

## $\mu$ -Opioid Receptor-mediated ERK Activation Involves Calmodulin-dependent Epidermal Growth Factor Receptor Transactivation\*

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**Phosphorylation of the MAPK isoform ERK by G protein-coupled receptors involves multiple signaling pathways. One of these pathways entails growth factor receptor transactivation followed by ERK activation. This study demonstrates that a similar signaling pathway is used by the  $\mu$ -opioid receptor (MOR) expressed in HEK293 cells and involves calmodulin (CaM). Stimulation of MOR resulted in both epidermal growth factor receptor (EGFR) and ERK phosphorylation. Data obtained with inhibitors of EGFR Tyr kinase and membrane metalloproteases support an intermediate role of EGFR activation, involving release of endogenous membrane-bound epidermal growth factor. Previous studies had demonstrated a role for CaM in opioid signaling based on direct CaM binding to MOR. To test whether CaM contributes to EGFR transactivation and ERK phosphorylation by MOR, we compared wild-type MOR with mutant K273A MOR, which binds CaM poorly, but couples normally to G proteins. Stimulation of K273A MOR with [D-Ala<sup>2</sup>,MePhe<sup>4</sup>,Gly-ol<sup>5</sup>]enkephalin (10–100 nM) resulted in significantly reduced ERK phosphorylation. Furthermore, wild-type MOR stimulated EGFR Tyr phosphorylation 3-fold more than K273A MOR, indicating that direct CaM-MOR interaction plays a key role in the transactivation process. Inhibitors of CaM and protein kinase C also attenuated [D-Ala<sup>2</sup>,MePhe<sup>4</sup>,Gly-ol<sup>5</sup>]enkephalin-induced EGFR transactivation in wild-type (but not mutant) MOR-expressing cells. This novel pathway of EGFR transactivation may be shared by other G protein-coupled receptors shown to interact with CaM.**

Calmodulin (CaM)<sup>1</sup> is a Ca<sup>2+</sup>-binding protein known to play

a role as an intracellular Ca<sup>2+</sup> sensor of many cellular processes such as cytoskeletal organization, vesicular trafficking, mitogenesis, and gene expression (1–3). Multiple interactions of CaM with proteins involved in signal transduction by G protein-coupled receptors (GPCRs) indicate that it plays an extensive role in signaling (4–15). However, evidence for direct binding of CaM to GPCRs has emerged only recently. A CaM-binding site has been located on the C-terminal region of subtypes 5 and 7 of the metabotropic glutamate receptors and the third intracellular loop of the dopamine D2 receptors MOR and DOR (8–11, 13). Wang *et al.* (10) proposed that peptide motifs for binding to G $\alpha$  and CaM are similar to each other, raising the possibility that GPCR-CaM interactions could represent a common phenomenon.

The ERK phosphorylation cascade ranks among the main signaling pathways involved in mitogenic responses to external stimuli. This cascade entails receptor tyrosine kinase (RTK) signaling pathways as well as a highly interconnected network of multiple signals including input from GPCRs (16). Diverse mechanisms are involved in these heterologous signaling pathways. The GPCR signaling pathways to RTK-ERK activation include adaptor proteins such as Grb2 and Shc; the GTP/GDP exchange factor SOS; and the low molecular weight G protein Ras, which complexes with Raf, a MEK kinase in many cell types.

The GPCR-mediated activation of the ERK phosphorylation cascade can be initiated by release of either heterotrimeric GTP-binding protein G<sub>i/o</sub>  $\beta\gamma$ -subunits or G<sub>q</sub>  $\alpha$ -subunits and often involves PKC along with non-receptor tyrosine kinases such as PYK2 and Src (17–21). Similar ERK activation pathways have been reported for the opioid family of GPCRs (22–28).

GPCR signaling can converge at an early stage of the RTK pathway. Recently, it has been reported that GPCR agonists can induce ERK activation via tyrosine phosphorylation of the RTK itself (29–35). Transactivation of the epidermal growth factor receptor (EGFR) appears to occur via a plasma membrane-bound metalloprotease involved in processing of EGF-like precursor molecules anchored on the cell surface (36, 37). A similar EGFR transactivation mechanism involving an auto-crine metalloprotease-dependent release of heparin-binding EGF resulting from insulin-like growth factor stimulation has been proposed (38).

Ca<sup>2+</sup>/CaM strongly influences MAPK pathways and particularly that of ERK, the MAPK isozyme implicated in cell proliferation (39–43). Ca<sup>2+</sup>/CaM can modulate Src activity and directly or indirectly affect Ras activity or signaling elements downstream of Ras. Ca<sup>2+</sup>/CaM can activate Ras by binding to guanine nucleotide exchange factors (44, 45). On the other hand, CaM inhibitors block EGF stimulation of ERK phosphorylation at an undefined site downstream of Src and Ras, but

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<sup>1</sup> The abbreviations used are: CaM, calmodulin; GPCR, G protein-coupled receptor; MOR,  $\mu$ -opioid receptor; DOR,  $\delta$ -opioid receptor; ERK, extracellular signal-regulated kinase; RTK, receptor tyrosine kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PKC, protein kinase C; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; DAMGO, [D-Ala<sup>2</sup>,MePhe<sup>4</sup>,Gly-ol<sup>5</sup>]enkephalin; GFX, GF 109203X (bisindolylmaleimide D); GTP $\gamma$ S, guanosine 5'-O-(3-thiotriphosphate).

upstream of Raf and MEK in HEK293 cells (39). CaM antagonists abolish wild-type Raf kinase activity, and CaM-Sepharose precipitates some (but not all) isoforms of Raf in PC12 cell lysates (46).

