγS binding can be regulated by DAMGO and EGF by convergent mechanisms and support the hypothesis that tyrosine phosphorylation within the DRY motif may reduce mu opioid receptor - G-protein coupling efficiency.
The mu opioid receptor (MOR; OPRM1) belongs to the Class A (rhodopsin family) Gi/o coupled family of G protein coupled receptors (GPCRs) and functions to reduce neuronal excitability primarily by increasing potassium conductance and inhibiting voltage-gated calcium channels (Law et al., 2000; Williams et al., 2001). The opioid system is usually described within the context of drug abuse and analgesic drug action, however the normal physiological role of the opioid system is to regulate pain sensitivity, endocrine functioning, gut motility and smooth muscle tone in response to physiological stressors (Drolet et al., 2001; Lopez et al., 1999). The regulation of mu opioid signaling is a dynamic and complex process (Law et al., 2000). A primary desensitization mechanism involving G-protein receptor kinase (GRK) and β-arrestin dependent internalization through cytoplasmic serine/threonine phosphorylation has been well described (Celver et al., 2001; Celver et al., 2004; Williams et al., 2001). In addition, MOR contains four highly conserved cytoplasmic tyrosine residues (Thompson et al., 1993), and tyrosine kinase mediated mechanisms regulating MOR signaling have also been described (Zhang et al., 2009; McLaughlin and Chavkin, 2001). Tyrosine phosphorylation may influence MOR trafficking and signaling (Pak et al., 1999), consistent with the effects of tyrosine phosphorylation on internalization and signaling of the delta and kappa opioid receptors (Kramer et al., 2000; Appleyard et al., 2000). A recent study by Law and colleagues showed that tyrosine phosphorylation of MOR at Y166 and Y336 controlled the switch from inhibition to stimulation of adenylyl cyclase following prolonged agonist application (Zhang et al., 2009). A prior receptor mutagenesis study from our group also showed that tyrosine phosphorylation of MOR regulates agonist coupling efficiency following heterologous gene expression of MOR in Xenopus oocytes (McLaughlin and Chavkin, 2001). The latter study demonstrated that the increase in K\textsubscript{ir}3-mediated potassium conductance evoked by mu agonist stimulation could be strongly suppressed by MOR tyrosine phosphorylation; this could be blocked by mutation of Y166 and Y106 to phenylalanines, whereas mutation of Y96 or Y336 had no effect on signaling (McLaughlin and Chavkin, 2001). Inhibition of the high basal level of tyrosine kinase activity and stimulation of tyrosine phosphatases in these cells robustly increased mu opioid activation of K\textsubscript{ir}3 induced by wild type MOR, but not MOR(Y106F) or MOR(Y166F) expressing cells (McLaughlin and Chavkin, 2001). These results
suggested that tyrosine phosphorylation of MOR at the 106 or 166 sites could reduce coupling efficiency, but the underlying mechanism and relevance to signal transduction in mammalian cells was not evident.

Tyrosine 166 is part of the highly conserved DRY motif among class A GPCRs (Rovati et al., 2007). The DRY motif, located at the boundary of transmembrane 3 and intracellular loop 2, is thought to be important for regulating conformational states of the GPCR and G-protein activation. Molecular modeling of class A GPCRs suggests that in the inactive conformation the arginine residue (D3.50) forms a double salt bridge with its neighboring aspartate (R3.49) and a charged residue on helix 6 (Rovati et al., 2007). Computational methods predict that agonist-induced conformational changes involve breaking the ionic lock between D3.50 and a glutamic acid on helix 6 (E6.30) (Bhattacharya et al., 2008a; Bhattacharya et al., 2008b). Mutation of the aspartic acid in many class A GPCRs, including D(3.49) in MOR leads to constitutive, agonist-independent activation of the receptor (Li et al., 2001b). Other research has shown that in some GPCRs non-conservative mutations lead to a loss of G-protein coupling (Rovati et al., 2007).

To better understand the role of phosphorylation of the tyrosine in the DRY motif, we generated a phospho-selective antibody for the μ-opioid receptor at tyrosine 166 and measured the effects of receptor phosphorylation in the DRY motif on G-protein coupling. We found that in HEK293 cells expressing GFP-tagged μ-opioid receptors prior receptor activation by agonist is required for receptor phosphorylation by tyrosine kinases. Phosphorylation of MOR-Y166, measured as an increase in immunoreactivity, was found to be dependent on activation of Src and that phosphorylation reduced agonist-induced G-protein activation.
Materials and Methods

Chemicals. Mouse Epidermal Growth Factor (EGF) and PP2 were from Calbiochem (Gibbstown, NJ). Hydrogen Peroxide (H₂O₂) was from VWR International (West Chester, PA). (D-Ala², N-Me-Phe⁴, glyol⁵)-Enkephalin (DAMGO) was from Peninsula Laboratories (Palo Alto, CA). Naloxone was from Sigma Chemical (St Louis, MO). Drugs were dissolved in distilled H₂O except for PP2, which was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in the assays did not exceed 0.1%.

HEK293 cell culture. Human embryonic kidney (HEK) 293 cells were cultured in a 1:1 mixture of Dulbecco’s Modified Eagle’s media and F12 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), L-Glutamine and Pen-Strep at 37°C, 95%O₂/5%CO₂.

Mutagenesis of MOR and transfection of HEK293 cells. Mutation of the mu-opioid receptor of tyrosine 166 to a phenylalanine was done as previously described (McLaughlin and Chavkin, 2001). Stable transfections of the MOR-GFP and MOR(Y166F)-GFP constructs into HEK293 cells were obtained as described previously (Celver et al., 2004).

