

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

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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.

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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

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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 $\text{Ca}^{2+}/\text{Mg}^{2+}$ -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% $\text{CO}_2/95\%$ 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% $\text{O}_2/5\%$ 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,

2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 25 D-glucose, 2 CaCl₂ and 1 MgCl₂, saturated with 95% O₂/5% CO₂. Slices were maintained at room temperature for at least 30 min and were then transferred to artificial cerebrospinal fluid at 32 °C in a submersion chamber for 1.5 h before stimulation with H₂O₂. To inhibit RyR, slices were pre-incubated for 60 min with ryanodine (50 μM) before addition of H₂O₂ (1 μM). Following pharmacological manipulations, slices were immediately frozen on dry ice.

2.4. Western blot analysis

Cells were incubated at 4 °C in 30 μl lysis buffer containing (in mM) 50 Tris-HCl, pH 7.4, 150 NaCl, 1 EDTA, 1% Nonidet P-40, 5 Na₃VO₄, 20 NaF, 10 Na₄P₂O₇, plus protease inhibitors (Calbiochem, La Jolla, CA) as described [9]. Cell lysates were scrapped from the culture dishes and were sonicated for 1 min, incubated on ice for 20 min, and sedimented at 15,000 × g for 20 min to remove debris. Hippocampal slices were sonicated in homogenization buffer (in mM: 10 HEPES, 1 EGTA, 1 EDTA, 10 Na₄P₂O₇, 150 NaCl, 50 NaF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 0.2 μM calyculin A and 1 μM microcystin). Protein concentration of lysates was determined using bovine serum albumin (BSA) as standard as described [2]; the Bradford method was used for hippocampal slices. Lysates were suspended in Laemmli buffer, separated in 10% SDS-polyacrylamide gels and transferred to PDVF membranes (Millipore Corp., Bedford, MA). Membranes were blocked at room temperature for 1 h in Tris-buffered saline containing 3% fat-free milk, with or without 0.05% Tween-20, and were then incubated overnight with the appropriate primary antibody. After washing with Tris-buffered saline, membranes were incubated at room temperature with the secondary antibody for 1.5 h. Immunoreactive proteins were detected using enhanced chemiluminescence reagents according to the manufacturer instructions (Amersham Biosciences UK Ltd.). The films were scanned and the Scion Image program (NIH) was employed for densitometric analysis of the bands. To correct for loading, membranes were stripped in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 50 mM β-mercaptoethanol, at 50 °C for 30 min, and re-probed with the corresponding control antibodies.

2.5. Immunohistochemistry

After incubation with H₂O₂ plus or minus 50 μM ryanodine, hippocampal slices were rapidly immersed in ice-cold 4% paraformaldehyde in PBS, pH 7.4. Sections (25 μm) were cut with a microtome after incubation at 4 °C overnight. Free-floating sections were blocked with 5% bovine serum albumin in PBS plus 0.7% Triton X-100 at 4 °C. cells or hippocampal neurons in primary culture, equilibrated for 60 min in Krebs-Ringer solution plus or minus 50 μM ryanodine, were incubated for 20 min with 200 μM H₂O₂ and rapidly covered with 4% paraformaldehyde in PBS, pH 7.4 and incubated overnight at 4 °C. Cells were blocked with

5% bovine serum albumin in PBS containing 0.2% Triton X-100 at 4 °C. Sections from hippocampal slices and cells were then incubated overnight at 4 °C either with anti-phospho-CREB (1:1000) or anti-phospho-ERK (1:200) antibodies. After primary antibody incubation, specimens were washed for 30 min three times with PBS plus Triton X-100 (0.7% Triton X-100 for sections, 0.2% Triton X-100 for cells), incubated for 2 h at room temperature with Alexa Fluor secondary antibodies (1:250) and washed for 45 min with PBS before mounting onto L-lysine-coated slides. Immunostained cells were viewed and imaged using a Nikon Eclipse E400 epi-fluorescence microscope system (Tokyo, Japan), which allowed collection of actual fluorescence. Hippocampal slices were viewed and imaged in a Zeiss LSM 510 META confocal microscope system (Zeiss, Oberkochen, Germany); the resulting images are presented as such or in pseudo color.

2.6. Antibodies

Antibodies against the dually phosphorylated forms of ERK-1 and ERK-2 (phospho-ERK1/2) and against phospho-CREB were from Cell Signaling Technology (Beverly, MA). ERK antibodies were from UBI (Lake Placid, NY); CREB antibodies were from Cell Signaling Technology (Beverly, MA) or from UBI (Lake Placid, NY). Anti-GSH antibodies were purchased from Virogen (Watertown, MA, USA) and anti-RyR from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor anti-rabbit and anti-mouse antibodies were from Molecular Probes (Eugene, Oregon) and horseradish peroxidase-conjugated anti-rabbit from Pierce (Rockford, IL).

2.7. Determination of intracellular Ca²⁺ signals

Images of intracellular Ca²⁺ signals in N2a cells or primary hippocampal neurons in culture were obtained with an inverted confocal microscope (Carl Zeiss LSM 5 Pascal, Oberkochen, Germany). N2a cells were maintained for 60 min under resting conditions in Krebs-Ringer in the presence or absence of 50 μM ryanodine, cells were preloaded for 30 min at 37 °C with 5 μM fluo 3-AM (Molecular Probes, Eugene, OR) and were washed for 10 min in Krebs-Ringer to allow complete dye de-esterification. Hippocampal cells were maintained for 60 min in neurobasal medium in the presence or absence of 50 μM ryanodine; cells were then transferred to modified Ca²⁺-free Hanks solution, preloaded for 30 min at 37 °C with 5 μM fluo 3-AM (Molecular Probes, Eugene, OR) and washed for 10 min in modified Ca²⁺-free Hanks solution to allow complete dye de-esterification. Cells attached to coverslips were mounted in a 1 ml capacity perfusion chamber and placed in the microscope for fluorescence measurements (excitation 488 nm, argon laser beam). After H₂O₂ addition, image data were taken in the time line scan mode, with the scan line oriented along segments of neuronal prolongations in hippocampal cells or along the cell body in N2a cells, avoiding cell nuclei. Fluorescence images were

collected every 10 ms and the average of four line scans was analyzed using the equipment data acquisition program. The Ca^{2+} signals are presented as $\Delta F/F_0$ values, where F_0 corresponds to the basal fluorescence obtained from 2000 or 4000 line scans. After addition of H_2O_2 , fluorescence was recorded during an additional period to collect 2000 or 4000 line scans. All experiments were done at room temperature (20–22 °C).

