p38 MAPK and β-Arrestin 2 Mediate Functional Interactions between Endogenous µ-Opioid and α2A-Adrenergic Receptors in Neurons*

Miao Tan, Wendy M. Walwyn, Christopher J. Evans, and Cui-Wei Xie

From the Department of Psychiatry and Biobehavioral Sciences, David Geffen School of Medicine, Semel Institute for Neuroscience and Human Behavior, University of California, Los Angeles, California 90024-1759

Received for publication, September 2, 2008, and in revised form, December 9, 2008 Published, JBC Papers in Press, January 6, 2009, DOI 10.1074/jbc.M806742200

Formation of receptor complexes between µ-opioid and α2A-adrenergic receptors has been demonstrated in transfected cells. The functional significance and underlying mechanisms of such receptor interactions remain to be determined in neuronal systems. We examined functional interactions between endogenous µ and α2A receptors in mouse dorsal root ganglion neurons. Acute application of the µ agonist [d-Ala²,N-MePhe⁴,Gly-ol⁵]enkephalin (DAMGO) or the α agonist clonidine inhibited voltage-gated Ca²⁺ currents in these neurons. Prolonged treatment with either DAMGO or clonidine induced a mutual cross-desensitization between µ and α2A receptor-mediated current inhibition. The cross-desensitization was closely associated with simultaneous internalization of µ and α2A receptors. Morphine, a µ agonist triggering little µ receptor endocytosis, induced neither cross-desensitization nor internalization of α2A receptors. Furthermore, inhibition of p38 MAPK prevented the cross-desensitization as well as cointernalization of µ and α2A receptors. Changes in receptor trafficking profiles suggested that p38 MAPK activity was required for initiating µ receptor internalization and maintaining possible µ-α2A association during their cointernalization. Finally, the µ-α2A cross-desensitization was absent in dorsal root ganglion neurons lacking β-arrin-2. These findings demonstrated p38 MAPK- and β-arrin 2-dependent cross-regulation between neuronal µ and α2A receptors. By promoting receptor cross-desensitization and cointernalization, such functional interactions may serve as negative feedback mechanisms triggered by prolonged agonist exposure to modulate the signaling of functionally related G protein-coupled receptors.

G protein-coupled receptors (GPCRs) interact with each other through formation of receptor complexes, including homo- or heterodimers and possibly higher order oligomers (1–4). Heterooligomerization of GPCRs has been shown to enable cross-regulation between different receptor systems, resulting in various changes in receptor binding, signaling, and trafficking. For example, dimerization of µ-opioid and NK1 (neurokinin type 1) receptors in HEK-293 cells promotes agonist-induced cross-phosphorylation and cointernalization of the two receptors, whereas receptor binding and functional coupling are relatively unaffected (5). A similar pattern has been observed in cells expressing heterodimers of δ-opioid and β2-adrenergic receptors (6). Formation of µ- and δ-opioid receptor complexes alters receptor properties, leading to synergistic enhancement of receptor binding and signaling by µ and δ ligands (7). The µ-δ heterodimer may form as early as in the endoplasmic reticulum during receptor processing and allows co-trafficking of the two receptors (8). Controversial evidence exists, however, for agonist-induced, separate endocytosis of µ and δ receptors (9). Although these studies and many others have underscored the dynamic nature and divergent roles of receptor heterodimerization in GPCR modulation, the molecular basis and regulatory mechanisms for such interactions remain to be elucidated. In particular, most studies addressing this issue have been conducted in heterologous cells or in systems where receptors are overexpressed, which may lead to interactions nonexistent with endogenously expressed receptors. Further studies are necessary to identify and characterize interactions between naturally existing GPCRs in primary neurons.

We examined interactions between endogenous µ-opioid and α2A-adrenergic receptors in mouse dorsal root ganglion (DRG) neurons. Both µ and α2A receptors are coupled to Gs and Gq proteins and induce similar cellular responses, such as inhibition of voltage-gated Ca²⁺ channels and activation of inwardly rectifying potassium channels. These cellular effects can lead to presynaptic inhibition of neurotransmitter release or hyperpolarization of postsynaptic neurons. Both are crucial mechanisms for opioid and adrenergic modulation of nociception. A functional synergy between the two systems has been demonstrated in vivo, evidenced by potentiation of morphine analgesia (10, 11) and alleviation of opiate withdrawal (12) by the α2A-adrenergic agonist clonidine. Studies in transgenic mice lacking functional α2A receptors further indicate that the α2A receptor is the principal subtype mediating α2 agonist-induced analgesia at the spinal level (13) and responsible for the synergistic potentiation of morphine analogues (14). The exact mech-
anisms for this adrenergic-opioid synergy, however, remain unclear. Recently, the $\mu$-$\alpha_{2A}$ receptor heterodimers have been detected in HEK-293 cells co-expressing both receptors (15, 16). It is of great interest to further explore whether heterodimerization of $\mu$ and $\alpha_{2A}$ receptors serves as a novel mechanism coordinating the function of both receptors in pain-processing pathways. Here we report that endogenous $\mu$ and $\alpha_{2A}$ receptors interact in DRG sensory neurons via p38 MAPK- and $\beta$-arrestin 2-dependent mechanisms, which promote agonist-selective cross-regulation of receptor signaling and internalization.

**EXPERIMENTAL PROCEDURES**

**DRG Cultures**—Primary DRG cultures were prepared as described previously (17). Briefly, the ganglia were collected from postnatal day 0–3 pups of C57 BL/6 mice, enzymatically dissociated for 30 min with minimal essential medium containing 0.25% trypsin at 37 °C, and triturated with fire-polished Pasteur pipettes. Dissociated neurons were plated onto glass coverslips coated with poly-l-ornithine and laminin. The cultures were maintained at 37 °C in 5% CO$_2$; fed with serum-free Neurobasal-A medium supplemented with B-27, l-glutamine, 2.5 mM CaCl$_2$, 10 mM tetraethylammonium chloride, 25 mM HEPES, 25 mM D-glucose, and 0.25 mM tetrodotoxin at pH 7.35. The patch electrode was filled with an internal solution composed of 105 mM CsCl, 40 mM HEPES, 5 mM D-glucose, 2.5 mM MgCl$_2$, 10 mM EGTA, 2 mM Mg-ATP, and 0.5 mM GTP at pH 7.2. Ca$^{2+}$ currents were evoked every 10 s by 40-ms voltage steps from -80 to +10 mV using an Axopatch 200A patch clamp amplifier. Capacitance and series resistance were corrected with the compensation circuitry on the amplifier. Series resistance was compensated by 80–90%. Leak currents were subtracted using a P/6 protocol. Recorded signals were acquired and analyzed using Axon pCLAMP version 8.0 software (Axon Instruments). The amplitude of peak Ca$^{2+}$ currents was determined using the peak detect feature of the software.

