ELSEVIER

Cell Calcium 41 (2007) 491–502

cell calcium

www.elsevier.com/locate/ceca

Calcium release by ryanodine receptors mediates hydrogen peroxide-induced activation of ERK and CREB phosphorylation in N2a cells and hippocampal neurons

Ulrike Kemmerling[a,b,c], Pablo Muñoz[d,a], Marioly Müller[a], Gina Sánchez[a], María L. Aylwin[e], Eric Klann[f], M. Angélica Carrasco[a,e], Cecilia Hidalgo[a,g,*]

[a] *Centro FONDAP de Estudios Moleculares de la Célula, Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[b] *Facultad de Ciencias de la Salud, Universidad de Talca, Talca, Chile*
[c] *Programa de Anatomía y Biología del Desarrollo, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[d] *Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile*
[e] *Programa de Fisiología y Biofísica, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile*
[f] *Departments of Molecular Physiology & Biophysics and Neuroscience, Baylor College of Medicine, Houston, TX, USA*
[g] *Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Casilla 70005, Santiago 7, Chile*

Received 16 May 2006; received in revised form 21 September 2006; accepted 2 October 2006
Available online 30 October 2006

Abstract

Hydrogen peroxide, which stimulates ERK phosphorylation and synaptic plasticity in hippocampal neurons, has also been shown to stimulate calcium release in muscle cells by promoting ryanodine receptor redox modification (*S*-glutathionylation). We report here that exposure of N2a cells or rat hippocampal neurons in culture to 200 μM H_2O_2 elicited calcium signals, increased ryanodine receptor *S*-glutathionylation, and enhanced both ERK and CREB phosphorylation. In mouse hippocampal slices, H_2O_2 (1 μM) also stimulated ERK and CREB phosphorylation. Preincubation with ryanodine (50 μM) largely prevented the effects of H_2O_2 on calcium signals and ERK/CREB phosphorylation. In N2a cells, the ERK kinase inhibitor U0126 suppressed ERK phosphorylation and abolished the stimulation of CREB phosphorylation produced by H_2O_2, suggesting that H_2O_2 enhanced CREB phosphorylation via ERK activation. In N2a cells in calcium-free media, 200 μM H_2O_2 stimulated ERK and CREB phosphorylation, while preincubation with thapsigargin prevented these enhancements. These combined results strongly suggest that H_2O_2 promotes ryanodine receptors redox modification; the resulting calcium release signals, by enhancing ERK activity, would increase CREB phosphorylation. We propose that ryanodine receptor stimulation by activity-generated redox species produces calcium release signals that may contribute significantly to hippocampal synaptic plasticity, including plasticity that requires long-lasting ERK-dependent CREB phosphorylation.

Keywords: Hippocampus; Reactive oxygen species; Redox modifications; *S*-glutathionylation; Intracellular stores; Calcium release channels; Synaptic plasticity

1. Introduction

Activity-dependent phosphorylation of the transcription factor cAMP/Ca^{2+} response element binding protein (CREB) induces the transcription of several neuronal genes [43,62]. CREB phosphorylation is considered critical to induce long-term potentiation (LTP) and for several forms of learning and memory [10,41,56]. CREB-dependent transcription of genes involved in synaptic plasticity entails long-term CREB phosphorylation by the Ca^{2+}-sensitive Ras/ERK (extracellular signal-regulated kinase) pathway [25,63]. Most studies on Ca^{2+}-dependent neuronal gene expression have focused on neuronal Ca^{2+} entry pathways. Yet, Ca^{2+} release from

* Corresponding author at: Programa de Biología Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Casilla 70005, Santiago 7, Chile. Tel.: +56 2 978 6510; fax: +56 2 777 6916.
E-mail address: chidalgo@med.uchile.cl (C. Hidalgo).

0143-4160/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ceca.2006.10.001

intracellular stores also contributes to activity-dependent gene expression [37,50,57]. In particular, Ca^{2+} release by ryanodine receptors (RyR) contributes to synaptic plasticity and neuronal gene expression [4,6,22,40,55].

Functional RyR are required for long-lasting long-term potentiation (LTP) and for activity-dependent increases in phosphorylated CREB (phospho-CREB) in hippocampal area CA1 postsynaptic neurons [40]. RyR activity is highly sensitive to direct redox modification by reactive oxygen and nitrogen species (ROS/RNS) [2,20,21,28,46]. Active neurons display increased metabolic activity and oxygen consumption, as well as increased generation of ROS/RNS [15,64]; moreover, ROS generation has been implicated on hippocampal LTP [54]. Cell-permeable scavengers of superoxide anion, a free radical, block LTP induction in hippocampal area CA1 [36], a region which also contains a ROS producing NADPH oxidase (NOX) that is required for *N*-methyl-D-aspartate (NMDA) receptor-dependent ERK activation [35,53,58]. NOX-generated superoxide anion dismutates into H_2O_2, a ROS that at low concentrations (1 μM) increases tetanic LTP 2-fold and also enhances NMDA-independent LTP [32,33]. Interestingly, catalase, which scavenges H_2O_2, attenuates LTP [59]. Although electrophysiological studies have yielded divergent results on the effects of H_2O_2 on hippocampal function, in some studies the use of non-physiological H_2O_2 concentrations in the mM range may have caused deleterious oxidative reactions unrelated to the potential physiological responses [33].

