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Induction of oxidative stress by long-term treatment of live HEK293 cells with therapeutic concentration of lithium is associated with down-regulation of δ-opioid receptor amount and function

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ABSTRACT

The functional state of δ-opioid receptor signaling cascade in live cells exposed to a therapeutic concentration of lithium for a prolonged period of time (weeks) is not known because the previous studies of Li interference with OR were oriented to μ-OR only. The same applies to the analysis of the prolonged effect of Li on oxidative stress in context with δ-OR function. HEK293 cells stably expressing δ-OR were cultivated in the presence or absence of 1 mM LiCl for 7 or 21 days, homogenized and the post-nuclear (PNS) and plasma membrane (PM) fractions prepared from all four types of cells. Level of δ-OR in PM was determined by specific radioligand [^{3}H]DADLE binding and immunoblot assays; the functional coupling between δ-OR and G proteins was determined as DADLE-stimulated high-affinity [^{35}S]GTPγS binding. In the whole cells, general oxidative stress was monitored by fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCF) and results verified by analysis of PNS and isolated PM. Generation of 4-hydroxy-2-nonenal (4-HNE)-protein adducts and malondialdehyde (MDA) level were determined as products of lipid peroxidation. Li-treated cells exhibited the decreased amount of δ-OR. This was evidenced by both [^{3}H]DADLE binding and immunoblot assays. The δ-OR-G protein coupling efficiency was diminished. Simultaneously, in Li-treated cells, the highly increased oxidative stress measured as DCF fluorescence intensity was noticed. Importantly, this result was detected in live cells as well as PNS and PM. Accordingly, production of 4-HNE-protein adducts and MDA was clearly increased in Li-treated cells. The general significance of our work lies in presentation of novel data indicating that prolonged exposure of live HEK293 cells to the therapeutic concentration of Li results in down-regulation of δ-OR protein level and attenuation of δ-OR function in parallel with increased oxidative stress and increased level of lipid peroxidation products.

1. Introduction

Lithium (Li) represents the standard for the long-term treatment of bipolar disorder (BD) despite the fact that the progress in understanding how Li produces its clinical effect has been slow and is not fully elucidated up to the present time [2–4]. Part of the biochemical and

Abbreviations: AAS, atomic absorption spectroscopy; Ab, antibody; BD, bipolar disorder; DADLE, (2-D-alanine-5-D-leucine)-enkephalin = Tyr-D-Ala-Gly-Phe-D-Leu; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate (synonym dichlorodihydrofluorescein diacetate); DPH, 1,6-diphenyl-1,3,5-hexatriene; δ-OR, δ-opioid receptor; δ-OR-G_i1α, cells HEK393 cells stably expressing δ-OR-G_i1α ($C^{351}I$) fusion protein; δ-OR-HEK293 cells, HEK293 cells stably expressing δ-OR; G proteins, heterotrimeric guanine nucleotide binding regulatory proteins; G_i/G_o, G proteins inhibiting adenylyl cyclase activity in pertussis toxin sensitive manner; GPCR, G protein-coupled receptors; GTPγS, guanosine 5′-*O*-[gamma-thio] triphosphate; [^{35}S]GTPγS, guanosine-5′-*O*-[γ-^{35}S]-triphosphate; HEK293, human embryonic kidney 293 cells; 4-HNE, 4 hydroxy-2-nonenal; HPLC, high pressure liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; Li, lithium; MD, molecular modeling/molecular dynamics; MDA, malondialdehyde; (+)NMDG, *N*-methyl-D-glutamine; NMR, nuclear magnetic resonance; PM, Percoll^{R}-purified plasma membranes; PMSF, phenylmethylsulfonyl fluoride; PNS, post nuclear supernatant; ROS, reactive oxygen species/substances (throughout the article, this term is used in case that the chemical composition of species is unknown or when ROS might represent one of a several implicated molecules without certainty [1]; TBARS, thiobarbituric acid reactive substances; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phanyl-1,3,5-hexatriene