Calcium also plays a role in GPCR cross-talk with growth factor pathways. However, the mechanisms involved in  $\text{Ca}^{2+}$ /CaM regulation of ERK via GPCRs and RTKs remain to be elucidated.  $\text{Ca}^{2+}$ /CaM-dependent pathways have been implicated in  $G_{i/o}$ - and  $G_q$ -coupled receptor-mediated Ras-dependent ERK activation (39, 41–43). Inhibition with  $\text{Ca}^{2+}$ /CaM-dependent kinases and phosphodiesterases does not interfere with 5-hydroxytryptamine type 1A receptor-mediated ERK activation or bradykinin-induced EGFR transactivation (39, 40). It remains to be clarified whether CaM affects GPCR-mediated ERK phosphorylation only at points downstream of RTKs, at steps involved in transactivation of RTKs, or within a GPCR pathway that bypasses RTKs. This question is addressed here with MOR.

Interactions between opioid receptors and  $\text{Ca}^{2+}$ /CaM occur in some signaling pathways. Morphine and enkephalin were shown to regulate cellular redistribution of CaM in brain and in NG108-15 cell cultures (47, 48). In NG108-15 cells, DOR stimulates cAMP phosphodiesterase activity via a  $\text{Ca}^{2+}$ /CaM-dependent enzyme (49). CaM kinase II, a serine/threonine-dependent protein kinase, can desensitize opioid receptors (50, 51). Morphine treatment increases the expression of CaM kinase II in rat hippocampus, but not in some other brain regions (52); and inhibition of CaM kinase II in rat hippocampus attenuates morphine tolerance and dependence (53). Wang *et al.* (10) discovered that by binding to the third intracellular loop of MOR and DOR,  $\text{Ca}^{2+}$ /CaM interferes with G protein coupling. Agonist binding to the receptor precipitates the release of CaM from the plasma membrane and its translocation to the nucleus (54). Upon agonist stimulation of a mutant MOR (K273A MOR) that binds CaM poorly, CaM is not released from the plasma membrane into the cytosol, whereas the wild-type and mutant MORs stimulate free cytosolic  $\text{Ca}^{2+}$  to a similar extent (10). Thus, a novel signaling pathway for opioid receptors has been proposed involving CaM as a possible second messenger.

Here we present evidence for the presence of a major CaM-dependent and a minor CaM-independent MOR-mediated ERK signaling pathway that involve transactivation of EGFR as an intermediate step. EGFR transactivation by MOR appears to involve direct CaM-MOR interactions.

#### EXPERIMENTAL PROCEDURES

**Chemicals**—Chemicals were purchased from Sigma with the following exceptions. DAMGO was obtained from Multiple Peptide Systems (San Diego, CA). Diprenorphine was from National Institute on Drug Abuse Drug Supply (Research Triangle, NC). [ $^3\text{H}$ ]Diprenorphine (58 Ci/mmol) was from PerkinElmer Life Sciences. EGF (human recombinant) was from Life Technologies, Inc. The CaM inhibitor W-7, tyrphostin AG 1478, and bisindolylmaleimide I (GF 109203X (GFX)) were from Calbiochem. Anti-phospho-ERK antibody from Cell Signaling Technology (Beverly, MA); anti-ERK1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); anti-EGFR antibody (sheep polyclonal or mouse monoclonal) was from Upstate Biotechnology, Inc. (Lake Placid, NY); and anti-phospho-EGFR antibody (activated form, mouse monoclonal) was from Transduction Laboratories (Lexington, KY). Both forms of the EGFR antibodies detect the human growth factor receptor. Protein G Plus A-Agarose suspension was purchased from Oncogene Research Products (Cambridge, MA).

**Cell Culture Growth**—HEK293 cells were grown at 37 °C in a humidified  $\text{CO}_2$  (5%) incubator in Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 containing 10% fetal bovine serum. Stably transfected HEK293 cells expressing either the empty vector (pcDNA3) or plasmids containing N-terminal FLAG-tagged human wild-type or mutant K273A MOR or rat MOR were maintained in medium containing 200  $\mu\text{g}/\text{ml}$  G418 as described (10). Stably transfected HEK293 cells with rat MOR and coexpressing CaM-Zeo(–)pcDNA3 (antisense; re-

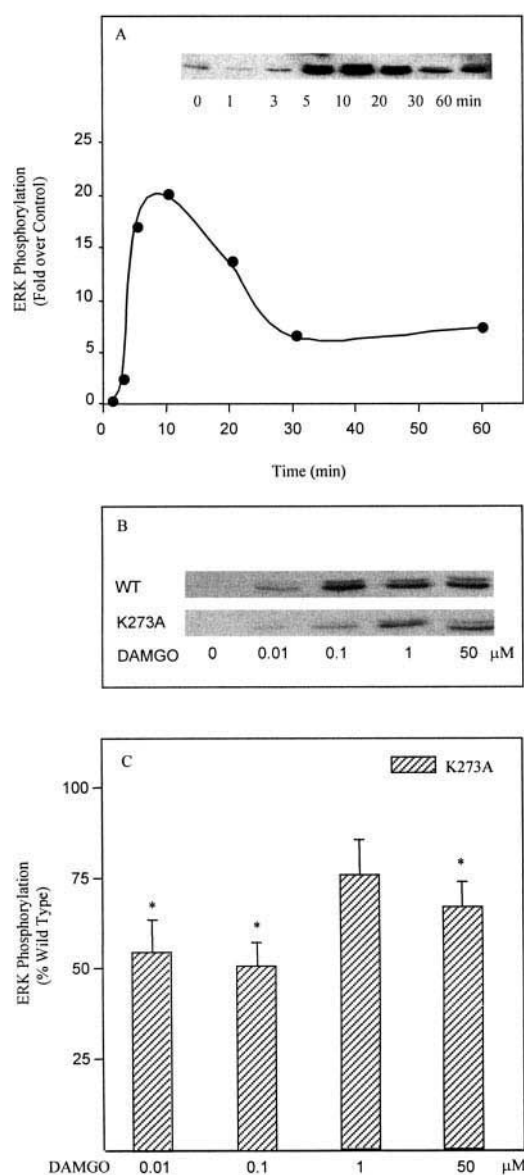
ferred to below as CaM-antisense) plasmids were maintained in medium containing 100  $\mu\text{g}/\text{ml}$  Zeocin<sup>TM</sup> (Invitrogen, Carlsbad, CA) as described (10). Cells were routinely seeded in six-well plates for at least 48 h prior to maintenance in serum-free medium for 24 h. For ERK experiments, serum-free medium was changed in all wells at the time of pretreatment with inhibitor.

