Morphine induces terminal μ-Opioid Receptor Desensitization by sustained Phosphorylation of Serine-375

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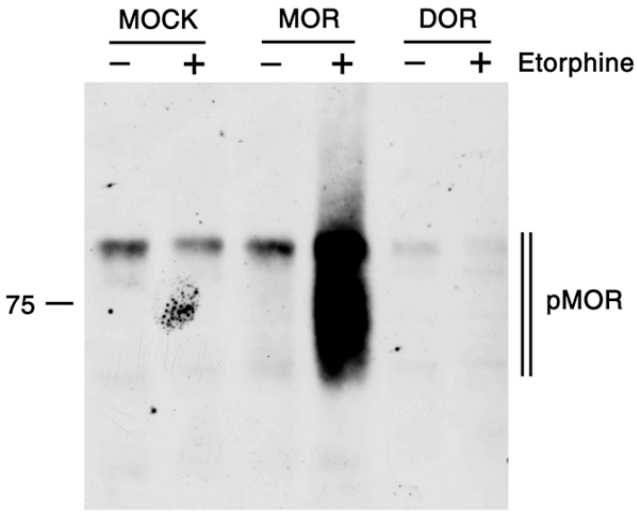
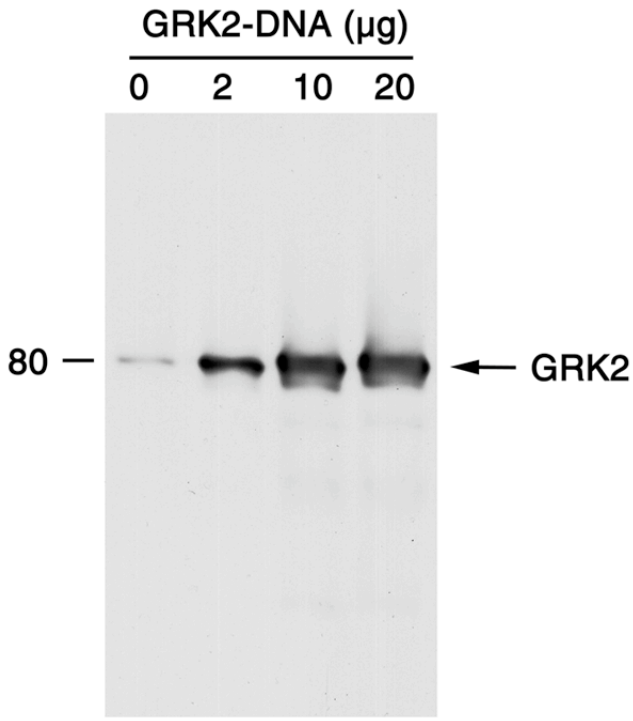
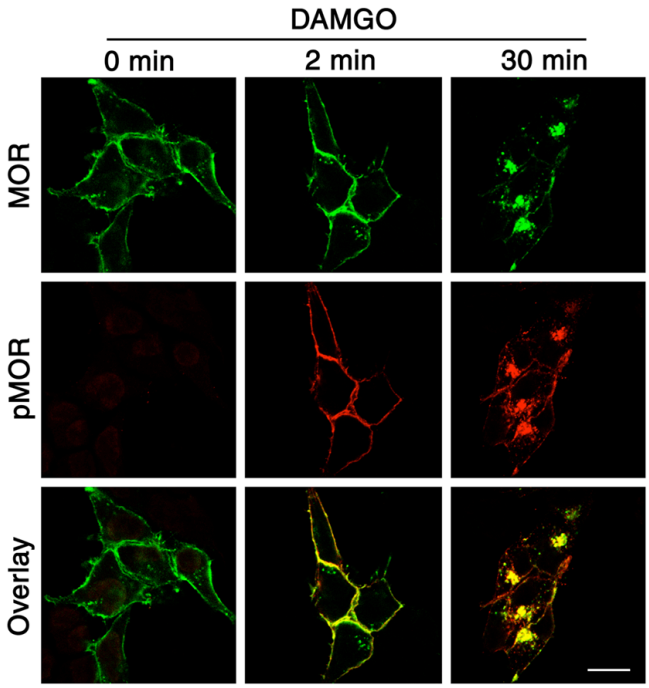
Supplementary Information



