Tyrosine Phosphorylation of $K_{ir}$3 following $\kappa$-Opioid Receptor Activation of p38 MAPK Causes Heterologous Desensitization*

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Prior studies showed that tyrosine 12 phosphorylation in the N-terminal, cytoplasmic domain of the G-protein-gated inwardly rectifying potassium channel, $K_{ir}$3.1 facilitates channel deactivation by increasing intrinsic GTPase activity of the channel. Using a phospho-selective antibody directed against this residue (pY12), we now report that partial sciatic nerve ligation increased pY12-$K_{ir}$3.1-immunoreactivity (ir) in the ipsilateral dorsal horn of wild-type mice, but not in mice lacking the $\kappa$-opioid receptor (KOR) or lacking the G-protein receptor kinase 3 (GRK3) genes. Treatment of AtT-20 cells stably expressing KOR-GFP with the selective KOR agonist U50,488 increased both phospho-p38-ir and pY12-$K_{ir}$3.1-ir. The U50,488-induced increase in pY12-$K_{ir}$3.1-ir was blocked by the p38 inhibitor SB203580. Cells expressing KOR(S369A)-GFP did not increase either phospho-p38-ir or pY12-$K_{ir}$3.1-ir following U50,488 treatment. Whole cell voltage clamp of AtT-20 cells expressing KOR-GFP demonstrated that p38 activation by U50,488 reduced somatostatin-evoked $K_{ir}$3 currents. This heterologous desensitization was blocked by SB203580 and was not evident in cells expressing KOR(S369A)-GFP. Tyrosine phosphorylation of $K_{ir}$3.1 was likely mediated by p38 MAPK activation of Src kinase. U50,488 also increased (pY418)Src-ir; this increase was blocked by SB203580 and not evident in KOR(S369A)-GFP expressing AtT20 cells; the Src inhibitor PP2 blocked the U50,488-induced increase in pY12-$K_{ir}$3.1-ir; and the heterologous desensitization of $K_{ir}$3 currents was blocked by PP2. These results suggest that KOR causes phosphorylation of Y12-$K_{ir}$3.1 and channel inhibition through a GRK3-, p38 MAPK- and Src-dependent mechanism. Reduced inward potassium current following nerve ligation would increase dorsal horn neuronal excitability and may contribute to the neuropathic pain response.

G-protein-gated inwardly rectifying potassium ($K_{ir}$3.x)$^2$ channels are important for controlling cellular excitability and contribute to the postsynaptic response of certain inhibitory transmitters and hormones (1). Release of the $G\beta\gamma$ subunit following activation of the $G_{i/o}$ class of G-protein-coupled receptors (GPCRs) directly activates $K_{ir}$3 channels, and channel deactivation occurs when the GTP bound to Go is hydrolyzed to GDP, and $G\beta\gamma$ dissociates from the channel (2, 3).

The activity of $K_{ir}$3 channels can be modulated by phosphorylation of tyrosine 185 in $K_{ir}$3.1 reduces open-state probability (4), and phosphorylation of tyrosine 16 on $K_{ir}$3.5 reduces in basal channel conductance by unmasking a GAP downstream of receptor (5). Research from our group has shown that after heterologous gene expression of the $K_{ir}$3.1 subunits and the TrkB receptor in Xenopus oocytes, activation of the receptor-tyrosine kinase with BDNF reduced channel current. This suppression was blocked by pretreatment with tyrosine kinase inhibitors and not seen after two N-terminal tyrosines (Tyr-12 and Tyr-67) were mutated to phenylalanines in $K_{ir}$3.1 (6).

Phosphorylation of tyrosine 12 on the N terminus of $K_{ir}$3.1 leads to a reduction in basal channel conductance by unmasking a GAP (7). Using an antibody specific for phosphorylation of tyrosine 12 on $K_{ir}$3.1, we previously found an increase in pY12-$K_{ir}$3.1-ir following exposure to behavioral stressors including acute inflammation, chronic neuropathic pain, and swim stress (8). The goal of this study was to identify the molecular mechanisms underlying phosphorylation of Y12-$K_{ir}$3.1 seen in these stress models.

The opioid receptors ($\mu$, $\kappa$, $\delta$) belong to the $G_{i/o}$ class of GPCRs that directly activate $K_{ir}$3 channels. There is a growing body of evidence suggesting that the dynorphin/$\kappa$-opioid receptor (KOR) system plays an important role in the behavioral responses to stress. For example, dynorphin knock-out animals or animals that had been treated with norBNI, a selective KOR antagonist, showed less immobility after a forced swim stress (9–11). Dynorphin is also important for the development and maintenance of neuropathic pain (12–14).