**Binding Assays**—HEK293 cells expressing MOR or MOR mutants were collected and spun at  $3000 \times g$  for 5 min, and the cell pellets were washed with ice-cold phosphate-buffered saline. After repeating the centrifugation, cells were suspended in ice-cold 50 mM Tris-HCl (pH 7.4) and immediately used in binding assays. Cells ( $0.3\text{--}3 \times 10^5$  cells/200  $\mu\text{l}$ ) were incubated with saturating concentrations (3–10 nM) of [ $^3\text{H}$ ]diprenorphine at 20 °C for 1 h in a final volume of 250  $\mu\text{l}$ . Nonspecific binding was measured in the presence of 10  $\mu\text{M}$  diprenorphine. Both tritiated and unlabeled ligands were diluted in 50 mM Tris-HCl (pH 7.4) containing 1% bovine serum albumin. Reactions were terminated by adding 5 ml of ice-cold buffer, followed by rapid filtration over GF/B filters using a Brandel cell harvester. Filters were washed twice with ice-cold 50 mM Tris-HCl (pH 7.4) and then counted. Proteins were extracted from cells with NaOH, and their levels were determined. Data were analyzed with the Newman-Keuls multiple comparison test using GraphPAD Prism software.

**ERK Assays**—ERK activity was measured by immunoblotting as described (26, 27, 55). Briefly, cultures were pretreated with different inhibitors, followed by DAMGO or EGF addition as described in the figure legends. Cells were then washed with cold phosphate-buffered saline and lysed with buffer containing 20 mM HEPES, 10 mM EGTA, 40 mM  $\beta$ -glycerophosphate, 2.5 mM  $\text{MgCl}_2$ , 2 mM sodium vanadate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 20  $\mu\text{g}/\text{ml}$  each aprotinin and leupeptin. Lysates were spun at  $14,000 \times g$  for 20 min at 4 °C, and the protein concentration of the supernatants was determined. Cell lysates (20–30  $\mu\text{g}$  of protein/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were blotted on Immobilon P<sup>TM</sup> polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Nonspecific sites were blocked with 5% milk in Tris-buffered saline and 0.2% Tween 20 (TBST). Blots were then washed three times with TBST and incubated with anti-phospho-ERK antibody, diluted 1:2000 in TBST for at least 15 h at 4 °C. After three washes with TBST, blots were incubated with 1:2000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) for 1 h at room temperature. Bands were visualized using an ECL detection system (Amersham Pharmacia Biotech) and exposure to Classic Blue sensitive x-ray film (Molecular Technologies, St. Louis, MO). For assurance of equivalent total ERK protein/lane, representative blots were stripped (0.2 M glycine (pH 2.5), 60 min at room temperature) and exposed to antibodies for ERK1, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. Band intensities were determined by densitometric analysis using an Eastman Kodak DC120 digital camera (1.2 megapixels), Kodak ds 1D Version 3.0.2 software (Scientific Imaging Systems, New Haven, CT), and Image software for Windows, a modification of NIH Image Version 1.62 (Scion Corp., Frederick, MD).

**EGFR Immunoprecipitation and Immunoblotting**—Cells were serum-starved for 24 h and treated with DAMGO (0.1  $\mu\text{M}$ , 10 min) or EGF (0.1  $\mu\text{g}/\text{ml}$ , 5 min). In some experiments, cells were pretreated with tyrphostin AG 1478 (0.1  $\mu\text{M}$ ) and then exposed to DAMGO (0.1  $\mu\text{M}$ , 10 min). Cultures were lysed using a modified radioimmune precipitation assay buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride; 1  $\mu\text{g}/\text{ml}$  each leupeptin and aprotinin, 1 mM  $\text{Na}_2\text{VO}_4$ , and 1 mM NaF. Cell lysates of 0.8–1.5 mg of protein (diluted to ~1  $\mu\text{g}/\mu\text{l}$ ) were used. EGFR was immunoprecipitated by adding 5–10  $\mu\text{g}$  of either mouse monoclonal or sheep polyclonal anti-EGFR antibody to the lysates and incubating overnight at 4 °C. This step was followed by addition of a 50- $\mu\text{l}$  suspension of protein G Plus A-Agarose beads per sample and incubation for 3–4 h at 4 °C. The beads were washed three times with phosphate-buffered saline, resuspended in SDS loading buffer, and boiled for 5 min before SDS-polyacrylamide gel electrophoresis. Proteins were blotted on Immobilon P<sup>TM</sup> polyvinylidene difluoride membranes with a 1:200 or 1:400 dilution of anti-phospho-EGFR antibody (active form) and then 1:2000 diluted horseradish peroxidase-conjugated goat anti-mouse IgG. As described above, bands were visualized using the ECL detection system.

**[ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  Binding Studies**—[ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  assays were conducted on membranes prepared from HEK293 cells as described (10). Cell membranes (10  $\mu\text{g}$ ) were incubated with different concentrations of morphine in assay buffer containing 10 mM HEPES, 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , 1 mM EDTA, 1 mM dithiothreitol, 0.2 nM [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$ , and 1  $\mu\text{M}$  GDP at 35 °C for 5 min. Reactions were stopped by centrifugation, and



**FIG. 1. Time course and concentration dependence of DAMGO modulation of ERK phosphorylation in wild-type and mutant K273A MOR-transfected HEK293 cells.** Cells were treated with varying concentrations of DAMGO and for different times as indicated. Cell lysates (20–30  $\mu$ g of protein) were resolved by 10% SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed with anti-phospho-ERK antibody. **A**, time dependence of DAMGO (1  $\mu$ M) modulation of ERK phosphorylation in wild-type MOR cells. The gel is a representative immunoblot showing the phosphorylated ERK bands. The graph shows a representative curve of quantified ERK phosphorylation. Data are the means  $\pm$  S.E. of four experiments. **B** and **C**, DAMGO concentration dependence of ERK phosphorylation in wild-type (WT) and mutant MOR cells. Cells were exposed to several different concentrations of DAMGO for 10 min before immunoblotting with anti-phospho-ERK antibody. **B** shows a representative immunoblot. In **C**, data are the means  $\pm$  S.E. of 4–11 experiments. \*, significantly different from ERK phosphorylation in wild-type MOR ( $p < 0.05$ ).

