THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 10, pp. 6270–6281, March 6, 2009

p38 MAPK and β-Arrestin 2 Mediate Functional Interactions between Endogenous μ-Opioid and α_{2A}-Adrenergic Receptors in Neurons*

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

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Formation of receptor complexes between μ-opioid and α_{2A}-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 α_{2A} receptors in mouse dorsal root ganglion neurons. Acute application of the μ agonist [D-Ala^2,*N*-MePhe^4, Gly-ol^5]enkephalin (DAMGO) or the α_2 agonist clonidine inhibited voltage-gated Ca^{2+} currents in these neurons. Prolonged treatment with either DAMGO or clonidine induced a mutual cross-desensitization between μ and α_{2A} receptor-mediated current inhibition. The cross-desensitization was closely associated with simultaneous internalization of μ and α_{2A} receptors. Morphine, a μ agonist triggering little μ receptor endocytosis, induced neither cross-desensitization nor internalization of α_{2A} receptors. Furthermore, inhibition of p38 MAPK prevented the cross-desensitization as well as cointernalization of μ and α_{2A} receptors. Changes in receptor trafficking profiles suggested that p38 MAPK activity was required for initiating μ receptor internalization and maintaining possible μ-α_{2A} association during their cointernalization. Finally, the μ-α_{2A} cross-desensitization was absent in dorsal root ganglion neurons lacking β-arrestin 2. These findings demonstrated p38 MAPK- and β-arrestin 2-dependent cross-regulation between neuronal μ and α_{2A} 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)[2] 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 β_2-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 α_{2A}-adrenergic receptors in mouse dorsal root ganglion (DRG) neurons. Both μ and α_{2A} receptors are coupled to G_i and G_o proteins and induce similar cellular responses, such as inhibition of voltage-gated Ca^{2+} 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 α_2-adrenergic agonist clonidine. Studies in transgenic mice lacking functional α_{2A} receptors further indicate that the α_{2A} receptor is the principal subtype mediating α_2 agonist-induced analgesia at the spinal level (13) and responsible for the synergistic potentiation of morphine analgesia (14). The exact mech-

* This work was supported, in whole or in part, by National Institutes of Health Grant P50DA05010. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; DRG, dorsal root ganglion; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; Gβγ, G protein βγ subunits; MAPK, mitogen-activated protein kinase; PPF, prepulse facilitation; β-arr2, β-arrestin 2; PBS, phosphate-buffered saline; NE, norepinephrine; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; CTAP, Cys^2, Tyr^3, Arg^5, Pen^7-amide; ERK, extracellular signal-regulated kinase.

anisms for this adrenergic-opioid synergy, however, remain unclear. Recently, the μ-α_{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 μ and α_{2A} receptors serves as a novel mechanism coordinating the function of both receptors in pain-processing pathways. Here we report that endogenous μ and α_{2A} receptors interact in DRG sensory neurons via p38 MAPK- and β-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, 2.5s nerve growth factor (0.1 μg/ml; Invitrogen), and 5-fluoxy-D-uridine (0.1 mg/ml; Sigma); and studied after 2–5 days *in vitro*.

DRG cultures were also prepared using postnatal day 0–3 mice lacking α_{2A} adrenergic receptors ($\alpha_{2A}{}^{-/-}$) or β-arrestin 2 (β-arr2$^{-/-}$) and their respective wild-type ($^{+/+}$) controls. The $\alpha_{2A}{}^{-/-}$ (stock number 004367; Jackson Laboratory) (18) and β-arr2$^{-/-}$ lines (19) have both been fully back-crossed to the C57 BL/6 background (10 generations). The +/+ mice used in the same experiments with the knockouts were within two generations of heterozygous mating.

Electrophysiological Recordings—The voltage-gated Ca^{2+} currents were recorded from DRG neurons with 15–30-μm diameters under whole-cell voltage clamp conditions, as described (17). Cells were perfused with an external solution containing 10 mM $CaCl_2$, 130 mM tetraethylammonium chloride, 5 mM HEPES, 25 mM D-glucose, and 0.25 μM 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 μ and α_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 μ or α_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 base-line 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 μ or α_2 agonist for 4 h. After extensive washing, whole-cell recording was performed in pretreated cells, and the acute inhibitory effect of μ or α_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 μ and α_{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 μ receptor (20) or of the α_{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 μ receptors and with biotinylated anti-goat IgG (Calbiochem) and Cy3-conjugated streptavidin (Calbiochem) to detect α_{2A} receptors. Control samples were prepared in the absence of the primary or secondary antibodies. The specificity of the anti-α_{2A} antibody was further confirmed by the absence of α_{2A} receptor labeling in neurons from the $\alpha_{2A}{}^{-/-}$ mice (see Fig. 4*B*). 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 μ Receptors—Internalization of μ 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% NaN_3. 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 FACScan 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 density of surface receptors of μ-positive cells reflected by their mean fluorescence intensity. Nonspecific background fluorescence was determined in control samples processed without the primary antibody and was subtracted to obtain mean relative 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 Thr^{180} and Tyr^{182} (Cell Signaling Technology) (25). After drug treatment, DRG cells were harvested from culture plates with PBS buffer containing 4 mM EDTA, 1 mM phenylmethylsulfonyl 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 resuspended with fresh PBS. After the final wash, the fluorescence signal of the cells was analyzed with a FACScan 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 Bonferroni *post hoc* test for pairwise comparisons. Statistical significance was defined as $p < 0.05$.

RESULTS

α_2-Adrenergic Agonist-induced Cross-desensitization to μ-Opioids—Brief bath application of norepinephrine (NE) at 10 μM induced acute inhibition of voltage-gated Ca^{2+} currents in DRG neurons (Fig. 1*A*). This effect was significantly attenuated by a selective α_2 antagonist yohimbine, confirming involvement of α_2 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 α_2 agonist, clonidine (10 μM), induced similar reductions in Ca^{2+} currents (20.0 ± 4.2%, $n = 12$) reversible by co-application of yohimbine (5.7 ± 1.6%, $n = 10$, $p < 0.01$ compared with clonidine alone). The effect of NE or clonidine was substantially reduced in neurons pretreated with the same agonist 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 neurons), indicating development of homologous desensitization to α_2 receptor-mediated effect. As expected, this desensitization was blocked by yohimbine added during NE or clonidine pretreatment (Fig. 1*A*).

We then determined the influence of prolonged α_2 agonist exposure on μ-opioid-induced Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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-treatment (Fig. 1*B*).