**Drug Application and Desensitization Protocols**—The $\mu$ and $\alpha_2$ receptor ligands (Sigma) were prepared as stock solutions in water, diluted with external solution to the final concentration for acute bath application, or added into culture medium for pretreatment. Various kinase inhibitors (Sigma) were dissolved in DMSO and diluted with culture medium for pretreatment with a final DMSO concentration of 0.1%. Cells pretreated with the medium containing 0.1% DMSO served as vehicle controls in these experiments. In additional control experiments, DAMGO- or clonidine-induced current inhibition was compared between untreated cells and cells pretreated with 0.1% DMSO for 4 h. No significant differences were observed between the two treatments (data not shown).

During recording, the external solution was continuously applied at 2 ml/min through a 0.5-ml recording chamber carrying the culture coverslip. After establishing a stable base line, the $\mu$ or $\alpha_2$ agonist was applied for up to 1 min to observe the maximal change in Ca$^{2+}$ currents. Agonist-induced current inhibition was measured as the maximal reduction in the peak current amplitude during drug perfusion and expressed as percentage changes from the baseline level. The voltage dependence of agonist effect was assessed using a prepulse facilitation (PPF) protocol consisting of two normal test pulses (P1 and P2) and in between a strong depolarizing prepulse ($-80$ to $+80$ mV, 40 ms) delivered 10 ms before P2. The PPF was expressed by the amplitude ratio of currents activated by the two test pulses (P2/P1). To induce chronic desensitization, DRG cultures were pretreated with either $\mu$ or $\alpha_2$ agonist for 4 h. After extensive washing, whole-cell recording was performed in pretreated cells, and the acute inhibitory effect of $\mu$ or $\alpha_2$ agonist on Ca$^{2+}$ currents was measured during a brief perfusion (0.5–1 min). The extent of desensitization was determined by the percentage reduction of the agonist effect in pretreated neurons relative to untreated neurons.

**Immunocytochemical Analysis and Fluorescence Confocal Microscopy**—Cellular distribution of $\mu$ and $\alpha_{2A}$ receptors in DRG cultures was determined by immunocytochemical double labeling. After drug treatment, the cultures were fixed with 4% paraformaldehyde for 10 min, washed in phosphate-buffered saline (PBS), permeabilized, and preblocked with PBS containing 10% normal donkey serum and 0.1% Triton X-100 for 2 h at room temperature. The cultures were then incubated overnight at 4 °C with the primary antibodies against the C-terminal sequence of the $\mu$ receptor (20) or of the $\alpha_{2A}$ receptor (21) (sc-1478 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); ab45871 (Abcam)). After washing, the cells were further incubated for 2 h with Alexa Fluor 488-labeled donkey anti-rabbit IgG for visualization of $\mu$ receptors and with biotinylated anti-goat IgG (Calbiochem) and Cy3-conjugated streptavidin (Calbiochem) to detect $\alpha_{2A}$ receptors. Control samples were prepared in the absence of the primary or secondary antibodies. The specificity of the anti-$\alpha_{2A}$ antibody was further confirmed by the absence of $\alpha_{2A}$ receptor labeling in neurons from the $\alpha_{2A}^{-/-}$ mice (see Fig. 4B). The images of neurons with receptor labeling were acquired using a Leica TCS-SP confocal laser-scanning microscope and processed either as a single scan or as maximum intensity projections of multiple scans taken at successive 1-μm depths.

**Flow Cytometric Measurement of Surface $\mu$ Receptors**—Internalization of $\mu$ receptors was further accessed in living neurons by quantifying cell surface receptors with flow cytometry as described previously (22). DRG cultures were treated with clonidine, DAMGO, morphine, or control medium for 4 h at 37 °C. Monensin (10 μM) was added during the treatment to block recycling of internalized receptors in all experiments. DRG cells were then harvested in PBS containing 2 mM EDTA,
spun at 300 × g for 5 min, and resuspended with PBS containing
1% normal goat serum and 0.1% NaN₃. Cell surface μ receptors
were labeled at 4°C for 30 min with a polyclonal antibody
against the third extracellular loop of the receptor (Chemicon
International) (23) and visualized with Alexa Fluor 488-labeled
donkey anti-rabbit IgG at 4°C for another 30 min. Cell surface
immunofluorescence was measured with a FACSscan flow
cytometer at 5,000–10,000 cells/sample. Data were acquired
and analyzed using Cell Quest version 3.0.1 (BD Bioscience).
The loss of cell surface μ receptors after agonist exposure was
quantified by the reduction in the proportion of cells expressing
detectable surface μ receptors (μ-positive cells) and in the den-
sity of surface receptors of μ-positive cells reflected by their
mean fluorescence intensity. Nonspecific background fluores-
cence was determined in control samples processed without
the primary antibody and was subtracted to obtain mean rela-
tive fluorescence intensity of experimental samples.

Measurement of Phospho-p38 MAPK—The cellular level of
phospho-p38 MAPK was measured with flow cytometry (24),
using a polyclonal antibody recognizing p38 MAPK dually
phosphorylated at Thr180 and Tyr182 (Cell Signaling Technol-
ogy) (25). After drug treatment, DRG cells were harvested
from culture plates with PBS buffer containing 4 mM EDTA, 1 mM
phenylmethanesulfonlfuryl fluoride, 0.1 mM calyculin A, 1 μg/ml
leupeptin, and protease inhibitor mixture tablet (Sigma) (26).
The cells were fixed in 2% formaldehyde for 10 min at room
temperature, permeabilized in ice-cold 90% methanol for 30
min, incubated with the primary antibody for 60 min at room
temperature, and labeled with an Alexa Fluor 488-conjugated
second antibody for another 60 min. In between these steps, the
cells were washed, centrifuged at 450 × g for 5 min, and resus-
pended with fresh PBS. After the final wash, the fluorescence
signal of the cells was analyzed with a FACSscan flow cytometer
at 5,000 cells/sample, and the data were processed with
CellQuest software.

