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MODULATION OF μ-OPIOID RECEPTOR DESENSITIZATION IN PERIPHERAL SENSORY NEURONS BY PHOSPHOINOSITIDE 3-KINASE γ

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Abstract—G protein-coupled opioid receptors undergo desensitization after prolonged agonist exposure. Recent in vitro studies of μ-opioid receptor (MOR) signaling revealed an involvement of phosphoinositide 3-kinases (PI3K) in agonist-induced MOR desensitization. Here we document a specific role of the G protein-coupled class IB isoform PI3Kγ in MOR desensitization in mice and isolated sensory neurons. The tail-withdrawal nociception assay evidenced a compromised morphine-induced tolerance of PI3Kγ-deficient mice compared to wild-type animals. Consistent with a role of PI3Kγ in MOR signaling, PI3Kγ was expressed in a subgroup of small-diameter dorsal root ganglia (DRG) along with MOR and the transient receptor potential vanilloid type 1 (TRPV1) receptor. In isolated DRG acute stimulation of MOR blocked voltage-gated calcium currents (VGCC) in both wild-type and PI3Kγ-deficient DRG neurons. By contrast, following long-term opioid administration the attenuating effect of MOR was strongly compromised in wild-type DRG but not in PI3Kγ-deficient DRG. Our results uncover PI3Kγ as an essential modulator of long-term MOR desensitization and tolerance development induced by chronic opioid treatment in sensory neurons.

Key words: MOR, PI3Kγ, DRG, VGCC, desensitization, opioid tolerance.

The powerful pain relieving effects of opioids were originally assigned to the activation of opioid receptors in the central nervous system but recent investigations also demonstrate an important role of peripheral mechanisms of opioid analgesia (Stein and Lang, 2009). Peripheral sensory neurons in the dorsal root ganglia (DRG) are among the critical targets of opioids acting on opioid receptors, which are abundantly expressed in the cell body and terminals of DRG neurons. Particularly the μ-opioid receptor (MOR) was found to induce heat analgesia (Scherrer et al., 2009). The interaction of opioids with MOR is accompanied by increasing desensitization of the G protein-coupled receptor (GPCR), which has been linked to the induction of tolerance, a phenomenon of extraordinary importance in medicine (Bailey et al., 2009). Mechanistically, opioid receptor desensitization involves receptor phosphorylation by different cytosolic protein kinases and interaction of the phosphorylated receptor with arrestin eventually leading to receptor internalization and recycling (Schulz et al., 2004; Koch et al., 2005; Koch and Höllt, 2008).

Previous studies have reported an involvement of phosphoinositide 3-kinase (PI3K) dependent signaling pathways in the sensitization of primary nociceptive neurons (Bonnington and McNaughton, 2003; Zhuang et al., 2004). For example, pharmacological investigations using broad specificity PI3K inhibitors have proposed a role for PI3K activity in MOR desensitization in sensory neurons (Tan et al., 2003), but the identity of the PI3K species involved is unknown. All four members of the class 1 PI3K family produce the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which controls a multitude of cellular functions (Hawkins et al., 2006). The only class IB member PI3Kγ was characterized as a major mediator of GPCR agonists. As holds true for other PI3K enzymes, PI3Kγ exhibits both a lipid kinase and protein kinase activity (Bondeva et al., 1998). As yet PI3Kγ has been documented in immune cells (Hirsch et al., 2000; Fruman and Bismuth, 2009) and cells of the cardiovascular system (Patrucco et al., 2004). In addition, a number of studies have reported expression of PI3Kγ in neuronal tissue (Narita et al., 2002; Cunha et al., 2010).

Given the proposed involvement of PI3Ks in MOR desensitization and the well-established function of PI3Kγ as a signaling mediator downstream of GPCR in leukocytes (Fruman and Bismuth, 2009), we tested the possibility that PI3Kγ might act as a mediator of desensitization processes induced by prolonged opioid treatment. Herein we report reduced pain relieving effects of morphine and strongly decreased tolerance development after systemic morphine application in mice deficient of PI3Kγ. Taken together with accompanying electrophysiological recordings in DRG, our findings disclose a novel, neuronal function of PI3Kγ as a specific mediator of opioid tolerance development and MOR desensitization in the peripheral nervous system.