2.8. Detection of RyR S-glutathionylation

Cells were lysed in the following lysis buffer (in mM): 225 NaCl, 1.5 EDTA, 5 Na_3VO_4 , 40 NaF, 10 $\text{Na}_4\text{P}_2\text{O}_7$, 200 *N*-ethylmaleimide, 75 Tris-HCl, pH 7.4, 4.5% Nonidet P-40, plus protease inhibitors (0.5 leupeptin, 0.116 pepstatin, 15.9 benzamide, 19.5 PMSF). Cell lysate fractions containing 50–70 μg of protein were dissolved (1:1) in 2-fold concentrated non-reducing loading buffer containing urea plus *N*-ethylmaleimide (12 M urea, 2% SDS, 0.04% Bromophenol blue, 96.4 mM NaH_2PO_4 , 34 mM Na_2HPO_4 , 10 mM *N*-ethylmaleimide), incubated at 60 °C for 20 min and separated by PAGE in 3.5–8% gradient polyacrylamide gels under non-reducing conditions using the Tris-Acetate buffer system (Novex NuPAGE[®], Invitrogen Life Technologies, Carlsbad, CA). After electrophoresis, proteins were transferred for 3 h at 100 V to PVDF membranes (Millipore Corp., Bedford, MA); membranes were blocked at 4 °C overnight in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.6, 140 mM NaCl) containing 5% BSA plus 0.2% Tween-20, and were then incubated at room temperature 1 h with anti-glutathione (anti-GSH) antibody (diluted 1/25,000 in TBS, 5% BSA, 0.2% Tween-20). After washing three times with 20 ml TBS, 0.2% Tween-20 membranes were incubated as above at room temperature with the secondary antibody for 1 h. After antigen-antibody reaction and detection with ECL, membranes were stripped and probed with anti-RyR antibody. For this purpose, membranes were stripped for 30 min at 50 °C with a solution containing 62.5 mM Tris-HCl, pH 6.7, 100 mM β -mercaptoethanol, 2% SDS. After washing three times with 20 ml of TBS plus 0.2% Tween-20, membranes were blocked for 1 h at room temperature in TBS, 5% non-fat milk, 0.2% Tween-20. After removing this solution, membranes were incubated at 4 °C overnight with anti-RyR antibody (diluted 1/2000) in TBS, 5% non-fat milk, 0.2% Tween-20. After washing three times with TBS, 0.2% Tween-20, membranes were incubated at room temperature with the secondary antibody for 1 h. After antigen-antibody reaction and detection with ECL, membranes were stripped again as above, and stained with Coomassie blue. For this purpose, membranes were washed for 1 min with 20 ml methanol, incubated 5 min with 20 ml of a solution containing 0.01% Coomassie Brilliant Blue R250, 50% methanol, 10% acetic acid, washed 1 min with 20 ml of 50% methanol, and air-dried. Blots were quantified by densitometric analysis using the Quantity One software (Bio-Rad Laboratories, Hercules, CA). Results are expressed as the ratio of anti-GSH/RyR band densities.

2.9. Statistics

Results are expressed as mean \pm S.E. The significance of differences was evaluated using Student's *t*-test for paired data or ANOVA followed by Dunnett's or Bonferroni's post-test as indicated.

3. Results

Activation of the Ras/ERK pathway is required for long-term CREB phosphorylation (Wu et al., 2001) [60], a prerequisite of sustained, long-lasting LTP in the hippocampus [41,56]. Accordingly, the study of the cellular factors that may affect CREB phosphorylation via the ERK pathway in hippocampal neurons is of special significance. In this work, we investigated how modifying the cellular redox state with H_2O_2 affected ERK and CREB phosphorylation in neuronal cells in culture or in hippocampal slices. We initially used the N2a neuroblastoma cell line and we then explored the effects of H_2O_2 on hippocampal neurons. In all cases, cell cultures treated with H_2O_2 presented the same proportion of live cells (>90%) as in control conditions.

3.1. Stimulation of ERK and CREB phosphorylation in N2a cells by H_2O_2

Incubation of N2a cells with 100–400 μM H_2O_2 in the presence of 3 mM external $[\text{Ca}^{2+}]$ produced a significant increase in both ERK and CREB phosphorylation; maximal levels were obtained after 10 min of incubation, the earliest time tested, and remained elevated for up to 30 min when using 200 μM H_2O_2 (data not shown). Accordingly, in all subsequent experiments cells were incubated with 200 μM H_2O_2 for 20 min. In these conditions, H_2O_2 increased significantly both ERK1/2 and CREB phosphorylation over the endogenous levels (Fig. 1A).

It is well established that ryanodine at concentrations >10 μM is an extremely specific RyR channel blocker that binds only to the open state of RyR channels [30]; this feature requires long incubations with ryanodine (30–60 min) to ensure RyR inhibition. Thus, N2a cells or hippocampal neurons were preincubated with 50 μM ryanodine for 60 min; in these conditions the stimulation of ERK and CREB phosphorylation produced by 200 μM H_2O_2 was drastically curtailed (Fig. 1A). These results suggest that H_2O_2 -induced ERK and CREB activation required functional RyR. Basal ERK phosphorylation in control cells also was slightly reduced by 50 μM ryanodine but this reduction was not statistically significant.