Statistical Analysis—All data are presented as means ± S.E.
One-way analysis of variance was applied for overall statistical
significance across multiple group means, followed by the Bon-
ferroni post hoc test for pairwise comparisons. Statistical signi-
ficance was defined as *p < 0.05.

RESULTS
α₂-Adrenergic Agonist-induced Cross-desensitization to
μ-Opioids—Brief bath application of norepinephrine (NE) at
10 μM induced acute inhibition of voltage-gated Ca²⁺ currents
in DRG neurons (Fig. 1A). This effect was significantly attenu-
ated by a selective α₂ antagonist yohimbine, confirming
involvement of α₂ receptors in NE action (27.2 ± 5.0% current
inhibition without yohimbine versus 9.7 ± 1.6% with 10 μM
yohimbine, n = 12 and 8, p < 0.05). Acute application of a
selective α₂ agonist, clonidine (10 μM), induced similar reduc-
tions in Ca²⁺ currents (20.0 ± 4.2%, n = 12) reversible by co-
application of yohimbine (5.7 ± 1.6%, n = 10, p < 0.01 com-
pared with clonidine alone). The effect of NE or clonidine was
substantially reduced in neurons pretreated with the same ago-
nist for 4 h (6.4 ± 1.7%, n = 9 for NE; 7.6 ± 1.1%, n = 8 for
clonidine, p < 0.01 compared with the effects in untreated neu-
rions), indicating development of homologous desensitiza-
tion to α₂ receptor-mediated effect. As expected, this desensitiza-
tion was blocked by yohimbine added during NE or clonidine
pretreatment (Fig. 1A).

We then determined the influence of prolonged α₂ agonist
exposure on μ-opioid-induced Ca²⁺ current inhibition. In
DRG neurons pretreated with 10 μM clonidine for 0.5 or 4 h, the
subsequent DAMGO application (1 μM, 1 min) reduced Ca²⁺
currents by 40.5 ± 6.7% (n = 4) and 37.4 ± 3.0% (n = 18),
respectively. The latter was significantly smaller than the
responses in untreated cells (0 h, 54.7 ± 2.5%, n = 62, p < 0.01),
indicative of a cross-desensitization to DAMGO. Similarly,
morphine application (1 μM, 1 min) reduced Ca²⁺ currents by
47.6 ± 6.2% in control cells (0 h, n = 10) but by only 27.2 ± 4.4% in
cells pretreated with clonidine for 4 h (n = 9, p < 0.05). Thus,
prolonged NE or clonidine exposure heterologously reduced the
effect of subsequently applied μ-opioid agonists. This
cross-desensitization was prevented by yohimbine co-treat-
ment (Fig. 1B).

Inhibition of neuronal Ca²⁺ channels by GPCRs is mediated
by both voltage-dependent and voltage-independent mecha-
nisms. The voltage-dependent inhibition requires direct bind-
ing of G-protein βγ subunits (Gβγ) to the Ca²⁺ channel, a
process reversible by strong depolarization. To determine
whether this direct Gβγ-channel interaction was affected by
clonidine, we examined voltage dependence of clonidine action
using the PPF protocol (Fig. 1, C and D). In cells acutely per-
fused with clonidine (10 μM, 1 min), the depolarizing prepulse
produced little relief of inhibition, with a P2/P1 ratio similar to
that under basal conditions (1.06 ± 0.04 versus 0.99 ± 0.02, n =
5 for each group, p > 0.05). Clonidine pretreatment for 4 h was
also without effect on the P2/P1 ratio subsequently tested either
during control perfusion medium (0.97 ± 0.02, n = 8) or during
acute clonidine test (1.06 ± 0.03, n = 8). These results sug-
gested that at the concentration we used, the effect of clonidine
was primarily mediated by voltage-independent mechanisms.
We then measured PPF induced by intracellular application of
0.1 mM GTPγS that can directly activate G proteins and release
Gβγ subunits. GTPγS-induced PPF was not significantly differ-
ent between untreated control cells and clonidine-pretreated
cells (1.68 ± 0.23 versus 1.68 ± 0.18 at 8 min after the applica-
tion, n = 12 for each group, p > 0.05) (Fig. 1, C and D). This
confirmed that clonidine-induced desensitization was not asso-
ciated with a diminished cell capacity for voltage-dependent
Gβγ-Ca²⁺ channel interactions.

DAMGO-induced Cross-desensitization to α₂A Adrenergic
Responses—As we previously reported (17, 22), application of 1
μM DAMGO or morphine strongly inhibited Ca²⁺ currents in
DRG neurons, but their effects were desensitized dramatically
following a 4-h pretreatment (Fig. 2A). The inhibitory effect of
10 μM NE or clonidine on Ca²⁺ currents also significantly
decreased in DAMGO-treated cells (11.4 ± 2.5%, n = 9 for NE;
5.3 ± 1.6%, n = 6 for clonidine, p < 0.05 for both as compared
with untreated cells). This cross-desensitization was prevented by
a selective μ receptor antagonist, Cys², Tyr⁴, Arg⁵, Pen⁷-
amicide (CTAP), added during DAMGO pretreatment (Fig. 2B).
Interestingly, the effect of NE or clonidine was not significantly
reduced in morphine-pretreated cells (21.7 ± 6.3%, n = 7 for
NE; 16.3 ± 3.3%, n = 11 for clonidine, p > 0.05 for both as

p38 MAPK and β-Arrestin 2 Mediate μ-α₂A Interactions

p38 MAP kinase (p38 MAPK) is a member of the mitogen-activated protein kinase (MAPK) family, which plays a crucial role in mediating cellular responses to various extracellular signals, including stress, inflammation, and cell growth. p38 MAPK is activated by a variety of stimuli and regulates a wide range of cellular processes, such as proliferation, differentiation, and survival. In particular, p38 MAPK is involved in the regulation of neuronal excitability and sympathetic nervous system function. Recent studies have shown that p38 MAPK is also upregulated by β-arrestin 2 (β-Arrestin 2), a protein that interacts with G-protein-coupled receptors (GPCRs) and plays a key role in receptor internalization and desensitization. β-Arrestin 2 interacts directly with Gβγ subunits, which are released from activated GPCRs, and this interaction contributes to the desensitization of GPCR-mediated signaling. Therefore, understanding the role of p38 MAPK and β-Arrestin 2 in GPCR signaling is important for developing new therapeutic strategies in the treatment of various neurological and psychiatric disorders.
compared with untreated cells), despite development of homologous morphine desensitization in these cells (Fig. 2A). Thus, the cross-desensitization to clonidine was induced by prolonged treatment with DAMGO but not morphine.