Agonist activation of KOR stimulates all three mitogen-activated protein kinases (MAPK) including extracellular signal-related kinase (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK in vitro (16–18). There is also evidence that activation of the dynorphin/KOR system after forced swim stress and
neuropathic pain leads to activation of downstream signaling effectors. For example, repeated swim stress activates ERK1/2 and p38 in a KOR-dependent manner (19, 20), and neuropathic pain produces KOR-dependent p38 MAPK activation (21). We hypothesized that activation of the dynorphin/KOR and subsequent activation of second messenger cascades following behavioral stress exposure may be responsible for tyrosine phosphorylation of K_\text{v}3.1.

In this study, we used partial sciatic nerve ligation (pSNL) to induce an increase in pY12-K_\text{v}3.1-ir. Then using pharmacological and genetic approaches, we defined the molecular signal transduction mechanisms linking activation of the dynorphin/KOR system to the potassium channel response. Using an \textit{in vitro} model, we found that KOR-dependent increases in pY12-K_\text{v}3.1-ir required GRK3 phosphorylation of KOR, p38 MAPK activation, and subsequent Src activation. As predicted from earlier studies using heterologous gene expression in \textit{Xenopus} oocytes (6–8), this KOR-dependent phosphorylation of K_\text{v}3.1 decreased channel conductance.

### EXPERIMENTAL PROCEDURES

**Chemicals**—Norbinaltorphimine HCl and (-) U50,488 were obtained from Tocris (Ellisville, MO) and the NIDA Drug Supply Program. SB203580 HCl and PP2 were obtained from Calbiochem (Gibbstown, NJ), and somatostatin was obtained from Sigma. Drugs were dissolved in water unless otherwise indicated.

**Cell Culture and Transfection of AtT-20 Cells**—AtT-20 cells expressing rat KOR-GFP and rat KOR(S369A)-GFP were generated as previously described (10) where ≥90% of both cell lines express the GFP-tagged receptor at roughly equal amounts (similar B_max) (16). KOR-GFP and KOR(S369A)-GFP AtT-20 cells were grown in Dulbecco's modified medium with 10% horse serum (Sigma).

**Antibody Purification**—The phosphospecific polyclonal antibody for tyrosine 12 in the N terminus of the G-protein-gated inwardly rectifying potassium channel, pY12-K_\text{v}3.1, was generated and affinity purified as described previously (8).

**Western Blot**—Untransfected AtT-20 cells or AtT-20 cells expressing KOR-GFP or KOR(S369A)-GFP were cultured in 6-well plates, incubated for 48 h, and then serum-starved overnight. The cells were either pretreated with norBNI or a vehicle control for 1 h prior to treatment with U50,488. In other experiments, cells were pretreated with SB203580 or a vehicle control for 30 min prior to agonist treatment. After different durations of agonist exposure, the medium was aspirated, the cells were washed with PBS (Invitrogen), and 250 μl of ice-cold lysis buffer (22) containing 1:100 dilutions of protease inhibitor mixture set 1 and of phosphatase mixture inhibitor set 1 (Calbiochem) was added to each well. Lysates were sonicated for 10 s and then centrifuged for 15 min (14,000 × g, 4 °C). The supernatant was transferred to a new tube, and the protein concentration determined by BCA protein assay (Thermo Scientific, Rockford, IL). 30–40 μg of protein were resolved by SDS-PAGE on 10% Bis-Tris NuPAGE (Invitrogen) gels and transferred to nitrocellulose. Blots were blocked in 5% nonfat milk in Tris-buffered saline (TBS), pH 7.4, for 2 h at room temperature then incubated in rabbit anti-pY12-K_\text{v}3.1, 30 μg/ml, or rabbit anti-K_\text{v}3.1 (Millipore, Billerica, MA), 1:200, diluted in blocking buffer. Following overnight incubation, blots were washed 4 × 10 min in TBS containing 1% Tween-20 (TBST), then incubated in IR-Dye\textsuperscript{TM} 800-conjugated affinity-purified anti-rabbit IgG (Rockland Immun diagonal, Gilbertsville, PA) at a dilution of 1:7500 in a 1:1 mixture of 5% milk/TBS and Li-Cor Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) for 1 h at room temperature. Membranes were then washed 3 × 5 min in TBST, 2 × 5 min in TBS and analyzed using the Odyssey infrared imaging system (Li-Cor Biosciences). Blots were also probed with anti-β-actin to control for equal protein loading, and band intensities were measured as described previously (16). In experiments probing for phospho-Src, the treatments were as described above except membranes were incubated in Src-pY416 (1:1000, Cell Signaling Technology Inc., Danvers, MA), diluted in 5% bovine serum albumin/TBST overnight at 4 °C.