Inhibition of neuronal Ca^{2+} channels by GPCRs is mediated by both voltage-dependent and voltage-independent mechanisms. The voltage-dependent inhibition requires direct binding of G-protein βγ subunits (Gβγ) to the Ca^{2+} 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 perfused 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 in control perfusion medium (0.97 ± 0.02, $n = 8$) or during acute clonidine test (1.06 ± 0.03, $n = 8$). These results suggested 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 different between untreated control cells and clonidine-pretreated cells (1.68 ± 0.23 *versus* 1.68 ± 0.18 at 8 min after the application, $n = 12$ for each group, $p > 0.05$) (Fig. 1, *C* and *D*). This confirmed that clonidine-induced desensitization was not associated with a diminished cell capacity for voltage-dependent Gβγ-Ca^{2+} channel interactions.

DAMGO-induced Cross-desensitization to α_{2A} Adrenergic Responses—As we previously reported (17, 22), application of 1 μM DAMGO or morphine strongly inhibited Ca^{2+} currents in DRG neurons, but their effects were desensitized dramatically following a 4-h pretreatment (Fig. 2*A*). The inhibitory effect of 10 μM NE or clonidine on Ca^{2+} 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, Cys^2, Tyr^3, Arg^5, Pen^7-amide (CTAP), added during DAMGO pretreatment (Fig. 2*B*). 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

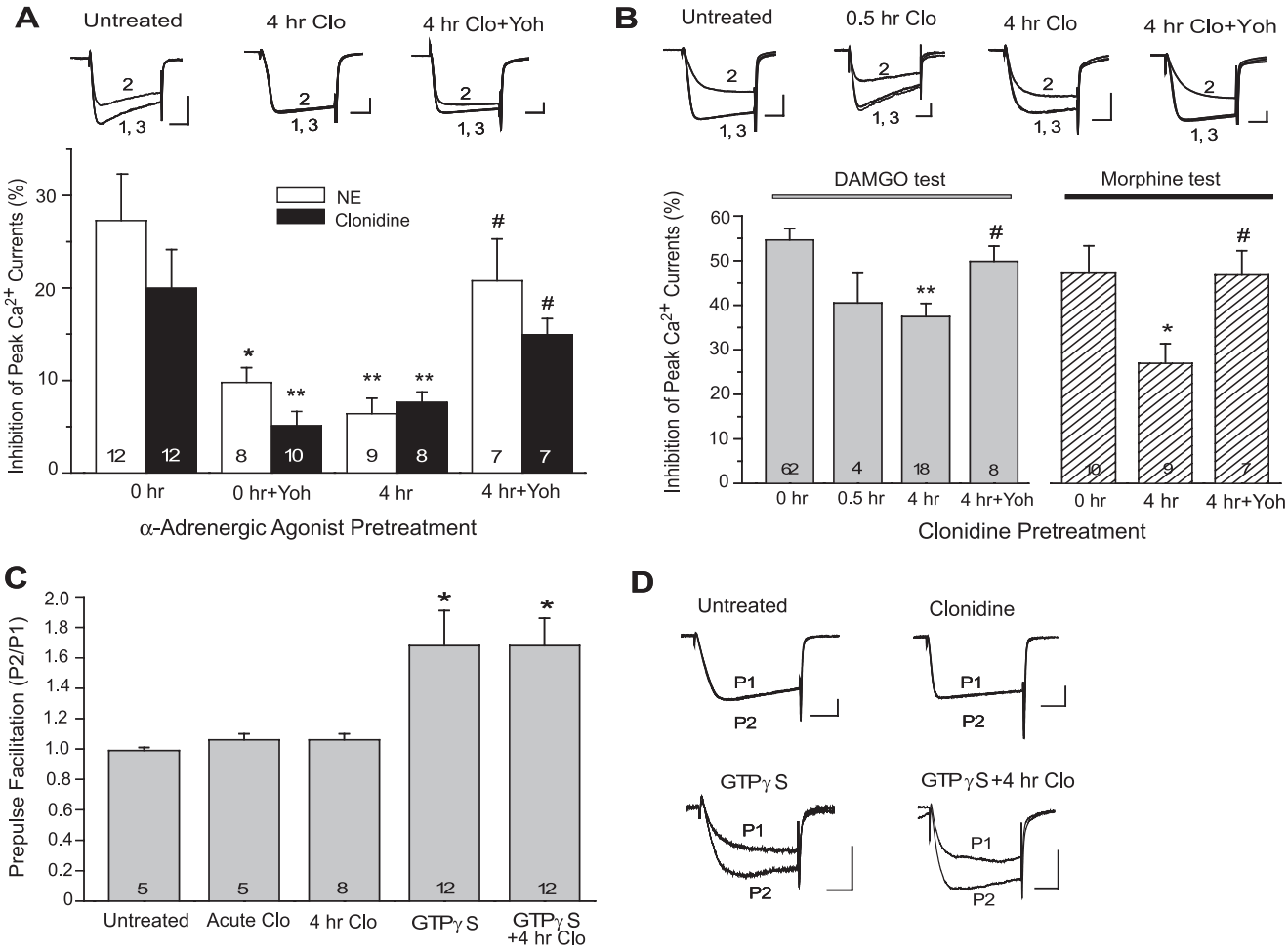

FIGURE 1. **The α_2-adrenergic agonist-induced cross-desensitization to μ-opioids in DRG neurons.** *A* and *B*, DRG cultures were pretreated with NE or the selective α_2 agonist clonidine at 10 μM for 4 h and then tested with the same agonist (*A*) or with the μ-opioid agonist DAMGO or morphine at 1 μM (*B*). Inhibition of whole-cell Ca^{2+} currents by α_2 or μ agonists was significantly reduced in clonidine-pretreated cells, suggesting development of homologous α_2 receptor desensitization and cross desensitization to μ-opioids. Both forms of desensitization were prevented by co-treatment with 10 μM yohimbine (*Yoh*), a selective α_2 antagonist. The number of cells in each group is displayed at the *bottom* of the *column*. *, $p < 0.05$; **, $p < 0.01$ compared with untreated neurons (0 h). #, $p < 0.05$ compared with neurons pretreated with clonidine alone (4 h). The *top panels* are representative current recordings collected before (*1*), during (*2*), and after (*3*) acute test with clondine (*A*) or DAMGO (*B*). Cells were pretreated as labeled. Calibration was 1 nA, 10 ms. *C*, PPF in clonidine- or GTPγS-treated cells. *D*, representative recordings of PPF. Ca^{2+} currents elicited by two test pulses, P1 and P2, were superimposed. P2 was preceded by a 40-ms depolarizing prepulse (−80 to +80 mV; data not shown). Calibration was 0.5 nA, 10 ms.

compared with untreated cells), despite development of homologous morphine desensitization in these cells (Fig. 2*A*). 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 α_2 receptors, we examined the role of α_{2A} receptors in the acute and chronic effects of clonidine. Acute application of 10 μM clonidine induced negligible current inhibition in DRG neurons derived from the $\alpha_{2A}{}^{-/-}$ mice (2.5 ± 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. 4*A*). These results suggested that in our preparation, the α_{2A} receptor was the major subtype responsible for clonidine-induced Ca^{2+} current inhibition and its cross-desensitization to DAMGO.

