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Functional Interactions between μ Opioid and α_{2A}-Adrenergic Receptors

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ABSTRACT

Adrenergic and opioid receptors belong to the rhodopsin family of G-protein coupled receptors, couple to analogous signal transduction pathways, and affect the nociceptive system. Although a number of previous studies have reported functional interactions between these two receptors, the basis for this has not been well explored. We propose that direct receptor-receptor interactions could account, in part, for opioid-adrenergic cross-talk. In this report, we have addressed this using biophysical, biochemical, and pharmacological studies. We show that μ opioid and α_{2A} adrenergic receptors reside in close proximity in live cells using the bioluminescence resonance energy transfer assay. These receptors colocalize to proximal dendrites in primary hippocampal neurons. μ-α_{2A} Receptor complexes can be isolated from heterologous cells or primary neurons coexpressing these receptors. In these cells, the activation of either μ or α_{2A} receptor leads to a significant increase in the level of immunoprecipitable μ-α_{2A} complexes, whereas activation of both receptors leads to a significant decrease. The implications of these effects on signaling were examined using the agonist-mediated increase in G-protein activity and mitogen-activated protein kinase activity. We find that activation of either μ or α_{2A} receptors leads to an increase in the extent of signaling, whereas activation of both receptors leads to a decrease. The increase in signaling by individual ligands and decrease by a combination of ligands is also seen in primary spinal cord neurons endogenously expressing these receptors. Taken together, these results suggest that physical associations between μ and α_{2A} receptors could play a role in the functional interactions between these receptors.

Adrenergic α_{2A} receptors are members of the family A of G-protein-coupled receptors (GPCRs) and couple to G_i/G_o proteins. The activation of these receptors leads to inhibition of adenylyl cyclase activity, activation of K^+ currents, inhibition of Ca^{2+} channels, and increases in MAP kinase phosphorylation (Morita and North, 1981; Limbird, 1988; Richman and Regan, 1998). The α adrenergic receptor system is important in promoting arterial vasoconstriction. However, it also has significant effects on the nociceptive system (Yaksh, 1979). Clonidine, a partial agonist at α adrenergic receptors, elicits an analgesic response (Paalzow, 1974).

μ Opioid receptors are also members of the family A of GPCRs and couple to secondary messenger systems similar to those coupled by α_{2A} adrenergic receptors (Jordan and Devi, 1998). These receptors are involved in many biological responses, including depression of neurotransmitter release in the spinal cord (Macdonald and Nelson, 1978). Activation of μ receptors by agonists such as morphine results in strong antinociceptive effects (Wigdor and Wilcox, 1987; Matthes et al., 1996).

A number of previous studies have noted functional interactions between these two receptor systems (Drasner and Fields, 1988; Ossipov et al., 1997). Direct evidence supporting an interaction between the two systems came from studies with mice lacking functional α_{2A} receptors (Stone et al., 1997). In these mice, there was a decrease in the analgesic potency of spinally administered morphine compared with wild-type mice, suggesting an interaction between opioid and adrenergic receptors (Stone et al., 1997). The molecular mechanisms that mediate these synergistic interactions have not yet been well characterized. It is possible that α_{2A} receptors physically associate with opioid receptors and this interaction modulates receptor func-

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; MAP, mitogen-activated protein kinase; BRET, bioluminescence resonance energy transfer; YFP, yellow fluorescent protein; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; PHG, PBS/HEPES/glucose; BSA, bovine serum albumin; HA, hemagglutinin; DTT, dithiothreitol; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; PAGE, polyacrylamide gel electrophoresis; ERK, extracellular-signal regulated kinase.

tion. Studies with a number of GPCRs support such a notion (Rios et al., 2001; Angers et al., 2002).

Recent studies using atomic force microscopy of rod outer segment membranes have shown that rhodopsin, a prototypical GPCR, exists in dimeric arrays in native tissue (Fotiadis et al., 2003; Liang et al., 2003). GPCRs of closely related as well as distantly related members of the family are able to associate with each other. In some cases, these associations are found to be necessary for functional activity (Marshall et al., 1999; Nelson et al., 2001). However, in other cases, these interactions modulate GPCR activity, leading to changes in ligand affinity, efficacy, trafficking, and/or desensitization properties (Gomes et al., 2001; Angers et al., 2002). Thus, GPCR dimerization seems to be an important regulatory mechanism of receptor function for a variety of GPCRs.

In this study, we explored physical interactions between α_{2A} and opioid receptors and examined the functional consequences of such interactions. We find that μ and α_{2A} receptors reside in close proximity in live cells, as determined by the energy transfer assay. The receptor complexes can be isolated from heterologous cells and primary neurons coexpressing these receptors. Treatment with μ or α_{2A} agonists leads to increases in the immunoprecipitable receptor complexes with a corresponding increase in the level of receptor signaling as measured by G-protein activation and MAP kinase phosphorylation. This increase in signaling is also seen in primary neurons endogenously expressing μ and α_{2A} receptors. These results suggest that receptor-receptor interactions, could at least in part, account for the previously observed functional interactions in vivo.

Materials and Methods

Construction of Plasmids and BRET Analysis. The μ opioid and α_{2A} adrenergic receptors with mutated stop codons were subcloned in frame into RLuc and YFP in the pRLuc-N3 (PerkinElmer Life Sciences, Boston, MA) and pEYFP-N1 (BD Biosciences Clontech, Palo Alto, CA) plasmids such that RLuc and YFP were present at the C termini. All sequences were confirmed by DNA sequencing. Human embryonic kidney (HEK) 293 cells were transfected with 1 μg of μ-RLuc along with 1 μg of YFP, 1 μg of CCR5-YFP, or 0.1 to 1 μg of α_{2A}-YFP using standard techniques, and the cells were subjected to the BRET assay 48 to 72 h after transfection as described previously (Gomes et al., 2003). The levels of receptor expression were determined using binding assays (described under *Receptor Ligand Binding*).