Incubation of cells with U0126, a specific inhibitor of the mitogen-activated protein kinase (MEK), the upstream ERK kinase, prevented ERK phosphorylation and decreased significantly the enhancement of ERK phosphorylation induced by H_2O_2 ; U0126 also abolished H_2O_2 -induced CREB stimulation (Fig. 1B), strongly suggesting that CREB activation

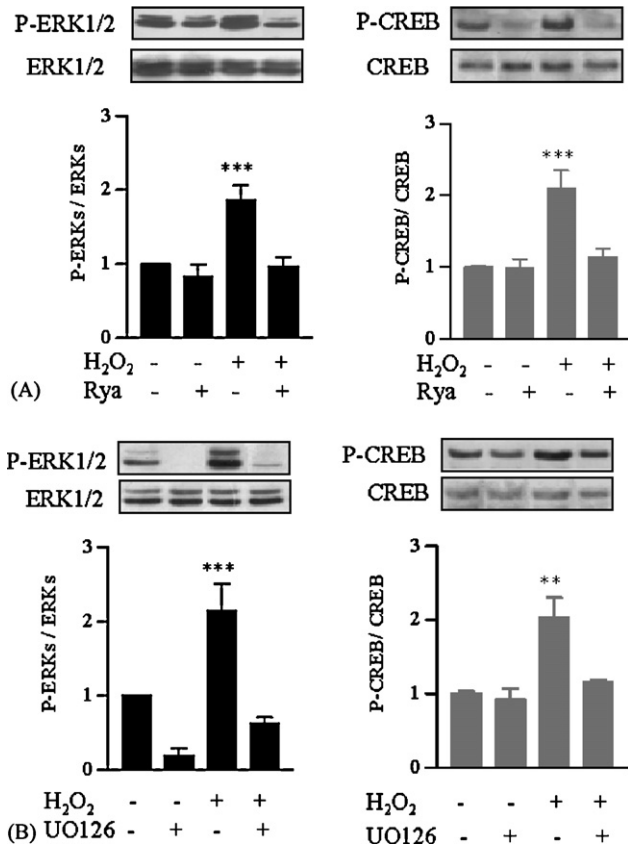


Fig. 1. Stimulation by H₂O₂ of ERK1/2 and CREB phosphorylation in N2a cells. Effects of ryanodine (A) or the MEK inhibitor UO126 (B). N2a cells were maintained for 60 min under resting conditions in Krebs–Ringer medium. When indicated, 50 μM ryanodine or 10 μM UO126 were added to this solution for the entire 60 min or for the last 30 min, respectively, of the preincubation period. After preincubation, 200 μM H₂O₂ was added for 20 min to control cells or to cells preincubated with ryanodine or UO126. In each panel, the upper part illustrates the Western blots for phospho-ERK1/2 (P-ERK in the figure) or phospho-CREB (P-CREB in the figure) and the total ERK or CREB levels, respectively, as loading controls. The bar graphs under the Western blots represent the ratios, normalized with respect to the values obtained in control cells, of phospho-ERK1/2 over total ERK (black solid bars) or of phospho-CREB over total CREB (gray solid bars). All values, given as mean ± S.E., correspond to at least 3 independent experiments carried out in duplicates. ***p* < 0.01; ****p* < 0.001.

was a consequence of the activation of the Ras/MEK/ERK pathway by H₂O₂.

In the absence of extracellular Ca²⁺, H₂O₂ also enhanced ERK and CREB phosphorylation to a similar extent as observed in the presence of Ca²⁺. In both conditions, preincubation with 50 μM ryanodine abolished the stimulatory effects of H₂O₂ (Fig. 2A). Cells incubated with thapsigargin (1 μM) in Ca²⁺-free conditions displayed similar basal phospho-ERK levels as controls but did not exhibit the H₂O₂-induced increase in ERK phosphorylation (Fig. 2B). Cells incubated with thapsigargin as above displayed a statistically significant increase in CREB phosphorylation levels; yet the phospho-CREB levels of cells incubated with thapsigargin alone or with thapsigargin plus H₂O₂ were not statistically different (Fig. 2B). These results indicate that H₂O₂ did not

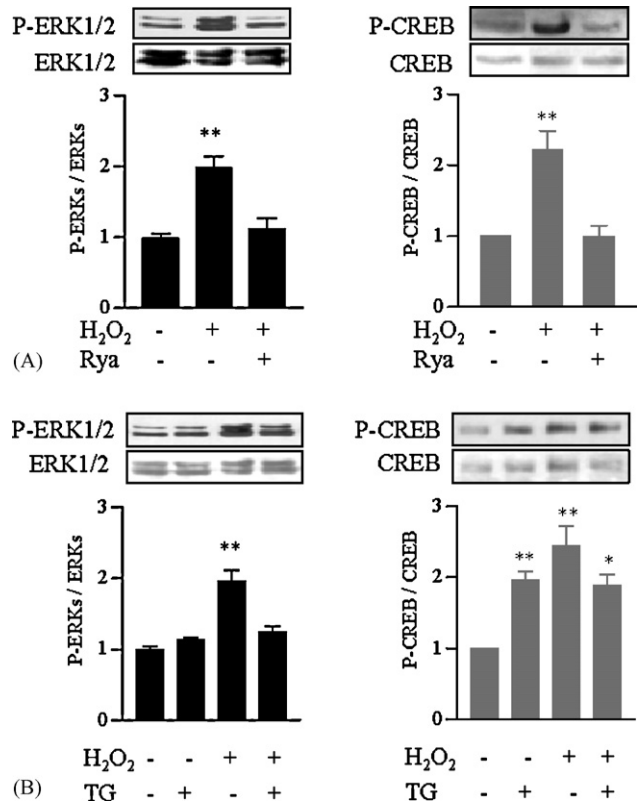


Fig. 2. Stimulation of ERK and CREB phosphorylation by H₂O₂ in N2a cells. Effects of external Ca²⁺ removal (A) or of thapsigargin (B). (A) 200 μM H₂O₂ was added for 20 min to cells incubated for 60 min in Ca²⁺-free solution; when indicated, 50 μM ryanodine was added at the start of the 60 min preincubation period. (B) Cells were incubated for 40 min in Ca²⁺-free solution plus or minus 1 μM thapsigargin. After this time, 200 μM H₂O₂ or vehicle was added to this solution and cells were incubated for an additional 20 min period. The upper part of each panel illustrates Western blots for phospho-ERK1/2 or phospho-CREB and total ERK or CREB, respectively, as loading controls. The bar graphs under the Western blots represent the ratios, normalized with respect to control values, of phospho-ERK1/2 over total ERK (black solid bars) or of phospho-CREB over total CREB (gray solid bars). All values, given as mean ± S.E., correspond to at least 3 independent experiments carried out in duplicates. **p* < 0.05; ***p* < 0.01.

stimulate ERK/CREB phosphorylation in cells with Ca²⁺-depleted ER.