Since clonidine is relatively nonselective to different subtypes of the \( \alpha_2 \) receptors, we examined the role of \( \alpha_{2A} \) receptors in the acute and chronic effects of clonidine. Acute application of 10 \( \mu M \) clonidine induced negligible current inhibition in DRG neurons derived from the \( \alpha_{2A}^- \) mice (2.5 \( \pm \) 1.1%, \( n = 6 \), \( p > 0.05 \)). In contrast to the wild-type neurons, the \( \alpha_{2A}^- \) neurons also displayed no cross-desensitization to DAMGO following clonidine pretreatment (Fig. 4A). These results suggested that in our preparation, the \( \alpha_{2A} \) receptor was the major subtype responsible for clonidine-induced Ca\(^{2+} \) current inhibition and its cross-desensitization to DAMGO.

**Agonist-induced Cointernalization of \( \mu \) and \( \alpha_{2A} \) Receptors—**

Next we determined whether the mutual cross-desensitization between \( \mu \) and \( \alpha_{2A} \) receptors was associated with changes in agonist-induced receptor internalization. Immunohistochemical double labeling and confocal microscopy showed that a substantial portion of \( \mu \) and \( \alpha_{2A} \) receptors were colocalized on the cell membrane throughout the cell body and processes of cultured DRG neurons under basal conditions (Fig. 3A). Incubation with DAMGO for 30 min to 4 h induced simultaneous internalization of both receptors, which was blocked by co-incubation with the \( \mu \) antagonist CTAP (Fig. 3C). Clonidine also elicited internalization of both \( \mu \) and \( \alpha_{2A} \) receptors, reversible by the \( \alpha_{2A} \) antagonist yohimbine (Fig. 3D). In contrast, morphine treatment up to 4 h failed to internalize either \( \mu \) or \( \alpha_{2A} \) receptors (Fig. 3B). Furthermore, clonidine did not induce \( \mu \) receptor endocytosis in \( \alpha_{2A}^- \) mice (Fig. 4B). Thus, similar to the cross-desensitization, the cointernalization was agonist-selective and dependent upon the presence of functional \( \mu \) and \( \alpha_{2A} \) receptors.

Fluorescence flow cytometry was then conducted in living DRG neurons to quantify agonist-induced \( \mu \) receptor internalization. Surface \( \mu \) receptors were detected in 77.7 \( \pm \) 3% of control cells. DAMGO treatment for 4 h reduced the portion of
cells expressing detectable levels of surface μ receptors (μ-positive cells) by 26.3 ± 5.8% (p < 0.05) relative to the controls (Fig. 5B). Furthermore, the intensity of surface μ receptor labeling in the μ-positive cells decreased by 43.1 ± 7.8% (p < 0.05) following DAMGO treatment (Fig. 5A). These changes confirmed loss of surface μ receptors due to DAMGO-induced receptor internalization. Consistent with previous reports in heterologous cells (20, 27), morphine was much less effective in triggering μ receptor internalization, causing little change in surface μ receptor expression after a 4-h treatment. Importantly, clonidine pretreatment reduced the portion of μ-positive cells by 16.7 ± 5.7% and the level of surface μ receptors by 30.8 ± 10.9% (p < 0.05 for both compared with the controls), and both effects were blocked by yohimbine (Fig. 5).

**Blockade of μ-α2A Cross-desensitization and Cointernalization by p38 MAPK Inhibitors**—To explore the signaling mechanisms underlying μ-α2A interactions, we examined potential involvement of several opioid-activated protein kinases in the cross-desensitization. DRG neurons were pretreated with clonidine (10 μM, 4 h) in the presence of a selective kinase inhibitor, washed, and tested for acute DAMGO responses. As shown in Fig. 6A, co-treatment with a selective p38 MAPK inhibitor PD169316 (26) attenuated clonidine-induced cross desensitization. Acute DAMGO responses were significantly greater in cells co-treated with 5 or 20 μM PD169316 (49.0 ± 4.8% (n = 8) or 54.9 ± 8.1% (n = 10)) compared with cells treated with clonidine alone (37.4 ± 3.0%, n = 18, p < 0.05 for both comparisons). Another selective p38 MAPK inhibitor, SB239063 (28), also significantly reduced the cross-desensitization when co-applied with clonidine. In contrast, inhibitors of several other protein kinases failed to prevent clonidine-induced cross-desensitization, which included the phosphoinositide 3-kinase inhibitor LY294002 (10 μM), the extracellular signal-regulated kinase (ERK) inhibitor PD98059 (10 μM), the protein kinase A inhibitor rpCAMP (1 mM), and the protein kinase C inhibitor Go6987 (0.1 μM). Acute DAMGO responses in cells co-treated with these inhibitors was 41.7 ± 2.7% (n = 14), 38.5 ± 6.3% (n = 7), 31.5 ± 4.5% (n = 7), and 36.8 ± 6.6% (n = 6), respectively, not significantly different from that in cells treated with clonidine alone.
In a separate set of experiments, DRG neurons were pretreated with DAMGO (1 μM, 4 h) in the presence of one of the kinase inhibitors and tested for acute responses to clonidine. Blocking p38 MAPK activity with PD169316 (5 and 20 μM) during pretreatment attenuated DAMGO-induced cross-desensitization in a dose-dependent manner (Fig. 6B). The responses to subsequent clonidine test were significantly greater in cells co-treated with 20 μM PD169316 compared with those treated with DAMGO alone (13.9 ± 2.4% versus 5.7 ± 0.8%, n = 17 and 18, p < 0.01). Co-treatment with another specific p38 inhibitor SB203580 (20 μM) (29) also significantly increased clonidine responses (Fig. 6B). Again, the inhibitors for phosphoinositide 3-kinase, ERK, protein kinase A, and protein kinase C were unable to prevent the cross-desensitization. Clonidine-induced current inhibition ranged from 4.0 to 7.5% in cells co-treated with one of these inhibitors, indistinguishable from those treated with DAMGO alone. None of the inhibitors examined above elicited significant changes in basal Ca\(^{2+}\) currents when applied acutely in the absence of μ or α2 agonist (2.2–4.9% current inhibition, n = 4–6, p > 0.05). In addition, pretreatment with PD169316 (20 μM) alone for 4 h did not affect the acute effect of DAMGO or clonidine (49.0 ± 4.1 and 19.8 ± 3.9%, n = 11 and 9, p > 0.05 compared with vehicle-treated controls). Thus, blockade of the cross-desensitization by p38 MAPK inhibitors was not due to any direct effect of the inhibitors per se on Ca\(^{2+}\) currents or to interference of acute DAMGO or clonidine action. Together, these results suggested that p38 MAPK activity was necessary for the μ-α2 interaction leading to the mutual cross-desensitization.