Cell Culture Transfections/Infection. HEK-293 or Madin-Darby canine kidney (MDCK) cells were transfected with 1 to 5 μg of Flag-tagged μ receptor cDNA, HA-tagged α_{2A} receptor cDNA, Flag-tagged CB1 receptor cDNA (all N-terminally tagged), or a combination of these and analyzed approximately 48 h after transfection as described previously (Gomes et al., 2002, 2003). The level of receptor expression was determined by ligand binding, and care was taken to ensure that the expression levels of each receptor type were comparable (as determined by ligand binding, see below). For studies examining ligand-induced changes, cells were seeded onto 24-well plates (10^5 cells/well) and treated with 100 nM morphine, clonidine, or both for 20 min before solubilization and immunoprecipitation (described under *Coimmunoprecipitations and Western Blotting*).

Primary Cell Cultures. Primary hippocampal cultures and spinal cord neurons were prepared as described by Osten et al. (1998) and Einheber et al. (1993). Briefly, E16 Sprague-Dawley rat pups were removed from the uterus of the mother and decapitated in a PBS/10 mM HEPES, pH 7.3/0.6% glucose (PHG) solution. Hippocampi were dissected out with the help of a dissection microscope, and spinal cords were removed by cutting open the spinal cavity with a fine ribbon scissor. The hippocampi or spinal cords were cut into 15 to 20 pieces and incubated for 15 min at 37°C with the PHG buffer containing 0.25% trypsin. They were then washed three times with PHG buffer and resuspended in buffer A consisting of 0.8 ml of plating media (minimal essential medium, 10% fetal bovine serum, 0.45% glucose, 1 mM sodium pyruvate, 25 μM glutamate, and 1× penicillin/streptomycin) along with 0.1 ml of BSA (4% in plating medium) and 0.1 ml of DNase (1 mg/ml). Cell suspension was prepared with the help of a 1-ml pipette and layered onto a 1-ml, 4% BSA/plating media cushion and centrifuged for 10 min at 300*g*. The pellet was resuspended in plating medium and cells plated for 3 to 4 h on wells coated with polyornithine/laminin. After 3 to 4 h, medium was changed to Neurobasal A supplemented with B-27 and nerve growth factor (50 ng/ml) (Invitrogen, Carlsbad, CA). Experiments were performed on cells grown for 1 to 2 weeks in culture.

Sindbis Virus-Mediated Transfection. Pseudovirions containing N-terminally Flag-tagged μ receptor cDNA and HA-tagged α_{2A} receptor cDNAs were generated as described in the Sindbis expression system manual provided by the supplier (Invitrogen). Baby hamster kidney cells were cotransfected with the recombinant pseudovirions and pSinRep Sindbis virus construct (Osten et al., 1998); typically, a 1:30 dilution of virions collected from the supernatant of these cells yielded ~10 to 20% infection of neurons. Levels of receptor expression were determined using binding assays (described below) and were found to be ~2- to 5-fold greater than endogenous levels.

Immunocytochemistry. MDCK cells coexpressing epitope-tagged μ and α_{2A} receptors were grown on poly-D-lysine–coated coverslips until they showed apical and basolateral polarity. These or primary neurons coexpressing epitope tagged μ and α_{2A} receptors were grown on poly-D-lysine–coated coverslips, washed twice with PBS, and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Permeabilization was carried out with 0.25% Triton X-100 in PBS for 10 min at room temperature. Nonspecific sites were blocked with either 3% BSA or 5% normal donkey serum in PBS for 1 h at room temperature. Cells were incubated for 1 h at room temperature with 1 μg/ml anti-Flag monoclonal or anti-HA polyclonal antibody and washed with PBS (three times for 5 min each), followed by a 1-h incubation with 1 μg/ml fluorescein isothiocyanate-labeled anti-mouse or rhodamine labeled anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Cells were washed three times for 5 min each with PBS and mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA) antifading media. All pictures were taken on a Nikon confocal imaging system.

Receptor Ligand Binding. Membranes from cells expressing μ and α_{2A} receptors or from frozen spinal cords (Pel-Freez) were prepared as described previously (Jordan and Devi, 1999; Gomes et al., 2002). For competition assays, 50 to 100 μg of membranes were incubated with increasing concentrations of [^{3}H]diprenorphine or [^{3}H]yohimbine in buffer containing 0.1% BSA and in a final volume of 1 ml for 1 h at 37°C. Nonspecific binding was determined in the presence of 1 μM diprenorphine or yohimbine. In the case of spinal cords, different concentrations of morphine or [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin, in the presence or absence of either 10 or 100 nM clonidine or yohimbine were used to compete this binding. The membranes were collected on Whatman GF/B filters, washed, and the radioactivity was determined. IC_{50} values or saturation binding curves were determined using Prism 2.0 (GraphPad, San Diego, CA).

Coimmunoprecipitations and Western Blotting. Neurons or HEKs coexpressing μ and α_{2A} receptors were lysed for 1 h in 50 mM Tris-Cl, pH 8.0, containing 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 150 mM NaCl (solubilization buffer) with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) and 100 mM iodoacetamide to prevent the spurious formation of disulfide bonds. Receptor complexes were immunoprecipitated using anti-Flag antibodies (rabbit; Sigma) or anti-HA antibodies (rabbit; Santa Cruz