Antibody generation and affinity purification. A polypeptide-containing residues 158-177 (KTMSVDRpYIAVCHPVKALD) of the mu-opioid receptor phosphorylated at tyrosine-166 was generated by PeptidoGenic Research & Co., Inc (Livermore, CA). Polyclonal antibody generation and affinity purification using 2-5 μmol of MOR-Y166p peptide was done as described previously for other phosphopeptide antibodies (Ippolito et al., 2005). Sera aliquots (4 ml) were added to the peptide conjugated Sepharose beads and incubated for 72 hrs at 4°C with gentle rocking. Unbound protein was washed off the beads using 20 ml of Tris (50 mM, pH 7.4) containing NaCl (150 mM) and NaN₃ (0.02%). Antibody was eluted with 5 ml MgCl₂ (5 M) in Tris/NaCl and concentrated using a Centriprep-30 (Millipore, Bedford, MA). Antibody aliquots were stored at -20°C in 30% glycerol. Protein concentrations were determined by the by bicinchoninic assay (BCA) (Pierce, Rockford, IL).

ELISA Assay. 96 well plates were incubated for 4 hr at room temperature with a 10 μg/ml solution of a peptide containing residues 159-177 of the mu opioid receptor, or with a peptide containing the mu
opioid receptor phosphorylated at the tyrosine residue at the 166 site. The wells were then incubated for 2 hr at room temperature in a blocking solution (3% bovine serum albumin in PBS). Wells were washed with PBS-T solution (PBS/0.05% Tween-20), then incubated with increasing concentrations of purified MOR-Y166p antibody in triplicate overnight at 4°C. Wells were then incubated in IgG/alkaline-phosphatase-conjugated secondary antibody (Promega, Madison, WI) diluted 1:3000 in 1x PBS-T solution. The alkaline phosphatase was detected by 1 mg/ml p-nitrophenyl phosphate in 50 mM sodium carbonate buffer (pH 9.8, with 1 M MgCl₂). NaOH (2.5 M) was added to terminate the reaction, and the absorbance was measured via spectrophotometer at 410 nm. The absorbance readings were compared to the antibody dilutions (triplicates were averaged) to establish the serum antibody titer.

**Immunocytochemistry.** HEK293 cells either expressing MOR-GFP or MOR(Y166F)-GFP were plated on poly-D-lysine coated coverslips (BD Biosciences, San Jose, CA) at 60% confluency and cultured for 24 to 48 hr as described above. Cells were washed with sterile phosphate buffered saline (PBS, Invitrogen) and serum starved overnight. After drug treatment, cells were washed once with sterile PBS and then fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. After three 5 min washes in PBS, cells were incubated in blocking buffer (PBS containing 0.3% gelatin and 0.025% Triton X-100) for 1 hr at room temperature. Cells were then incubated with rabbit anti-MOR-Y166p (15 μg/ml) and mouse anti-Src-pY418 (1:300, Calbiochem) diluted in blocking buffer for 72 hr at 4°C. After primary antibody incubation, cells were washed 4x 10 min in PBS, pH 7.4, and then incubated in Alexa 555® anti-rabbit (1:500, Molecular Probes, Eugene, OR) and Alex 633® anti-mouse (1:500, Molecular Probes) diluted in blocking buffer for 2 hr at room temperature. After three 5 min washes with PBS and two 5 min washes in 0.1M phosphate buffer (PB), slides were allowed to air dry for 10 min and then mounted onto coverslips with Vectashield (Vector Laboratories, Burlingame, CA). In other experiments, the same protocol was followed except that cells were incubated with mouse anti-Src-pY418 (1:300, Calbiochem) and then incubated in Alexa 555® anti-mouse (1:500, Molecular Probes). Slides were viewed by Leica confocal microscopy. Pixel intensity was quantified using Metamorph Imaging System software (Universal Imaging Corporation, Downingtown, PA), in which the membrane and cell body
(excluding the nucleus) were outlined for >30 cells per field, and the average pixel intensity of the defined region was determined.

**Membrane Preparation.** Untransfected HEK293 cells or cells expressing either MOR-GFP or MOR(Y166F)-GFP were plated on 100 mm culture plates at 50% confluency and harvested when 80% confluent. Cells were cultured as described above for 24 to 48 hr, washed once with sterile PBS and then serum starved overnight. DAMGO-stimulated $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding and western blotting were assayed in HEK293 membrane homogenates as previously described (Carroll et al., 2005). Briefly, cells were treated with vehicle, DAMGO (1 μM), EGF (50 ng/ml), or DAMGO + EGF at specified time points. After treatment cells were washed 2x with PBS. Cells were scraped into 2 ml membrane buffer containing (300 mM NaCl, 1 mM EDTA, 1 mM Na$_3$VO$_4$, 1 mM NaF, 1x protease and 1x phosphatase inhibitor cocktails (Calbiochem). Cells were then homogenized with a Polytron homogenizer for 2 x 10 sec pulses. Homogenates were centrifuged at 30,000 xg at 4°C for 20 min. Supernatant was discarded and pellet was washed in membrane buffer, rehomogenized, and recentrifuged one more time before freezing the pellet at -80°C until use. In western blot experiments the remaining membrane pellet was resuspended in a buffer containing (in mM) 150 NaCl, 1 CaCl$_2$, 1 MgCl$_2$, 10% glycerol and 1% n-Dodecyl β-D-maltoside with 1x protease and 1x phosphatase cocktail inhibitor sets (Calbiochem) for 2 hr on ice then stored at -80°C.