Since the cross-desensitization was closely associated with cointernalization of μ and α2A receptors, we investigated whether reversal of the cross-desensitization by PD169316 was accompanied by blockade of receptor cointernalization. The p38 MAPK inhibitor was found to completely block internalization of both μ and α2A receptors in DAMGO-treated cells (Fig. 6C). Interestingly, PD169316 selectively blocked internalization of μ but not α2A receptors in clonidine-treated cells. This effect led to separation of the two receptors during clonidine treatment, with α2A receptors being internalized but μ receptors remaining on the plasma membrane of DRG neurons (Fig. 6C and D). These results suggested that activation of p38 MAPK was necessary for μ receptor endocytosis as well as for supporting cointernalization of μ and α2A receptors.

Activation of p38 MAPK by DAMGO and Clonidine, but Not by Morphine—To obtain direct biochemical evidence for p38 activation by μ and α2A agonists, we measured p38 MAPK phosphorylation in DRG neurons. In a time course analysis, DAMGO treatment (1 μM) induced rapid and sustained activa-
p38 MAPK and β-Arrestin 2 Mediate μ-α2A Interactions

In agreement with differential activation of p38 MAPK by the two opioid agonists, PD169316 selectively blocked DAMGO but not morphine-induced homologous desensitization (Fig. 7B). Acute DAMGO responses in cells co-treated with PD169316 (41.4 ± 7.7%, n = 9) were significantly greater than those pretreated with DAMGO alone (18.4 ± 3.8%, n = 7, p < 0.05) and comparable with untreated controls (49.4 ± 7.5%, n = 11, p > 0.05). In contrast, morphine-induced current inhibition was similar between cells treated with morphine alone and those co-treated with PD169316 (22.3 ± 2.1% versus 28.3 ± 2.9%, n = 19 and 21, p > 0.05). Furthermore, although it blocked DAMGO-induced cross-desensitization to clonidine, PD169316 did not prevent homologously induced clonidine desensitization (Fig. 7C). This indicated that p38 MAPK activity was also differentially involved in the homologous versus heterologous desensitization of α2A receptors.

The Essential Role of β-Arrestin 2 in μ-α2A Cross-desensitization—β-Arrestins are key adaptor proteins involved in receptor endocytosis, intracellular sorting, and MAPK regulation. We determined involvement of β-arrestin 2 in μ and α2A receptor desensitization using DRG neurons from β-arrestin 2−/− mice and their wild-type controls (Fig. 8). As expected, the 4-h DAMGO or clonidine treatment induced both homologous and cross-desensitization in β-arrestin 2−/− neurons. Similar to our previous observations (30), acute responses to DAMGO were smaller in untreated β-arrestin 2−/− neurons compared with their wild-type counterparts. This, however, did not affect development of homologous DAMGO desensitization in β-arrestin 2−/− neurons (Fig. 8E). Importantly, the cross-desensitization was completely absent in β-arrestin 2−/− neurons following either DAMGO or clonidine treatment (Fig. 8, A–C). Thus, similar to p38 MAPK, β-arrestin 2 was required for the receptor interactions leading to the mutual cross-desensitization. Different from the effect of p38 inhibition, however, genetic deletion of β-arrestin 2 abolished clonidine- but not DAMGO-induced homologous desensitization (Fig. 8, D and E).

DISCUSSION

Cross-regulation of μ and α2A Receptor Signaling in Neurons—Although formation of μ and α2A receptor complexes has been convincingly demonstrated in cell lines and transfected neurons (15), functional significance and regulatory mechanisms for the μ-α2A interaction remain to be clarified. The initial
report showed that heterodimerization of μ and α2A receptors in HEK293 cells enhanced receptor signaling in response to acute application of morphine or clonidine (15). A recent study, however, demonstrates a direct conformational cross-talk between μ and α2A receptors within the heterocomplex that allows rapid inactivation of one receptor by the other with subsecond kinetics (31). In locus coeruleus neurons naturally expressing high levels of α2A and μ receptors, analysis of the effect of co-applied μ and α2A agonists reveals no functional interactions between the two receptors (32). These findings raise the questions as to whether and how naturally existing μ and α2A receptors in neurons could interact with each other to regulate cellular function. The present study demonstrated strong functional interactions between endogenous μ and α2A receptors in sensory neurons. Such interactions required p38 MAPK activity and β-arrestin 2 and promoted receptor co-trafficking and cross desensitization.

Desensitization of GPCR signaling involves modifications at the receptor level and in downstream signal transduction pathways. We previously reported that chronic homologous DAMGO desensitization in DRG neurons was partially mediated by phosphoinositide 3-kinase- and ERK-mediated changes in voltage-dependent Gβγ-Ca2+ channel interactions (17). In the present study, however, neither acute nor prolonged DAMGO treatment altered PPF, a direct measure of voltage-dependent Gβγ effect on Ca2+ channels. The μ-α2A cross-desensitization was also unaffected by the selective inhibitors for phosphoinositide 3-kinase or ERK. These results suggest that modification of Gβγ-Ca2+ channel interactions by these two kinases did not play a significant role in the cross-desensitization. Another scenario for the cross-desensitization to occur at the post-receptor level would be desensitization of signaling pathways shared by both receptors. Our results did not support this possibility either. p38 MAPK and β-arrestin 2 differentially regulated homologous μ and α2A desensitization, suggesting that divergent rather than common signaling pathways were engaged by μ and α2A receptors.