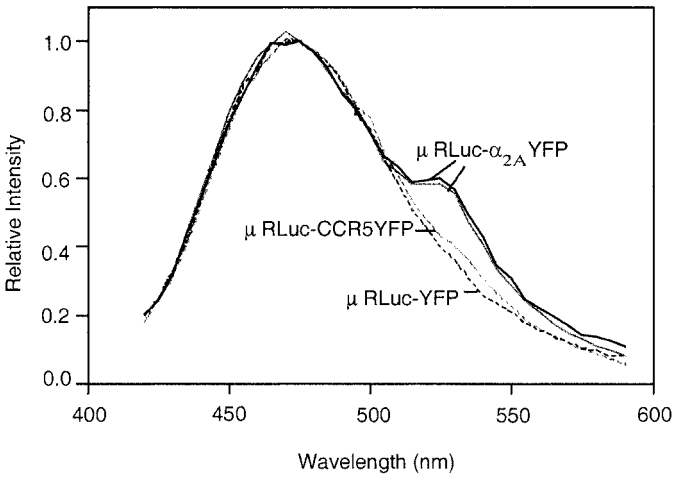

Fig. 1. μ Opioid and α_{2A} adrenergic receptor interactions in live cells. Light emission was monitored from HEK-293 cells transfected with 1 μg of μ RLuc and 1 μg of untagged YFP (μ RLuc-YFP; black dashed line) or CCR5 YFP (gray dashed line). This is compared with light emission from cells cotransfected with 0.1 μg (solid gray line) or 1 μg (solid black line) of α_{2A} YFP (μ RLuc−α_{2A} YFP) receptors. The peak of light emission by luciferase is seen at 470 nm and the peak resulting from the BRET between μ RLuc−α_{2A} YFP receptors is seen at 530 nm. A representative sample of 3 to 4 experiments is shown in the figure.

Biotechnology, Santa Cruz, CA), collected with protein A-Sepharose beads, and analyzed by Western blotting using anti-HA antibodies (mouse; Roche Molecular Biochemicals, Indianapolis, IN) or anti-Flag antibodies (mouse, M1; Sigma) as described previously (Gomes et al., 2003). To disrupt higher order oligomers (i.e., ~200-kDa band), the sample buffer was treated with 8 M urea and 50 mM DTT before separation by gel electrophoresis (Figs. 3D and 4). For the isolation and detection of cell-surface complexes, HEK cells transiently coexpressing μ and α_{2A} receptors were washed twice with PBS and incubated with 5 μg/ml of anti-Flag antibody for 2 h at 4°C. The cells were lysed with the solubilization buffer and the lysate was incubated with protein A-beads overnight. Beads were washed, resuspended in sample loading buffer, and processed for SDS-PAGE and Western blot analysis with anti-HA antisera as described previously (Jordan and Devi, 1999).

[^{35}S]GTPγS Binding in Permeabilized Cells. HEK-293 cells transiently expressing either μ receptors alone or in combination with α_{2A} receptors or CB1 receptors were collected and washed in a GTPγS assay buffer (50 mM Tris-Cl, pH 7.5, containing 5 mM $MgCl_2$, 100 mM NaCl, and 0.2 mM EGTA). Cells expressing comparable levels of μ receptors expressed alone or coexpressed with α_{2A} or CB1 receptors were used for these studies. Cells were resuspended in the assay buffer containing 0.5% CHAPS for 20 to 30 min for permeabilization. For the GTPγS binding assay, permeabilized cells representing ~40 μg of protein were washed with the assay buffer and incubated in the same buffer containing 100 μM GDP, 0.1 nM [^{35}S]GTPγS, and 0 to 10 μM morphine (in the absence or presence of 100 nM clonidine) in a final volume of 1 ml. Basal binding was assessed in the presence of GDP and absence of morphine. Nonspecific binding was determined in the presence of 10 μM GTPγS. After 1 h at 30°C, samples were filtered through Whatman GF/B filters (Schleicher and Schuell, Keene, NH), washed, and their radioactivity was determined. EC_{50} values were determined using Prism 2.0. The basal values of [^{35}S]GTPγS binding in cells expressing only μ receptors (9.2 ± 0.09 fmol/10^7 cells) were not significantly different from levels in cells coexpressing μ and α_{2A} (8.9 ± 0.07 fmol/10^7 cells). In the presence of 100 nM clonidine, there was a ~1.5-fold increase in the basal signal; this was taken as 100% while calculating the effect of clonidine on morphine signaling (Fig. 5).

MAP Kinase Assays. Approximately 1×10^5 spinal cord neurons or HEK cells transiently expressing receptors were treated with indicated concentrations of ligands for 30 s to 5 min. Only those cells expressing the same number of μ receptors when expressed alone or in combination with α_{2A} receptors (as determined by ligand binding) were used for these studies. The cells were lysed with ~100 μl of 2% SDS in 50 mM Tris-Cl, pH 6.8, and ~10 μg of protein were subjected to analysis by SDS-PAGE and Western blotting using monoclonal antibodies against phospho-ERK1/2 (Cell Signaling Technologies, Beverly, MA). For standardization, the membranes were stripped