**Western blot.** Membrane samples were thawed and protein concentration was determined by BCA protein assay (Thermo Scientific, Rockford, IL). Fifteen to 20 μg of protein were resolved by SDS-PAGE on 4-12% Bis-Tris NuPAGE (Invitrogen) gels and transferred to nitrocellulose. Blots were blocked in 5% nonfat milk in Tris-buffered saline (TBS), pH 7.4, for 1.5 hr at room temperature and then incubated in primary antibody (rabbit anti-MOR-Y166p, 15 μg/ml) diluted in blocking buffer overnight at room temperature. Blots were then washed four x 10 min in TBS containing 1% Tween-20 (TBST) and then incubated in IR-Dye™ 800-conjugated affinity-purified anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) at a dilution of 1:7500 in a 1:1 mixture of 5% milk/TBS and Li-Cor Blocking Buffer (Li-Cor Biosciences, Lincoln, NE, USA) for 1 hr at room temperature. After
incubation cells were washed 3 x 5 min in TBST and 2 x 5 min in TBS and analyzed using the Odyssey infrared imaging system (Li-Cor Biosciences). Blots were then re-probed with mouse anti-β-actin (AbCam, Cambridge, MA) 1:5000 diluted in 5% nonfat milk in TBS for 2 hr at room temperature and washed 3x 5 min with TBST. Blots were then incubated in Alexa Fluor® 680 anti-mouse (Molecular Probes) at a dilution of 1:7500 in a 1:1 mixture of 5% nonfat milk/TBS and Li-Cor Blocking Buffer for 1 hr at room temperature, washed 3x 5 min in TBST and 2x 5 minutes in TBS. Immunoblots were scanned using the Odyssey Infrared imaging system and band intensities were measured as described previously (Bruchas et al., 2006).

[^35S]GTPγS Binding. For the GTPγS assay, membranes containing 20 mg protein were incubated with DAMGO (0.1-1000 nM) in 50 mM binding buffer containing (50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 0.1% BSA and 1 mM DTT, pH 7.4) at 30°C for 1hr in the presence of 0.1 nM[^35S]GTPγS and 10 mM GDP. Bound radioligand was separated from free by rapid filtration using a Brandel cell harvester (Gaithersberg, MD) onto GF/B filters (Whatman). Filters were washed 3x in a buffer containing 50 mM Tris, pH 7.4, with 0.1% BSA. Bound radioligand was measured using Econoscient XR (National Diagnostics, Atlanta, GA, USA) scintillation fluid and counted using a Packard Tri-Carb 2200 CA liquid scintillation analyzer. Triplicate determinations were normalized to percent of vehicle binding. Concentration-response curves were plotted using non-linear regression analysis (Graph Pad Prism, 4.0, San Diego, CA).
Results

A phosphopeptide containing residues 159-177 of the MOR sequence phosphorylated at tyrosine 166 was used to generate an affinity-purified polyclonal antibody (Fig. 1A). The resulting reagent demonstrated a high degree of selectivity for the phosphopeptide compared with the unphosphorylated version in the Elisa assay (Fig. 1B). Using membrane proteins isolated from HEK293 cells stably expressing a GFP-tagged μ-opioid receptor (MOR-GFP), we found that basal MOR-Y166p-ir was low as visualized by western blot (Fig. 2A). Treatment of the cells with hydrogen peroxide (H$_2$O$_2$, 4.5 mM) for 15 min did not increase MOR-Y166p-ir (Fig. 2A, lane 2). This result was surprising since H$_2$O$_2$ is a known activator of tyrosine kinases and inhibitor of tyrosine phosphatases that leads to an overall increase in tyrosine phosphorylation within the cell (Takano et al., 2002; Thakali et al., 2007). Similarly, treatment with the MOR-specific agonist, DAMGO (1 μM), for 30 min alone did not increase MOR-Y166p-ir (Fig. 2A, lane 3). In contrast, 30 min treatment with 1 μM DAMGO followed by subsequent co-treatment with 4.5 mM H$_2$O$_2$ for 15 min caused a significant increase in MOR-Y166p-ir (Fig. 2A, lane 4). The apparent molecular weight of the immunoreactive band was approximately 82 kDa (supplemental Fig. 1), which is consistent with prior determinations of MOR mobility in western blots (Petraschka et al., 2007). The MOR-Y166p-ir protein band showed the same mobility as the band detected by anti-mouse GFP and peroxide treatment did not affect MOR-GFP protein expression. Quantification of replicate images indicate that the MOR-Y166p-ir band intensity significantly increased 3.38 ± 0.56 fold (n= 5) following combined DAMGO-H$_2$O$_2$ treatment, but was not significantly affected by treatment with either DAMGO or H$_2$O$_2$ alone (Fig. 2A, left).

Specificity of the MOR-Y166p antibody was assessed using HEK293 cells transfected with the MOR-Y166F-GFP, having tyrosine-166 mutated to phenylalanine (McLaughlin and Chavkin, 2001). Although these cells were shown to express equivalent amounts of MOR, treatment with DAMGO and H$_2$O$_2$ alone or in combination failed to significantly increase MOR-Y166p-ir (Fig. 2B). Similarly, untransfected HEK293 cells did not show MOR-Y166p-ir (Fig. 2C). These results suggest that the
tyrosine-166 residue is occluded in the basal state, and that agonist activation by DAMGO unmasks the phosphorylation site.