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pharmacological research in this area was focused on information transduction initiated by GPCR and mediated by trimeric G proteins [5]. Evidence that δ-OR represent an attractive target for the treatment of brain disorders was strengthened in recent years [6]. This receptor is broadly expressed in the brain, binds endogenous opioid peptides and exhibits the functional profile distinct from those of μ-OR and κ-OR. The beneficial effects of δ-OR agonists were clearly established in treatment of a variety of emotional responses and mood disorders. Although opioids were traditionally associated with pain-related behavior [7] and opioid dependence [8,9], increasing evidence links OR [10,11] and OR-mediated activation of G proteins [12] to the psychiatric disorders and suicide.

Over the years, the original evidence for negative, inhibitory effect of Li on G protein function [13,14] was strengthened and later considered to be involved in the pathogenesis as well as therapy of mood disorders. Determination of (responding) G protein levels and activities was tested as a biochemical marker of various types of mental disorders [15–18]. It was suggested that Li^+ interaction with G proteins proceeds as direct interaction with magnesium ions bound to the low-affinity Mg^{2+}-sites in Gα subunits [19]. Competition between Li^+ and Mg^{2+} ions as an underlying mechanism for the pharmacological action of Li salts in BD was suggested by Mota de Freitas et al. [20]. Results of Dudev et al. [21] indicated that conformation of ATP in solution does not change significantly when Li^+ binds to Mg^{2+}-loaded ATP. Consequently, ATP-Mg-Li, like Mg^{2+}-ATP can fit into the ATP-binding site of the host enzyme/receptor and activate corresponding signaling pathways. Recently, trimeric G proteins as the targets of Li action received a strong support with the observation that Li inhibits expression of the gene for $G_i\alpha$ in conjunction with inhibition of $G_s\alpha$ function [22].

The Li concentrations, which affects the function of a GPCR including OR *in vitro* are much higher than therapeutic plasma levels in patients [23], but the evidence that low concentrations of Li might interact with GPCR under *in vivo* conditions was strengthened by results obtained by pharmacological, crystallographic, mutagenesis and MD studies indicating that Li^+ binds to allosteric regulatory site of receptor molecule which is energetically more favourable than Na^+-site [24]. Thus, in similarity with conventional GPCR ligands, the GPCR-specific structure-activity relationships for various cations are recently emerging.

It was also demonstrated that, among many contributing factors, the generation of oxidative stress plays an important role in the pathophysiology of BD, major depression and psychosis [25,26] and that the modulation of oxidative stress may be relevant to the mood-stabilizing effects of Li [27–30]. The influence of Li and other antipsychotic drugs on oxidative stress parameters was under intensive examination [31–34], however, the elucidation of interaction between the mood stabilizing therapy and regulation of oxidative stress needs further investigations as contradictory results are reported in this area [35,36].

The present knowledge about effects of oxidative stress on the OR system is also rather limited [37]. It was demonstrated that oxidative stress caused significant decrease in the function and protein level of OR in opioid-responsive SK-N-SH neuronal cells and that the intensity of oxidative stress may exert a different effect on μ-, δ- and κ-OR [38,39].

Numerous studies were performed with the aim to elucidate the relationship between GPCR function, membrane lipid composition and the biophysical properties of PM such as membrane bilayer viscosity/fluidity (for review see Ref. [40]). Most recently, peroxidation of membrane lipids was shown to exert a significant alteration of the structure and dynamics of lipid membranes including the increase of water permeability, decrease of the bilayer thickness and change of the lipid membrane order and fluidity [41].