Agonist-induced Cointernalization of μ and α_{2A} Receptors—Next we determined whether the mutual cross-desensitization between μ and α_{2A} receptors was associated with changes in agonist-induced receptor internalization. Immunohistochemical double labeling and confocal microscopy showed that a substantial portion of μ and α_{2A} receptors were colocalized on the cell membrane throughout the cell body and processes of cultured DRG neurons under basal conditions (Fig. 3*A*). Incubation with DAMGO for 30 min to 4 h induced simultaneous internalization of both receptors, which was blocked by co-incubation with the μ antagonist CTAP (Fig. 3*C*). Clonidine also elicited internalization of both μ and α_{2A} receptors, reversible by the α_2 antagonist yohimbine (Fig. 3*D*). In contrast, morphine treatment up to 4 h failed to internalize either μ or α_{2A} receptors (Fig. 3*B*). Furthermore, clonidine did not induce μ receptor endocytosis in $\alpha_{2A}{}^{-/-}$ neurons (Fig. 4*B*). Thus, similar to the cross-desensitization, the cointernalization was agonist-selective and dependent upon the presence of functional μ and α_{2A} receptors.

Fluorescence flow cytometry was then conducted in living DRG neurons to quantify agonist-induced μ receptor internalization. Surface μ receptors were detected in 77.7 ± 3% of control cells. DAMGO treatment for 4 h reduced the portion of

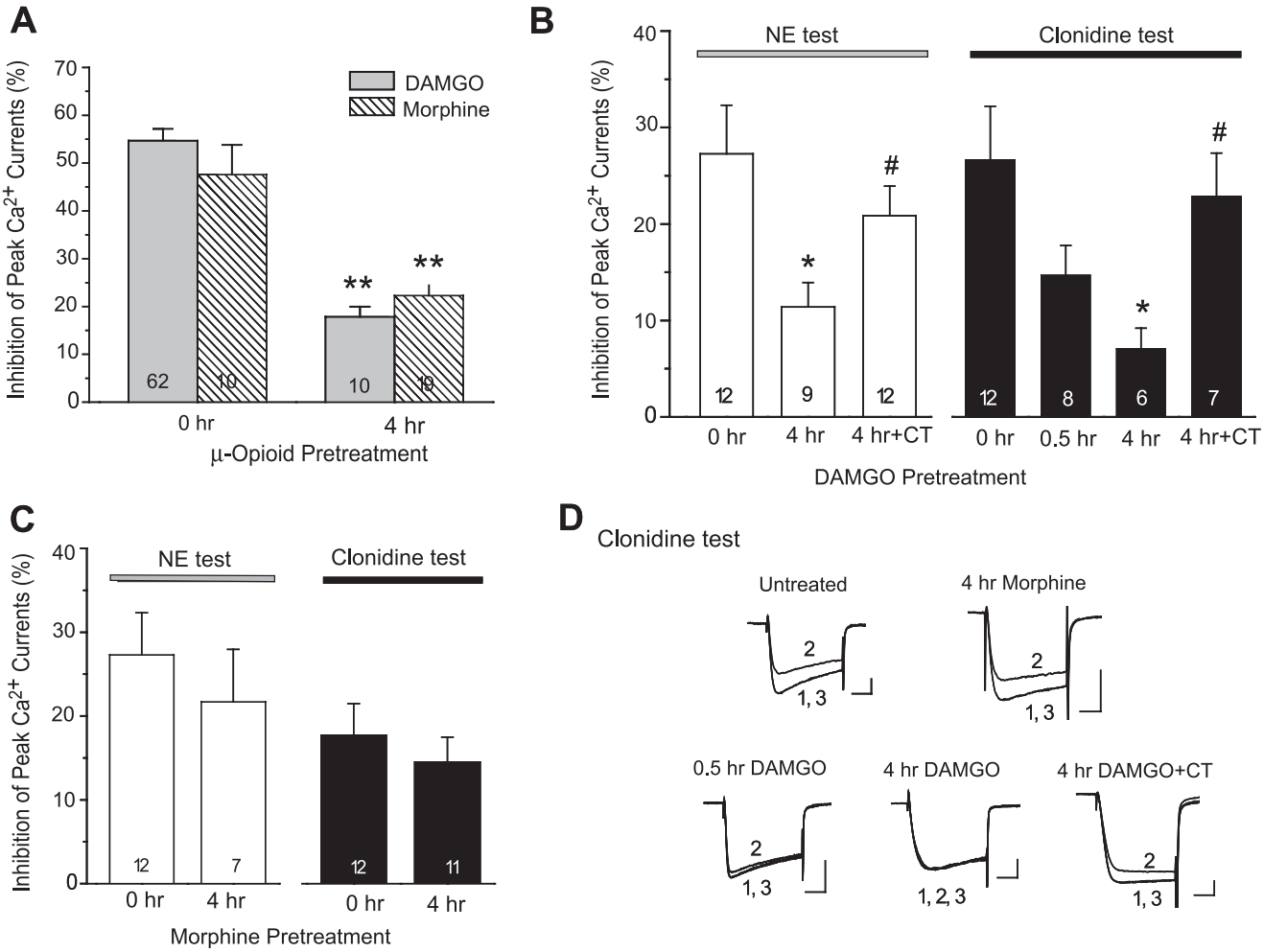

FIGURE 2. **The μ agonist-induced cross desensitization to α_2-adrenergic responses in DRG neurons.** *A*, DRG cultures were pretreated with either DAMGO or morphine at 1 μM for 4 h and tested with the same agonist after washing. Both μ agonists induced homologous μ receptor desensitization. *B* and *C*, cultures were pretreated with a μ agonist and tested with 10 μM NE or clonidine. DAMGO but not morphine induced cross-desensitization to the effect of α_2 agonists. The selective μ antagonist CTAP (*CT*; 1 μM) applied with DAMGO blocked induction of cross-desensitization. *, $p < 0.05$; **, $p < 0.01$ compared with untreated neurons (0 h). #, $p < 0.05$ compared with neurons pretreated with DAMGO alone (4 h). *D*, representative current recordings collected before (*1*), during (*2*), and after (*3*) acute clonidine test. Cells were pretreated as labeled. Calibration was 0.5 nA, 10 ms.

cells expressing detectable levels of surface μ receptors (μ-positive cells) by 26.3 ± 5.8% ($p < 0.05$) relative to the controls (Fig. 5*B*). 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. 5*A*). 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-α_{2A} Cross-desensitization and Cointernalization by p38 MAPK Inhibitors—To explore the signaling mechanisms underlying μ-α_{2A} 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. 6*A*, 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.

