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β-Arrestin 2-mediated heterologous desensitization of IGF-IR by prolonged exposure of SH-SY5Y neuroblastoma cells to a mu opioid agonist

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ABSTRACT

Prolonged (12 h) exposure of SH-SY5Y neuroblastoma cells to the mu-opioid receptor (MOPr) agonist [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) causes homologous desensitization as well as heterologous desensitization of the extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation induced by insulin-like growth factor (IGF)-I. Brief (15 min) but not prolonged exposure to DAMGO transregulates the insulin-like growth factor-I (IGF-I) receptor, as evidenced by its phosphorylation in the absence of IGF-I. Silencing of β-arrestin 2 uncouples the crosstalk between the two receptors, thus maintaining IGF-I-mediated receptor phosphorylation and ERK 1/2 activation even after prolonged DAMGO exposure. Furthermore, MOPr-induced activation of IGF-I receptor requires the tyrosine kinase c-Src.

1. Introduction

Opioids are commonly used for pain relief. However, prolonged administration of opioids may cause tolerance and dependence, which limits their use [1]. At the molecular level, opioid-mediated analgesia is mainly achieved by agonist-induced activation of the mu-opioid receptor (MOPr), a G-protein coupled receptor (GPCR). This event generates a cascade of intracellular signals that affect several cytoplasmic and nuclear targets [1]. An important signaling pathway activated by MOPr is the extracellular signal-regulated kinase (ERK) pathway, specifically ERK 1/2, belonging to the family of mitogen-activated protein-kinases (MAPK) [2]. This pathway targets multiple transcriptional factors for expression or repression of genes, and produces changes in cellular phenotypes, contributing to opioid-induced cellular responses [3]. MOPr-stimulated ERK 1/2 phosphorylation closely resembles signaling by receptor tyrosine kinases (RTKs) [3]. A key finding in unraveling this puzzle was the discovery that MOPr agonists are able to transactivate at least one RTK, that for epidermal growth factor (EGFR) [4]. This effect is shared with δ-opioid receptor agonists [5] and other GPCR agonists [2].

A very important intermediate of the signaling flow from GPCR to RTK are β-arrestins, which play a fundamental role in GPCR signaling, participating in cytoplasmic signal transduction [6,7]. Based on the properties of β-arrestins as signaling adaptors and scaffolds, Xiao et al. [8] extensively analyzed their molecular interactions, and identified them as hub proteins in cytoplasmic signal distribution and progression. Bohn et al. [9] reported that β-arrestin 2 knockout mice display increased analgesia in response to morphine, demonstrating the crucial role of this protein in opioid receptor function. In fact, β-arrestin 2 regulates the constitutive activity and recycling of MOPr through acting as a scaffold for the tyrosine kinase c-Src or for ERK 1/2 [10]. Myiatake et al. [11] recently reported that the MOPr stimulation-dependent phosphorylation of EGFR strictly relies on β-arrestin 2 signaling pathways in astrocytes.

β-Arrestins contribute to opioid receptor desensitization, a phenomenon that reflects the gradual decrease in receptor activity during prolonged agonist treatment and that has been implicated in tolerance development [12]. Therefore, it could be hypothesized that β-arrestin translocation to receptors exposed to agonists [13]

Abbreviations: ANOVA, analysis of variance; DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin; DMSO, dimethylsulfoxide; EGFR, epidermal growth factor receptor; ERK 1/2, extracellular signal-regulated kinase 1/2; FCS, fetal calf serum; GPCR, G-protein coupled receptor; IGF-I, insulin-like growth factor-I; IGF-IR, insulin-like growth factor I receptor; MAPK, mitogen-activated protein-kinases; MOPr, mu-opioid receptor; PBS, phosphate-buffered saline; pERK 1/2, phosphorylated extracellular signal-regulated kinase 1/2; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-D]pyrimidine; pIGF-IR, phosphorylated insulin-like growth factor I receptor; RTK, receptor tyrosine kinase; siRNA, small interfering RNA; tERK 1/2, total extracellular signal-regulated kinase 1/2; tIGF-IR, total insulin-like growth factor I receptor

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may dampen signal transduction processes or contribute to transactivation of RTKs.

The insulin-like growth factor I receptor (IGF-IR) belongs to the RTK family. IGF-I and IGF-II bind to this receptor and mediate its autophosphorylation, which allows downstream recruitment of adaptor proteins capable of activating signaling cascades including the MAPK pathway [14]. IGF-IR is present in a variety of brain regions, and IGF-I plays an important role in neurogenesis, development and differentiation of the nervous system, and has protective effects in neurodegeneration and trauma [15,16]. By contrast, chronic opioid treatment affects hippocampal neurogenesis and plasticity in neuronal circuits [17]. It has been suggested that growth factors, including IGF-I, could be used to reverse the adverse effects of morphine or other opioids in humans [16]. Recently, we demonstrated that MOPr expression is dependent on IGF-IR stimulation in neuroblastoma cells, suggesting a crosstalk between these two receptor systems [18]. Possible transactivation mechanisms at signal transduction level, in neuronal cells expressing both receptors, have been poorly investigated.

In this study, we investigated the crosstalk between MOPr and IGF-IR in SH-SY5Y neuroblastoma-derived cells, a suitable model of neuronal cells expressing both these receptors [18].