We confirmed these results using confocal microscopy of HEK293 cells stably expressing MOR-GFP. Treatment with 1 µM DAMGO for 30 min alone induced receptor internalization (green), as shown by the comparison between the cell surface labeling (Fig. 3A) and cytosolic localization of the green fluorescence in panel B. Treatment with DAMGO alone did not increase MOR-Y166p-ir (red) (Fig. 3B). In contrast, co-treatment with 1 µM DAMGO and H₂O₂ caused both internalization of MOR-GFP and a significant increase in MOR-Y166p–ir (Fig. 3C). HEK293 cells stably expressing MOR(Y166F)-GFP show equivalent plasma membrane localization of GFP (Fig. 3D) and robust internalization following DAMGO treatment (Fig. 3E). Previous reports have shown that mutation of the aspartate residue, a part of the DRY triad, resulted in agonist-independent internalization of MOR (Li et al., 2001a; Li et al., 2001b). However, mutation of the tyrosine-166 residue did not promote agonist-independent internalization of MOR(Y166F)-GFP (Fig. 3D). Neither treatment with 1 µM DAMGO alone or with 4.5 mM H₂O₂, increased MOR-Y166p-ir in MOR(Y166F)-GFP expressing cells (Fig. 3E,F). Quantification of replicate images confirmed that MOR-Y166p-ir was significantly increased by combined DAMGO and H₂O₂ treatment of cells expressing MOR-GFP, but not cells expressing MOR(Y166F)-GFP (Fig. 3G). These results further support the conclusion that the antibody generated is able to detect phosphorylation of tyrosine-166 and that prior activation of the mu-opioid receptor is required for this phosphorylation event to occur.

Peroxide treatment is a fairly nonselective tyrosine kinase activator and activates several different tyrosine kinase cascades (Klann & Thiels, 1999). HEK293 cells endogenously express the EGF-receptor tyrosine kinase system (Kramer et al., 2002), and other groups have shown that EGF receptor and MOR interact (Belcheva et al., 2003; Chen et al., 2008). We next asked if activation of the EGF receptor could also increase MOR-Y166p-ir. Treatment of HEK293 cells stably expressing MOR-GFP with 50 ng/ml EGF alone for 5 min did not lead to tyrosine phosphorylation of the receptor (Fig. 4B, row 2). However, co-treatment of the cells with DAMGO (1 µM, 30 min) and EGF (50 ng/ml, 5 min) did cause a robust
increase in MOR-Y166p-ir that appeared to co-localize with the green fluorescence of the receptor (Fig. 4C, row 2 and 4). Quantification of replicate images confirmed that EGF alone did not significantly increase MOR-Y166p-ir, whereas co-treatment with DAMGO and EGF did increase staining by 1.72 ± 0.12 fold (n= 6) (Fig. 4D).

These results support the hypothesis that agonist-induced activation of MOR by DAMGO leads to a conformational change that allows access of the receptor tyrosine kinase initiated cascade to the tyrosine 166 residue; however, the identity of the kinase directly responsible for the increase in MOR(Y166p)-ir was not established. Recent studies have suggested that Src can be activated by agonist stimulated MOR and that this can result in Y166p (Zhang et al., 2009); however they concluded that Y336 phosphorylation is functionally important and Y166 somehow regulates the phosphorylation state of Y336 but that Y166 was not phosphorylated by Src. To assess the role of Src, we measured pSrc(Tyr418)-ir, which probes for the activated state of Src, in HEK293 cells that express either MOR-GFP or MOR(Y166F)-GFP. We found that treatment with DAMGO, EGF, or co-treatment with DAMGO and EGF led to a significant increase staining intensity (Fig. 5) in both cell lines. Notably, pretreatment of either cell line with the μ-opioid receptor antagonist, naloxone prevented increases in pSrc(Tyr418)-ir seen after co-treatment with DAMGO and EGF (Fig. 5A, column 5; Fig. 5B and C). Similarly, co-treatment with DAMGO and H2O2 leads to an increase in MOR-Y166p-ir that was blocked by pretreatment with either naloxone (1 μM) or the Src inhibitor, PP2 (5 μM) (Fig. 6A). Quantification of replicate experiments demonstrated that both naloxone and PP2 significantly blocked the increase in MOR-Y166p-ir caused by co-treatment with DAMGO and H2O2 (Fig. 6B). These results suggest that activation of Src may be responsible for the increased MOR-Y166p-ir.

As previously discussed, the DRY motif has been suggested to have a significant role in agonist-dependent activation of G proteins (Rovati et al., 2007). To assess the effects of tyrosine-166 phosphorylation on G-protein activation by MOR, we used the [35S]GTPγS binding assay to measure changes in DAMGO efficacy (Harrison and Traynor, 2003). Membranes isolated from MOR-GFP expressing HEK293 cells show a DAMGO concentration-dependent increase in [35S]GTPγS binding that
was not evident in untransfected cells (Fig. 7A). Pretreatment of the HEK293 cells with DAMGO (1 μM) for 30 min prior to membrane isolation significantly reduced the maximal increase in [35S]GTPγS binding caused by DAMGO without affecting the EC50 of DAMGO (Fig. 7A; Table 1). This observation is consistent with prior reports of DAMGO-induced receptor desensitization (Virk and Williams, 2008; Celver et al., 2001) and with images shown in Fig 3B of DAMGO-induced receptor internalization. Treatment of the cells with EGF alone also reduced the maximal effect of DAMGO-induced G-protein activation (Fig. 7A; Table 1). EGF treatment did not induce MOR-GFP internalization, but prior studies suggest that activation of receptor tyrosine kinases can reduce opioid receptor signaling (Chen et al., 2008). Co-treatment of the cells with DAMGO and EGF led to a near complete block of DAMGO-induced G-protein activation (Fig. 7A; Table 1).