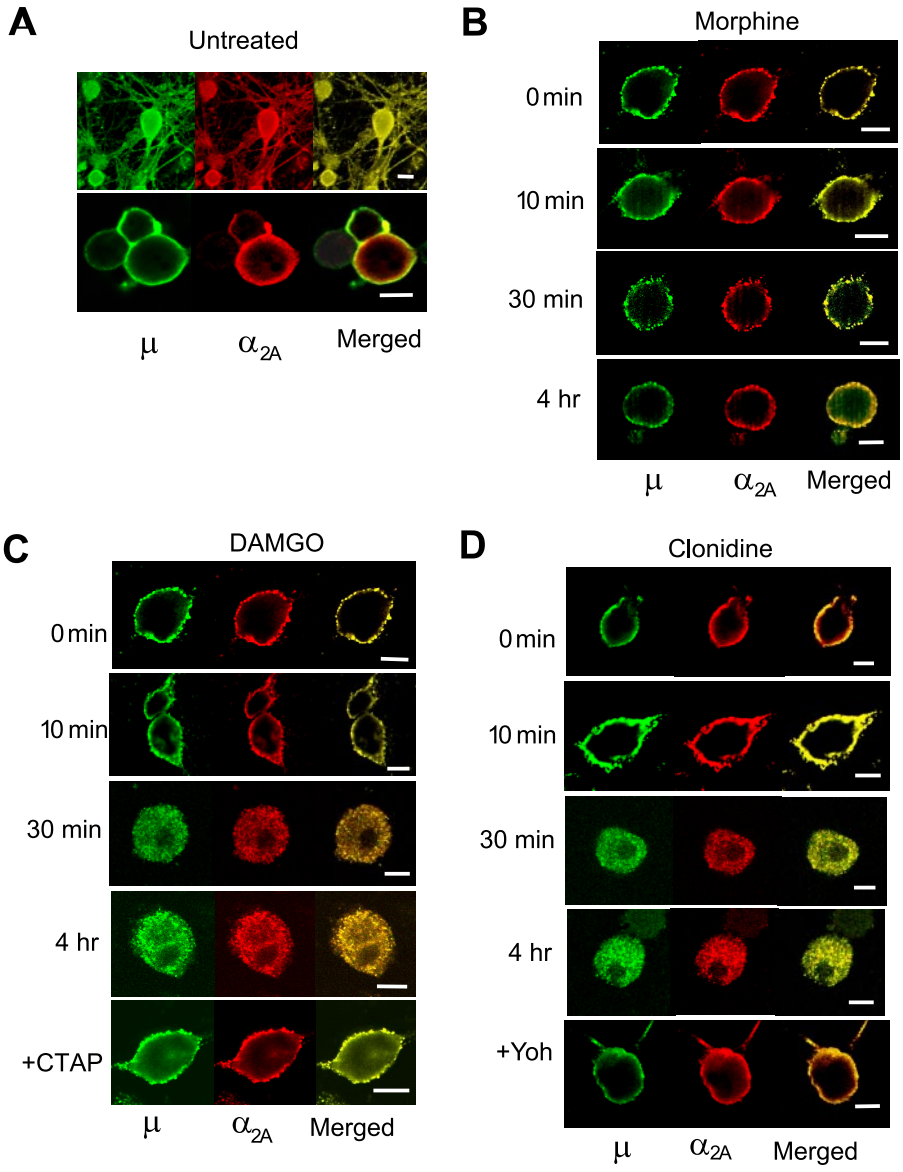

FIGURE 3. **Confocal microscopic images of DRG neurons showing colocalization and cointernalization of μ and α_{2A} receptors.** Cultured neurons were fixed, permeabilized, and labeled with polyclonal antibodies against native μ and α_{2A} receptors. The μ receptor was visualized with Alexa Fluor 488-labeled anti-rabbit IgG (*green*), and the α_{2A} receptor was detected with biotinylated anti-goat IgG and Cy3-conjugated streptavidin (*red*). *A*, in untreated neurons, μ and α_{2A} receptors were colocalized in plasma membrane throughout cell bodies and processes as shown by multiple scan images (*top*). Single scan images (*bottom*) reveal a substantial but not complete overlapping of μ and α_{2A} receptor localization in cell membrane. *C* and *D*, treatment with DAMGO (1 μM) or clonidine (10 μM) induced simultaneous internalization of μ and α_{2A} receptors, which was blocked by the selective μ antagonist CTAP or the α_2 antagonist yohimbine (*Yoh*), respectively. *B*, morphine (1 μM) failed to trigger internalization of either μ or α_{2A} receptors. All experiments were repeated at least three times, and representative samples are shown here. *Scale bars*, 10 μm.

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. 6*B*). 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. 6*B*). 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 α_2 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-α_2 interaction leading to the mutual cross-desensitization.

Since the cross-desensitization was closely associated with cointernalization of μ and α_{2A} 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 α_{2A} receptors in DAMGO-treated cells (Fig. 6*C*). Interestingly, PD169316 selectively blocked internalization of μ but not α_{2A} receptors in clonidine-treated cells. This effect led to separation of the two receptors during clonidine treatment, with α_{2A} receptors being internalized but μ receptors remaining on the plasma membrane of DRG neurons (Fig. 6, *C* 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 α_{2A} receptors.