These combined findings suggest that H₂O₂-induced increases in CREB phosphorylation in N2a cells are due primarily to Ca²⁺-dependent activation of the Ras/MEK/ERK pathway. Activation of this pathway by H₂O₂, which was independent of extracellular Ca²⁺, seems to require functional RyR since it was not observed after RyR inhibition with ryanodine.

3.2. Stimulation by H₂O₂ of ERK and CREB phosphorylation in hippocampal neurons

We investigated next the effects of H₂O₂ on ERK and CREB phosphorylation in hippocampal neurons. For this purpose, we used rat hippocampal neurons in primary cultures or mouse hippocampal slices. As observed in N2a cells,

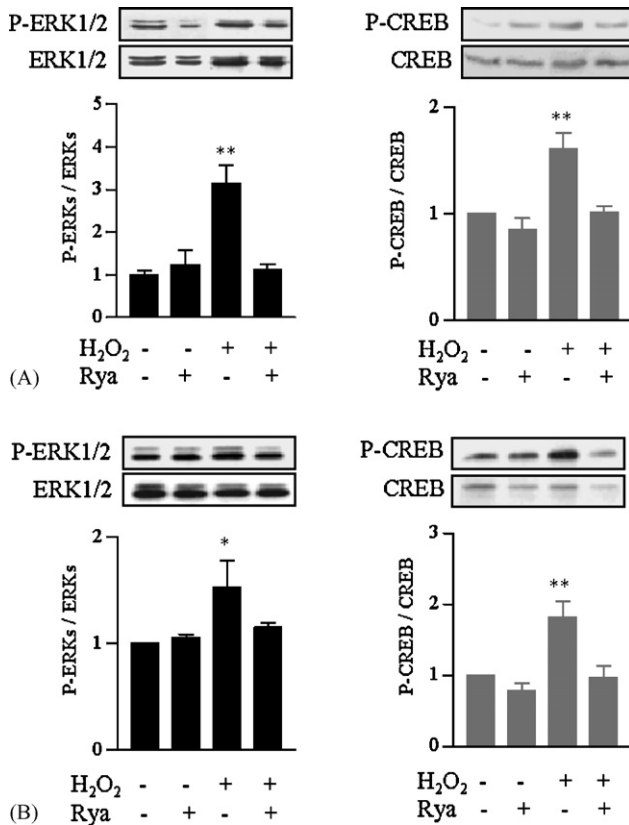


Fig. 3. Stimulation of ERK and CREB phosphorylation by H₂O₂ in hippocampal cells in primary culture (A) or in hippocampal slices (B). (A) 200 μ M H₂O₂ was added for 20 min to hippocampal neurons in primary culture, previously maintained for 60 min under resting conditions in Krebs–Ringer medium. When indicated, 50 μ M ryanodine was present during the entire preincubation period. (B) Hippocampal slices (400 μ m) were exposed to 1 μ M H₂O₂ for 20 min. Slices were preincubated with or without 50 μ M ryanodine for 60 min in Krebs–Ringer solution. The upper part of each panel illustrates Western blots for phospho-ERK1/2 or phospho-CREB and for total ERK or CREB as loading controls. The bar graphs under the Western blots represent the ratios (mean \pm S.E.) phospho-ERK1/2 over total ERK (black solid bars) or phospho-CREB over total CREB (gray solid bars). All values, normalized to the control values, correspond to at least 3 independent experiments carried out in duplicates. * $p < 0.05$; ** $p < 0.01$.

addition of 200 μ M H₂O₂ to hippocampal neurons in primary cultures caused significant stimulation of ERK1/2 and CREB phosphorylation that was abolished by 50 μ M ryanodine (Fig. 3A).

In mouse hippocampal slices addition of 1 μ M H₂O₂ produced significant stimulation of ERK1/2 and CREB phosphorylation (Fig. 3B), while 10- to 20-fold higher H₂O₂ concentrations were required in hippocampal neurons or N2a cells in culture. Cells grown in primary culture were exposed for several days to 95% air/5% CO₂ and had to adapt to a higher oxygen concentration than present in the tissue; this adaptation may have made them less sensitive to H₂O₂ than hippocampal slices, which were placed in an atmosphere saturated with 95% O₂/5% CO₂ only for a few h after dissection. As seen in N2a cells and hippocampal neurons in culture, 50 μ M ryanodine abolished the stimulatory effects of H₂O₂

on ERK1/2 and CREB phosphorylation in hippocampal slices (Fig. 3B).

Immunocytochemical analysis of phospho-ERK and phospho-CREB in N2a cells and hippocampal neurons in culture revealed that H₂O₂ stimulated ERK and CREB phosphorylation in all cases (Fig. 4). Immunohistochemical analysis revealed once again that 20-fold lower H₂O₂ concentrations (1 μ M) stimulated CREB phosphorylation in hippocampal slices when compared to N2a cells or hippocampal neurons in culture. In agreement with the Western blot results illustrated in previous figures, the stimulatory effects of H₂O₂ were largely abolished by 50 μ M ryanodine (Fig. 4). Control slices incubated with 50 μ M ryanodine were not ostensibly different from controls incubated only with vehicle (not shown).

3.3. Generation of cytoplasmic Ca²⁺ signals by H₂O₂ in N2a cells and hippocampal neurons in culture

To explore if H₂O₂ stimulated RyR-mediated Ca²⁺ release in N2a cells or hippocampal neurons in primary culture, we investigated the effects of H₂O₂ addition on cytoplasmic Ca²⁺, using fluo 3 as fluorescent Ca²⁺ indicator. As illustrated in Fig. 5A, N2a cells displayed low basal fluo 3 fluorescence in their cytoplasm; addition of 200 μ M H₂O₂ caused a marked increase in fluorescence. In contrast, fluo 3 fluorescence did not increase following addition of H₂O₂ to N2a cells preincubated with 50 μ M ryanodine, suggesting strongly that the Ca²⁺ increase produced by H₂O₂ was due to RyR-mediated Ca²⁺ release. Addition of 200 μ M H₂O₂ to hippocampal neurons in primary culture also stimulated the emergence of Ca²⁺ signals, evidenced as fluo 3 fluorescence increase (Fig. 5B). Preincubation with ryanodine (50 μ M) decreased to a large extent (>70%) the increase in fluo 3 fluorescence induced by H₂O₂, suggesting that in hippocampal cells in culture the Ca²⁺ increase induced by H₂O₂ was mostly, but not exclusively, due to RyR-mediated Ca²⁺ release.