Cells expressing MOR(Y166F)-GFP also showed a DAMGO concentration-dependent increase in [35S]GTPγS binding (Fig. 7B). Pretreatment with EGF did not significantly affect either the EC50 or Emax of DAMGO stimulated [35S]GTPγS binding to membranes from MOR(Y166F)-GFP expressing cells (Fig. 7B; Table 1). As expected pretreatment of the cells with DAMGO still caused a significant decrease in subsequent DAMGO-stimulated G-protein activation (Fig. 7B and Table 1). Both MOR-GFP and MOR(Y166F) expressing cells show equivalent receptor internalization (Fig. 3), and prior results showed that Y166F mutation did not affect MOR receptor activation of G-protein gated inwardly rectifying potassium currents (McLaughlin and Chavkin, 2001). Co-treatment of MOR(Y166F)-GFP expressing cells with both EGF and DAMGO did not produce a significantly greater reduction in Emax than did pretreatment with DAMGO alone (Fig. 7B; Table 1). This result suggest that tyrosine phosphorylation of residue 166 in the DRY motif strongly reduces G-protein coupling efficiency of the mu opioid receptor.
Discussion

In this study we investigated the mechanism and effects of phosphorylation of MOR-tyrosine 166, a residue previously found to be important in regulating receptor efficacy (McLaughlin and Chavkin, 2001). To elucidate the mechanism of receptor phosphorylation, we generated and characterized an affinity-purified antipeptide antibody selective for tyrosine-166 phosphorylation: MOR-Y166p. We found that increases in MOR-Y166p-ir were evident only if the receptor had been activated by DAMGO prior to treatment with either H2O2 or EGF, known activators of tyrosine kinase cascades. Furthermore, we found that phosphorylation of the tyrosine residue resulted in a decrease in agonist-mediated efficacy as measured using the GTPγS binding assay.

Tyrosine residue 166 is part of the highly conserved DRY motif found among class A GPCRs. The DRY motif, located in the second intracellular loop, is important in regulating conformation states of GPCRs and is important for agonist induced G-protein activation (Rovati et al., 2007). A wide body of research has used mutational analyses to understand the role of this triad sequence. For example, mutation of the aspartic acid (D3.49) of the β2 adrenergic receptor results in structural instability of the receptor and constitutive activation (Rasmussen et al., 1999). Likewise, mutation of the aspartic acid (D3.49) of the MOR results in constitutive activation and receptor internalization in the absence of agonist stimulation (Li et al., 2001a; Li et al., 2001b). A charge-conserving mutation of the arginine residue (R3.50) of the α1beta-adrenergic receptor led to constitutive activity of the receptor and an increase in binding affinity (Scheer et al., 2000), whereas mutation of this arginine residue in the α2alpha adrenergic receptor led to a decrease in binding affinity (Chung et al., 2002). While much research has investigated the role of the aspartate and arginine residues in the DRY motif, previously there was little understanding of the role of the tyrosine.

We generated a mutant MOR where tyrosine residue 166 was changed to a phenylalanine and found no evidence that this caused constitutive activity. There was no internalization of the receptor in the absence of agonist, and the receptor appeared to internalize normally in the presence of DAMGO. Also, in the GTPγS binding assay, the mutant receptor was not different from the wild type MOR in DAMGO-
induced G-protein activation, EC₅₀ values, or desensitization profile. These results are also consistent with the finding that MOR(Y166F) and the wild type receptor show comparable activation of G-protein gated inwardly rectifying potassium (GIRK, Kir3) currents in response to DAMGO (McLaughlin and Chavkin, 2001). The tyrosine and phenylalanine amino acids are structurally similar and this conservative mutation is advantageous when focusing on the effects of phosphorylation, however future studies using alternative substitutions at this site might induce constitutive activity similar to that produced by mutation of the adjacent arginine (Rovati et al., 2007).

The conclusion that the affinity-purified antibody generated for this study was phosphoselective was based on a series of control experiments. First, the affinity purified MOR-Y166p antibody had a higher affinity for the phosphopeptide than the non-phosphopeptide in the ELISA assay. Also, co-treatment of HEK293 cells that express MOR(Y166F) or untransfected cells with DAMGO and H₂O₂ showed no increase in immunoreactivity as measured by either western blot or confocal microscopy. Pretreatment with the MOR antagonist, naloxone, before co-treatment with DAMGO and H₂O₂ prevented the increase in MOR-Y166p-ir. The apparent molecular weight of the MOR-Y166p-ir was the same as that identified by anti-GFP-ir staining of MOR-GFP expressed by HEK293 cells. Further development of this tool will enable us to extend our research from transfected cells to in vivo models.

Increases in immunoreactivity were not seen in cells that had been treated with DAMGO alone but required co-activation of tyrosine kinases, by treatment with either H₂O₂ or EGF. This is likely due to a difference in the conformational state of the activated receptor and dissociation of the G-protein that would otherwise occlude the DRY motif. Alternatively, tyrosine phosphorylation of MOR may require β-arrestin recruitment which has been demonstrated to be necessary for Src activation (Luttrell et al., 1999; Walwyn et al., 2007).