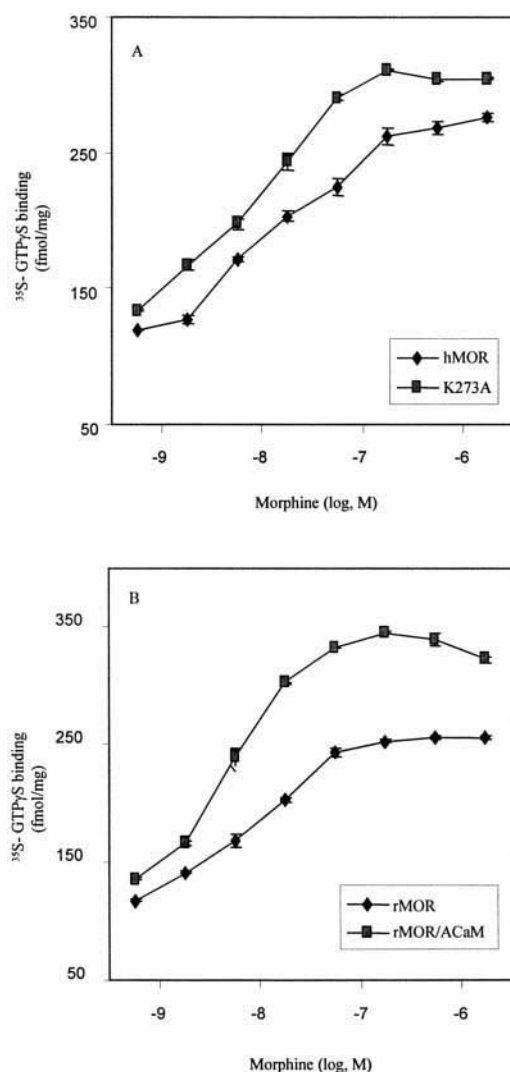
radioactivity was measured by liquid scintillation counting.

**cAMP Assays**—cAMP measurements were performed by radioimmunoassay in suspensions of HEK293 cells as described (56).

**Protein Assay and Statistical Analysis**—Protein concentrations were determined by the method of Bradford (57) with bovine serum albumin (1 mg/ml) as the standard. Statistical determinations were made by Student's *t* test analysis using GraphPAD Prism software.

## RESULTS

### Time and Concentration Dependence of DAMGO Modulation of ERK Phosphorylation in Wild-type and Mutant K273A MOR-



**FIG. 2. G protein coupling to human wild-type and K273 mutant MOR-transfected (A) and rat MOR-transfected and rat MOR/CaM-antisense-cotransfected (B) HEK293 cells.** Cell membranes (10  $\mu$ g) were treated with different concentrations of morphine in assay buffer at 35  $^{\circ}$ C for 5 min as described under "Experimental Procedures." Data are the means  $\pm$  S.D. ( $n = 3$ ). hMOR, human wild-type MOR; rMOR, rat MOR; ACaM, CaM-antisense.

**transfected HEK293 Cells**—Cells were treated with DAMGO for the times indicated in Fig. 1A and then collected, lysed, and assayed for ERK phosphorylation by immunoblotting. Wild-type MOR showed a time course of ERK phosphorylation by DAMGO with a maximum between 5 and 10 min. Mutant K273A MOR gave a similar time course (data not shown). To compare wild-type and mutant MOR-mediated ERK phosphorylation at different DAMGO concentrations, experiments on the two cell lines were run on the same gel (Fig. 1B). At all concentrations tested, DAMGO-induced ERK phosphorylation was greater in wild-type than in mutant K273A MOR-transfected cells (Fig. 1C). At nanomolar levels of DAMGO, the CaM-insensitive mutant MOR stimulated less ERK phosphorylation, whereas at micromolar levels, differences between the wild-type and mutant MORs were attenuated.

Since differences in efficacy between the wild-type and mutant MORs may be due to the presence of different levels of expressed MOR, we determined binding parameters for both receptors. Binding data generated using wild-type and mutant MOR-expressing cells and the antagonist [ $^3$ H]diprenorphine suggested the presence of similar levels of expressed MOR in

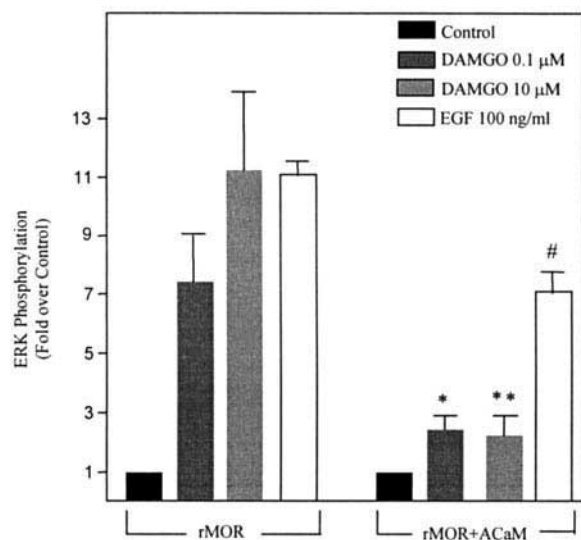


TABLE I

Inhibition of forskolin-induced cAMP production by morphine

HEK293 cell suspensions were incubated with 10  $\mu\text{M}$  forskolin for 10 min at 37  $^{\circ}\text{C}$  in the presence of different concentrations of morphine ( $10^{-9}$  to  $10^{-5}$  M). Cells were harvested and lysed, and cAMP levels were measured. hMOR, human MOR; rMOR, rat MOR; ACaM, CaM-antisense.