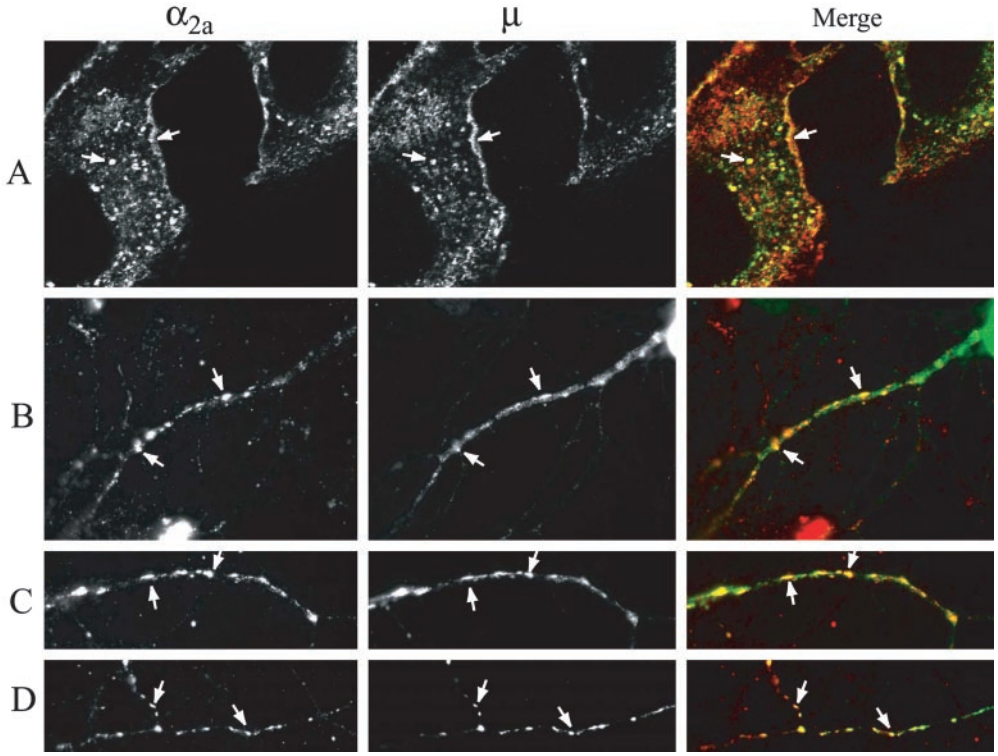

Fig. 2. Colocalization of μ and α_{2A} receptors. MDCK cells (A) or primary hippocampal neurons (B, C, and D) expressing μ and α_{2A} receptors were fixed, permeabilized with 0.25% Triton X-100 (A, C, and D) and labeled with rabbit polyclonal anti-HA (to label α_{2A}) and mouse monoclonal anti-Flag antibody (to label μ). Receptors were visualized with rhodamine conjugated anti-rabbit antibodies for α_{2A} receptors and with fluorescein isothiocyanate conjugated anti-mouse to detect the location of μ receptors. Arrows point to representative areas of colocalization. B, colocalization at the cell surface in a nonpermeabilized cell. The pictures were taken with a 60× objective with a Nikon confocal system. Experiments were repeated four times and representative samples are shown in the figure.

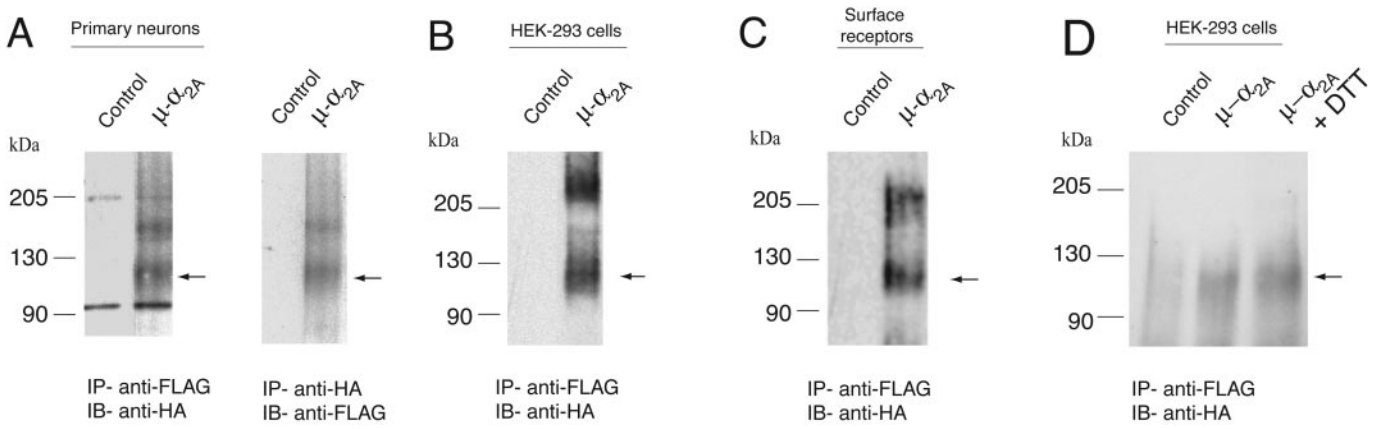

Fig. 3. Coimmunoprecipitation of μ opioid and α_{2A} adrenergic receptors. Neurons or HEK cells coexpressing μ and α_{2A} receptors or individually expressing the receptors were solubilized and subjected to immunoprecipitation with rabbit anti-Flag or anti HA-antibodies and Western blotting with mouse anti-HA or anti-Flag antibodies, respectively as described under *Materials and Methods*. A, a band of ~120 kDa and a higher molecular band of ~160 kDa are seen upon coexpression of the two receptors in neuronal cells (μ-α_{2A}). B, μ-α_{2A} interacting complexes in HEK cells. A band of ~120 kDa and another band of >200 kDa are seen only upon coexpression of the two receptors and not in a mixture of cells individually expressing these receptors (control). C, a band of ~120 kDa and a higher molecular mass band of >200 kDa are also seen when cell surface μ-α_{2A} receptor complexes are isolated as described. D, μ-α_{2A} interacting complexes isolated from HEK cells were treated with 8 M urea before Western blotting. This treatment leads to the disruption of the higher order complexes enabling the visualization of the α_{2A} receptor dimer. Treatment of cells with 50 mM DTT before solubilization and immunoprecipitation does not significantly affect the level of immunoprecipitable α_{2A} receptor dimers (μ-α_{2A} + DTT). All experiments were repeated four times and representative samples are shown in the figure.

and reprobed with anti-tubulin antibody (Sigma Chemical Co., St. Louis, MO). Densitization was carried out on multiple exposures of the autoradiograms from six individual experiments using NIH Image software (version 6.2).

Results

Interaction between μ and α_{2A} Receptors in Living Cells. To examine μ-α_{2A} receptor interactions in living cells, we used the proximity-based BRET assay. For this, RLuc or YFP was genetically fused to the C terminus of μ and α_{2A} receptors to generate μ-RLuc and α_{2A}-YFP donor/acceptor combinations and the tagged receptors expressed in HEK-293 cells. When μ-RLuc was coexpressed with α_{2A}-YFP, two peaks of light emission were observed; a peak at 470 nm corresponding to the emission maximum by RLuc and a second peak at 530 nm corresponding to the BRET signal (Fig. 1). The peak at 530 nm was not detected when μ-RLuc was coexpressed with either untagged YFP or chemokine CCR5-YFP (Fig. 1, black and gray dashed lines). The intensity of the BRET signal did not vary significantly when the level of expression of the μ-RLuc and α_{2A}-YFP receptors was varied 10-fold (Fig. 1, black and gray solid lines). In these experiments, the level of receptor expression as determined by [^{3}H]diprenorphine and [^{3}H]yohimbine binding was 500 fmol/mg of protein for μ-RLuc and 500 or 5000 fmol/mg of protein for α_{2A}-YFP. Taken together, these results demonstrate that μ-RLuc and α_{2A}-YFP receptors reside in close enough proximity in the membranes of live cells, which could allow direct associations between these receptors.