A summary of the results of several experiments done in N2a cells or hippocampal neurons in culture is presented in Fig. 6. Following H₂O₂ addition N2a cells displayed an immediate increase in fluo 3 fluorescence while the fluorescence of hippocampal cells in primary culture reached its maximum at 25 s. These differences may arise from the line scan source, since the fluorescence line scans of N2a cells were recorded in the cell body, avoiding the nucleus, whereas in hippocampal cells they were recorded in neuronal prolongations that may vary in RyR content. Alternatively, the two cell types may possess intrinsic redox defense systems that may endow them with different redox sensitivities. Further experiments are needed to test this point.

3.4. Enhanced RyR S-glutathionylation induced by H₂O₂ in hippocampal cells in culture

The above results suggest that enhanced RyR-mediated Ca²⁺ release causes the stimulation of ERK/CREB phospho-

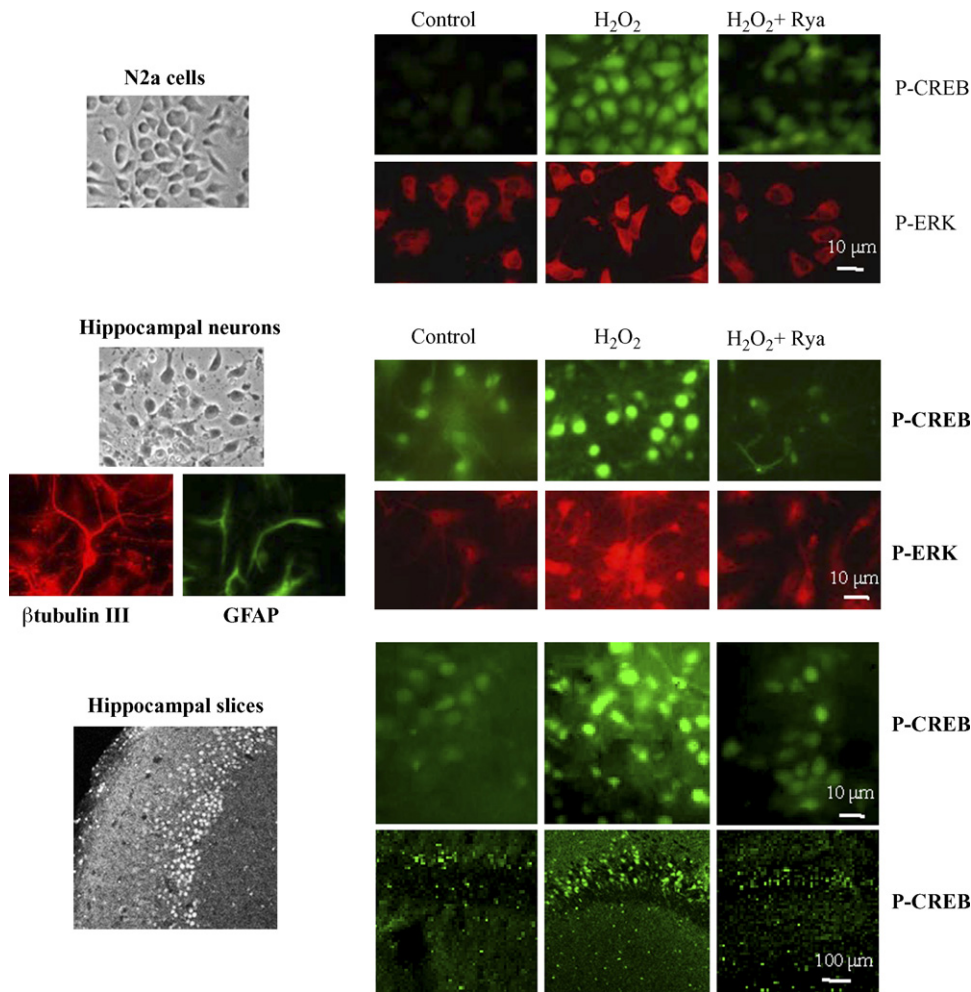


Fig. 4. Stimulation by H₂O₂, visualized by immunocytochemical or immunohistochemical analysis, of phospho-ERK1/2 or phospho-CREB in N2a cells, hippocampal cells in primary culture, or hippocampal slices. Phospho-ERK1/2 or phospho-CREB staining was obtained with their corresponding fluorescent secondary antibodies, as detailed in the text. Neurons or slices were fixed after 60 min preincubation with or without 50 μM ryanodine, followed by 20 min incubation with H₂O₂; 200 μM H₂O₂ was added to cells in culture and 1 μM H₂O₂ to hippocampal slices. Fluorescent antibodies against β tubulin III to stain neurons, or against the glial fibrillary acidic protein GFAP to stain glial cells, were used as controls for hippocampal cells in culture. Fluorescent images were obtained as detailed in the text; transmitted images for N2a cells or hippocampal cells in primary culture are also shown.

rylation produced by H₂O₂. To test whether H₂O₂-induced RyR redox modifications that might stimulate RyR-mediated Ca²⁺ release, we measured the levels of RyR S-glutathionylation before and after addition of H₂O₂. As illustrated in Fig. 7, incubation of either N2a cells or hippocampal neurons in culture with 200 μM H₂O₂ enhanced significantly (>2-fold) RyR S-glutathionylation over the endogenous basal levels. RyR S-glutathionylation was lost after incubation of samples in vitro with dithiothreitol (not shown), indicating that reducing agents can readily reverse this redox modification.

4. Discussion

The results described herein suggest strongly that stimulation of RyR-mediated Ca²⁺ release, presumably through H₂O₂-induced redox modifications of the RyR protein, is

responsible for the ERK/CREB activation displayed by N2a cells and hippocampal neurons exposed to H₂O₂. The physiological relevance of these results lies in their possible relevance to long-lasting LTP, which in the hippocampus requires ROS production and the Ca²⁺-induced Ras/ERK activation necessary for long-lasting CREB phosphorylation and CREB-dependent gene transcription.