HEK293 cell type	hMOR	K273A	rMOR	rMOR/ACaM
IC <sub>50</sub> (nM)	1.62 $\pm$ 0.15	2.1 $\pm$ 0.09	3.45 $\pm$ 0.11	4.2 $\pm$ 0.07
I <sub>max</sub> (%)	68.1 $\pm$ 2.3	82 $\pm$ 3	74.8 $\pm$ 8.8	97 $\pm$ 3.5

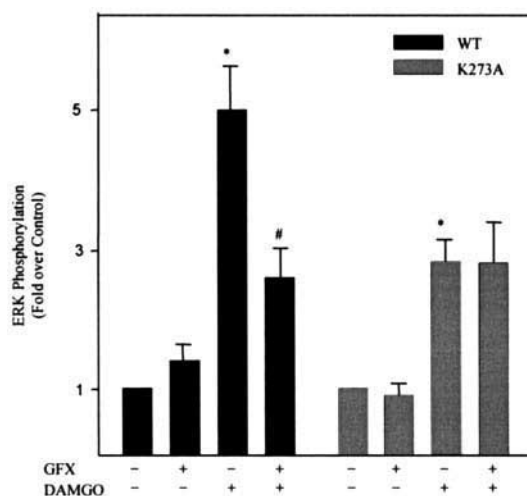


**FIG. 3. Abatement of DAMGO and EGF stimulation of ERK phosphorylation in rat MOR-transfected and rat MOR/CaM-antisense-cotransfected HEK293 cells.** Cells transfected with rat wild-type MOR (rMOR) with or without CaM-antisense (ACaM) were treated with 0.1 or 10  $\mu\text{M}$  DAMGO or 100 ng/ml EGF for 5–10 min, and ERK phosphorylation was measured. Data are the means  $\pm$  S.E. of four to six experiments. \*, significantly different from 0.1  $\mu\text{M}$  DAMGO-treated rat MOR-expressing cells ( $p < 0.05$ ); \*\*, significantly different from 10  $\mu\text{M}$  DAMGO-treated rat MOR-expressing cells ( $p < 0.05$ ); #, significantly different from EGF-treated rat MOR-expressing cells ( $p < 0.05$ ).

wild-type ( $1.24 \pm 0.28$  pmol/mg of protein) and mutant K273A ( $1.57 \pm 0.19$  pmol/mg of protein) cell lines. This finding confirmed previous binding studies performed with the same cells by Wang *et al.* (10).

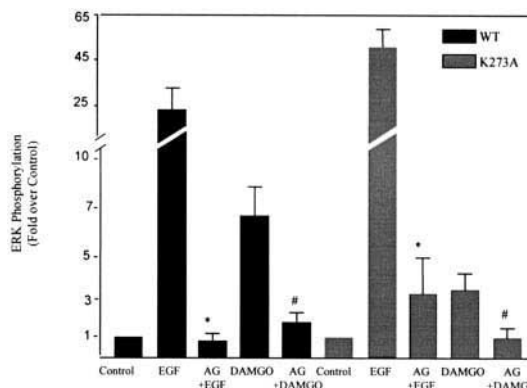
[ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  experiments were performed on both wild-type and mutant K273A MOR-transfected cells and compared. The mutant receptor proved to be just as efficient as the wild-type receptor, if not better, in mediating both [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  binding activity (Fig. 2A) and inhibition of forskolin-induced cAMP production (Table I). Thus, the mutation adversely affects neither G protein coupling nor cAMP modulation of MOR, consistent with earlier results (10).

**DAMGO- and EGF-induced ERK Phosphorylation Is Reduced in CaM-antisense- and Rat MOR-cotransfected HEK293 Cells**—Since CaM binds to MOR and appears to influence ERK signaling, we conducted [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  binding, cAMP production, and ERK phosphorylation experiments with HEK293 cells expressing CaM-antisense and rat MOR. The expression of MOR and the reduced levels of CaM in these cells have been previously characterized (10). Cells cotransfected with CaM-antisense and rat MOR displayed greater activity in both [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  binding and cAMP assays than those overexpressing rat MOR alone (Fig. 2B and Table I). In contrast, the presence of CaM-antisense significantly reduced (but did not abolish) MOR-mediated ERK phosphorylation (Fig. 3). Assuming the involvement of growth factor signaling intermediates, one might ask whether there are CaM-dependent steps in the



**FIG. 4. The PKC inhibitor GFX differentially affects DAMGO-induced ERK phosphorylation in wild-type and mutant K273A MOR-transfected HEK293 cells.** Cells were pretreated with GFX (0.1  $\mu\text{M}$ ) for 30 min before exposure to DAMGO (0.1  $\mu\text{M}$ ) for 10 min. ERK phosphorylation is expressed as -fold over control in the presence or absence of inhibitor. Data are the means  $\pm$  S.E. of four to five experiments. \*, significantly different from control cells ( $p < 0.01$ ); #, significantly different from DAMGO ( $p < 0.01$ ). WT, wild-type MOR.

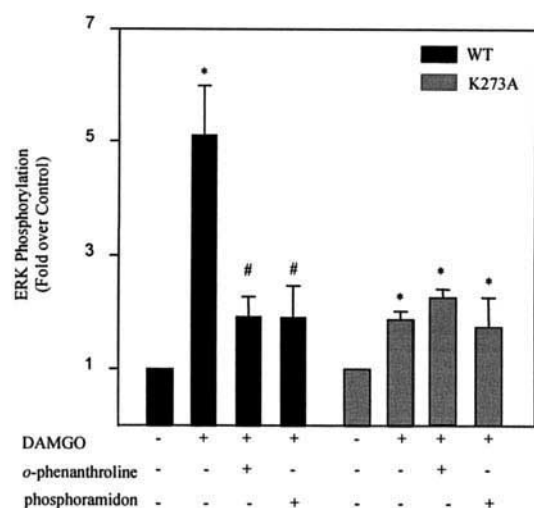
RTK segment of this heterologous pathway. To answer this question, we determined whether CaM-antisense blocks EGF-stimulated ERK phosphorylation in the rat MOR- and CaM-