**Immunocytochemistry**—Untransfected, KOR-GFP, and KOR(S369A)-GFP expressing AtT-20 cells were plated onto poly-d-lysine-coated coverslips (BD Biosciences, San Jose, CA) and incubated for 24 h. The cells were then serum-starved overnight and treated with drugs as described above. After drug treatment, the medium was aspirated, and cells were washed once with PBS and fixed in 4% paraformaldehyde (PFA) for 20 min. Postfixation, cells were washed 2 × 5 min in PBS, incubated in blocking buffer (0.3% gelatin/0.025% Triton X-100 in PBS) for 2 h at room temperature, and then incubated 72 h at 4 °C in primary antibody (rabbit anti-pY12-K_\text{v}3.1, 6 μg/ml) diluted in blocking buffer. In some experiments, cells were incubated in rabbit anti-pY12-K_\text{v}3.1 (see dilution above) and mouse anti-Src(pY418) at a dilution of 1:300 in blocking buffer (Calbiochem) for 72 h at 4 °C. Cells were then washed 4 × 10 min in PBS and incubated in Alexa Fluor\textsuperscript{®} 633 anti-mouse and/or Alexa Fluor\textsuperscript{®} 555 anti-rabbit antibody (Molecular Probes, Eugene, OR) at a dilution of 1:1000 in blocking buffer for 2 h at room temperature. After four 10-min washes in PBS and two 5-min washes in 0.1 M phosphate buffer (PB), pH 7.4, coverslips were air dried and then mounted on slides with Vectashield (Vector Laboratories, Burlington, CA). Coverslips were viewed by Leica SL confocal microscopy. Quantification of pixel intensity was performed as described previously (8) in which the plasma membrane was outlined, and average pixel intensity was determined from the defined region. Quantitation of pixel intensity for a group of >25–30 cells per coverslip was averaged and treated as an individual determination; the number of independent replicate determinations (n) is given for each experiment.

**Animals and Housing**—Male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) weighing 25–35 g were housed in a 12:12 light/dark cycle in groups of two or four. Animal procedures and housing were approved by the University of Washington Institutional Animal Care and Use Committee. Homozygous KOR knock-out (-/-), GRK3 knock-out (-/-), and respective wild-type (+/+ ) littermate control mice were prepared by heterozygous crosses and genotyped as described earlier (15).

**Partial Sciatic Nerve Ligation (pSNL)**—pSNL was used as a model of neuropathic pain (23). Animals were anesthetized with isoflurane and pSNL or sham ligation surgery were done as described previously (8). Seven days after surgery, animals were

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**K_\text{v}3 Regulation by p38 MAPK**
Kir3 Regulation by p38 MAPK

**Increase in pY12-Kir3.1-ir following pSNL Requires KOR and GRK3 Expression**—Previous studies have shown that phosphorylation of tyrosine 12 on Kir3.1 occurs after exposure to behavioral stressors including repeated forced swim, acute pain induced by formalin injection in the hind paw, and chronic neuropathic pain induced by partial sciatic nerve ligation (pSNL) (8). There is evidence that the dynorphin/KOR system is also activated by these stressors (15). However, it has not been established that increased pY12-Kir3.1-ir seen after behavioral stress is KOR dependent. To address this question, KOR(−/−) and wild-type littermate mice received unilateral pSNL or sham ligation, then spinal cord sections were stained with a phospho-selective antibody 7 days later. This antibody was previously characterized and shown to require Kir3.1 expression (8). Consistent with previous findings, wild-type animals showed an increase in pY12-Kir3.1-ir in the dorsal horn ipsilateral to the side of injury (Fig. 1A”). This same increase in immunoreactivity was not seen on the contralateral side to injury or in animals that received a sham ligation (Fig. 1A). Interestingly, animals

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**Immunohistochemistry**—After cardioperoxipulmonary perfusion, spinal cords were dissected and placed in 4% PFA for 1 h then put in 30% sucrose solution and incubated 2–3 days at 4 °C. Spinal cords were sliced into 30-μm floating sections using a microtome (Leica, Bannockburn, IL) and stored in 0.1 M PB at 4 °C. Sections were then washed 3 × 10 min in PBS, incubated for 1 h at room temperature in blocking buffer (5% goat serum, 0.3% Triton X-100 in PBS), and then incubated in primary antibody (rabbit anti-pY12-Kir3.1, 12 μg/ml) diluted in blocking buffer for 72 h at 4 °C. After four 10-min washes in PBS, sections were incubated in Alexa Fluor® 488 anti-rabbit antibody (Molecular Probes) at a dilution of 1:500, in blocking buffer for 2 h at room temperature. Sections were washed 3 × 10 min in PBS and 2 × 10 min in PB and then mounted on slides and allowed to dry overnight. After treating with Vectashield, sections were coverslipped, and photographic images were taken using a Nikon upright microscope.