Colocalization of μ and α_{2A} Receptors in MDCK Cells and Primary Neurons. We next examined the localization of μ and α_{2A} receptors when coexpressed in MDCK cells or primary hippocampal neurons. MDCK cells have been used to characterize the localization of a variety of receptors including α_{2A} receptors because they show a clear apical-basal polarization (Saunders and Limbird, 1999). We found that α_{2A} receptors colocalized with μ receptors at the plasma membrane as well as within vesicles of MDCK cells (Fig. 2A). Interestingly, there was no preferential targeting of these receptors to either the apical or basolateral surfaces. We found that μ and α_{2A} receptors colocalize primarily at proximal dendrites in both permeabilized (Fig. 2, C and D) and nonpermeabilized neurons (Fig. 2B). These areas of colocalization did not correspond to spines or areas adjacent to synapses as assessed by immunostaining with antibodies to the GluR2 subunit of the glutamate receptor, synaptophysin or the SV2 synaptic vesicle marker (B. A. Jordan and L. A. Devi, unpublished observations). The identity of the subcellular structure in which μ-α_{2A} receptors colocalize is currently under investigation. Taken together, these results indicate that μ and α_{2A} receptors colocalize to proximal dendrites of primary neurons.

Characterization of μ and α_{2A} Interacting Complexes. To isolate and characterize μ and α_{2A} receptor-interacting complexes, we coexpressed differentially tagged receptors (Flag-tagged μ and HA-tagged α_{2A}) in neurons or HEK cells and subjected the cell lysates to immunoprecipitation with anti-Flag antibodies. We used a Sindbis virus expression system to introduce tagged receptors into cultured primary hippocampal neurons. This facilitated the selective and efficient immunoprecipitation of receptor complexes. The results showed that anti-Flag immunoprecipitates contained HA-tagged α_{2A} receptors (Fig. 3A). Reciprocally, anti-HA immunoprecipitates contained Flag-tagged μ receptors (Fig. 3B). A band at ~160 kDa observed in these studies could represent heteromeric complexes bound to additional proteins in neurons. Coexpression of μ and α_{2A} receptors in HEK cells also showed that the μ-opioid receptor immunoprecipitates contained the α_{2A} receptor dimer (~120 kDa) and a higher order oligomer (>200 kDa) (Fig. 3B). No monomeric α_{2A} receptors were observed suggesting that μ receptors interact with α_{2A} receptor dimers and oligomers. In these experiments, we expressed receptors at levels roughly comparable with those found in cells endogenously expressing these receptors (~450 fmol/mg of protein). No interactions were observed when a mixture of cells individually expressing μ

and α_{2A} receptors were subjected to identical immunoprecipitation conditions (Fig. 3B). Surface labeling studies to identify oligomeric complexes revealed the presence of the expected dimers as well as higher order complexes (Fig. 3C). These results suggest that μ-α_{2A} receptors could associate in heterologous cells and in neurons and that the complexes are present at the cell surface. Next, we examined whether treatment of interacting complexes with chaotropic agents such as urea would disrupt the higher order complexes. We find that this treatment leads to a significant disruption of the higher order complexes, leading to the clear visualization of a single band at ~120 kDa representing α_{2A} receptor dimers (Fig. 3D). Treatment of cells before immunoprecipitation with DTT did not affect the level of α_{2A} receptor dimers (Fig. 3D). Taken together, these results support the notion that hydrophobic rather than covalent interactions play an important role in associations between μ and α_{2A} receptors.

Ligand-Mediated Changes in Receptor-Receptor Associations. To examine whether μ and α_{2A} receptor associations could be affected by receptor activation, cells coexpressing these receptors were treated, before lysis and immunoprecipitation, with either morphine or clonidine (μ and α_{2A} ligands, respectively) or a combination of the two (Fig. 4). Immunoprecipitated material was subjected to SDS-PAGE under reducing conditions using 8 M urea and 50 mM DTT in the sample buffer to disrupt higher molecular mass oligomeric complexes; this led to the detection of only the ~120-kDa band representing α_{2A} receptors. We find that treatment with morphine or clonidine led to an increase in the level of α_{2A} receptors associated with μ receptors by ~70 and 60%, respectively. In contrast, treatment with both ligands led to a decrease in the levels of association to a level below the basal level (Fig. 4). Thus, it seems that the simultaneous presence of μ and α_{2A} ligands leads to changes in the strength of interaction between these receptors. In HEK-293 cells, agonist-mediated changes in the level of oligomers were seen only when the level of receptors were between 400 to 450 fmol/mg of protein. We evaluated a range of densities from 200 to 2000 fmol/mg of protein and found that a density of ~450 fmol/mg of protein is ideally suited for the detection of ligand-mediated changes in the immunoprecipitable complexes. At higher expression levels, ligand-mediated changes were not consistently discernible, and at lower expression levels, immunoprecipitated receptors could not be readily visualized. Thus, it seems that μ and α_{2A} receptors associate even when expressed at fairly low levels and the strength of their association can be modulated by a ligand to either receptor.