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

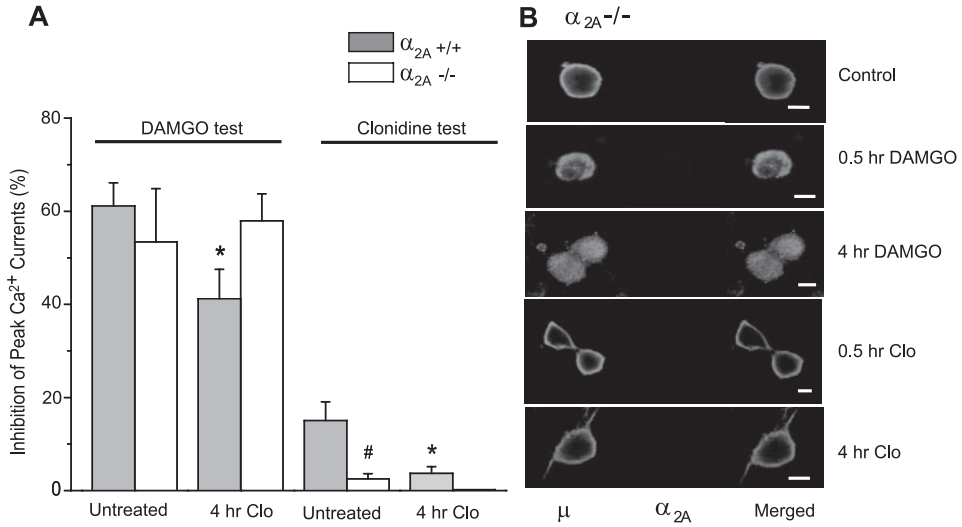

FIGURE 4. **Clonidine does not induce μ receptor internalization and cross-desensitization in $\alpha_{2A}^{-/-}$ neurons.** *A*, DRG cultures derived from the $\alpha_{2A}^{-/-}$ and $\alpha_{2A}^{+/+}$ mice were pretreated with 10 μM clonidine for 4 h and tested with DAMGO or clonidine. No cross-desensitization to DAMGO was observed in $\alpha_{2A}^{-/-}$ neurons. Also note the lack of responses to clonidine in $\alpha_{2A}^{-/-}$ neurons. $n = 4–8$ for each group; *, $p < 0.05$ compared with untreated neurons of the same genotype. #, $p < 0.05$ compared with cells with different genotype but receiving the same treatment. *B*, confocal microscopic images of $\alpha_{2A}^{-/-}$ neurons processed for double labeling of μ and α_{2A} receptors. Note the absence of α_{2A} receptor labeling and the inability of clonidine to induce μ receptor endocytosis in these neurons. *Scale bars*, 10 μm.

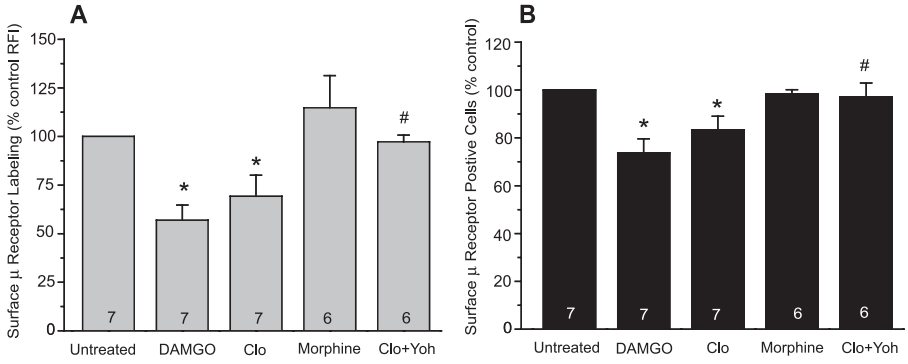

FIGURE 5. **Flow cytometric analysis of μ receptor internalization induced by μ or α_2 agonists.** DRG cultures were treated with DAMGO (1 μM), morphine (1 μM), or clonidine (10 μM) for 4 h. Cell surface μ receptors were labeled with an antibody recognizing the third extracellular loop of the μ receptor. Agonist-induced μ receptor internalization was quantified by percentage changes in the mean relative fluorescence intensity (*RFI*) of surface μ receptor labeling (*A*) and in the proportion of the cells expressing a detectable level of surface μ receptors (μ-positive cells) (*B*). Both DAMGO and clonidine significantly reduced surface μ receptor labeling, whereas morphine failed to do so. The effect of clonidine was blocked by co-treatment with yohimbine (*Clo + Yoh*). *, $p < 0.05$ compared with untreated cells. #, $p < 0.05$ compared with cells treated with clonidine alone (*Clo*).

tion of p38 MAPK, increasing the phospho-p38 MAPK level to 200, 207, 378, and 336% of the control level after 5, 15, 30, and 60 min of exposure, respectively. When comparing the peak effect of each agonist after a 30-min exposure, DAMGO (0.3 and 1 μM) or clonidine (3 and 10 μM) induced dose-dependent increases in phospho-p38 MAPK levels, and their effects were blocked by the p38 inhibitor PD169316 (Fig. 7*A*). Morphine (1 μM) produced a small and insignificant increase in p38 MAPK phosphorylation (136 ± 16%, $p > 0.05$ compared with vehicle controls).

In agreement with differential activation of p38 MAPK by the two opioid agonists, PD169316 selectively blocked DAMGO- but not morphine-induced homologous desensitization (Fig. 7*B*). 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. 7*C*). This indicated that p38 MAPK activity was also differentially involved in the homologous *versus* heterologous desensitization of α_{2A} receptors.

The Essential Role of β-Arrestin 2 in μ-α_{2A} 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 α_{2A} receptor desensitization using DRG neurons from β-arr2$^{-/-}$ 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-arr2$^{+/+}$ neurons. Similar to our previous observations (30), acute responses to DAMGO were smaller in untreated β-arr2$^{-/-}$ neurons compared with their wild-type counterparts. This, however, did not affect development of homologous DAMGO desensitization in β-arr2$^{-/-}$ neurons (Fig. 8*E*). Importantly, the cross-desensitization was completely absent in β-arr2$^{-/-}$ 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-arr2 abolished clonidine- but not DAMGO-induced homologous desensitization (Fig. 8, *D* and *E*).