**Electrophysiology**—AtT-20 cells stably expressing KOR-GFP or KOR(S369A)-GFP were grown on poly-d-lysine coated coverslips (BD Biosciences) for 48 h prior to recording. Cells were then either untreated, treated with U50,488 (10 μM, 60 min) or treated with the p38 inhibitor, SB203580 (1 μM, 30 min) prior to treatment with U50,488. In other experiments, cells were treated with DMSO (0.4%, 30 min) or pretreated with DMSO (0.4%, 30 min) or PP2 (5 μM, 30 min) prior to treatment with U50,488 (10 μM, 60 min). Cells were then used in patch clamp experiments in the whole cell configuration with a pipette solution containing 130 mM KCl, 20 mM HEPES, 10 mM EGTA, 5 mM MgCl2, 3 mM ATP-Na2, and 0.6 mM GTP, pH 7.25 in an external solution containing 25 mM HEPES, 10 mM glucose, 40 mM KCl, 110 mM NaCl, and 1 mM CaCl2, pH 7.35. Whole cell currents were obtained using an Axopatch 200 (Molecular Devices, Union City, CA) and analyzed using Clampex 8.2 software (Molecular Devices). Cells were held at −45 mV and were hyperpolarized to −100 mV for 50 ms every 5 s for the duration of the experiment. Membrane currents were recorded for 5 min in the external solution before adding external solution containing somatostatin (1 μM) for an additional 5 min. Inward potassium currents were blocked by adding external solution containing 10 mM BaCl2.

**Data Analysis**—Immunoblots were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Band intensity was measured using the Odyssey software, which subtracts background and calculates band density in pixels. Data were normalized to a percentage of control, vehicle-treated sample band intensity (basal, 100%) and plotted using Prism 4.0 (GraphPad Software, San Diego, CA) software. Statistical significance was taken as p < 0.05, or p < 0.01 as determined by the Student’s t test or ANOVA followed by a Dunnet’s or Tukey’s post-hoc test where appropriate.

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The images shown are representative of 3–5 independent replicates.
Kα3 Regulation by p38 MAPK

Non-phospho Kα3.1-ir

WT

KOR⁻/⁻

GRK3⁻/⁻

Sham

200 μm

pSNL

200 μm

FIGURE 2. Partial sciatic nerve ligation does not affect Kα3.1 expression. Images of spinal cord sections from wild-type (A), KOR(−/−) (B), and GRK3(−/−) (C) mice 7 days after a unilateral sham ligation, or pSNL (primed letters). The ipsilateral side is oriented to the right of the image, and the contralateral side was marked by a ventral incision during the dissection. Higher magnification of the side ipsilateral to nerve injury for wild-type (A), KOR(−/−) (B), and GRK3(−/−) (C) mice (double prime letters). The images shown are representative of three independent replicates.

FIGURE 3. KOR stimulation results in an increase in pY12-Kα3.1-ir, whereas untransfected AtT-20 cells and cells expressing KOR(S369A)-GFP do not increase pY12-Kα3.1-ir. A, representative Western blot of pY12-Kα3.1-ir in AtT-20 cells after 0, 5, 15, 30, 60, or 90 min of U50,488 (10 μM) exposure. B, representative Western blot of pY12-Kα3.1-ir in AtT-20 cells after pretreatment with norBNI (1 μM, 60 min) and subsequent exposure to U50,488 (10 μM) for 0, 5, 15, 30, 60, or 90 min. C, quantification of band intensities. D, quantification of pY12-Kα3.1-ir from Western blots of either untransfected AtT-20 cells or AtT-20 cells expressing KOR-GFP or KOR(S369A)-GFP that had been treated with U50,488 (10 μM, 60 min). D, quantification of non-phospho-Kα3.1-ir from Western blots of either untransfected AtT-20 cells or cells expressing KOR-GFP or KOR(S369A)-GFP that had been treated with U50,488 (10 μM, 60 min). All data points are normalized to β-actin-ir and shown as a fold change from the zero time point.
with the side contralateral to injury (2B’, C’). However, despite slight increases in K_{ir}3.1 protein expression, there is no increase in pY12-K_{ir}3.1-ir evident in KOR(−/−) and GRK3(−/−) animals after pSNL (Fig. 1, B and C), which supports the conclusion that the lack of increase in pY12-K_{ir}3.1-ir in KOR(−/−) and GRK3(−/−) animals was not due to a decrease in K_{ir}3.1 protein expression.