Although the c-terminal tail and 3rd loop have been implicated in G-protein activation (Johnson and Siderovski, 2007), it is also believed that agonists can act by changing the conformation of the GPCR 2nd loop to activate the switch on the G-protein, remove the GDP block required for G-protein activation, or stabilize nucleotide exchange (Rovati et al., 2007; Wacker et al., 2008). It is therefore possible that the
prior treatment with DAMGO opens the second loop’s DRY motif for Src phosphorylation causing subsequent inhibition of G-protein activation. Computational research of agonist-induced conformational states of rhodopsin predicts a disruption of the ionic lock between R3.50 and E6.30 that results in an increase in the distance between R3.50 and Y3.51 (Bhattacharya et al., 2008a). Indeed, research on the ligand-stabilized conformational states of the β2-adrenergic receptor has shown that agonist-induced changes include conformational changes of transmembrane helices 3, 5 and 6 and breaking of the ionic lock between R3.50 and E6.30. Notably, when comparing agonists, it was found that while all agonists tested were able to induce transmembrane conformational changes, there was a difference in disruption of the ionic lock. Treatment with the full agonist, norepinephrine, was able to induce disruption of the ionic lock whereas treatment with catechol, a weak agonist, was not able to break the ionic lock (Bhattacharya et al., 2008b). In our experiments robust phosphorylation of MOR-Y166 was seen when we co-treated the cells with DAMGO, a full agonist, and H₂O₂ or EGF. In the future it would be interesting to compare this response with co-treatment with a weak or partial agonist.

We found that co-treatment of cells with DAMGO and EGF resulted in a significant increase in activation of the non-receptor tyrosine kinase, Src. We also found that pharmacological inhibition of Src blocked DAMGO and H₂O₂ induced increases in MOR-Y166p-ir. It is known that activation of both the EGFR and MOR increase Src activity (Walwyn et al., 2007). However, treatment with DAMGO or EGF alone was not able to increase MOR-Y166p-ir, despite the ability of both agonists to lead to a significant increase in Src activation. These results suggest that activation of both pathways results in a robust increase in Src activity and that this combined stimulus is needed for phosphorylation of MOR-Y166 to occur. Previous reports have also shown that in some cases activation of both GPCR and RTK pathways are needed for full stimulation of an effector (Wetzker and Böhmer, 2003). For example, activation of mitogen-activated protein kinase (MAPK) by the Bradykinin (B2) receptor requires both PKC activation and transactivation of the EGFR (Adomeit et al., 1999). Also, MOR-mediated activation of extracellular signal-regulated kinase (ERK1/2) by DAMGO in cortical astrocytes also requires transactivation of the
EGFR (Belcheva et al., 2003). Our results may represent another example of multiple input coordinated signal transduction.

In order to better understand the functional implications of phosphorylation of MOR-Y166, we used the GTPγS binding assay. Studies have shown the DRY motif to be important for regulating G-protein coupling and activation, and previous studies have shown this tyrosine phosphorylation event to be important for regulating receptor efficacy (Rovati et al., 2007; McLaughlin and Chavkin, 2001). First, we found that pretreating the membranes with DAMGO resulted in a significant decrease in subsequent DAMGO induced G-protein activation. It is well known that agonist treatment of GPCRs results in desensitization and internalization of the receptor and a reduction in further G-protein activation. We found that co-treatment of membranes with DAMGO and EGF, a stimulus that results in tyrosine phosphorylation at residue 166, resulted in a complete obstruction of GTPγS binding with an Emax that was significantly less than the vehicle control and the DAMGO treated group. This result suggests that phosphorylation of MOR-Y166 results in a decrease in agonist efficacy, and it is consistent with the hypothesis that dephosphorylation of this residue results in an increase in agonist efficacy (McLaughlin and Chavkin, 2001).

Interestingly, we found that treatment of membranes with EGF alone resulted in a decrease in agonist-mediated G-protein activation that was comparable to the DAMGO treated group. This result is consistent with research showing that activation of receptor tyrosine kinase cascades transregulate GPCRs (Delcourt et al., 2007). It has been shown that activation of the EGFR in HEK293 cells results in phosphorylation and activation of GRK2. Activation of GRK2 then promotes internalization of opioid receptors and phosphorylation of DOR at the GRK phosphorylation site (Chen et al., 2008). Our results complement these studies by suggesting that activation of EGFR leads to a reduction in G-protein coupling. It is notable that the decrease in G-protein binding after EGF treatment was not seen in the mutant receptor, MOR(Y166F).

In summary, we generated and characterized an antibody that can selectively detect phosphorylation of tyrosine 166 of the mu opioid receptor. Our data suggests that agonist activation of
the receptor in combination with activation of tyrosine kinase cascades results in an increase in MOR-Y166p-ir. Phosphorylation of tyrosine 166 was dependent on Src activation and resulted in a block of agonist-mediated G-protein activation. This mechanism of regulation of opioid receptor signaling by coincident activation of opioid and tyrosine kinase cascades may provide a novel explanation for the reduction in opioid sensitivity during chronic stress.
Acknowledgments

We thank Hernan Navarro (Research Triangle Institute), Nephi Stella, and Faith Reyes (UW) for procedural advice.
References


20
K(ir)3.1 in spinal cord is induced by acute inflammation, chronic neuropathic pain, and behavioral stress.  

Johnston CA and Siderovski DP (2007) Receptor-mediated activation of heterotrimeric G-proteins:

species: implications for hippocampal synaptic plasticity. Prog Neuropsychopharmacol Biol Psychiatry. 
23: 359-76.

