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EGFR dependent subcellular communication was responsible for morphine mediated AC superactivation

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ABSTRACT

Compensatory adenylyl cyclase (AC) superactivation has been postulated to be responsible for the development of morphine tolerance and dependence, the underlying mechanism was demonstrated to comprise c-Src-dependent upregulation of AC5 within the lipid rafts. In the present study, we demonstrated that chronic morphine treatment sensitized EGFR signaling by augmenting EGFR phosphorylation and translocation into ER, which was essential for CRT-MOR tethering within the lipid rafts and AC5 superactivation. Intriguingly, synaptic clustering of CRT-MOR was dependent on EGFR phosphorylation and presumed to implicate in alignment and organization of synaptic compartments. Taken together, our data raised the possibility that an adaptive change in MOR and EGFR signal systems might establish CRT related subcellular communication, the signaling network within brain synaptic zone was proposed to implicate in morphine tolerance and dependence.

1. Introduction

Opioid receptors belong to the family of seven-transmembrane receptors that regulate their appropriate intracellular effector systems via inhibitory G proteins [1]. Acute activation of an opioid receptor leads to the inhibition of adenylyl cyclase (AC) and subsequently a reduction of intracellular cAMP levels [2]. During chronic opioid treatment, however, initially attenuated cAMP levels begin to recover and, in some cell systems and brain areas, the increase in AC activity were observed, which is generally referred to as AC sensitization. This compensatory AC activation has been postulated to be responsible for the development of drug tolerance and dependence [3], up to date, several regulatory changes in the quantity of stimulatory receptors, G proteins, as well as other signaling molecules were reported to comprise in this process [4–7].

Previous study have demonstrated that morphine mediated AC superactivation was dependent on the compartmentalization of cAMP signaling within lipid rafts, which provided a platform for concentrating a growing number of G protein-coupled receptors (GPCRs), ion channels, and receptor tyrosine kinases (RTK) [8,9]. Localization of c-Src to the lipid rafts could facilitate temporal and spatial restricted signaling produced by chronic morphine treatment [10–12], by which assembled AC complexes [13,14]. So far, accumulating evidence has documented that growth factor receptors were critically involved c-Src activation, and of principal importance in many GPCR activities [15,16], for example, long-term MOR signaling entailed inhibition of epidermal growth factor (EGF) induced ERK activation [17–19]. It was further reported that after activation, EGFR could be internalized and translocated into endoplasmic reticulum (ER), in which specific signals might be oriented and potentiated allowing access to downstream gene transcription [20–23]. Thus, we assumed that chronic morphine treatment might recruit c-Src signaling, by which to amplify EGFR effector system and fine-tune cAMP signaling cascades.

Many studies to elucidate the intracellular distribution of calreticulin (CRT) has shown that CRT, a ER chaperone, to be localized not only to ER, but also to cell surface, importantly, it seemed that signaling pathways from ER lumen to the lipid rafts existed to assembly adhesion related contact formation and differentiation [24–26]. As such, it was imperative to dissect the cross-talk of MOR and EGFR, the signaling network was presumed to enhance subcellular communication and promote synaptic specializations during morphine withdrawal [27,28].

2. Materials and methods

2.1. Plasmids and adenovirus construction

Constructs with the full-length wild-type EGFR, and a mutation in which tyrosine was substituted for phenylalanine at position 845 were introduced by means of a site-directed mutagenesis kit (QuikChange XL, Stratagene). Fragment containing point mutation was swapped with the corresponding sequence in wild-type EGFR that had been subcloned into plasmid DNA (pcDNA3.1). A FLAG tag was added to the 3′ end of

Abbreviations: MOR, mu opioid receptor; GPCR, G protein coupled receptor; HA, hemagglutinin; TCA, trichloroacetic acid; FACS, fluorescence-activated cell sorting; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PTX, pertussis toxin.