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Abbreviations: DAMGO, D-Ala[2],N-Me-Phe[4],Gly[5]-ol-enkephalin; DRG, dorsal root ganglion; GPCR, G protein-coupled receptor; MOR, μ-opioid receptor; PI3K, phosphoinositide 3-kinase, class 1; TRPV1, transient receptor potential vanilloid type 1; VGCC, voltage-gated calcium channel.

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EXPERIMENTAL PROCEDURES

Nociceptive assay, dose–response data and tolerance induction

The *in vivo* experimental procedures were performed on wild-type and PI3K$\gamma^{-/-}$ mice (10 male and female each, 11–13 weeks of age, 24±4 g). Animals were housed four to six to a cage with same sex littermates and maintained at 12-h dark/light cycle in a temperature-controlled environment with unrestricted food and water. The wild-type and PI3K$\gamma^{-/-}$ mice were derived by 10 generations of successive backcrosses of heterozygous male knockout mice from chimeric C57BL6/129Sv PI3K$\gamma^{-/-}$ mice (Hirsch et al., 2000) with C57BL/6 females (Jackson Laboratories, USA). Experiments were approved by the committee of the Thuringian State Government on Animal Research. All testing was conducted near mid-photophase (9 AM to 4 PM) to reduce circadian effects on nociceptive and analgesic sensitivity (Kavaliers and Hirst, 1983). Following a 30 min habituation to the testing room, mice were assessed for baseline nociceptive sensitivity on the 45 °C tail-withdrawal test. In this assay of acute, thermal nociception, the mouse is gently restrained and the distal half of the tail is immersed in water maintained at 45.0±0.2 °C by an immersion circulator pump. Latency to reflexive withdrawal of the tail was measured twice to the nearest 0.1 s, with each determination separated by a minimum of 20 s. The two determinations were averaged. The tail-withdrawal test was chosen because of its stability even after repeated exposures to noxious water temperatures (D'Amour and Smith, 1941). A cut-off latency of 45 s was employed to prevent tissue damage.

Immediately following baseline latency assessment, subjects were injected with an initial dose of morphine (1.0 mg/kg), followed in succession with increasing doses (2.0, 3.6, 6.5, and 11.7 mg/kg). For tolerance development, morphine was applied at increasing dosages (20, 40 mg/kg) at two following days with three injections each day. At day 4, repeated tail-withdrawal test was performed with the same escalating morphine administrations. Percentage of the maximum possible effect (% MPE) was calculated by the formula: 100%×[(agonist response time−basal response time)/(cut off time−basal response time)]=% MPE.

Immunohistochemistry

Sections (5 μm) of paraformaldehyde-fixed and paraffin-embedded DRG tissues from adult rats (Wistar), wild-type and PI3K$\gamma^{-/-}$ mice were labeled with anti-PI3Kγ monoclonal antibody (Leopoldt et al., 1998) and mouse anti-IgG2a (Sigma) as isotype negative control. Colocalization of PI3Kγ was analyzed on adjacent slices with anti-TRPV1 polyclonal antibody (diluted 1:100, raised in rabbit against a 15 AA peptide of rat transient receptor potential vanilloid type 1 (TRPV1) receptor of the cytoplasmatic C-terminus of the receptor, Alpha Diagnostics, Germany) and anti-μ-opioid receptor polyclonal antibody (diluted 1:5, raised in rabbits). Dewaxed sections were rinsed with ddH_2O and transferred to an autoclavable cuvette with 10 mM citrate buffer. Heating for 15 min at 120 °C was used for antigen retrieval followed by PBS wash and blocking step with 2% serum and 0.3% Triton X-100 in PBS for 30 min at room temperature. Primary antibodies incubated over night at 4 °C and were detected using biotinylated secondary antibodies (diluted 1:100, Dako, Denmark) and the ABC-Vectastain-KS Kit (Vector Laboratories) following manufacturers instructions. Visualization followed with application of the peroxidase substrate Jenchrom (MobiTech, Germany). In the case of PI3Kγ colocalization with MOR, we used secondary antibodies labeled with Cy2 and Cy3 (diluted 1:200, Dianova, Germany). Control experiments were carried out with omission of all primary antibodies.