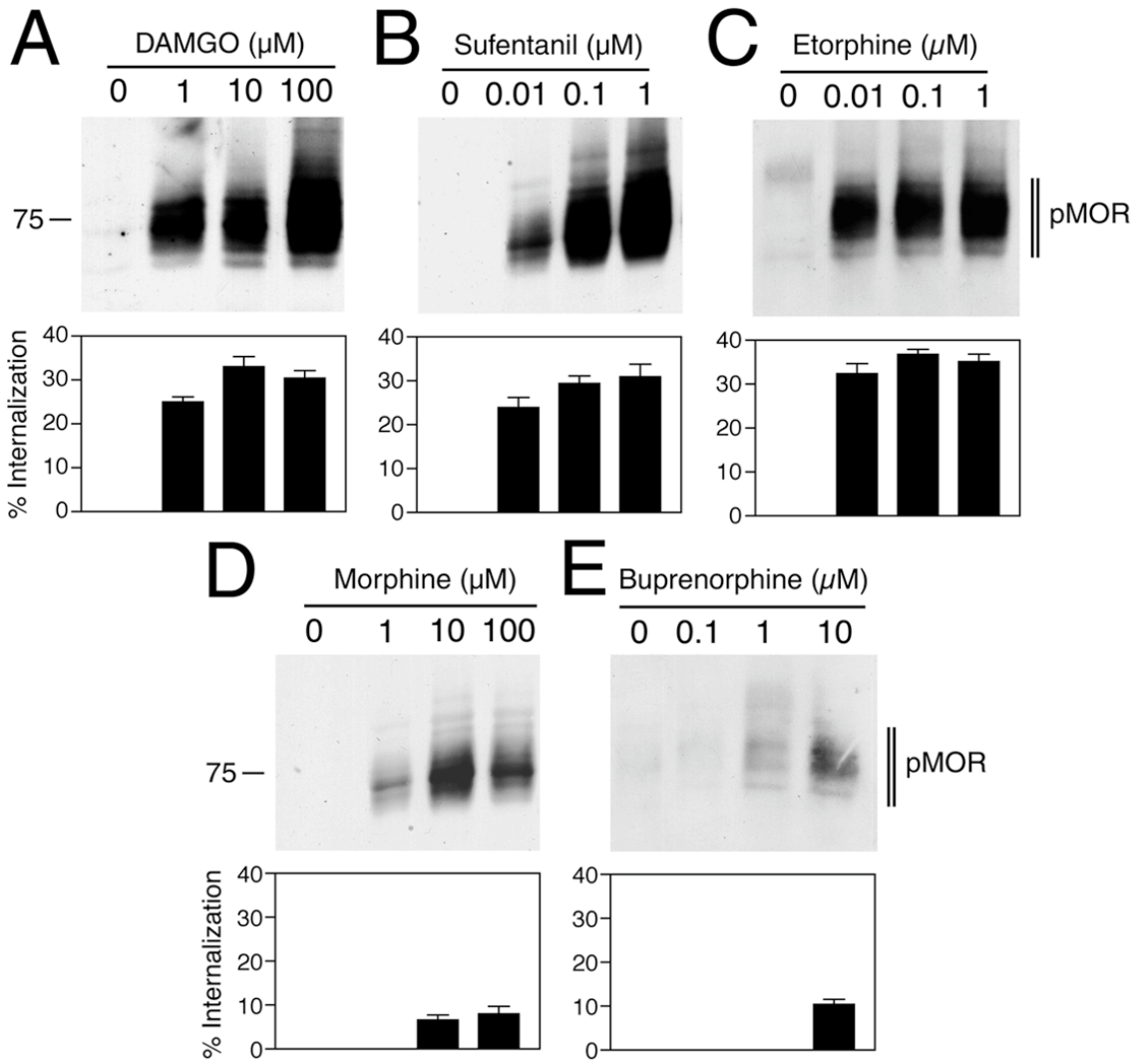
Sup_Fig. 1 Specificity and selectivity of the anti-phospho-Ser^{375} antibody. HEK 293 were transfected with empty vector (*MOCK*), μ-opioid receptor (*MOR*) or δ-opioid receptor (*DOR*). Cells were either not treated or treated with 1 μM etorphine for 30 min, lysed and immunoblotted with an antibody specific for the Ser^{375}-phosphorylated MOR (*pMOR*). Note, the anti-phospho-Ser^{375} antibody selectively recognizes MORs but not DORs which had been activated by the universal opioid agonist etorphine. The positions of molecular mass markers are indicated on the left (in kDa).