To identify the potential mechanisms responsible for KOR-dependent increase in pY12-K_{ir}3.1-ir in vivo, we next used a mouse pituitary cell line, AtT-20 cells, transfected with KOR-GFP (10). AtT-20 cells were selected because they endogenously express both K_{ir}3.1 and somatostatin receptors (24, 25). AtT-20 cells stably expressing GFP-tagged KOR were treated with the selective KOR agonist, U50,488 (10 μM) to characterize the time course of response (Fig. 3). Western blot analysis demonstrated that agonist stimulation of KOR significantly increased pY12-K_{ir}3.1-ir after 15 min and that this increase returned to baseline following 2 h of U50,488 treatment (Fig. 3C). The return to basal levels despite sustained agonist was likely caused by KOR desensitization and internalization (10), although alternative mechanisms were not excluded. The increase was not evident after cells were pretreated with the selective KOR antagonist, norBNI (1 μM) (Fig. 3, B and C), and untransfected AtT-20 cells did not respond to U50,488 (Fig. 3D). Importantly, there was no change in the levels of the unphosphorylated form of K_{ir}3.1 in both untransfected and KOR-GFP-expressing cells after 60 min of U50,488 treatment (Fig. 3E).

Using confocal microscopy to confirm these results, we found that treatment of AtT-20 cells with 10 μM U50,488 for 30 and 60 min resulted in activation and internalization of KOR as seen by punctate intracellular staining (Fig. 4A). A significant increase in channel phosphorylation occurred in KOR-GFP-expressing cells (Fig. 4, A and B) after 30 and 60 min of agonist exposure. This same increase in pY12-K_{ir}3.1-ir was not seen in norBNI-pretreated (Fig. 4B) or in untransfected cells (Fig. 4, A and B). These data suggest that agonist stimulation of KOR activates downstream signaling events that lead to tyrosine phosphorylation of K_{ir}3.1 in AtT-20 cells.

The KOR receptor that has serine 369 mutated to alanine, KOR(S369A)-GFP, does not recruit β-arrestin (16) and does not internalize or desensitize after agonist exposure (10). Interestingly, stimulation of this mutant receptor with U50,488 also failed to increase pY12-K_{ir}3.1-ir at any time point tested; the lack of effect at 60 min is shown (Fig. 3D). This difference in pY12-K_{ir}3.1-ir induced by KOR compared with KOR(S369A) was not due to changes in K_{ir}3.1 expression (Fig. 3E). Additionally, 30 and 60 min of agonist exposure failed to induce receptor internalization or increase pY12-K_{ir}3.1-ir as shown by confocal microscopy (Fig. 4, A and B). These data mirrored the results seen after pSNL in GRK3(−/−) animals and further substantiated the conclusion that a KOR-dependent increase in channel phosphorylation required GRK3 phosphorylation of KOR.

**K_{ir}3 Regulation by p38 MAPK**

**FIGURE 4.** pY12-K_{ir}3.1 immunoreactivity is increased in AtT-20 cells expressing KOR-GFP. A, representative confocal images taken of either AtT-20 cells that express KOR-GFP, KOR(S369A)-GFP or untransfected cells. Cells shown were either treated with a vehicle control or U50,488 (10 μM) for 60 min. Green represents the GFP-tagged receptor and red represents pY12-K_{ir}3.1-ir. Arrows indicate areas of increased pY12-K_{ir}3.1-ir. Data are shown as a fold change over the vehicle control for each group, n = 4–5. One-way ANOVA, p < 0.001 with Dunnett’s multiple comparison test. All bars were compared with the respective vehicle control (shown as a dotted line). *, p < 0.05; **, p < 0.01.

**FIGURE 5.** Block of p38 MAP kinase prevents κ-opioid receptor-mediated increases in pY12-K_{ir}3.1 immunoreactivity. A, representative Western blots of pY12-K_{ir}3.1 in AtT20 cells. Cells expressing KOR-GFP were pretreated with either vehicle or SB203580 (1 μM) for 30 min prior to treatment with U50,488 (10 μM, 60 min). B, quantification of band intensities. All data were normalized to β-actin-ir and shown as a fold change from the zero time point (denoted by the horizontal dashed line), n = 5 for each group. *, p < 0.05 by Student’s t test.
Kir3 Regulation by p38 MAPK

KOR-mediated Phosphorylation of Tyrosine 12 of K\textsubscript{\(\beta\)3.1} through Activation of p38 MAP Kinase Leads to Reduced Channel Conductance—

To investigate the physiological implications of KOR-mediated tyrosine phosphorylation of K\textsubscript{\(\beta\)3.1}, we used whole-cell voltage clamp techniques to assess channel conductance in AtT-20 cells stably expressing KOR-GFP or KOR(S369A)-GFP. AtT-20 cells endogenously express the somatostatin receptor, a G\textsubscript{i/o}-coupled G-protein-coupled receptor that also activates K\textsubscript{\(\beta\)}3 channels (24, 25). Prior work showed that U50,488 treatment of these AtT20 cells expressing KOR-GFP or KOR(S369A)-GFP produced equivalent activation of inwardly rectifying potassium currents (10). Somatostatin (1 \(\mu\)M) also induced a barium-sensitive, inwardly rectifying current in AtT-20 cells expressing either KOR-GFP or KOR(S369A)-GFP (Fig. 6A, inset). Pretreatment of KOR-GFP-expressing cells with 10 \(\mu\)M U50,488 for 60 min, a time when significant channel phosphorylation was evident, induced a profound suppression of the somatostatin-evoked current (Fig. 6A, middle black trace). In contrast, somatostatin-induced currents were unchanged by U50,488 pretreatment in AtT20 cells expressing KOR(S369A)-GFP (Fig. 6A, middle gray trace). The reduction in the somatostatin response in KOR-GFP-expressing cells was not caused by residual U50,488; the recording chamber was perfused with drug-free buffer for 30 min following U50,488 pretreatment and acute channel activation reversed within 5 min of drug washout (data not shown) and Ref. 10.