**FIG. 5. Tyrphostin AG 1478 inhibits DAMGO- and EGF-induced ERK phosphorylation in wild-type and mutant MOR-transfected HEK293 cells.** Cells were pretreated with tyrphostin AG 1478 (0.1  $\mu\text{M}$ ) for 20 min before exposure to EGF (0.1  $\mu\text{g/ml}$ ) for 5 min or DAMGO (0.1  $\mu\text{M}$ ) for 10 min. ERK phosphorylation is expressed as -fold over control in the presence or absence of inhibitor. Data are the means  $\pm$  S.E. of four to seven experiments. \*, significantly different from EGF alone ( $p < 0.01$ ); #, significantly different from DAMGO alone ( $p < 0.05$ ). WT, wild-type MOR.

antisense-cotransfected cell lines. CaM-antisense attenuated ERK phosphorylation stimulated by EGF. The inhibitory effect was less than that of CaM-antisense on DAMGO-elicited ERK (Fig. 3). Thus, reduced levels of CaM accompany the attenuation of ERK phosphorylation in both the GPCR and RTK pathways.

**The PKC Inhibitor GFX Differentially Affects DAMGO-induced ERK Phosphorylation in Wild-type and Mutant MOR-transfected HEK293 Cells**—Since PKC, like CaM, is an important  $\text{Ca}^{2+}$ -binding protein that has been implicated in GPCR activation of MAPKs, its possible involvement in this MOR signaling pathway was tested. GFX, a relatively selective inhibitor of PKC, was added to the media of wild-type and mutant MOR-transfected cells prior to DAMGO exposure. Al-

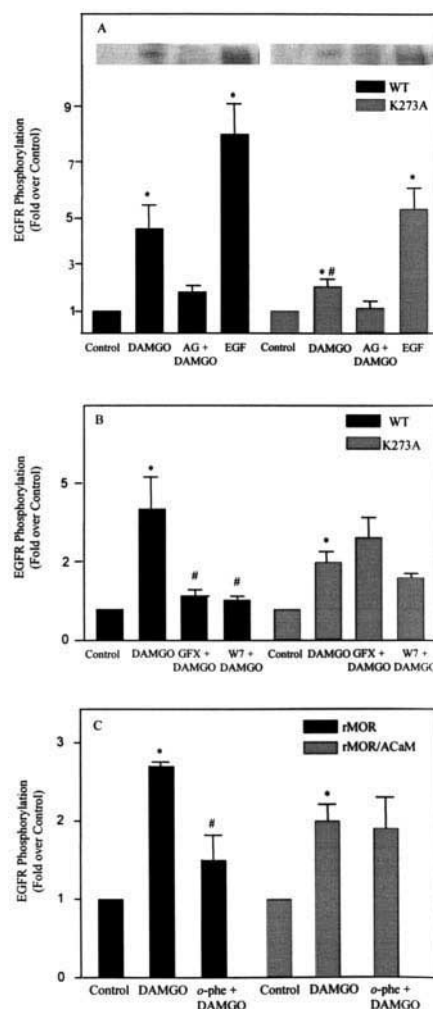


**FIG. 6. The metalloprotease inhibitors *o*-phenanthroline and phosphoramidon attenuate DAMGO-induced ERK phosphorylation in wild-type (but not mutant) MOR-transfected HEK293 cells.** Cells were pretreated with either *o*-phenanthroline (200  $\mu$ M) or phosphoramidon (300  $\mu$ M) for 1 h before exposure to DAMGO (0.1  $\mu$ M) for 10 min. ERK phosphorylation is expressed as -fold over control in the presence or absence of inhibitor. Data are the means  $\pm$  S.E. of three to six experiments. \*, significantly different from control cells ( $p < 0.05$ ); #, significantly different from DAMGO alone ( $p < 0.05$ ). WT, wild-type MOR.

though GFX alone did not affect basal levels, it inhibited DAMGO-induced phosphorylation of ERK in the wild-type MOR-transfected cells (Fig. 4). In contrast to wild-type MOR, GFX did not reduce mutant MOR signaling.

**Tyrphostin AG 1478 Inhibits DAMGO- and EGF-induced ERK Phosphorylation in Wild-type and Mutant K273A MOR-transfected HEK293 Cells**—To determine whether MOR-mediated activation of ERK requires EGFR transactivation, we treated cells with the EGFR Tyr kinase inhibitor tyrphostin AG 1478, followed by DAMGO, and measured ERK phosphorylation (Fig. 5). Again, DAMGO was less effective in stimulating ERK activation via the mutant compared with wild-type MOR. Tyrphostin AG 1478 alone attenuated basal levels of ERK phosphorylation by 50% ( $n = 4-7$ ;  $p < 0.05$ ), suggesting the existence of tonic autocrine RTK activation. Similarly, tyrphostin AG 1478 reduced DAMGO-stimulated ERK phosphorylation in both wild-type and mutant MOR-expressing cells, suggesting that an EGFR transactivation mechanism may be involved in both cases.

**The Metalloprotease Inhibitors *o*-Phenanthroline and Phosphoramidon Inhibit DAMGO-induced ERK Phosphorylation in Wild-type (but Not Mutant K273A) MOR-transfected HEK293 Cells**—As discussed in the Introduction, metalloproteases are involved in the shedding of some endogenously expressed growth factor-like ligands from their plasma membrane anchor. The use of metalloprotease inhibitors could provide additional information on the involvement of growth factor receptor activation by endogenously released ligands. Therefore, wild-type and mutant K273A MOR-transfected HEK293 cells were pretreated with either *o*-phenanthroline or phosphoramidon for 1 h before exposure to DAMGO. Both inhibitors when used alone diminished basal levels of ERK phosphorylation by 40–60% ( $n = 3-6$ ;  $p < 0.05$ ), consistent with the existence of tonic autocrine RTK activation as seen with tyrphostin. Moreover, as shown in Fig. 6, *o*-phenanthroline or phosphoramidon affected DAMGO stimulation of ERK phosphorylation in the wild-type line, but not in the mutant line. These data support the hypothesis that wild-type MOR signals to ERK via at least two EGFR transactivation mechanisms. A major EGFR-dependent