Depolarizing stimuli activate Ca²⁺ entry through postsynaptic NMDA receptors or L-type voltage-dependent Ca²⁺ channels [16,17]. Calcium entry may also stimulate Ca²⁺ release from intracellular stores [6,61], resulting in amplification and propagation of the initial Ca²⁺ entry signal via Ca²⁺-induced Ca²⁺ release (CICR). An increase in intracellular free Ca²⁺ concentration is a requisite for LTP induction. Through Ras stimulation [for reviews see 14,60] Ca²⁺ activates the Ras/MEK/ERK1/2 cascade. It has been demonstrated that ERK activation is required for sustained CREB phosphorylation and CREB-dependent transcription of the

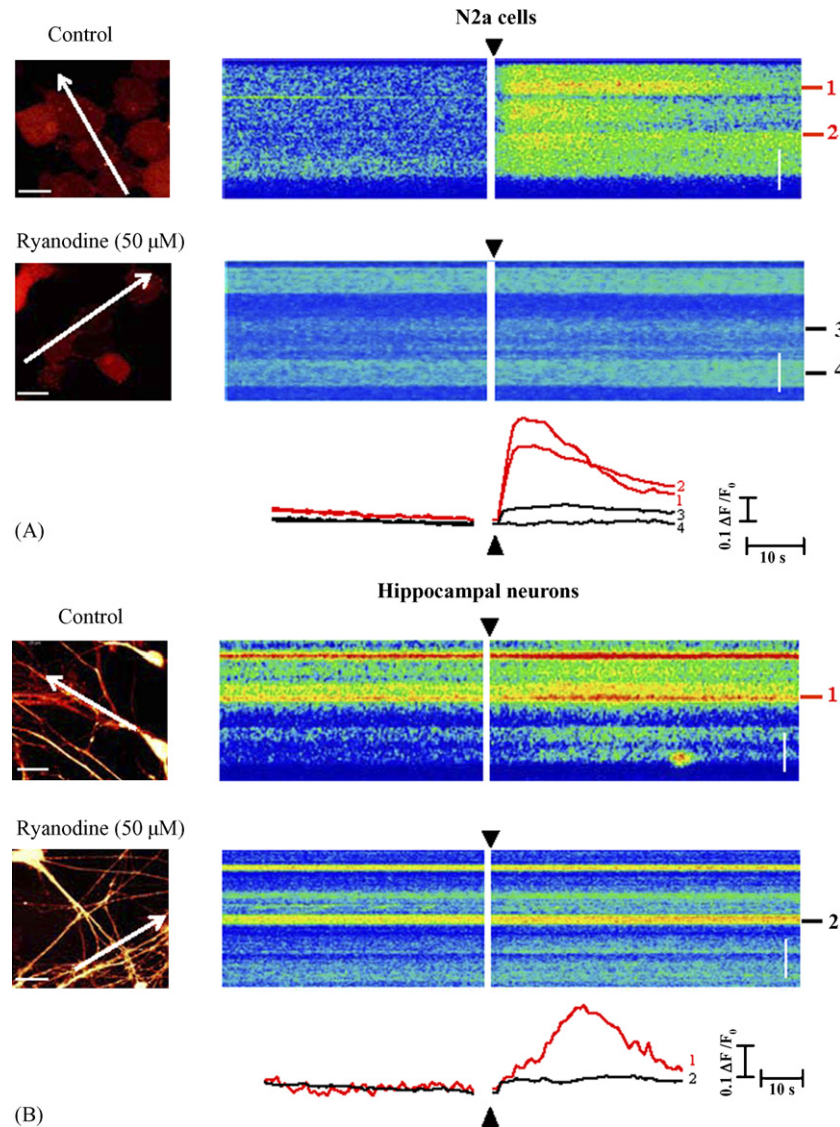


Fig. 5. Determination of Ca^{2+} signals in N2a cells or hippocampal neurons in culture exposed to H_2O_2 . To determine intracellular Ca^{2+} levels, cells were preloaded with 5 μM fluo 3-AM as detailed in the text. When indicated, cells were preincubated with 50 μM ryanodine for 60 min. All fluorescence image data were taken at room temperature (20–22 $^{\circ}C$) in the time line scan mode; line scan images were collected every 10 ms and the average of four line scans was obtained as detailed in the text. Image collection was stopped after collecting a set of 2000–4000 line scans; after a few seconds, scan collection was reinitiated concomitantly with the addition of 200 μM H_2O_2 (arrowheads). The left panels in (A) and (B) show images of the cell cultures (horizontal calibration bar = 10 μm) used to obtain the line scan panels shown at right for control cells (upper panels) or for cells incubated with ryanodine (lower panels); vertical calibration bar = 10 μm . (A) For N2a cells in culture, the scan lines were oriented along the cell body, avoiding cell nuclei. The lower part of (A) shows the time course of fluorescence changes recorded at two specific points in control or ryanodine-treated cells (indicated at the right of line scan images). (B) For hippocampal neurons in primary culture, line scans were oriented along segments of neuronal prolongations. The lower part of (B) shows the time course of fluorescence changes recorded from a single point in control or ryanodine-treated cells (indicated at the right of line scan images). In (A) and (B), fluorescence changes are plotted as $\Delta F/F_0$, where F_0 corresponds to the basal fluorescence obtained from the 2000 or 4000 line scans collected before H_2O_2 addition.

genes required for long-lasting synaptic plasticity and long-term memory acquisition [16,25,26,31,39,60,62,63]. Both, activity-dependent postsynaptic Ca^{2+} entry and Ca^{2+} release signals contribute to LTP induction in the hippocampus [41]. Yet, specific ERK stimulation by Ca^{2+} signals generated via RyR-mediated Ca^{2+} release has not been reported.