DISCUSSION

Cross-regulation of μ and α_{2A} Receptor Signaling in Neurons—Although formation of μ and α_{2A} receptor complexes has been convincingly demonstrated in cell lines and transfected neurons (15), functional significance and regulatory mechanisms for the μ-α_{2A} interaction remain to be clarified. The initial

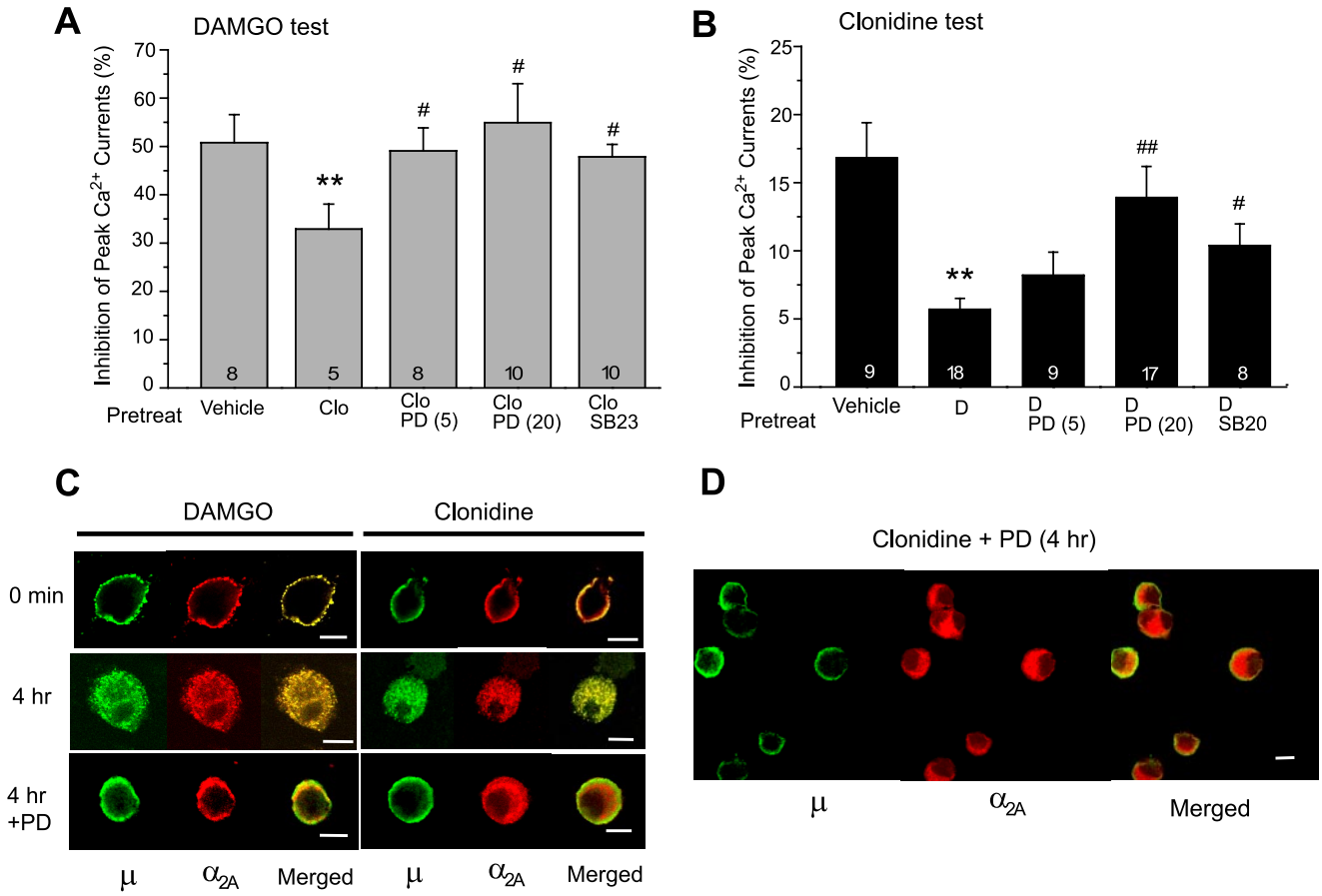

FIGURE 6. **p38 MAPK activity is required for μ-α_{2A} cross-desensitization and cointernalization.** *A* and *B*, DRG cultures were pretreated with 1 μM DAMGO (*D*) or 10 μM clonidine (*Clo*) for 4 h in the presence or absence of a selective p38 inhibitor and examined for the cross-desensitization. Clonidine-induced cross-desensitization to DAMGO was completely prevented by 5 or 20 μM PD169316 (*PD*; *5* and *20*), or by 20 μM SB239063 (*SB23*). Similarly, DAMGO-induced cross-desensitization to clonidine was dose-dependently reduced by PD169316 and significantly attenuated by another p38 inhibitor SB203580 (*SB20*; 20 μM). **, $p < 0.01$ compared with vehicle controls pretreated with medium containing 0.1% DMSO. #, $p < 0.05$; ##, $p < 0.01$ compared with cells pretreated with clonidine or DAMGO alone. *C* and *D*, PD169316 (20 μM) blocked DAMGO-induced cointernalization of μ and α_{2A} receptors. The p38 inhibitor did not block clonidine-induced α_{2A} receptor internalization but prevented cointernalization of μ receptors, leading to dissociation of the two receptors. Immunocytochemical labeling of μ and α_{2A} receptors was performed as described in the legend to Fig. 3. *Scale bars*, 10 μm.

report showed that heterodimerization of μ and α_{2A} 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 α_{2A} receptors within the heterocomplex that allows rapid inaction of one receptor by the other with subsecond kinetics (31). In locus coeruleus neurons naturally expressing high levels of α_{2A} and μ receptors, analysis of the effect of co-applied μ and α_{2A} agonists reveals no functional interactions between the two receptors (32). These findings raised the questions as to whether and how naturally existing μ and α_{2A} receptors in neurons could interact with each other to regulate cellular function. The present study demonstrated strong functional interactions between endogenous μ and α_{2A} 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-Ca^{2+} channel interactions (17). In the present study, however, neither acute nor prolonged clonidine treatment altered PPF, a direct measure of voltage-dependent Gβγ effect on Ca^{2+} channels. The μ-α_{2A} cross-desensitization was also unaffected by the selective inhibitors for phosphoinositide 3-kinase or ERK. These results suggest that modification of Gβγ-Ca^{2+} 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 α_{2A} desensitization, suggesting that divergent rather than common signaling pathways were engaged by μ and α_{2A} receptors.