These results suggest that KOR activation produced a heterologous desensitization of the somatostatin-evoked activation of K\textsubscript{\(\beta\)3}. The block in this effect by serine 369 mutation to alanine in KOR suggests that the heterologous desensitization required phosphorylation of K\textsubscript{\(\beta\)3.1} through GRK3-mediated p38 MAPK activation.

phorylation of KOR and subsequent \(\beta\)-arrestin recruitment (16). To assess the role of this MAPK, we pretreated AtT-20 cells expressing KOR-GFP with the selective p38 inhibitor, SB203580 (1 \(\mu\)M), for 30 min prior to KOR agonist exposure. We found that inhibition of p38 blocked KOR-mediated increases in pY12-K\textsubscript{\(\beta\)3.1}-ir (Fig. 5). These data suggest that agonist stimulation of KOR results in tyrosine phosphorylation of K\textsubscript{\(\beta\)3.1} through GRK3-mediated p38 MAPK activation.
or the mitogen-activated protein kinases including MEK, ERK, or JNK (21, 26–28). In both types of cells somatostatin was able to induce inward potassium currents that were unaffected by SB203580 (data not shown), and the heterologous desensitization effect of prior U50,488 exposure of KOR-GFP-expressing cells was blocked by SB203580 (Fig. 6A, right traces). Panel 6A shows representative traces, and panels 6, B and C summarize the results of 6–9 independent replicates. In KOR-GFP-expressing AtT-20 cells, 10 μM U50,488 pretreatment significantly suppresses subsequent somatostatin evoked potassium currents, and this effect was completely blocked by SB203580 (Fig. 6B). In contrast, 10 μM U50,488 pretreatment of KOR(S369)-GFP expressing AtT-20 cells did not significantly affect subsequent somatostatin evoked potassium currents (Fig. 6C). These results suggest that KOR-mediated phosphorylation of Kir3.1 results in reduced channel conductance and that this effect can be blocked by inhibiting p38 MAP kinase.

**FIGURE 7. κ-opioid receptor-mediated activation of Src requires the GRK3 phosphorylation site and is p38 MAPK dependent.** A, representative confocal images of AtT20 cells expressing KOR-GFP. Cells were pretreated with either a vehicle, norBNI (1 μM, 60 min), SB203580 (1 μM, 30 min), PP2 (5 μM, 30 min), or DMSO before treatment with U50,488 (10 μM, 60 min). The GFP-tagged receptor is shown in green, pY12-Kir3.1-ir in red and phospho-Src (pY418) immunoreactivity in gray. B, quantification of pixel intensity of pSrc(pY418)-ir. Data are shown as a fold change over basal, n = 3–5. One-way ANOVA, p < 0.01. Using the Tukey’s multiple comparison post-hoc test we found that the norBNI- and SB203580-pretreated groups were significantly different from the vehicle-treated group and the PP2-treated group was significantly different from the DMSO-pretreated group, *, p < 0.05. C, quantification of pSrc(pY416)-ir from Western blots of either untransfected AtT20 cells or cells that express KOR-GFP or KOR(S369A)-GFP, n = 8–9. All data were normalized to β-actin-ir and shown as a fold change of the zero time point. One-way ANOVA, p < 0.001 with Tukey’s multiple comparison test. **, p < 0.01 when compared with the zero time point within the group. †, p < 0.05 when compared with the matching time point between groups.

or the mitogen-activated protein kinases including MEK, ERK, or JNK (21, 26–28). In both types of cells somatostatin was able to induce inward potassium currents that were unaffected by SB203580 (data not shown), and the heterologous desensitization effect of prior U50,488 exposure of KOR-GFP-expressing cells was blocked by SB203580 (Fig. 6A, right traces). Panel 6A shows representative traces, and panels 6, B and C summarize the results of 6–9 independent replicates. In KOR-GFP-expressing AtT-20 cells, 10 μM U50,488 pretreatment significantly suppresses subsequent somatostatin evoked potassium currents, and this effect was completely blocked by SB203580 (Fig. 6B). In contrast, 10 μM U50,488 pretreatment of KOR(S369)-GFP expressing AtT-20 cells did not significantly affect subsequent somatostatin evoked potassium currents (Fig. 6C). These results suggest that KOR-mediated phosphorylation of Kir3.1 results in reduced channel conductance and that this effect can be blocked by inhibiting p38 MAP kinase.