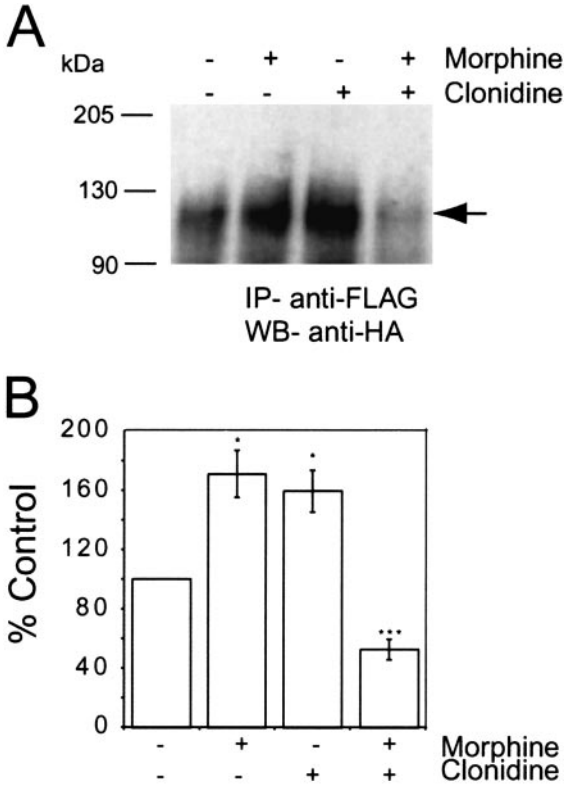

Fig. 4. Ligand-induced changes in μ-α_{2A} receptor interactions. HEK-293 cells coexpressing μ and α_{2A} receptors were treated with 100 nM morphine or clonidine for 20 min at 37°C before lysis and immunoprecipitation as described under *Materials and Methods*. A, the arrow points to the dimeric species of the α_{2A} receptor. B, the quantitation of ligand-induced changes in the level of dimers was carried out by densitizing multiple exposures of autoradiograms from four independent experiments using NIH Image 6.2. The bar graph shows that treatment with either morphine or clonidine leads to ~60 to 70% increase in the level of immunoprecipitable complexes compared with untreated cells. In contrast, treatment with a combination of two ligands leads to a ~50% decrease in the level of these receptors. Statistically significant differences between untreated and treated cells were determined by the student's t test. *, $p < 0.05$; ***, $p < 0.001$.

Modulation of Function by μ-α_{2A} Interactions. We next examined the functional significance of ligand-induced changes in interacting receptors by determining the G-protein activity (agonist-mediated increase in radiolabeled GTPγS binding) upon activation of individual or both receptors. Treatment with morphine resulted in a significant enhancement of GTPγS binding in cells coexpressing μ-α_{2A} receptors compared with cells expressing μ receptors alone (Fig. 5A), indicating that the mere presence of α_{2A} receptors is sufficient to potentiate μ receptor signaling. This effect is α_{2A} receptor selective, because coexpression of CB1 cannabinoid receptors (that also couple to G_i proteins) with μ receptors did not alter the morphine-mediated GTPγS binding (Fig. 5A). Next, we examined the effect of a combination of drugs on G-protein activity in cells coexpressing μ and α_{2A} receptors. As seen in Fig. 5B, we find that the treatment with clonidine leads to a decrease in the extent of morphine-mediated receptor activity. These results are consistent with the decrease in immunoprecipitable μ-α_{2A} complexes seen upon treatment with the combination of ligands suggesting a role for the strength of receptor interaction in signaling.

Next, we examined the effect of treatment with a single or combination of ligands on the agonist-mediated activation of the MAP kinase pathway. In cells expressing only μ receptors, morphine exhibited a dose-dependent increase in the phosphorylation of ERK 1/2 kinases (Fig. 5C). In cells coexpressing μ-α_{2A} receptors, there was a substantial (~3-fold) increase in the level of phosphorylated MAP kinase in response to morphine compared with cells expressing only μ receptors. The dramatic difference between the GTPγS assay and phospho-MAP kinase assay is most presumably a result of signal amplification, which is measured by the latter assay. The GTPγS binding assay measures G-protein activa-