Kramer HK, Andria ML, Esposito DH, and Simon EJ (2000) Tyrosine phosphorylation of the delta-
opioid receptor. Evidence for its role in mitogen-activated protein kinase activation and receptor

opioid activation of the mitogen-activated protein kinase cascade does not require transphosphorylation of
receptor tyrosine kinases. BMC Pharmacol 2: 5.


Li J, Chen C, Huang P, and Liu-Chen LY (2001) Inverse agonist up-regulates the constitutively active
D3.49(164)Q mutant of the rat mu-opioid receptor by stabilizing the structure and blocking constitutive

opioid receptor by mutation of D3.49(164), but not D3.32(147): D3.49(164) is critical for stabilization of
the inactive form of the receptor and for its expression. Biochemistry 40: 12039-12050.


Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K,
Luttrell DK, Caron MG, Lefkowitz RJ. (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src

McLaughlin JP and Chavkin C (2001) Tyrosine phosphorylation of the mu-opioid receptor regulates

-independent down-regulation of the mu opioid receptor. The receptor is a direct substrate for protein-

and Chavkin C (2007) The absence of endogenous beta-endorphin selectively blocks phosphorylation and
desensitization of mu opioid receptors following partial sciatic nerve ligation. Neuroscience 146: 1795-
1807.

highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural


Footnotes

This study was supported by USPHS grant DA11672 from the National Institute on Drug Abuse.
Legends

Fig 1. MOR-Y166p antibody is phosphoselective and specific for the μ-opioid receptor. (A) Cartoon of the μ-opioid receptor (MOR) phosphorylated at tyrosine 166. (B) ELISA assay showing that the MOR-Y166p affinity purified antibody is specific for the phosphorylated MOR-Y166 peptide (●) over the unphosphorylated peptide (○).

Fig. 2. Phosphorylation of the μ-opioid receptor at tyrosine 166 requires prior activation of the receptor. HEK293 cells were treated either with the vehicle, hydrogen peroxide (H₂O₂, 4.5 mM, 15 min), DAMGO (1 μM, 30 min) or co-treated with DAMGO and H₂O₂. (A) A representative western blot of HEK293 cells stably expressing MOR-GFP and quantification of band intensities, n = 4-7. One-way ANOVA, p = 0.0007, with Tukey’s Multiple Comparison Test. **, p < 0.01 when compared to the H₂O₂ treated group or the DAMGO treated group. (B) A representative western blot of HEK293 cells stably expressing MOR(Y166F)-GFP and quantification of band intensities, n = 5. One-way ANOVA, ns. (C) A representative western blot of untransfected HEK293 cells and quantification of band intensities, n = 5. One-way ANOVA, ns. Band intensities were normalized to actin and shown as fold change over the vehicle treated band.

Fig 3. Co-treatment of HEK293 cells expressing MOR-GFP with DAMGO and H₂O₂ results in increased MOR-Y166p IR that co-localizes with the receptor. A-C. Confocal images of HEK293 cells stably expressing MOR-GFP. Cells were treated with either the vehicle (A), DAMGO (B) or DAMGO + H₂O₂ (C). D-F. Confocal images of HEK293 cells stably expressing MOR(Y166F)-GFP. Cells were treated with either the vehicle (D), DAMGO (E), or DAMGO + H₂O₂ (F). Scale bar represents 10 μm. Green represents the GFP-tagged receptor and red represents MOR-Y166p IR. (G) Quantification of MOR-Y166p pixel intensity. Data is shown as a fold change over the vehicle control for each group, n= 4-6.
One-way ANOVA, \( p < 0.0001 \), with a Tukey’s Multiple Comparison post-hoc. ***, \( p < 0.0001 \) when compared to the vehicle control. †††, \( p < 0.0001 \) when compared to the DAMGO treated group.

**Fig. 4.** Co-treatment of HEK293 cells expressing MOR-GFP with DAMGO and EGF results in increased MOR-Y166p IR. (A) HEK293 cells stably expressing MOR-GFP treated with a vehicle control. (B) HEK293 cells stably expressing MOR-GFP treated with EGF (50 ng/ml, 5 min). (C) HEK293 cells stably expressing MOR-GFP co-treated with DAMGO (1 \( \mu \)M, 30 min) and EGF (50 ng/ml, 5 min). Green represents the GFP-tagged receptor and red represents MOR-Y166p IR. Scale bar represents 10 \( \mu \)m. (D) Quantification of MOR-Y166p pixel intensity. Data is shown as a fold change over the vehicle control group, \( n = 6 \). One-way ANOVA, \( p < 0.0001 \), with Tukey’s Multiple Comparison post-hoc. ***, \( p < 0.001 \) when compared with the vehicle control.