Thus, as presented in this short summary of up-to-date literature data, numerous reports suggest a causative, bi-directional linkage between Li-induced alteration of OR signaling and manifestation of oxidative stress and vice versa. At present time and to our best knowledge, there are no available experimental results which would test this linkage for δ-OR and their cognate G proteins. Therefore, the aim of our present study was to examine the effects of the long-term (weeks) exposition of live cells to therapeutic, 1 mM concentration of LiCl on δ-OR level and function in model cell line stably expressing this type of OR. Manifestation of oxidative stress, PM lipid peroxidation and extra and intracellular Li concentrations were determined in parallel experiments.

2. Materials and methods

2.1. Materials and chemicals

The 35 mm glass bottom dish, dish size 35 mm, well size 20 mm, #1.5 cover glass (D35-20-1.5-N) was purchased from Cellvis (Mountain View, California, USA) and Nunc™ Cell Culture Treated Flasks with Vent/Close Caps 80 cm^2 (153732) were from Thermo Scientific (Waltham, Massachusetts, USA). GF/B (1821-915 and 1821-025) and GF/C (1822-915 and 1822-025) filters were from Whatman (Maidstone, Kent, UK). The fluorescence probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA, D6883) was from Sigma-Aldrich (St. Louis, Missouri, USA). δ-OR agonist [^{3}H]DADLE (41.9 Ci/mmol, NET648250UC) and [^{35}S]GTPγS (1250 Ci/mmol, NEG030H001MC) were purchased from Perkin Elmer (Waltham, Massachusetts, USA). Antibodies oriented against δ-OR (H-60: sc-9111, N-terminus), actin (I-19, sc-1616) and goat anti-rabbit IgG-HRP (sc-2004) were from Santa Cruz Biotechnology (Dallas, Texas, USA), antibody oriented against $G_i1,2\alpha$ (ab3522) and Anti-4 Hydroxynonenal antibody (ab46545) were from Abcam (Cambridge, UK). SuperSignalTM West Dura Extended Duration Substrate (34075) was from Thermo Scientific. Complete Protease Inhibitor Cocktail Tablets (1697498001) were from Roche Diagnostics (Indianapolis, Indiana, USA). All other materials and chemicals were either from Sigma-Aldrich, SERVA (Heidelberg, Germany) or Invitrogen-GIBCO® (Waltham, Massachusetts, USA) and were of the highest purity available.

2.2. Cell culture

HEK293 cells stably expressing FLAG-δ-OR (δ-OR-HEK293) were used in our experiments. The cDNA encoding NH_2-terminally FLAG-tagged rat δ-OR was kindly donated by T. Costa (Istituto Superiore Sanità, Rome, Italy). For detailed information about generation of HEK293 cells stably expressing FLAG-δ-OR see Brejchova et al. [42].

Li has a very narrow therapeutic window. Concentration of 1 mM Li corresponds to therapeutic serum level generally used in the treatment of mood disorders and this level has to be closely monitored as small differences in dose or blood concentrations may lead to serious therapeutic failures or adverse effects. Therefore, Li concentration was used in our experiments at only a single, 1 mM, concentration and δ-OR-HEK293 cells were cultivated in absence or presence of 1 mM LiCl as described in details before [43].

Briefly, δ-OR-HEK293 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM (0.584 g/l) L-glutamine and 9% (v/v) fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37 °C. Geneticin (400 μg/ml) was included in the course of cell cultivation to maintain appropriate selection pressure. The cells were grown to 60–80% confluence in 80 cm^2 tissue culture flasks impregnated with 2% gelatine in the absence (control) or presence of 1 mM LiCl (Li-treated) for 7 or 21 days.

On the days 7 or 21 since the beginning of cultivation, cells were washed 2-fold with PBS to remove cultivation medium, mechanically detached from the surface and harvested by centrifugation for 10 min at 1000 × *g*. The cell sediments were snap frozen in liquid nitrogen and stored at −80 °C until use. The frozen cell sediments were used for isolation of PNS and PM fractions (Section 2.5) and also for determination of Li concentration by ICP-MS (Section 2.4). Control and Li-treated cells prepared under each of these two experimental conditions