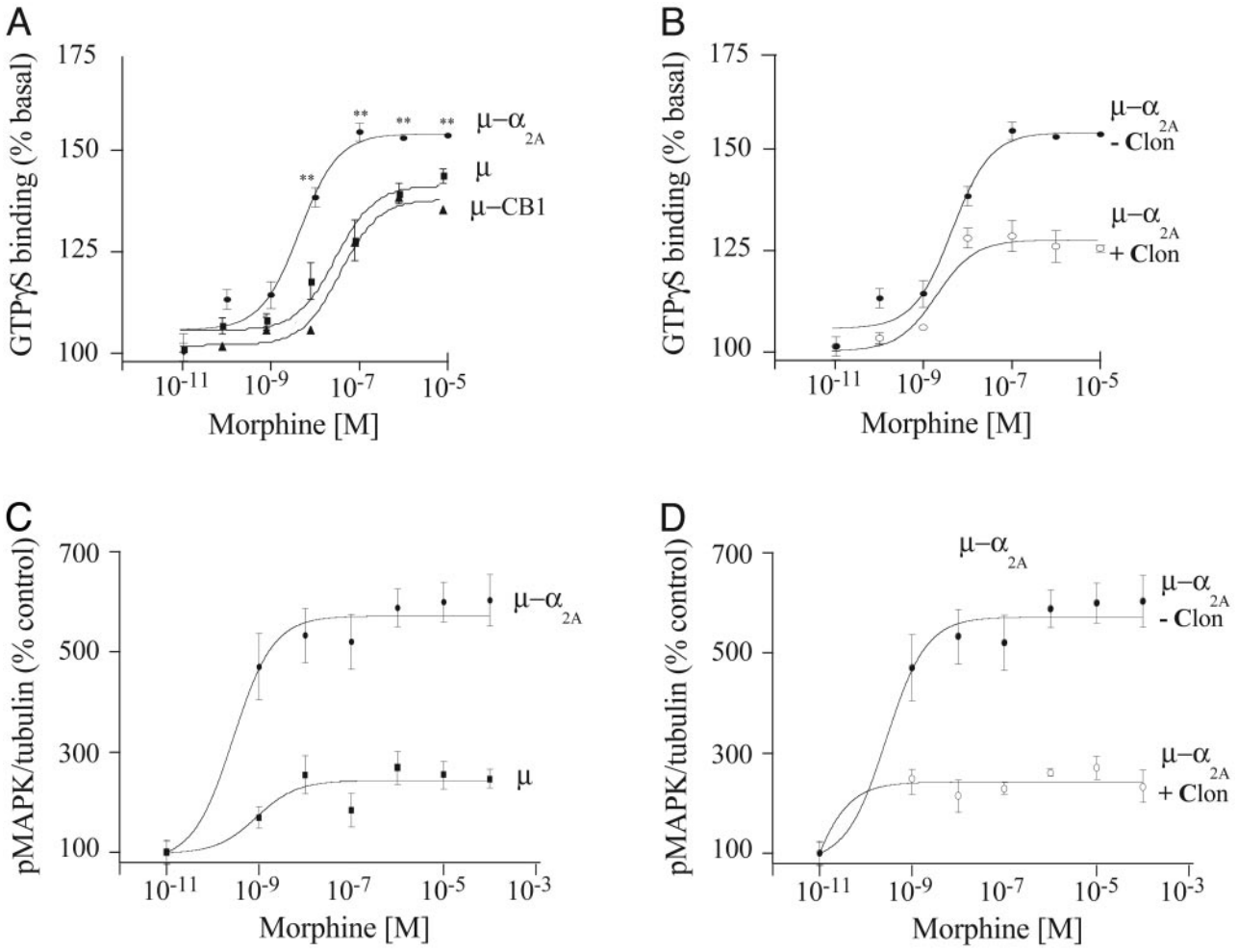

Fig. 5. Signaling in cells expressing μ receptors alone or in combination with α_{2A} receptors. A and B, agonist-mediated activation of GTPγS binding. HEK-293 cells expressing either μ receptors alone or in combination with α_{2A} or CB1 receptors were treated with indicated concentrations of morphine and subjected to a permeabilized cell [^{35}S]GTPγS binding assay as described under *Materials and Methods* (A). Cells coexpressing μ and α_{2A} were treated with indicated concentrations of morphine in the absence or presence of 100 nM clonidine and subjected to a permeabilized cell [^{35}S]GTPγS binding assay as described under *Materials and Methods* (B). The data represent mean ± S.E.M. from four to six experiments. **, $p < 0.001$, Student's *t* test. C and D, agonist-mediated increase in phosphorylated MAP kinases. HEK-293 cells expressing either μ receptors alone or in combination with α_{2A} were treated with indicated concentrations of morphine and subjected to phospho-MAP kinase assay as described under *Materials and Methods* (C). Cells coexpressing μ and α_{2A} receptors were treated with indicated concentrations of morphine in the absence or presence of 100 nM clonidine and subjected to phospho-MAP kinase assay as described under *Materials and Methods* (D). pMAPK/tubulin refers to the ratio of phospho-MAPK levels to the tubulin levels; the level in untreated cells is taken as 100. The data represent mean ± S.E.M. from six experiments.

tion—the most proximal event in the receptor activation—whereas the MAP kinase phosphorylation is a distal event and is the product of a cascade of steps, including a number of phosphorylation steps, leading to a significant amplification of the signal. As seen in the case of the GTPγS assay, a combination of ligands (100 nM clonidine in addition to morphine) resulted in a significant decrease in phosphorylated MAP kinase levels (Fig. 5D). Taken together, these results suggest that the presence of inactive α_{2A} receptors enhances μ receptor signaling and this effect is abolished by the simultaneous activation of both receptors.

Modulation of Function by μ-α_{2A} Interactions in Neurons. To examine whether the alteration of the signaling properties of the interacting receptors expressed in heterologous cells can be seen in neurons, we used spinal cord neurons (that endogenously express μ and α_{2A} receptors) and examined the agonist-mediated phosphorylation of ERK1/2 kinases. We found that treatment with morphine led to a significant increase in ERK 1/2 phosphorylation at low doses and a decrease at higher doses (Fig. 6A) and/or longer time of incubation (Fig 6B), suggesting a rapid desensitization of μ opioid receptors in this system. In contrast to treatment with either morphine or clonidine, which led to a significant increase in the levels of phosphorylated MAP kinases, treatment with a combination of both drugs led to a decrease in the levels to near basal levels (Fig. 6C). These results are consistent with the findings in HEK cells, where simultaneous activation of μ and α_{2A} receptors led to a significant decrease in signaling. It seems that the changes in receptor activity are not caused by changes in the relative affinities of the ligands, because we found that the affinities of the μ agonists ([D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin, 13.2 ± 1.3 nM; morphine, 12.1 ± 1.2 nM) were not changed by the presence of clonidine or yohimbine in spinal cord membranes. Taken together, these results suggest that in spinal cord neurons as well as in heterologous cells, the signaling by μ receptors can be significantly regulated by α_{2A} receptors.