**Receptor Cointernalization Contributes to μ-α2A Cross-desensitization**—An important feature of the cross-desensitization was its close association with agonist-induced cointernalization of μ and α2A receptors. It is well documented that both μ and α2A receptors undergo agonist-induced rapid endocytosis via clathrin coated-pits, a process regulated by receptor phosphorylation and binding with nonvisual β-arrestins (27, 28). p38 MAPK and β-arrestin 2 were strongly expressed in DRG neurons (35). In the present study, however, neither acute nor prolonged DAMGO treatment altered PPF, a direct measure of voltage-dependent Gβγ effect on Ca2+ channels. The μ-α2A cross-desensitization was also unaffected by the selective inhibitors for phosphoinositide 3-kinase or ERK. These results suggest that modification of Gβγ-Ca2+ channel interactions by these two kinases did not play a significant role in the cross-desensitization. Another scenario for the cross-desensitization to occur at the post-receptor level would be desensitization of signaling pathways shared by both receptors. Our results did not support this possibility either. p38 MAPK and β-arrestin 2 differentially regulated homologous μ and α2A desensitization, suggesting that divergent rather than common signaling pathways were engaged by μ and α2A receptors.

**Receptor Cointernalization Contributes to μ-α2A Cross-desensitization**—An important feature of the cross-desensitization was its close association with agonist-induced cointernalization of μ and α2A receptors. It is well documented that both μ and α2A receptors undergo agonist-induced rapid endocytosis via clathrin coated-pits, a process regulated by receptor phosphorylation and binding with nonvisual β-arrestins (27, 28).
by

oid desensitization developed in hours can be greatly affected seconds to minutes. Evidence exists, however, that chronic opi-

sium channels (40) when measured on the time scale of several

/H9262

internalization of

/H9262

down-regulation and more persistent signaling reduction (44,

ized receptor to lysosomes for degradation, causing receptor
desensitization (41, 42). Alternatively, if internalized receptors
tor resensitization, effectively reducing the extent of apparent
desensitization (43).

Extended or repeated agonist exposure can also target internal-

ated receptors to lysosomes for degradation, causing receptor
down-regulation and more persistent signaling reduction (44,

co-localization of the two receptors in DRG neurons and a rela-
tively low percentage of μ receptors cointernalized by clonidine

mediated by p38 MAPK and β-arrestin (45). Therefore, the func-
tional consequence of receptor internalization may vary consid-
erably in different model systems, depending upon the rate and extent of endocytosis as well as its coupling with distinct
intracellular sorting pathways that determine the postendocytic fate of

Receptor oligomerization has added a new dynamic to the com-
plex relationship between internalization and desensitization. Studies
in HEK293 cells show that formation of heterocomplexes between μ
receptors and other GPCRs often alters receptor trafficking profiles.
In many cases, co-expressed recep-
tors are both internalized in response to a single selective agonist
(5, 6, 8), suggesting that ligand-acti-

ated receptor may “drag” another receptor in the same complex to the
endocytic pathway (46). Similarly, we demonstrated that μ and α₂A
receptors colocalized on the plasma membrane of untreated DRG
neurons and underwent simultaneous internalization when either recep-
tor was activated. These findings were in agreement with the pres-
ence of μ-α₂A complexes. Never-
theless, a fraction of μ and α₂A
receptors were likely to exist as homodimers or monomers (4, 8), as
indicated by the less than complete

33, 34). How this event regulates GPCR signaling, especially in
the case of opioid desensitization, has been a subject of intense
investigation (35–37). Several studies in primary neurons and
AtT20 cells indicate that receptor internalization does not con-
tribute to rapid desensitization of μ receptors coupled to volt-
age-gated Ca²⁺ channels (38, 39) or inwardly rectifying potas-
sium channels (40) when measured on the time scale of several
seconds to minutes. Evidence exists, however, that chronic opioid
desensitization developed in hours can be greatly affected by μ receptor internalization and recycling (41). Continued
internalization of μ receptors during prolonged agonist treat-
ment can attenuate opioid responses via physical removal of the
receptor from cell surface, but it also can promote receptor dephosphorylation and recycling. When rapid recycling occurs,
the internalization is considered an important means for recep-
tor desensitization, effectively reducing the extent of apparent
desensitization (41, 42). Alternatively, if internalized receptors
are trapped in endosomes, significant loss of surface receptors
can occur, leading to enhancement of desensitization (43).

Extended or repeated agonist exposure can also target internal-
ized receptor to lysosomes for degradation, causing receptor
down-regulation and more persistent signaling reduction (44,
p38 MAPK and β-Arrestin 2 Mediate μ-α2A Interactions

A Clonidine test

β-arrestin 2+/+

β-arrestin 2−/−

B Clonidine test

C DAMGO test

D Clonidine test

E DAMGO test

FIGURE 8. The crucial role of β-arrestin 2 in μ-α2A cross-desensitization. DRG neurons derived from the β-arrestin 2−/− and β-arrestin 2+/+ mice were pretreated with 1 μM DAMGO or 10 μM clonidine for 4 h and tested for the cross-desensitization and homologous desensitization. The μ-α2A cross-desensitization induced by DAMGO (B) or clonidine (C) was clearly seen in β-arrestin 2+/+ neurons but completely absent in β-arrestin 2−/− neurons. Representative current recordings (A) were collected before (1), during (2), and after (3) acute clonidine test. Calibration was 2 nA, 20 ms. Clonidine-induced α2A receptor desensitization was also absent in β-arrestin 2−/− neurons (D), but DAMGO-induced μ receptor desensitization remained intact in these neurons (E). *, p < 0.05; **, p < 0.01 compared with untreated neurons with the same genotype.

their recycling and resensitization could be significantly delayed.

p38 MAPK and β-Arrestin 2 Are Key Regulators of μ-α2A Cross-regulation—We observed strong activation of p38 MAPK by DAMGO and clonidine. The enhanced p38 MAPK activity may play two different roles in the cross-regulation of μ-α2A receptors. First, activation of p38 MAPK is known to facilitate μ receptor internalization by enhancing the function of endocytic machinery regulated by the small GTPase Rab5 (47, 48). By driving μ receptor endocytosis, p38 MAPK activity may trigger simultaneous internalization of those α2A receptors that are directly or indirectly associated with μ receptors. This scenario can well explain our findings that inhibition of p38 MAPK effectively blocked DAMGO-initiated internalization of both μ and α2A receptors. Second, our results indicated that p38 activity was not required for clonidine-initiated α2A receptor internalization but was necessary for co-trafficking of μ receptors with activated α2A receptors. Thus, activation of p38 MAPK may be essential for maintaining μ-α2A association during the cointernalization.