Analysis of immunohistochemical data

The sections were examined with a light microscope (Axioplan 2, Zeiss, Germany) coupled to a CCD video camera and an image analyzing system (KS 300, Zeiss, Germany). The mean area, diameter, and mean grey value were determined for each neuronal soma. To take into account differences in the basal grey values of individual sections, a relative grey value of each neuron was calculated by dividing the mean grey value of the neuron by the grey value of the cover slip background. For an unbiased discrimination of cells with or without positive labeling with antibodies, all neurons were considered as positive if they showed a relative grey value above that of neurons from the control incubations, which were not treated with the primary antibodies. Proportions of labeled neurons are expressed as the mean±SD. In double labeling experiments sections were additionally analyzed with a standard fluorescence filter. Neurons were classified as unlabeled, single, and double labeled.

PCR

Total-RNA extracts of murine 48 h primary DRG culture, WEHI (mouse myelomonocytic leukemia) and MEF cells (mouse embryonic fibroblasts) were isolated with RNeasy-Kit (Qiagen, Germany) and cDNA was produced using TaqMan Kit (Applied Biosystems) following manual instructions. Polymerase chain reaction for PI3Kγ (forward: 5′-GGAGAACTATGAACAACCGG, reverse: 5′-ATCTCACTTCGCAGGAAC, product 2100 bp) and β-actin (forward: 5′-GAGGTATCCTGACCCTGAAG, reverse: 5′-CAGAGGCATACAGGGACAG, product 250 bp) ran 95 °C 30 s, 59 °C 30 s, and 72 °C 2 min for 40 cycles.

Primary DRG culture

DRG were dissected from whole spinal cord of wild-type and PI3K$\gamma^{-/-}$ mice (C57/B6J) and collected in DMEM/F12 (Invitrogen). Digestion followed with collagenase (type II, 400 U/ml, PAA, Austria) for 45 min and Trypsin/EDTA (0.05%, PAA, Austria) for 10 min at 37 °C. DRG were suspended in DMEM/F12 and cells were dispersed by mechanical trituration with a fire-polished Pasteur pipette. The suspension was then plated on Poly-L-Lysine (50 μg/ml, Sigma) coated coverslips in DRG medium (DMEM/F12+10% heat-inactivated FCS supplemented with 1 mM L-Glutamine (Invitrogen), 1% PenStrep (PAA, Austria) and 100 ng/ml nerve growth factor (ProSpec, Israel). Measurements were performed within 48 h of culture.

Transfection of primary DRG neurons

Freshly isolated DRG cell suspensions (ca. 10^5 cells per transfection) were electroporated using Amaxa Rat Nucleofection Kit and Amaxa Nucleofector I (Lonza, Germany) following manual instructions. Plasmid DNA of pEGFP-N1 encoding wild-type PI3Kγ (1 μg, PIK3CG, GeneID: 5294) were combined with plasmid DNA encoding for the transmembrane glycoprotein CD8 (0.3 μg). After electroporation cells were seeded dropwise onto Poly-L-Lysine coated coverslips. Positively transfected DRG neurons were preselected by CD8 specific Dynabeads (Deutsche Dynal, Germany) and thereafter identified via green fluorescence.

Electrophysiology

Whole-cell voltage-clamp recordings were performed with an EPC-9 amplifier (HEKA Elektronik, Germany). Pulse protocol generation and data acquisition was controlled with PatchMaster software (HEKA Elektronik, Germany). Series resistance errors were compensated to 70%. Correction for leak currents and capacitive transients was executed using a P/6 method. Data were low-pass filtered at 5 kHz. For whole-cell voltage-gated calcium current measurements external solution was composed of (mM): 140