κ-Opioid Receptor-mediated Increases in Src Activation Require the GRK3 Phosphorylation Site and p38 MAPK Activation—p38 MAPK is a Ser/Thr kinase and is unlikely to be able to directly phosphorylate the tyrosine 12 residue in Kir3.1 (29). The recruitment of β-arrestin has been shown to be important for not only internalization and desensitization of GPCRs, but also as a scaffold for several signaling proteins including the non-receptor tyrosine kinase Src (16, 30–34). It has also been shown that the KOR is able to activate Src in human monocytic THP1 cells, but the mechanism of that effect was not established (18).

To assess whether KOR activation of Src was mediated by GRK3-dependent p38 MAPK activation, AtT20 cells expressing KOR-GFP were treated with 10 μM U50,488 (Fig. 7). As evident from these co-labeled images, U50,488 treatment simultaneously induced KOR-GFP internalization (green), increased pY12-Kir3.1-ir (red), and increased phospho-Src-ir (white) (Fig. 7A). These changes were blocked by pretreatment with either norBNI, SB203580, or PP2. In contrast, the DMSO vehicle for PP2 had no effect on the response to U50,488 (Fig. 7A). Quantitation of pixel intensity changes in replicate experiments confirmed that phospho-Src-ir was significantly increased by 10 μM U50,488 treatment of KOR-GFP expressing AtT-20 cells, and that this increase was completely blocked by either norBNI, SB203580, or PP2 pretreatment (Fig. 7B).

Parallel experiments using Western blot resolution showed that U50,488 significantly increased Src phosphorylation at 30 and 60 min (Fig. 7C). This same increase in phospho-Src-ir was...
not evident after 10 μM U50,488 treatment of untransfected cells or cells that express the mutant receptor KOR(S369A)-GFP lacking the GRK3 phosphorylation site (Fig. 7C). The results suggest that KOR-mediated increases in pY12-Kir3.1-ir resulted from p38-induced Src activation.

To assess the role of Src activation in Kir3 heterologous desensitization, AtT-20 cells stably expressing KOR-GFP were pretreated with U50,488 (10 μM) for 60 min before somatostatin (1 μM) was added to the external buffer. Pretreatment of cells expressing KOR-GFP with U50,488 (10 μM, 60 min) or pretreatment with either U50,488 (n = 6) or PP2 + U50,488 (n = 6) show a reduced inward current in response to somatostatin (middle trace). One-way ANOVA, p < 0.001.

**DISCUSSION**

There were two principal findings in this study. First, activation of the κ-opioid receptor during behavioral stress leads to phosphorylation of Y12-Kir3.1. We found that increases in pY12-Kir3.1-ir seen after partial sciatic nerve ligation were absent in KOR(−/−) animals, and agonist stimulation of KOR expressed in AtT-20 cells also lead to tyrosine phosphorylation of the ion channel. Second, KOR-mediated increase in phospho-Y12-Kir3.1-ir suppressed a subsequent somatostatin-evoked response. These results suggest that activation of the dynorphin/KOR system during behavioral stress leads to tyrosine phosphorylation of Kir3.3, and this channel phosphorylation results in heterologous desensitization.

Although KOR activation of p38 MAPK was previously demonstrated (19), we were surprised to find that the increase in pY12-Kir3.1-ir induced by pSNL was mediated by through a p38 MAPK-dependent mechanism. The increased pY12-Kir3.1-ir seen in dorsal horn of the spinal cord after partial sciatic nerve ligation was dependent on both KOR and GRK3 expression. Involvement of p38 MAPK was also suggested by the in vitro studies: activation of the mutant KOR receptor lacking the GRK3 phosphorylation site failed to induce pY12-Kir3.1-ir and the increase was blocked by p38 MAPK inhibition. These results are consistent with previous findings showing that the GRK3 phosphorylation site is needed for KOR-mediated activation of p38 MAPK (16). The suggestion that p38 MAPK was required for Src activation is also a novel finding of this study. κ-opioid receptor-mediated p38 MAPK activation led to increased activation of the non-receptor tyrosine kinase, Src, and subsequent increase in pY12-Kir3.1-ir. Interpretation of these results is dependent on the selectivities of the inhibitors used and establishing the validity of this proposed cascade will require additional direct analysis. However, the results obtained in this study suggest a novel mechanism for potassium channel regulation.