Receptor Cointernalization Contributes to μ-α_{2A} Cross-desensitization—An important feature of the cross-desensitization was its close association with agonist-induced cointernalization of μ and α_{2A} receptors. It is well documented that both μ and α_{2A} receptors undergo agonist-induced rapid endocytosis via clathrin coated-pits, a process regulated by receptor phosphorylation and binding with nonvisual β-arrestins (27,

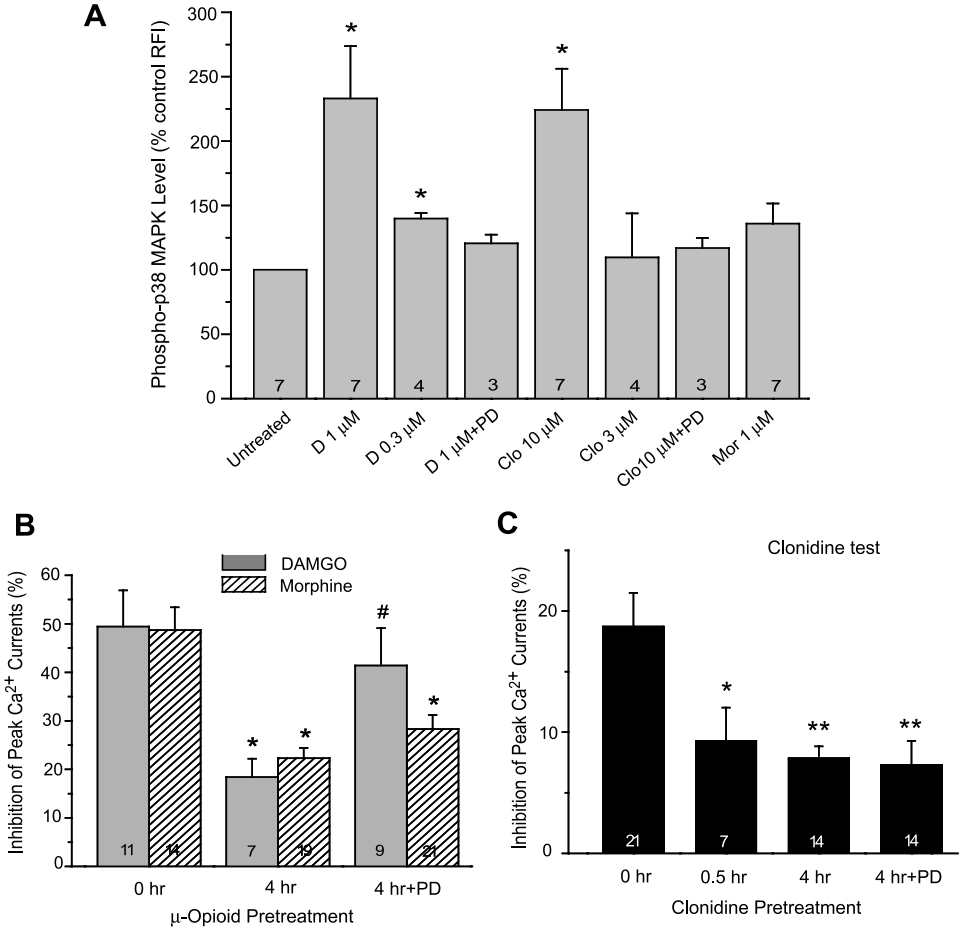

FIGURE 7. **Agonist-selective activation of p38 MAPK and its differential roles in homologous μ and α_{2A} receptor desensitization.** *A*, a 30-min treatment with DAMGO (1 μM) or clonidine (10 μM), but not morphine (1 μM), induced significant increases in the level of phospho-p38 MAPK in DRG neurons. *B* and *C*, DRG neurons were pretreated and tested with the same agonist to measure the homologous μ or α_{2A} receptor desensitization. Co-treatment with the p38 inhibitor PD169316 (20 μM) prevented DAMGO desensitization but not morphine or clonidine desensitization. *, $p < 0.05$; **, $p < 0.01$ compared with untreated neurons (0 h). #, $p < 0.05$ compared with neurons pretreated with the same agonist in the absence of the p38 inhibitor (4 h).

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 contribute to rapid desensitization of μ receptors coupled to voltage-gated Ca^{2+} channels (38, 39) or inwardly rectifying potassium 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 treatment 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 receptor resensitization, 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 internalized receptor to lysosomes for degradation, causing receptor down-regulation and more persistent signaling reduction (44, 45). Therefore, the functional consequence of receptor internalization may vary considerably 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 the receptor.

Receptor oligomerization has added a new dynamic to the complex 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 receptors are both internalized in response to a single selective agonist (5, 6, 8), suggesting that ligand-activated receptor may "drag" another receptor in the same complex to the endocytic pathway (46). Similarly, we demonstrated that μ and α_{2A} receptors colocalized on the plasma membrane of untreated DRG neurons and underwent simultaneous internalization when either receptor was activated. These findings were in agreement with the presence of μ-α_{2A} complexes. Nevertheless, a fraction of μ and α_{2A} receptors were likely to exist as homodimers or monomers (4, 8), as indicated by the less than complete colocalization of the two receptors in DRG neurons and a relatively low percentage of μ receptors cointernalized by clonidine measured with flow cytometry.

Importantly, the propensity of μ and α_{2A} agonists to promote cross-desensitization was closely related to their ability to induce receptor cointernalization. Exposure to DAMGO or clonidine induced both cointernalization and cross-desensitization, whereas morphine treatment resulted in neither. Furthermore, blocking μ-α_{2A} cointernalization with p38 MAPK inhibitors effectively prevented the cross-desensitization. These findings strongly supported a crucial role of receptor cointernalization in μ-α_{2A} cross desensitization. It is conceivable that a substantial portion of μ and α_{2A} receptors coexist on cell surface as receptor complexes and internalize together via p38 MAPK-dependent mechanisms. During chronic agonist treatment, receptor cointernalization may be coupled with delayed recycling, which reduces cell surface receptors and attenuates signaling. Indeed, μ receptors are known to traffic through both early and late sorting endosomes in DRG neurons, two sorting pathways differing significantly in the rate of recycling (39). If interactions with α_{2A} receptors promote μ receptors to traffic through the slower late sorting pathway,