In hippocampal neurons, NMDA receptor activation also promotes the generation of ROS/RNS [7,12,29,48]. Recent evidence indicates that ROS have an important role in ERK

activation and in the induction of long-lasting LTP in the hippocampus [35,54]. Furthermore, hippocampal neurons possess an intrinsic NOX activity that produces superoxide anion [35,54,58]. By enzymatic or chemical dismutation, superoxide anion readily generates H_2O_2 as a by-product. Pharmacological and genetic manipulations that lead to NOX inhibition abolish NMDA receptor-induced ERK activation [35]. These results suggest that NOX-dependent ROS production forms part of the signaling cascades linking stimulation

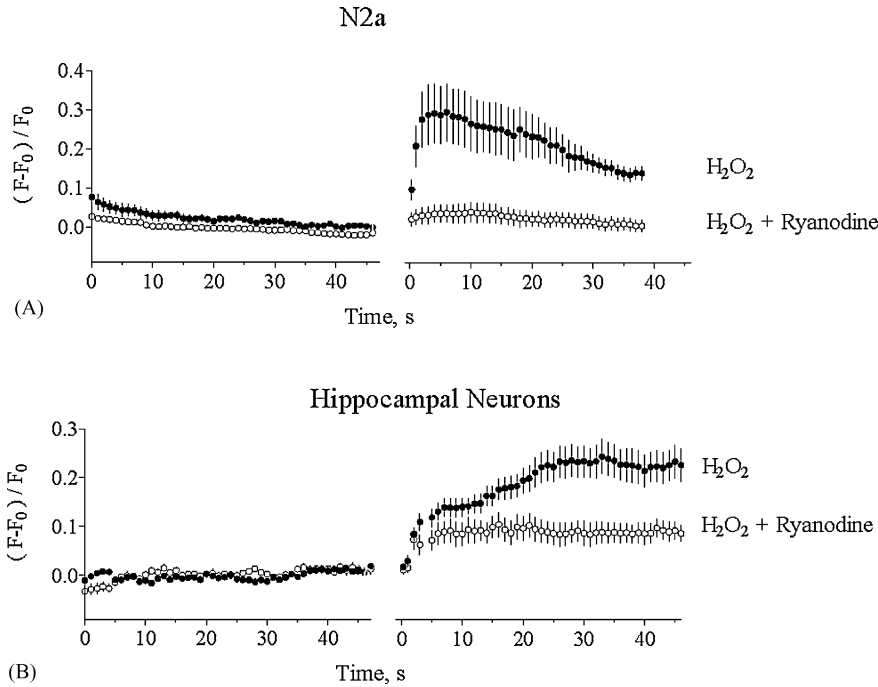


Fig. 6. Time course of H_2O_2 -induced changes in intracellular free $[Ca^{2+}]$, determined as fluo 3 fluorescence, in N2a cells (A) or hippocampal neurons in primary culture (B). At time zero, $200 \mu\text{M } H_2O_2$ was added to the external solution. For N2a cells, values were obtained from three different cultures, with $n = 7$ both for control cells (closed circles) and for cells preincubated with $50 \mu\text{M}$ ryanodine (open circles). For hippocampal cells in culture, values were obtained from five different cultures, with $n = 23$ for control cells (closed circles) and $n = 8$ for cells preincubated with $50 \mu\text{M}$ ryanodine (open circles). All values are given as mean \pm S.E. Conditions were as detailed in the legend to Fig. 5.

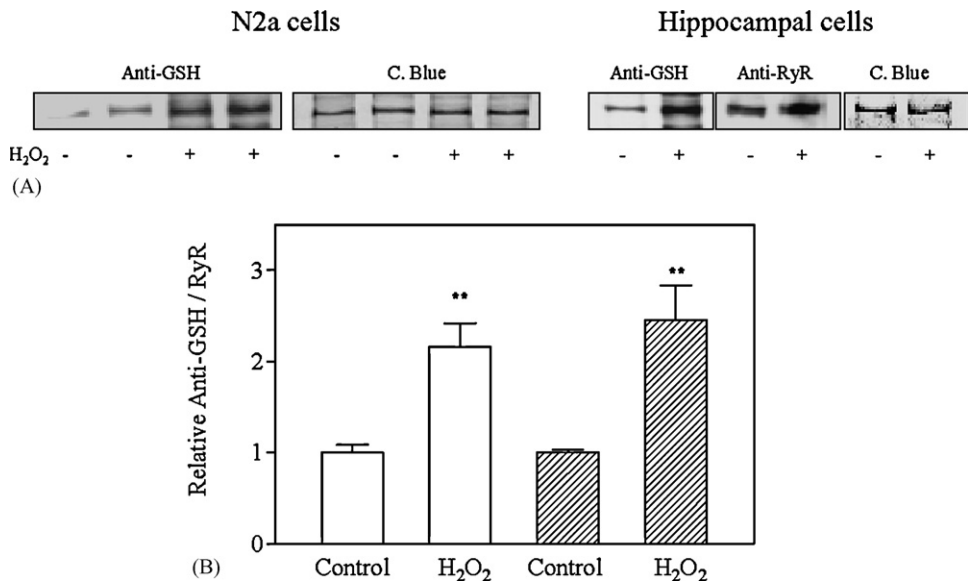


Fig. 7. Determination of RyR S-glutathionylation in N2a cells or hippocampal neurons. Non-reducing gels from control cells or from cells incubated for 20 min with $200 \mu\text{M } H_2O_2$ were transferred to PVDF membranes and sequentially probed with anti-GSH and anti-RyR antibodies; after stripping, membranes were stained with Coomassie blue as detailed in the text. (A) Images correspond to a representative PVDF membrane of N2a cells or hippocampal neurons probed with anti-GSH or anti-RyR antibodies, or stained with Coomassie blue. (B) The bar graph represents the ratios between band densities obtained with anti-GSH or anti-RyR. Ratios obtained from at least 3 independent experiments were determined from blots like those shown in upper part of the figure. Values are given as mean \pm S.E. Open bars: N2a cells ($n = 5$); hatched bars: hippocampal neurons ($n = 7$). ** $p < 0.01$.

of NMDA receptors with ERK activation in hippocampal neurons.