Another key regulator of the μ-α2A cross-modulation identified in this study was β-arrestin 2. Deletion of β-arrestin 2 in DRG neurons prevented μ-α2A cross-desensitization, an effect similar to that of p38 MAPK inhibitors. Studies have shown that
p38 MAPK and β-Arrestin 2 Mediate α2A Interactions

β-arrestins act as scaffold proteins to regulate spatial distribution and activity of MAPK cascades (49) and that activation of p38 MAPK by GPCRs requires the presence of β-arrestin isoforms (50–52). Thus, β-arrestin 2 may regulate the cross-desensitization via its control over p38 MAPK. Such a serial pathway could well explain the requirement of both molecules for the cross-desensitization. Our data also showed that β-arrestin and p38 MAPK clearly differed in regulating the homologous desensitization. β-arrestin 2 but not p38 MAPK was required for clonidine-induced α2A desensitization, and their roles were reversed for DAMGO-induced μ desensitization. One possibility is that homologous desensitization was primarily mediated by endocytosis of μ or α2A homodimers and monomers, which have distinct requirement for p38 MAPK and β-arrestins as compared with μ-α2A heterodimers.

In untreated α2Aβ2−/− neurons, we observed less current inhibition by DAMGO or clonidine compared with the wild-type controls. The reduced μ agonist effect was reported previously in these neurons and explained by decreased constitutive internalization and recycling of μ receptors that are constitutively coupled with Ca2+ channels. Such receptors remain on the cell membrane, reducing the pool of receptors available for ligand activation (30). A similar reduction in constitutive recycling of α2A receptors may be responsible for decreased clonidine action in β-arr2−/− neurons. Such changes, however, would not account for the lack of the cross-desensitization in β-arr2−/− neurons, since DAMGO-induced μ desensitization remained intact in these neurons.

The nonvisual β-arrestins (1 and 2) are multifunctional proteins regulating diverse cellular functions in addition to their best-recognized roles in initiating GPCR internalization. Heterodimerization of μ receptors with other GPCRs can alter receptor interaction with β-arrestin 2, leading to delayed recycling of cointernalized receptors (5) or a shift in the activation pattern of ERK pathways (53). Therefore, the absence of μ-α2A cross-desensitization in β-arr2−/− neurons could be the result of a lack of cointernalization, altered recycling, or activation of specific signaling cascades independent of internalization. A possible model congruent with our data and these recent findings is β-arrestin 2-dependent formation of a macromolecular signaling complex. The complex contains both μ and α2A receptors, either present as heterooligomers or indirectly associated with each other through binding with β-arrestins. Receptor activation leads to recruitment of specific signaling pathways, such as p38 MAPK, to the complex in an agonist-selective manner, which in turn modulates receptor trafficking and desensitization. Lack of β-arrestin 2 prevents the formation of the complex or destabilizes it, thus disrupting μ-α2A cross-regulation.

In summary, functional interactions between neuronal μ and α2A receptors can lead to mutual cross-desensitization and receptor cointernalization. p38 MAPK and β-arrestin 2 serve as two key regulators of such interactions. These findings provide new insight into the complex relationship between opioid and adrenergic systems in the pain processing pathways and functional significance of GPCR signaling complexes.

Acknowledgments—We thank Dr. David J. Jentsch for providing the α2A−/− and α2A+/- mice and Dr. Robert J. Lefkowitz for β-arr2−/− and β-arr2+/- mice.