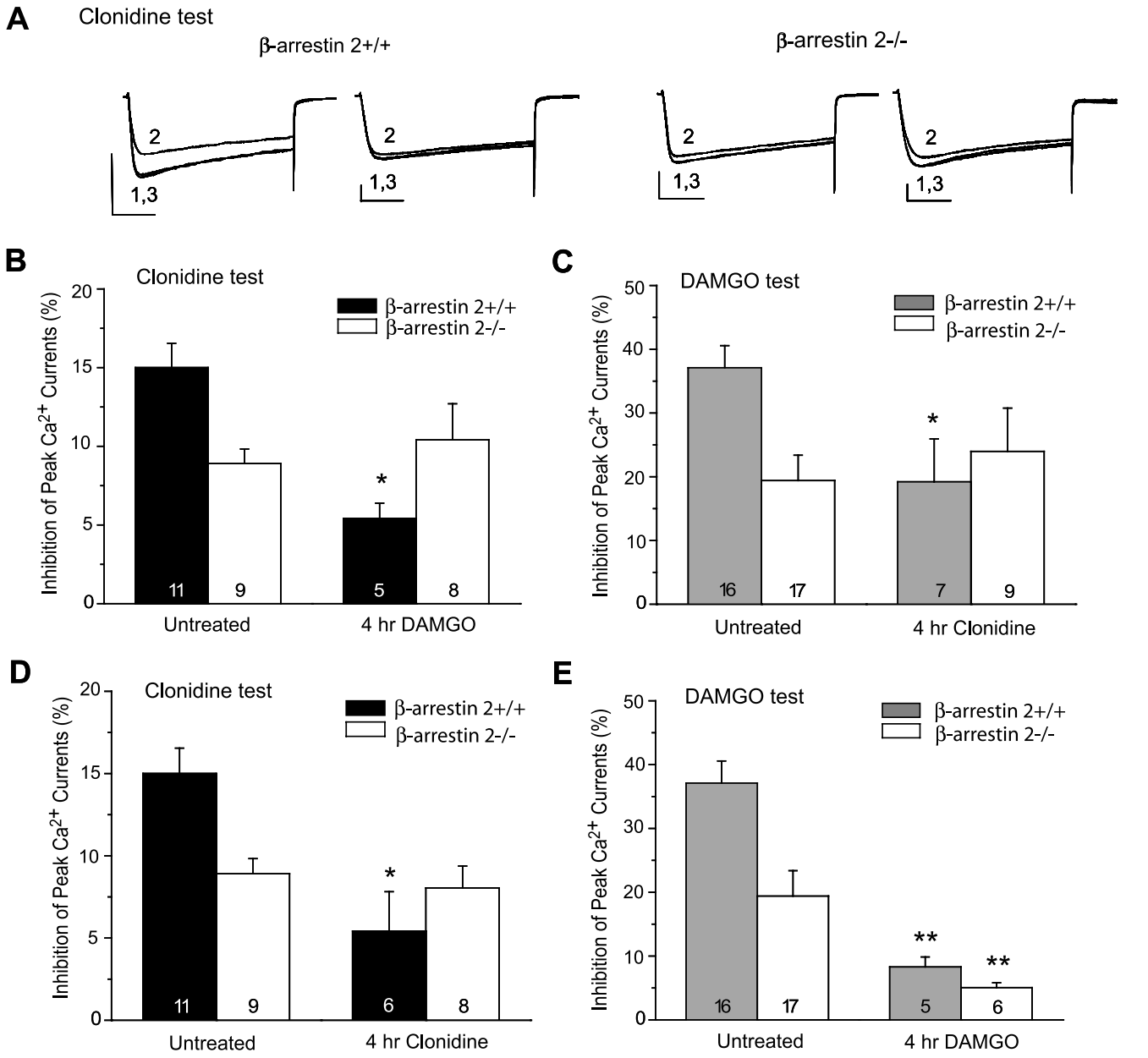

FIGURE 8. **The crucial role of β-arrestin 2 in μ-α_{2A} cross-desensitization.** DRG neurons derived from the β-arrestin $2^{-/-}$ and β-arrestin $2^{+/+}$ mice were pretreated with 1 μM DAMGO or 10 μM clonidine for 4 h and tested for the cross-desensitization and homologous desensitization. The μ-α_{2A} cross-desensitization induced by DAMGO (*B*) or clonidine (*C*) was clearly seen in β-arrestin $2^{+/+}$ neurons but completely absent in β-arr$2^{-/-}$ 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 α_{2A} receptor desensitization was also absent in β-arr$2^{-/-}$ 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-α_{2A} 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 and α_{2A} 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 α_{2A} 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 α_{2A} receptors. Second, our results indicated that p38 activity was not required for clonidine-initiated α_{2A} receptor internalization but was necessary for co-trafficking of μ receptors with activated α_{2A} receptors. Thus, activation of p38 MAPK may be essential for maintaining μ-α_{2A} association during the cointernalization.

Another key regulator of the μ-α_{2A} cross-modulation identified in this study was β-arrestin 2. Deletion of β-arrestin 2 in DRG neurons prevented μ-α_{2A} cross-desensitization, an effect similar to that of p38 MAPK inhibitors. Studies have shown that

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 α_{2A} 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 α_{2A} homodimers and monomers, which have distinct requirement for p38 MAPK and β-arrestins as compared with μ-α_{2A} heterodimers.

In untreated β-arr2$^{-/-}$ 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 Ca^{2+} 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 α_{2A} 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-α_{2A} 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 α_{2A} 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-α_{2A} cross-regulation.

In summary, functional interactions between neuronal μ and α_{2A} 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 $\alpha_{2A}{}^{-/-}$ and $\alpha_{2A}{}^{+/+}$ 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., Schroder, H., Hollt, V., and Schulz, S. (2003) *J. Biol. Chem.* **278,** 51630–51637
6. Jordan, B. A., Trapaidze, N., Gomes, I., Nivarthi, R., and Devi, L. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 343–348
7. Gomes, I., Jordan, B. A., Gupta, A., Trapaidze, 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., Alijaniaram, M., Rasenick, M. M., O'Dowd, B. F., and George, S. R. (2007) *Biochemistry* **46,** 12997–13009
9. Law, P. Y., Erickson-Herbrandson, L. J., 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. Maldonado, R. (1997) *Neurosci. Biobehav. Rev.* **21,** 91–104
13. Lakhlani, 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, J., 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) *Ann. N. Y. Acad. Sci.* **881,** 265–271
19. Bohn, L. M., Lefkowitz, R. J., Gainetdinov, R. R., Peppel, 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., Monteillet-Agius, 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., Kieffer, 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., Rao, P. K., and Heidenreich, K. A. (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., Bochnowicz, S., Webb, E. F., Rieman, D. J., Lee, J. C., Romanic, A. M., Adams, J. L., Hay, D. W., and Griswold, D. E. (2000) *Am. 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) *FEBS 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

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. L., 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., Schroder, H., Wolf, R., Raulf, E., and Hollt, 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 Gruenberg, 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