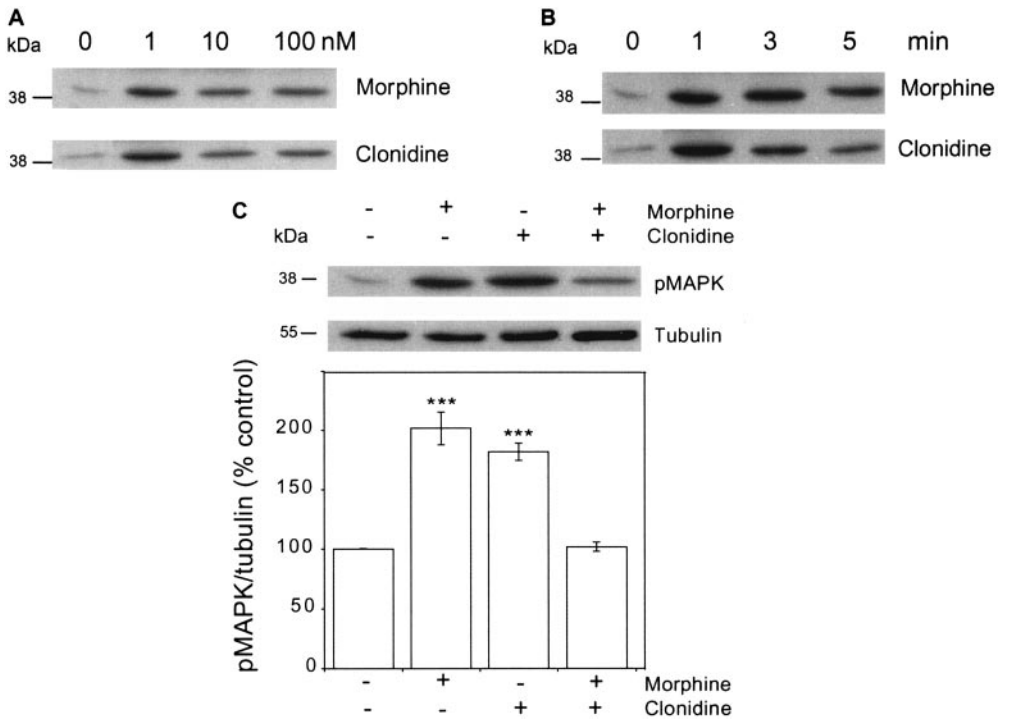

Fig. 6. MAP kinase signaling in primary spinal cord neurons endogenously expressing μ and α_{2A} receptors. A, primary spinal cord neurons were treated with indicated concentrations of morphine or clonidine for 30 s, and the extent of phosphorylated ERK 1/2 (pMAPK) was determined by Western blot analysis. B, primary spinal cord neurons were treated with 1 nM morphine or clonidine for 0 to 5 min and the extent of phosphorylated ERK 1/2 (pMAPK) was determined by Western blot analysis. C, primary spinal cord neurons were treated with 1 nM morphine, clonidine, or a combination of both for 30 s and the extent of phosphorylated ERK 1/2 (pMAPK) was determined by Western blot analysis as described. pMAPK/tubulin refers to the ratio of phospho-MAPK levels to the tubulin levels; the level in untreated cells is taken as 100. The data represent mean ± S.E.M. from six different experiments. Statistically significant differences from basal levels were determined by the Student's *t* test. ***, $p < 0.001$ represents differences in levels between ligand treated and untreated cells.

Discussion

There is considerable evidence for functional interactions between the opioid and adrenergic systems in the spinal cord (Yaksh, 1979; Roerig et al., 1984; Wigdor and Wilcox, 1987; Stone et al., 1997). Our data suggests that μ opioid and α_{2A} adrenergic receptors can physically interact, because the receptor complexes can be isolated from heterologous cells and from neurons by coimmunoprecipitation. This interaction can be enhanced by the addition of selective ligands for either system but not by the addition of both ligands. This is probably caused by conformational changes that render the complexes more stable (with individual agonists) or less stable (with a combination of agonists) to detergents. The alteration of hetero-oligomeric stability by ligands in vitro is an important indication of their ability to induce significant changes in the conformation of the complex, which may be responsible for the observed effects on receptor function. This is supported by our findings with the signaling assays. We find that the presence of α_{2A} receptors is sufficient to substantially increase the efficacy of morphine in this assay, because this occurs in the absence of α_{2A} ligands. It is possible that the interaction of the μ receptor with the inactive α_{2A} receptor could lead to the stabilization of μ receptors upon ligand binding to a conformationally active state that efficiently activates G-proteins in response to morphine. Coactivation of α_{2A} and μ receptors could lead, in turn, to the destabilization of these interactions (supported by our biochemical studies, Fig. 4), leading to a decrease in the efficacy of G-protein turnover by the two receptors (as seen in the signaling studies, Fig. 5). Taken together, these results are consistent with the notion that the physical interaction between μ and α_{2A} receptors plays an important role in modulating their signaling and this could be brought about, at least in part, by the agonist-induced changes in receptor conformation and/or association.

The phosphorylation of MAP kinase in response to specific ligands has been extensively used as an assay to test the function of some GPCRs. We find that the presence of α_{2A} receptors is also sufficient to potentiate the phosphorylation of MAP kinases in response to morphine and that the combination of ligands abrogates this effect. It is also possible that adrenergic receptors, by binding to scaffolding proteins, could bring additional signaling complexes, such as the components of the MAP kinase pathway to the proximity of the μ receptors, thus enhancing its activity. It is also possible that physical associations lead to changes in the localization of receptors to areas enriched in signaling molecules and that α_{2A} receptors at least in part, via a physical interaction, help target μ receptors to "active" areas, such as the lipid rafts. This is not unreasonable, given the case of $GABA_B$ receptors that require associations between the R1 and R2 subunits for proper targeting to the plasma membrane (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). For these events to be feasible, the two receptors should be in close proximity, thus influencing their ability to transduce signals. Our results with BRET and colocalization studies support such a notion. Finally, Stone et al. (1997) showed that in mice harboring mutant α_{2A} receptors, there was a considerable decrease in the potency of spinally administered morphine, supporting the hypothesis that μ receptors require functional α_{2A} receptors for proper functioning in the spinal cord.

Studies with a variety of inflammatory pain models have suggested that the phosphorylation of MAP kinase is important in mediating pain signals (Ciruela et al., 2003; Dai et al., 2002). A number of studies have correlated increases in phosphorylated ERK kinases with pain transmission. Our results show that spinal cord neurons are very responsive to opiate or adrenergic stimulation because fairly low doses and short-term treatment lead to a significant increase in the levels of phosphorylated ERK kinases, and these responses are rap-

idly desensitized. This and the fact that exposure to a combination of ligands lead to a decrease in phosphorylated ERK kinases suggests additional levels of regulation of these receptors in the modulation of analgesia.

In summary, physical interactions between μ and α_{2A} receptors provide a novel mechanism for modulation of receptor function that could have profound effects in the development of analgesia to opioid and adrenergic drugs.

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