There is a strong connection between the mechanisms that we defined in transfected cells in vitro and differentiated cells in spinal cord. First, both AtT20 cells and spinal cord showed KOR-dependent activation of p38 MAPK through a GRK3-me-
diated mechanism (16, 21), p38 MAPK activation was required for increases in pY12-Kr3.1-ir in both AtT20 and spinal cord. The mechanism of Src activation by p38 was shown in AtT20, but additional work is required to define the signaling pathway in vivo. Src is a reasonable candidate, and the present study suggests a novel mechanism linking KOR activation and channel tyrosine phosphorylation.

In a previous study (8), we reported that H$_2$O$_2$ treatment of mouse atrial myocytes also increased pY12-Kr3.1-ir. Hydrogen peroxide is a known activator of both p38 and Src (35, 36). But in that same study, we found that treatment of atrial myocytes with the p38 activator, anisomycin failed to increase pY12-Kr3.1-ir (8). The basis for this discrepancy is not clear, but suggests that differences in cell type or organization of the receptor signaling complex may be important for tyrosine phosphorylation of the ion channel. Signaling specificity can be achieved by either compartmentalization within spatially discrete membrane microdomains or by organization of the GPCR signaling units through interactions with scaffolding proteins such as β-arrestin (37). We found that KOR activation of Src was dependent upon GRK3 phosphorylation of the receptor, which is important for arrestin recruitment. Previous research has also shown that p38 MAPK activation by the κ-opioid receptor is GRK3 and arrestin dependent (16). Together, these data suggest that KOR-mediated increases in pY12-Kr3.1-ir occur through a signaling complex that includes β-arrestin, p38 MAPK, and Src.

Classically, p38 MAPK is thought to be activated by cellular stress and cytokine stimulation, and p38 MAPK activation is known to be important for normal immune and inflammatory responses (38). Like other members of the MAPK family, p38 is typically thought to exert its effects by activation of other protein kinases or altering gene expression by acting on transcription factors (29). However, p38 MAPK has also been shown to modulate the function of many cytoplasmic and membrane proteins as well. For example, activation of p38 MAPK regulates μ-opioid receptor endocytosis through phosphorylation of the small GTPase, Rab5. Rab5 in turn recruits EEA1, a protein involved in the tethering, docking, and fusion of early endosomes (39). p38 is also able to modulate the plasma membrane serotonin transporter (SERT) activity through activation of protein phosphatase 2 (PP2A), which leads to dephosphorylation and enhancement of SERT transport activity (40). p38 MAPK regulation of ion channel activity has also been documented. Kv2.1, a delayed rectifying potassium channel found in neurons, is the key exit route of potassium ions during apoptosis. Phosphorylation of the ion channel by p38 MAPK during apoptosis leads to membrane insertion and an enhancement of potassium currents (41). The results presented in this paper are another example of p38 MAPK regulation of a membrane protein through activation of Src, which leads to tyrosine phosphorylation of K_r3.1.

K_r3 channels are broadly distributed throughout the central nervous system (42). They are important for regulating the excitability of neurons and are also expressed by astrocytes where they are important for extracellular potassium buffering in brain. In the spinal cord, K_r3.1 was found almost exclusively on postsynaptic excitatory interneurons in lamina II of the dorsal horn (43). K_r3.1 channels show a high degree of co-localization with cells that express the μ-opioid receptor and GABA$_B$ receptors (44). These two receptor systems are well known for their ability to reduce excitatory neurotransmission. We have shown previously that phosphorylation of Y12-Kr3.1 occurs in both astrocytes and GABAergic neurons in the dorsal horn of the spinal cord (8). We also know that phosphorylation of Y12-Kr3.1 leads to decreased basal conductance (6, 7) and heterologous desensitization. The phosphorylation seen after pSNL may contribute to an overall hyperexcitability through decreased basal conductance and lack of response to agonists that decrease excitability through activation of this channel. This hypothesis is supported by data suggesting that lack of inward rectifying potassium channels leads to an increased susceptibility to seizures and spontaneous pain-like behaviors (45, 46). Furthermore, specific knockdown of K_r3.1 leads to hyperalgesia, blunted analgesia in response to opioids and an increase in motor activity (47, 48).

In summary, using an antibody selective for pY12-K_r3.1, we were able to propose a possible mechanism for the increased immunoreactivity seen in behavioral models of stress. Our data suggest that activation of dynorphin/κ-opioid receptor system during exposure to stress leads to tyrosine phosphorylation of K_r3.1 through GRK3-mediated activation of p38 MAPK and subsequent recruitment of Src kinase. We also found that channel phosphorylation results in heterologous desensitization of potassium currents. Importantly, we were able to prevent channel desensitization by blocking p38 activity either through pharmacological inhibition or by reducing the ability of GRK3 to phosphorylate the receptor. Future experiments would include using slice electrophysiological techniques after behavioral stress to understand the importance of tyrosine phosphorylation of K_r3.1 in vivo.

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