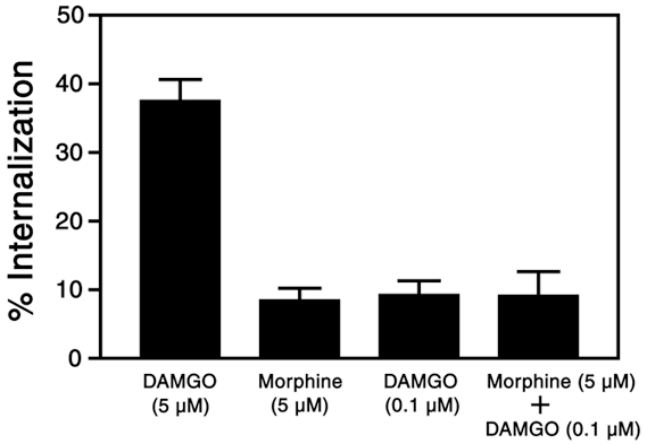
Sup_Fig. 2 Overexpression of GRK2. HEK 293 cells were transiently transfected with 0, 2, 10 or 20 µg GRK2. Two days later, cells were lysed and immunoblotted with an antibody specific for GRK2 (C15, Santa Cruz Biotechnology). Note, (I) GRK2 is detectable in nontransfected cells, (II) transfection with 20 µg of plasmid encoding GRK2 resulted in a ~15-fold increase in cellular levels of GRK2 protein, (III) transfection efficiency was > 80%. The positions of molecular mass markers are indicated on the left (in kDa).



Sup_Fig. 3 Trafficking of Ser^{375}-phosphorylated MOR receptors. HEK 293 cells expressing MOR were exposed to 10 µM DAMGO for 0, 2 or 30 min. Cells were subjected to dual immunofluorescent labeling using a mixture of rat anti-HA (*MOR, green, upper panels*) and rabbit anti-phospho-Ser^{375} (*pMOR, red, middle panel*) antibodies and examined under a confocal microscope. Note, (I) Ser^{375}-phosphorylated MOR receptors were not detectable in untreated cells (*0 min*), confined to the plasma membrane after 2 min and localized to perinuclear clusters of vesicles after 30 min of DAMGO treatment, (II) extensive colocalization of pMOR- and MOR-immunoreactivities (*Overlay, yellow, lower panel*) strongly suggesting the detection of two epitopes of the same molecule. Shown are representative images from one of two independent experiments performed in duplicate. Scale bar, 20 µm.



Sup_Fig. 4 Dose-response-relationship of various opioids for the induction of Ser^{375} phosphorylation and internalization of MOR. HEK 293 cells expressing MOR were treated with 0, 1, 10 or 100 μM DAMGO (*A*); 0, 0.01, 0.1 or 1 μM sufentanil (*B*); 0, 0.01, 0.1 or 1 μM etorphine (*C*), 0, 1, 10 or 100 μM morphine (*D*) or 0, 0.1, 1 or 10 μM buprenorphine (*E*) for 30 min. For determination of receptor phosphorylation, cells were lysed and immunoblotted with an antibody specific for the Ser^{375}-phosphorylated MOR (*upper panels*). 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 (*lower panels*). Note, morphine was much less potent than the full agonists DAMGO, sufentanil and etorphine but more potent than the partial agonist buprenorphine. The positions of molecular mass markers are indicated on the left (in kDa).



Sup_Fig. 5 Lack of synergistic activity of DAMGO and morphine. HEK 293 cells expressing MOR were treated with 5 μM DAMGO, 5 μM morphine, 0.1 μM DAMGO or a combination of 5 μM morphine plus 0.1 μM DAMGO. 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.