REFERENCES

1. Bouvier, M. (2001) Nat. Rev. Neurosci. 2, 274–286
2. Marshall, F. H. (2001) Curr. Opin. Pharmacol. 1, 40–44
3. Devi, L. A. (2001) Trends. Pharmacol. Sci. 22, 532–537
4. Gurevich, V. V., and Gurevich, E. V. (2008) Trends Neurosci. 31, 74–81
5. Pfeiffer, M., Kirscht, S., Stumm, R., Koch, T., Wu, D., Laugsch, M., Schroeder, H., Poller, H., and Schulz, S. (2003) J. Biol. Chem. 278, 51630–51637
6. Jordan, B. A., Traapaizde, N., Gomes, I., Nivarthi, R., and Devi, L. A. (2001) Prog. Natl. Acad. Sci. U. S. A. 98, 343–348
7. Gomes, I., Jordan, B. A., Gupta, A., Traapaizde, N., Nagy, V., and Devi, L. A. (2000) J. Neurosci. 20, RC110, 1–5
8. Hasbi, A., Nguyen, T., Fan, T., Cheng, R., Rashid, A., Aljaniaram, M., Rasenick, M. M., O’Dowd, B. F., and George, S. R. (2007) Biochemistry 46, 12997–13009
9. Law, P. Y., Erickson-Herbrandson, L. K., Zha, Q. Q., Solberg, J., Chu, J., Sarre, A., and Loh, H. H. (2005) J. Biol. Chem. 280, 11152–11164
10. Ossipov, M. H., Harris, S., Lloyd, P., and Messineo, E. (1990) J. Pharmacol. Exp. Ther. 255, 1107–1116
11. Fairbanks, C. A., and Wilcox, G. L. (1999) J. Pharmacol. Exp. Ther. 288, 1107–1116
12. Maldonato, R. (1997) Neurosci. Biobehav. Rev. 21, 91–104
13. Lakkhini, P. P., MacMillan, L. B., Guo, T. Z., McCool, B. A., Lovinger, D. M., Maze, M., and Limbird, L. E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9950–9955
14. Stone, L. S., MacMillan, L. B., Kitto, K. F., Limbird, L. E., and Wilcox, G. L. (1997) J. Neurosci. 17, 7157–7165
15. Jordan, B. A., Gomes, I., Rios, C., Filipovska, I., and Devi, L. A. (2003) Mol. Pharmacol. 64, 1317–1324
16. Zhang, Y. Q., and Limbird, L. E. (2004) Biochem. Soc. Trans. 32, 856–860
17. Tan, M., Groszer, M., Tan, A. M., Pandya, A., Liu, X., and Xie, C. W. (2003) J. Neurosci. 23, 10292–10301
18. Hein, L., Limbird, L. E., Eglen, R. M., and Kobilka, B. K. (1999) Annu. N. Y. Acad. Sci. 881, 265–271
19. Bohn, L. M., Lefkowitz, R. J., Gainetdinov, R. R., Peppler, K., Caron, M. G., and Lin, F. T. (1999) Science 286, 2495–2498
20. Keith, D. E., Anton, B., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Monteleit-Aguis, G., Stewart, P. L., Evans, C. J., and von Zastrow, M. (1998) Mol. Pharmacol. 53, 377–384
21. Nasser, Y., Ho, W., and Sharkey, K. A. (2006) J. Comp. Neurol. 495, 529–553
22. Walwyn, W. M., Keith, D. E., Jr., Wei, W., Tan, A. M., Xie, C. W., Evans, C. J., Kiefier, B. L., and Maidment, N. T. (2004) Neuroscience 123, 111–121
23. Williams, J. P., Thompson, J. P., McDonald, J., Barnes, T. A., Cote, T., Rowbotham, D. J., and Lambert, D. G. (2007) Anesth. Analg. 105, 998–1005
24. Perez, O. D., and Nolan, G. P. (2002) Nat. Biotechnol. 20, 155–162
25. Uddin, S., Lekmine, F., Sharma, N., Majchrzak, B., Mayer, I., Young, P. R., Bokoch, G. M., Fish, E. N., and Platanias, L. C. (2000) J. Biol. Chem. 275, 27634–27640
26. Kummer, J. L., Wei, W., and von Zastrow, M. (1997) J. Biol. Chem. 272, 20490–20494
27. Whistler, J. L., and von Zastrow, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9914–9919
28. Underwood, D. C., Osborn, R. R., Bochowicz, S., Webb, E. F., Riemann, D. I., Lee, J. C., Romanic, A. M., Adams, J. L., Hay, D. W., and Griswold, D. E. (2000) Ann. J. Physiol. 279, L895–L902
29. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) FEBBS Lett. 364, 229–233
30. Walwyn, W., Evans, C. J., and Hales, T. G. (2007) J. Neurosci. 27, 5092–5104
31. Vilardaga, J. P., Nikolaev, V. O., Lorenz, K., Ferrandon, S., Zhuang, Z., and Lohse, M. J. (2008) Nat. Chem. Biol. 4, 126–131

6280 JOURNAL OF BIOLOGICAL CHEMISTRY
VOLUME 284 • NUMBER 10 • MARCH 6, 2009

ASHBURNER
p38 MAPK and β-Aरrestin 2 Mediate μ-α2A Interactions

MARCH 6, 2009 • VOLUME 284 • NUMBER 10

JOURNAL OF BIOLOGICAL CHEMISTRY 6281

32. Stone, L. S., and Wilcox, G. L. (2004) Neurosci. Lett. 361, 265–268
33. Zhang, J., Ferguson, S. S., Barak, L. S., Bodduluri, S. R., Laporte, S. A., Law, P. Y., and Caron, M. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7157–7162
34. DeGraff, J. I., Gagnon, A. W., Benovic, J. L., and Orsini, M. J. (1999) J. Biol. Chem. 274, 11253–11259
35. Connor, M., Osborne, P. B., and Christie, M. J. (2004) Br. J. Pharmacol. 143, 685–696
36. Marie, N., Aguila, B., and Allouche, S. (2006) Cell. Signal. 18, 1815–1833
37. Martini, L., and Whistler, J. L. (2007) Curr. Opin. Neurobiol. 17, 556–564
38. Borgland, S. L., Connor, M., Osborne, P. B., Furness, J. B., and Christie, M. J. (2003) J. Biol. Chem. 278, 18776–18784
39. Walwyn, W. M., Wei, W., Xie, C. W., Chiu, K., Kieffer, B. L., Evans, C. J., and Maidment, N. T. (2006) Neuroscience 142, 493–503
40. Arttamangkul, S., Torrecilla, M., Kobayashi, K., Okano, H., and Williams, J. T. (2006) J. Neurosci. 26, 4118–4125
41. Qiu, Y., Law, P. Y., and Loh, H. H. (2003) J. Biol. Chem. 278, 36733–36739
42. Koch, T., Schulz, S., Schröder, H., Wolf, R., Rauf, E., and Holtz, V. (1998) J. Biol. Chem. 273, 13652–13657
43. Law, P. Y., Erickson, L. J., El-Kouhen, R., Dicker, L., Solberg, J., Wang, W., Miller, E., Burd, A. L., and Loh, H. H. (2000) Mol. Pharmacol. 58, 388–398
44. Afify, E. A., Law, P. Y., Riedl, M., Elde, R., and Loh, H. H. (1998) Brain Res. Mol. Brain Res. 54, 24–34
45. Tsao, P. I., and von Zastrow, M. (2000) J. Biol. Chem. 275, 11130–11140
46. He, L., Fong, J., von Zastrow, M., and Whistler, J. L. (2002) Cell 108, 271–282
47. Mace, G., Miaczynska, M., Zerial, M., and Nebreda, A. R. (2005) EMBO J. 24, 3235–3246
48. Cavalli, V., Vilbois, F., Corti, M., Marcote, M. J., Tamura, K., Karin, M., Arkinstall, S., and Grunesberg, J. (2001) Mol. Cell 7, 421–432
49. McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J., and Lefkowitz, R. J. (2000) Science 290, 1574–1577
50. Sun, Y., Cheng, Z., Ma, L., and Pei, G. (2002) J. Biol. Chem. 277, 49212–49219
51. McLaughlin, N. J., Banerjee, A., Kelher, M. R., Gamboni-Robertson, F., Hamiel, C., Sheppard, F. R., Moore, E. E., and Silliman, C. C. (2006) J. Immunol. 176, 7039–7050
52. Bruchas, M. R., Macey, T. A., Lowe, J. D., and Chavkin, C. (2006) J. Biol. Chem. 281, 18081–18089
53. Rozenfeld, R., and Devi, L. A. (2007) FASEB J. 21, 2455–2465