**Fig. 5.** Treatment of HEK293 cells expressing MOR-GFP or MOR(Y166F)-GFP with EGF, DAMGO or co-treatment with EGF and DAMGO results in increased pSrc(Tyr418)-ir. (A) Confocal images taken of HEK293 cells expressing MOR-GFP (top row) or MOR(Y166F)-GFP (bottom row) treated with a vehicle, EGF (50 ng/ml, 5 min), DAMGO (1 \( \mu \)M, 30 min), co-treated with DAMGO and EGF, or pretreated with naloxone (1 \( \mu \)M, 30 min) and then co-treated with DAMGO and EGF. Green represents the GFP tagged receptor and red represents pSrc(Tyr418)-ir. Scale bar represents 10 \( \mu \)m. (B) Quantification of pSrc(Tyr418) pixel intensity in cells expressing MOR-GFP. Data is shown as a fold change over the vehicle control group, \( n = 5-8 \). One-way ANOVA, \( p < 0.01 \), with a one-sample t test. *, \( p < 0.05 \), **, \( p < 0.01 \) when compared to the vehicle control. ††, \( p < 0.01 \) when compared to the naloxone pre-treated group. (C) Quantification of pSrc(Tyr418) pixel intensity in cells expressing MOR(Y166F)-GFP. Data is shown as a fold change over the vehicle control group, \( n = 4-5 \). One-way ANOVA, \( p < 0.01 \), with a Tukey’s Multiple Comparison post-hoc.
ANOVA, p < 0.01, with a one-sample t test. *, p < 0.05; **, p < 0.01 when compared to the vehicle control.

**Fig. 6.** Tyrosine phosphorylation of the μ-opioid receptor is dependent on receptor activation and activation of the non-receptor tyrosine kinase, Src. (A) A representative western blot of MOR-Y166p and actin in HEK293 cells stably expressing MOR-GFP. Cells were pretreated with the vehicle control, naloxone (1 μM, 30 min) or PP2 (5 μM, 30 min) and subsequently co-treated with DAMGO (1 μM, 30 min) and H2O2 (4.5 mM, 15 min). (B) Quantification of band intensities. All data points were normalized to actin and shown as a fold change over the untreated control group, n = 4-5. One-way ANOVA, p < 0.01 with a Newman-Keuls Multiple Comparison test. **, p < 0.01 when compared to the untreated control. †, p < 0.05 when compared to the Naloxone and PP2 pretreated groups.

**Fig. 7.** Phosphorylation of MOR-Y166 by co-treatment with DAMGO and EGF show reduced G-protein activation in response to DAMGO. Dose-response relationship of DAMGO-induced G-protein activation on membranes from untransfected HEK293 cells (□) or cells stably expressing MOR-GFP (A) or MOR(Y166F)-GFP (B). Cells were pre-treated with a vehicle (■), DAMGO (▲), EGF (○) or co-treated with DAMGO and EGF (●) and then isolated as described in the Experimental Methods section. [35S]GTPγS binding was then measured after the membranes had incubated with various concentrations of DAMGO.
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>$E_{\text{max}}$ (%) Vehicle</th>
<th>EC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MOR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>96 ± 4</td>
<td>1.9 ± 0.21</td>
</tr>
<tr>
<td>DAMGO</td>
<td>*61 ± 10</td>
<td>1.7 ± 0.39</td>
</tr>
<tr>
<td>EGF</td>
<td>*56 ± 8</td>
<td>0.65 ± 0.37</td>
</tr>
<tr>
<td>DAMGO+EGF</td>
<td>***-#23 ± 8</td>
<td>na</td>
</tr>
<tr>
<td><strong>Y166F</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>100 ± 2</td>
<td>1.5 ± 0.23</td>
</tr>
<tr>
<td>DAMGO</td>
<td>*71±6</td>
<td>2.0 ± 0.22</td>
</tr>
<tr>
<td>EGF</td>
<td>95 ± 15</td>
<td>0.98 ± 0.26</td>
</tr>
<tr>
<td>DAMGO+EGF</td>
<td>*60 ± 13</td>
<td>2.1 ± 0.38</td>
</tr>
</tbody>
</table>

Pharmacological data for DAMGO-stimulated GDP$\gamma$S binding was collected on membranes taken from HEK cells stably transfected with either wild type MOR (MOR) or the MOR mutant Y166F (Y166F). GDP$\gamma$S binding was measured and analyzed as described under Materials and Methods. Results represent the means ± S.E.M. of 4-8 independent experiments performed in triplicate. Data were analyzed by One-way ANOVA followed by Tukey’s Post-Hoc, *, $P < 0.05$, ***, $P < 0.001$, # $P < 0.05$ for DAMGO vs. DAMGO+EGF. EC$_{50}$ values were determined from non-linear regression analysis (Prism v4.0).
**Figure 1**

**A**

MOR

\[ \text{NH}_2 \]

\[ \text{DRpYIAVCHPVKALDFRTPRNK} \]

\[ \text{COOH} \]

**B**

- MOR-Y166
- MOR-Y166p

OD(410) vs. Antibody Concentration (mg/ml)
Figure 2

A  MOR

MOR-Y166p
Actin
DAMGO  -  -  +  +
H₂O₂  -  +  -  +

B  MOR(Y166F)

MOR-Y166p
Actin
DAMGO  -  -  +  +
H₂O₂  -  +  -  +

C  Untransfected

MOR-Y166p
Actin
DAMGO  -  -  +  +
H₂O₂  -  +  -  +
Figure 4

Vehicle

MOR-GFP

MOR-Y166p IR

Merge

EGF

DAMGO + EGF

Fold Change MOR-Y166p IR

EGF

DAMGO + EGF

***
Figure 6

A

MOR-Y166p

Actin

DAMGO + H₂O₂  -  +  +  +

Pretreatment  -  -  Nal  PP2

B

Fold Change MOR-Y166p IR

Post DAMGO + H₂O₂

Vehicle  Naloxone  PP2

†  **
Figure 7

A  Wild Type

B  Y166F

Log M [DAMGO]