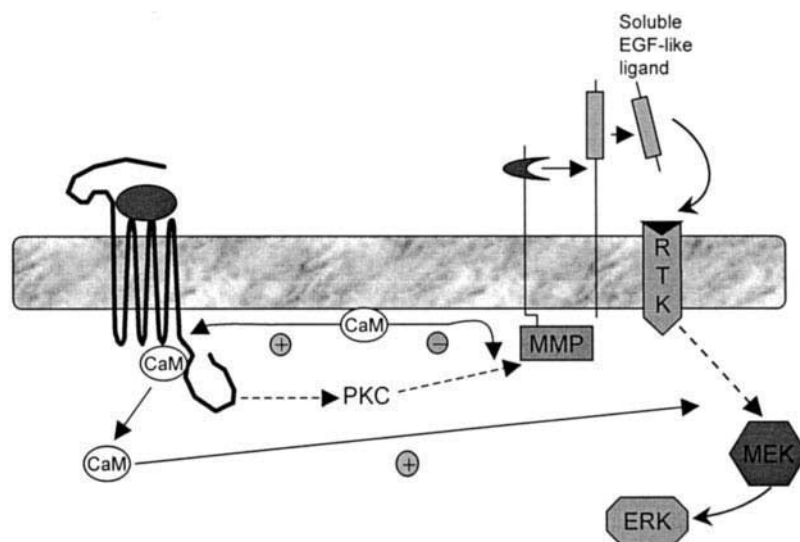


**FIG. 7. Dependence of DAMGO-induced EGFR phosphorylation on CaM and PKC.** A, DAMGO induces EGFR phosphorylation in wild-type and mutant MOR-transfected HEK293 cells. Cells were treated with DAMGO (0.1  $\mu$ M) or EGF (0.1  $\mu$ g/ml) for 10 min. In some experiments, cells were pretreated with tyrphostin AG 1478 (0.1  $\mu$ M) for 20 min before exposure to DAMGO (0.1  $\mu$ M) for 10 min. EGFR was immunoprecipitated with anti-EGFR antibody, and immunoblotting was performed with anti-phospho-EGFR antibody as described under "Experimental Results." Data are the means  $\pm$  S.E. of four to six experiments, except for the tyrphostin treatment, where  $n = 2$ . \*, significantly different from control cells ( $p < 0.05$ ); #, significantly different from the DAMGO effect in wild-type cells (WT) ( $p < 0.05$ ). B, DAMGO-induced EGFR phosphorylation is CaM- and PKC-sensitive in wild-type (but not mutant) MOR-transfected HEK293 cells. Cells were pretreated with either GFX (0.1  $\mu$ M) or W-7 (50  $\mu$ M) for 30 min before exposure to DAMGO (0.1  $\mu$ M) for 10 min. EGFR immunoprecipitation and immunoblotting were performed as described for A. Data are the means  $\pm$  S.E. of four to six experiments. \*, significantly different from control cells ( $p < 0.05$ ); #, significantly different from the DAMGO effect in wild-type cells ( $p < 0.05$ ). C, the metalloprotease inhibitor *o*-phenanthroline differentially attenuates DAMGO-induced EGFR phosphorylation in rat MOR (rMOR)-transfected and rat MOR/CaM-antisense-cotransfected HEK293 cells. Cells were pretreated with *o*-phenanthroline (*o*-phe; 200  $\mu$ M) for 1 h before exposure to DAMGO (0.1  $\mu$ M) for 10 min. EGFR immunoprecipitation and immunoblotting were performed as described for A. Data are the means  $\pm$  S.E. of two to four experiments. \*, significantly different from control cells ( $p < 0.05$ ); #, significantly different from the DAMGO effect in rat MOR cells ( $p < 0.05$ ).

pathway to ERK appears to involve CaM-MOR interactions and membrane metalloproteases.

**DAMGO Induces Different Levels of EGFR Phosphorylation in Wild-type and Mutant K273A MOR-transfected HEK293 Cells**—To further support the hypothesis that DAMGO-induced MOR-stimulated ERK occurs via a mechanism involving transactivation of EGFR, we determined whether the  $\mu$ -agonist

**FIG. 8. Model of CaM-mediated transactivation of EGFR and ERK phosphorylation.** Membrane-localized CaM either can bind to the third intracellular loop of the opioid receptor or block the matrix metalloprotease (MMP)-catalyzed limited proteolysis of membrane-anchored EGF-like ligands and thereby EGFR transactivation. Opioid agonist binding causes CaM bound to the third intracellular loop of the receptor to be released into the cytoplasm, where it can modulate a step just upstream of MEK. Dashed arrows show multiple or uncharacterized steps.



induces phosphorylation of the growth factor receptor. For this purpose, wild-type and mutant K273A MOR-transfected HEK293 cells were exposed to DAMGO or EGF, and EGFR was immunoprecipitated. Immunoblotting performed with anti-phospho-EGFR antibody revealed that DAMGO stimulated a 4-fold increase in EGFR phosphorylation in wild-type MOR cell lines compared with control cells (Fig. 7A). If cells were pretreated with tyrphostin AG 1478 before exposure to DAMGO, the EGFR phosphorylation by DAMGO was diminished. DAMGO-induced EGFR phosphorylation was only 30% in cells expressing the CaM binding-deficient mutant MOR in comparison with the wild-type MOR (Fig. 7A). The data are in good agreement with the differential rates of DAMGO-induced ERK phosphorylation by wild-type and mutant MORs (Fig. 1, B and C).

**Inhibitors of CaM and PKC Reduce DAMGO-induced EGFR Phosphorylation in Wild-type (but Not Mutant K273A) MOR-transfected HEK293 Cells**—Since CaM binding to MOR appears to regulate its signaling to ERK, it is important to determine whether there is a CaM-dependent step preceding EGFR transactivation. To this end, wild-type and mutant MOR-transfected cells were preincubated with the CaM inhibitor W-7, and DAMGO-induced EGFR phosphorylation was measured. W-7 significantly attenuated EGFR phosphorylation by DAMGO in wild-type MOR-transfected cells, but failed to do so in mutant cells (Fig. 7B). Similarly, the PKC inhibitor GFX also attenuated MOR-mediated EGFR phosphorylation in wild-type MOR-transfected cells, but not in mutant cells. These findings support the notion of a CaM- and PKC-dependent step in the MOR signaling pathway leading to EGFR transactivation in HEK293 cells.