2. Materials and methods

2.1. Cell culture and materials

Human neuroblastoma SH-SY5Y cells (Health Protection Agency Culture Collections, Salisbury, Wiltshire, UK) were grown as monolayers in minimum essential media (MEM) and Ham's F12 (1:1) medium supplemented with 10% (v/v) fetal calf serum (FCS) (Lonza, Basel, Switzerland), 2 mM L-glutamine, non-essential aminoacids solution, 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified environment containing 5% CO_2 and 95% air. For experiments, cells were first seeded into 6-well or 24-well dishes containing complete cell culture medium with FCS for at least 24 h, and subsequently cultured in serum-free medium for 16–18 h before experiments were carried out. Recombinant human IGF-I peptide (Preprotech Inc., Rocky Hill, NJ, USA), [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) and naloxone hydrochloride (both Tocris, Bristol, Avon, UK) were dissolved in phosphate-buffered saline (PBS).

Control cells were treated with the vehicle alone (PBS). The c-Src-specific inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo[3,4-D]pyrimidine (PP2) (Tocris) was dissolved in dimethylsulfoxide (DMSO; maximum 0.1% v/v). The mouse monoclonal antibody to human IGF-IR (1H7) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other reagents were of the highest grade of purity available and were purchased from Sigma (St. Louis, MO, USA), and the plastic consumables were purchased from Sarstedt (Nümbrecht, Germany).

2.2. Small interfering RNA (siRNA)

A pre-designed and validated small interfering (si)RNA for β-arrestin 2 and a control (CTRL) siRNA (Applied Biosystems, Foster City, CA, USA) were transfected using a commercial reagent (Lipofectamine RNAi MAX; Invitrogen) following the manufacturer's recommendations. SH-SY5Y cells were assayed 2 days after transfection.

2.3. Western blotting analysis

At the end of the experiments, cell monolayers were placed on ice, washed with ice-cold PBS and incubated in lysis buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 2 mmol/L sodium orthovanadate, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride) supplemented with 1X protease inhibitor cocktail (Sigma) for 30 min at 4 °C. Insoluble material was removed by centrifugation at 20,800×*g* for 10 min at 4 °C and protein concentrations were estimated by a commercial bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Protein extracts (50 μg) were denatured at 95 °C for 3 min before being loaded and separated in SDS–PAGE gels. The membranes were incubated overnight at 4 °C with anti-phospho-ERK 1/2 or anti-total ERK 1/2 antibodies (1:5000) (Promega, Madison, WI, USA) or anti-phospho-IGF1-R or anti-total (not phosphorylated) IGF1-R antibody (1:1000) (Cell Signaling, Boston, MA, USA) and, thereafter with the appropriate peroxidase-conjugated secondary antibody. Digital images were acquired and analyzed [18].

3. Results

3.1. MOPr and IGF-IR signaling converge on the ERK 1/2 pathway

It is well established that several GPCRs are able, upon agonist activation, to stimulate the phosphorylation of MAPK by exploiting different pathways [2]. In this study, we first evaluated the ability of DAMGO (10 nM–1 μM) to induce the phosphorylation of ERK 1/2 (phosphorylated extracellular signal-regulated kinase 1/2 (pERK 1/2)), as determined by western blotting, in SH-SY5Y cells. The experiments confirmed that this MOPr agonist induces a concentration-dependent increase of pERK 1/2, and the maximum effect was observed in cells exposed to 1 μM DAMGO for 15 min. We found that this effect was antagonized by 10 μM naloxone hydrochloride added 10 min before DAMGO exposure (data not shown).

The western blot analysis of time-dependent DAMGO treatment of SH-SY5Y cells (Fig. 1a) showed that pERK 1/2 increased after 3 min of exposure to DAMGO, and peaked at 15 min, with pERK 1/2 levels returning to basal activity in cells exposed to DAMGO for 30 min. Likewise, stimulation of IGF-IR wit 10 nM IGF-I also increased pERK 1/2 accumulation in a time-dependent fashion, reaching maximum levels after 15 min and returning to baseline levels after 30 min (Fig. 1b). Neither DAMGO nor IGF-I altered the total extracellular signal-regulated kinase 1/2 (tERK 1/2) protein level.

3.2. Prolonged DAMGO exposure causes homologous and heterologous desensitization of the ERK 1/2 signaling pathway

For investigation of homologous desensitization of ERK 1/2, SH-SY5Y cells were pre-treated with 1 μM DAMGO for 12 h, then, after washing out the ligand, the cells were briefly re-exposed (15 min) to fresh cell culture medium containing the vehicle alone or 1 μM DAMGO before being harvested and lysed. Using western blot analysis of protein extracts, we found that sustained exposure to 1 μM DAMGO caused homologous desensitization of this signaling pathway; there was no detectable increase in pERK 1/2 (Fig. 1c).

The heterologous desensitization of ERK 1/2 was also investigated. In cells pre-treated with DAMGO for 12 h, exposure to 10 nM IGF-I for 15 min after washing with PBS and adding fresh medium did not increase pERK 1/2 (Fig. 1c). Therefore, prolonged exposure to DAMGO produces both homologous and heterologous desensitization of this second messenger pathway.

We then tested the reversibility of this signaling desensitization by exposing SH-SY5Y cells to 1 μM DAMGO for 12 h and, subsequently washing them with PBS and incubating them for 12 h in fresh medium. Afterwards, SH-SY5Y cells were re-exposed to 1 μM DAMGO for 15 min or IGF-I (10 nM for 5, 15, 30, and