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the EGFR coding region. For recombinant adnovirus construction, cDNA for EGFRY845F were cloned into adenoviral shuttle vector pDE1sp1A (Microbix Biosystems, Inc. Canada). After homologous recombination in vivo with the backbone vector PJM17, plaques resulting from viral cytopathic effects were selected and expanded in 293 cells.

Human influenza virus HA epitope-tagged mouse wild type μ-opioid receptor (MOR) and mutant MOR-276RRITR280 (lacking G protein binding sites) were digested with Asp718 and XbaI and cloned into the pShuttle vector, plasmids from the identified and isolated recombinants were digested with PacI before transfecting into HEK293 for virus production. Titer of the amplified virus was determined using the Adeno-X™ rapid titer kit (BD Biosciences).

For construction of EGFR, CRT and AC5 siRNA, constructs of siRNA were cloned into pShuttle vector with siRNA sequence designed by GenScript siRNA design center siRNA Target Finder and siRNA Construct Builder. Following transfection, Neuroblastoma 2A (N2A) cells, primary neurons or brain slice were cultured for another 48 h prior to experiments.

2.2. Cell culture

N2A cells stably expressing either wild type FLAG-EGFR or FLAG-EGFR:Y845F were cultured with advanced modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum (HyClone), 2 mM glutamine (GlutaMAXTM, Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 0.2 mg/ml G418 (Geneticin, Invitrogen) in a 5% CO_2 atmosphere at 37 °C.

For primary neuron culture, A 25-mm glass coverslip (thickness, 0.08 mm) was inserted into a 35-mm Petri dish. Dissociated neuronal cultures from mice (the whole cortex) at postnatal days 1 and 2 were prepared and plated onto such 35-mm Petri dishes at a density of 1×10^6 cells. 10 days later, neurons were undergone the indicated treatment.

2.3. Fluorescence flow cytometry

The MOR or EGFR located on the plasma membrane was quantified by fluorescence-activated cell sorting (FACS) analysis. In brief, N2A cells were treated with 1 μM morphine for 5 min, 1 h, and 4 h, or addiction of naloxone (10 μM, 10 min) following 4 h of morphine incubation. After rinsed twice with serum-free DMEM, cells were incubated at 4 °C for 60 min in serum-free DMEM with anti-HA (Convance; Richmond, CA; 1:500) or anti-FLAG (Calbiochem; San Diego, CA; 1:500). Afterward, cells were washed and then incubated with Alexa488-labeled goat anti-mouse IgG secondary antibody (BD Biosciences; San Jose, CA; 1:500) at 4 °C for an additional 1 h. The cells were washed and fixed with 3.7% formaldehyde before quantifying the receptor immunofluorescence with FACS (FACScan; BD Biosciences). Fluorescence intensity of 10,000 cells was collected for each sample. CellQuest software (BD Bioscience) was used to calculate the mean fluorescence intensity of the cell population. All FACS analyses were conducted three times with triplicate in each experiment.

2.4. Subcellular fractionation

For lipid rafts separation, cells or tissues were washed twice with phosphate-buffered saline (pH 7.4) at 4 °C and dissolved into 2 ml of 500 mM sodium carbonate (pH 11.0) solution with 400 μl protease inhibitor cocktail (Merck Corporate; Whitehouse Station, NJ) followed by homogenization with three 10-s bursts of a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY) at the maximum setting. The lysate was sonicated with one 30-s burst at setting 4 and one 30-s burst at setting 8 (Heat Systems-Ultrasonics, Inc., Plainview, NY) and adjusted to 45% sucrose by adding 2 ml of 90% sucrose prepared in MBS buffer (25 mM MES, pH 6.5, and 0.15 M NaCl) and placed at the bottom of an ultracentrifuge tube. Then 4 ml of 35% sucrose and 4 ml of 5% sucrose (both in MBS buffer containing 250 mM sodium carbonate) were loaded to form a 5–45% discontinuous sucrose gradient. The sample was centrifuged at 160,000 ×*g* for 16 h in a Beckman L7 ultracentrifuge with an SW-41Ti rotor. Fractions of 1 ml each were collected from the top. A light-scattering band between 5 and 35% sucrose gradient were considered to be lipid raft-rich fractions.