**The Metalloprotease Inhibitor *o*-Phenanthroline Differentially Attenuates DAMGO-induced EGFR Phosphorylation in Rat MOR-transfected and Rat MOR/CaM-antisense-cotransfected HEK293 Cells**—To obtain additional evidence for the CaM dependence of DAMGO-induced EGFR phosphorylation and to determine the combined effect of low CaM levels and the presence of the metalloprotease inhibitor in the process, experiments were also performed with HEK293 cells expressing rat MOR cotransfected with the CaM-antisense construct. Pretreatment of cells with *o*-phenanthroline reduced DAMGO-induced EGFR phosphorylation in the wild-type rat MOR line, but not in cells cotransfected with CaM-antisense (Fig. 7C). The data support our previous evidence for CaM-dependent MOR transactivation of EGFR.

## DISCUSSION

The data presented here provide evidence that direct CaM interactions with MOR play a role in opioid modulation of ERK activation in HEK293 cells. The MOR-mediated pathway proceeds via a CaM- and PKC-sensitive transactivation of EGFR and appears to involve the activation of metalloproteases and release of endogenous EGF-like factors (Fig. 8). This novel signaling pathway is supported by the following findings of this study. 1) DAMGO-induced ERK phosphorylation was greater in wild-type than in mutant K273A MOR-transfected cells. 2) CaM-antisense inhibited DAMGO-induced ERK phosphorylation. 3) Inhibition by tyrphostin AG 1478, *o*-phenanthroline, and phosphoramidon implicated EGFR transactivation in MOR signaling to ERK. 4) More definitive evidence for MOR-mediated EGFR transactivation was gained by demonstrating that DAMGO induced tyrosine phosphorylation of EGFR.

Why does mutant MOR display less DAMGO-induced ERK phosphorylation than the wild-type receptor even though it couples to GTP $\gamma$ S, inhibits cAMP production, and activates cytosolic free Ca<sup>2+</sup> to a comparable extent? Wang *et al.* (10, 54) found that CaM binds to MOR and that, upon agonist activation, wild-type MOR releases CaM into the cytosol, where it can interact with other target proteins or undergo further trafficking to the nucleus. It has been shown that morphine treatment of striatum causes  $\geq 50\%$  increases in cytosolic and intracellular membrane CaM content (47–48). Since CaM translocation does not occur in mutant MOR-expressing lines, less CaM may become available in the cytosol and other cellular compartments. As discussed in the Introduction, the Ras-Raf complex may well be one of the sites of CaM stimulation of ERK phosphorylation in the EGF pathway to ERK in HEK293 cells. If the released cytosolic CaM is needed to optimally activate this Ca<sup>2+</sup>/CaM-dependent opioid signaling step, a decrease in ERK phosphorylation may ensue for mutant MOR. The inhibition of ERK phosphorylation in CaM-antisense-expressing cells is in accordance with this hypothesis.

Together with previous results implicating membrane-bound metalloproteases, the data reported here indicate that MOR stimulation leads mainly to transactivation of EGFR and only secondarily to ERK activation. CaM inhibitors have been shown to promote shedding of membrane-bound growth factors via matrix metalloproteases (58, 59). CaM antagonists were found to stimulate the cleavage of several membrane proteins, including EGFR-binding ligands in Chinese hamster ovary and human epithelial cells, and this process was PKC-independent.



In addition, Bosch *et al.* (60) reported that the CaM inhibitor W-13 alone induced an increase in Ras, Raf, and ERK activation in cultured NIH3T3 and normal rat kidney cells. The stimulation of ERK by W-13 alone may be explained by its induction of the release of endogenous plasma membrane-bound EGFR-binding ligands, leading to the activation of this receptor and subsequent activation of Ras-Raf. In accordance with these previous results, the CaM inhibitor W-7 attenuated DAMGO stimulation of EGFR phosphorylation in the wild-type MOR cells. However, in K273A MOR cells, the major CaM-dependent pathway was not operative; and therefore, W-7 had no effect. Therefore, a minor pathway of ERK activation exists in mutant MOR cells, which appears to be CaM-independent.

How is it possible to reconcile the opposing actions of CaM on ERK activation? When CaM is localized in the plasma membrane, it can block heterologous signaling of MOR by preventing G protein coupling and retarding release of membrane-bound EGF-like ligands (Fig. 8). Under these conditions, the cell can respond to exogenous EGF, and there is sufficient intracellular CaM to interact with the Ras-Raf complex to activate ERK. As discussed above, a morphine-induced increase in intracellular CaM may additionally enhance the activation of the Ras-Raf complex. CaM release from the plasma membrane, initiated by opioid agonist binding to MOR, may alleviate the inhibitory effect of plasma membrane CaM on both MOR and metalloprotease, thereby stimulating shedding of endogenously expressed EGF-like ligands. The lack of inhibition by a metalloprotease inhibitor of DAMGO-induced EGFR phosphorylation in the CaM-antisense line supports the existence of a CaM-independent minor pathway. Since the mutant MOR cell line does not release CaM from the plasma membrane, it does not effectively utilize the EGF transactivation process in signaling to ERK. This is consistent with our observation that mutant MOR-mediated activation of ERK is insensitive to inhibitors of metalloproteases and PKC.

Taken together, the data suggest that wild-type MOR stimulates ERK phosphorylation via a CaM- and PKC-dependent pathway that entails EGFR transactivation. It appears that  $\text{Ca}^{2+}$ /CaM plays a role not only at the level of MOR, but also at additional site(s) in the EGF segment of the pathway. However, as shown by the CaM mutant MOR studies, minor pathways, independent of CaM and EGFR transactivation, may also lead to activation of ERK. The components of such putative signaling pathway(s), their possible compartmentation on scaffolds, and their prevalence remain to be determined.

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