We investigated here whether RyR channels participate in H_2O_2-induced ERK phosphorylation in N2a cells or hippocampal neurons. We found that H_2O_2 modified RyR redox state, increasing its *S*-glutathionylation. H_2O_2 also stimulated Ca^{2+} release and increased sequentially ERK and CREB phosphorylation, while specific RyR inhibition by 50 μM ryanodine drastically reduced the stimulation of Ca^{2+} release and of ERK/CREB phosphorylation induced by H_2O_2. We propose that ROS generated during hippocampal LTP induction stimulate RyR, enhancing Ca^{2+} release and the Ca^{2+}-dependent ERK/CREB phosphorylation cascade required for CREB-dependent gene transcription.

2. Materials and methods

2.1. Cell cultures

Cell culture media were obtained from InVitrogen (Grand Island, NY). Mouse neuroblastoma (N2a) cells (CCL-131, American Type Culture Collection, Rockville, MD), were plated on 35 mm culture dishes in Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 110 mg/l sodium pyruvate and pyridoxine hydrochloride adjusted to contain 3.7 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 5% fetal bovine serum, antibiotics and antimycotics, and maintained at 37 °C. The culture medium was changed every 2 days. Primary rat hippocampal cultures were prepared as described [47]. Briefly, the hippocampus from Sprague–Dawley rats at embryonic day 18 was dissected in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, and 0.5% glucose and was rinsed twice with HBSS by allowing the tissue to settle to the bottom of the tube. After the second wash, the tissue was resuspended in HBSS containing 0.25% trypsin and was incubated for 5 min at 37 °C. After three rinses with HBSS, the tissue was resuspended in MEM supplemented with 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate and 2 mM L-glutamine (MEM-10) and was mechanically dissociated by gentle passage through Pasteur glass pipettes. Undisrupted tissue fragments were allowed to settle, cells in suspension were transferred to a new tube and viable cells were counted using 0.2% trypan blue. Cells were initially plated in MEM-10 media and maintained at 37 °C in a humid atmosphere with 5% CO_2/95% air. Three hours after plating, the MEM-10 medium was removed and serum-free Neurobasal medium supplemented with N-2 was added and changed every 72 h.

2.2. Cell incubation

For experiments, 5–6-day-old N2a cells or 10–12-day-old hippocampal cells were cultured for 12 h in serum-free or supplement-free medium, respectively. Cells were washed with phosphate buffered saline (PBS) and maintained for 60 min under resting conditions in Krebs–Ringer (in mM: 20 HEPES-Tris, pH 7.4, 118 NaCl, 4.7 KCl, 3 $CaCl_2$, 1.2 $MgCl_2$ and 10 glucose) in the absence or presence of 50 μM ryanodine to inhibit RyR. For H_2O_2 stimulation, cells were exposed to 200 μM H_2O_2 for 20 min unless otherwise indicated. For Ca^{2+}-free conditions, cells were incubated in the presence or absence of 50 μM ryanodine for 60 min in Ca^{2+}-free Krebs–Ringer supplemented with 0.5 mM EGTA and 3 mM $MgCl_2$ (4.2 mM total $MgCl_2$). To analyze the effects of inhibition of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase pump, N2a cells were incubated 40 min in Ca^{2+}-free solution with thapsigargin (1 μM) plus an additional 20 min period after addition of H_2O_2, or 60 min only with thapsigargin. All experiments were matched with vehicle-treated controls. Both control and experimental cells went through the same bath changes to discard differences by handling.

2.3. Preparation of hippocampal slices

Brains obtained from 6- to 8-week-old C57/Bl6 male mice, sacrificed by decapitation, were dissected rapidly and placed into ice-cold cutting saline containing (in mM): 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 5 D-glucose, 0.5 $CaCl_2$, 7 $MgCl_2$ and 0.6 ascorbic acid, saturated with 95% O_2/5% CO_2. A tissue chopper or a vibratome was used to prepare 400 μm transverse slices, which were transferred immediately into a 1:1 mix of cutting saline and artificial cerebrospinal fluid containing (in mM): 125 NaCl,