Several reports indicate that exogenously added H₂O₂ increases ERK phosphorylation in PC12 cells [5,24,65,66] and cortical neurons [13]. In hippocampal slices, 10 mM H₂O₂ increases ERK1/2 phosphorylation; this increase is blocked by the antioxidant *N*-acetylcysteine [34]. The possible participation of Ca²⁺ release from intracellular stores in H₂O₂-induced ERK activation has not been reported. The present findings, which show that μM H₂O₂ concentrations stimulate ERK phosphorylation in N2a cells and hippocampal neurons, confirm the stimulatory role of H₂O₂ on this reaction. In addition, we report here for the first time that H₂O₂ also can stimulate CREB phosphorylation in hippocampal neurons in primary culture or in hippocampal slices, as we reported previously for N2a cells [11]. Yet, in comparison to cells in culture 10- to 20-fold lower H₂O₂ concentrations were required in mouse hippocampal slices to produce significant stimulation of ERK1/2 and CREB phosphorylation. As mentioned in Section 3, this difference may be due to the adaptation of cells grown in culture to a significantly higher oxygen concentration than present in brain tissue, an adaptation which may have made cells in culture less sensitive to redox agents than freshly dissected hippocampal slices. The stimulation of ERK and CREB phosphorylation induced by H₂O₂ in N2a cells did not require extracellular Ca²⁺ and was drastically curtailed by U0126 or by thapsigargin-induced depletion of endoplasmic reticulum Ca²⁺.

Exposure of N2a cells or hippocampal neurons to H₂O₂ generated ryanodine-sensitive intracellular Ca²⁺ signals, indicating that H₂O₂ stimulated RyR-mediated Ca²⁺ release. Likewise, in N2a cells or hippocampal neurons a concentration of ryanodine that blocks RyR-mediated Ca²⁺ release exerted a powerful inhibitory effect or suppressed the ERK/CREB stimulation produced by H₂O₂. These combined results strongly suggest that H₂O₂ stimulates RyR-mediated Ca²⁺ release from the ER, and that the resulting Ca²⁺ concentration increase activates the Ras/MEK/ERK cascade, promoting ERK-dependent CREB phosphorylation.

Endogenous ROS, including H₂O₂, stimulate RyR-mediated Ca²⁺ release in vitro while reducing agents have the opposite effects [2,20,21,46]. In particular, highly reduced single RyR channels from neurons barely respond in vitro to activation by Ca²⁺ [42], even in the presence of ATP [8]. Presumably, this redox sensitivity makes RyR-mediated CICR highly dependent on cellular redox state [28]. Resting neurons have cytoplasmic GSH/GSSG ratios ≥60 [44]; the resulting highly reducing potential of the neuronal cytoplasm [52] is bound to keep RyR in a reduced state, presumably hindering RyR activation by Ca²⁺. We propose that modification of RyR redox state by ROS generated during neuronal activation makes possible efficient RyR activation by the concomitant Ca²⁺ entry signals. In fact, each RyR subunit possesses highly reactive cysteine residues, susceptible to modification by oxidation, *S*-nitrosylation, *S*-glutathionylation

and alkylation at physiological pH. Thus, H₂O₂, molecular oxygen, nitrosoglutathione, glutathione disulfide and NO or NO donors all enhance the activity of mammalian RyR [28]. We have shown here that H₂O₂ increased RyR *S*-glutathionylation, a redox modification that enhances RyR-mediated CICR in skeletal and cardiac muscle [2,51] and which is likely to enhance CICR in neuronal cells as well.

Redox modifications of the Ras protein by ROS/RNS stimulate Ras activity [1,27]. Accordingly, direct Ras stimulation might also contribute to the stimulation of ERK phosphorylation induced by H₂O₂. The results obtained with ryanodine, which when used in conditions that ensure selective RyR inhibition essentially abolished all the stimulatory effects of H₂O₂, make unlikely this possibility and strongly suggest that functional RyR are the primary targets of H₂O₂ both in N2a cells and hippocampal neurons.

4.1. Physiological implications

The hippocampus of rats trained in an intensive water maze task displays increased expression of the RyR2 isoform, suggesting that RyR-mediated Ca²⁺ release signals may be involved in memory processing after spatial learning [67]. In hippocampal neurons, RyR activation enhances activity-dependent release of brain derived neurotrophic factor (BDNF) [3] and elicits changes in spine morphology, producing a significant increase in spine area [37]. Additionally, BDNF treatment (3–6 h) of cultured hippocampal neurons-induced mRNAs encoding several synapse-associated proteins, including RyR2 [49]. Functional RyRs are required to elicit NMDA receptor-mediated Ca²⁺ signals in hippocampal postsynaptic dendritic spines [19], albeit opposing results have also been presented [38]. RyR inhibition with 10 μM ryanodine significantly reduces late LTP induction and activity-dependent CREB phosphorylation in postsynaptic neurons, while lower ryanodine concentrations that activate RyR shift the induction of early LTP to late LTP [40]. We have found that preincubation of hippocampal neurons in culture with 50 μM ryanodine blocks both the intracellular Ca²⁺ increase and the stimulation of ERK phosphorylation induced by NMDA.¹ As discussed above, stimulation protocols that lead to long-lasting LTP induction activate ROS generation in the hippocampus. Based on these observations, we propose that in the hippocampus joint activation of RyR-mediated Ca²⁺ release by Ca²⁺ and ROS may be one of the early events in the postsynaptic signaling cascade that is initiated by NMDA receptor activation and that culminates in long-lasting LTP.

In summary, we propose that RyR channels act as coincidence detectors of the concomitant Ca²⁺ and ROS increase induced by NMDA receptor activation, and thus allow cross talk between Ca²⁺ signaling and redox signaling cascades in postsynaptic hippocampal neurons. Conditions that promote

¹ P. Muñoz, A. Kirkwood, E. Klann, C. Hidalgo and M.T. Núñez, manuscript in preparation.

oxidative stress, such as aging [18], may imbalance this cross communication, resulting in excessive stimulation of Ca^{2+} release that, if not controlled, could induce pathological conditions or even neuronal death [45]. Noteworthy, inhibition of RyR-mediated Ca^{2+} release in hippocampal CA1 neurons reduced or eliminated age-induced differences of several biomarkers that are Ca^{2+} -dependent [23]. These results suggest that excessive ROS production in aging neurons [18] may cause faulty Ca^{2+} homeostasis through over stimulation of RyR-mediated CICR.

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