For ER separation, cells or tissues were homogenized in 2 ml of 10 mM Tris–HCl (pH 7.5) and 150 mM sucrose, the resulting cell lysate was mixed with 6.5 ml of 1 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 63.5% sucrose and layered under a sucrose density gradient (1.5/1.7/2.1 M sucrose). Gradients were centrifuged at 100,000 ×*g* for 3 h and ER fractions were collected manually. ER fractions were washed with 2 vol. of 10 mM Tris–HCl (pH 7.5) with centrifugation, and resuspended in 10 mM Tris–HCl (pH 7.5) with 150 mM sucrose, then stored for later analysis.

2.5. Immunoprecipitation and western blotting

Proteins were combined and diluted with 15 ml of buffer A (100 mM NaCl and 10 mM Tris, pH 7.4) and concentrated with a Centriplus™ YM-30 centrifugal filter column (Millipore; Bedford, MA) to 1 ml at a speed of 3500 g. The concentrated solution was transferred to a new tube with the addition of 0.1% digitonin (Sigma-Aldrich; Milwaukee, WI) and respective antibodies including anti-HA, anti-Gαi2 (BD Biosciences), anti-MOR (Abcam; Cambridge, CB) at the concentration of 1:200. The mixture was incubated at 4 °C overnight with slow rotation. Sixty microliters of protein G-agarose beads (Invitrogen; Carlsbad, CA) were added, and the mixture was incubated at 4 °C for 3 h with slow rotation. The protein G-agarose beads were then pelleted by centrifuging at 12,000 ×*g* for 15 min at 4 °C and washed five times with buffer A. Protein samples were eluted from the beads with SDS sample buffer (75 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol).

For western blot analysis, proteins were resolved in SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NY). The membrane was probed in the presence of various specific primary antibodies including anti-CRT (1:1000, Millipore), anti-MOR (1:1000), anti-AC5 (Santa Crutz Biotechnology; Santa Cruz, CA), anti-EGFR/pEGFR (1:1000, Abcam) antibodies. Then the membrane was incubated with secondary antibody conjugated with alkaline phosphatase (BioRad, Hayward, CA), the protein bands were detected by ECF substrate (GE Healthcare; Piscataway, NJ) and scanned in the Storm 860 Imaging System (GE Healthcare). The band intensities were quantified and analyzed with the ImageQuant software (GE Healthcare).

2.6. In vitro Src kinase activity assay

After various treatments, ER fraction was separated from N_2A cells and incubated with 2 μg of anti-cSrc polyclonal antibody (Santa Cruz Biotechnology, Inc.) overnight at 4 °C followed by addition of 20 μl of protein G-agarose beads (Invitrogen) and incubation for another 3 h. The agarose beads were washed once with lysis buffer, then three times with washing buffer (0.5 M LiCl and Tris–HCl, pH 7.5), and one time with 25 mM Tris–HCl, pH 7.5. The immunoprecipitates were incubated at 30 °C with 5 μg of Src substrate peptide KVEKIGEGTYGVVYK, corresponding to amino acids 6–20 of p34cdc2 (Upstate; Albany, NY) in kinase buffer (total volume of 50 μl) containing 5 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences; Boston, MA), 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 25 μM ATP, 1 mM dithiothreitol, and 100 μM Na_3VO4. Ten-microliter aliquots were taken out at time points of 20 min, and the reaction was immediately terminated by the addition of 10 μl of 40% trichloroacetic acid, and the sample was spotted onto P81 cellulose phosphate paper (Upstate). The paper was washed extensively with 1% phosphoric acid three times and one time with