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Inhibitory ryanodine prevents ryanodine receptor-mediated Ca^{2+} release without affecting endoplasmic reticulum Ca^{2+} content in primary hippocampal neurons

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A B S T R A C T

Ryanodine is a cell permeant plant alkaloid that binds selectively and with high affinity to ryanodine receptor (RyR) Ca^{2+} release channels. Sub-micromolar ryanodine concentrations activate RyR channels while micromolar concentrations are inhibitory. Several reports indicate that neuronal synaptic plasticity, learning and memory require RyR-mediated Ca^{2+}-release, which is essential for muscle contraction. The use of micromolar (inhibitory) ryanodine represents a common strategy to suppress RyR activity in neuronal cells: however, micromolar ryanodine promotes RyR-mediated Ca^{2+} release and endoplasmic reticulum Ca^{2+} depletion in muscle cells. Information is lacking in this regard in neuronal cells; hence, we examined here if addition of inhibitory ryanodine elicited Ca^{2+} release in primary hippocampal neurons, and if prolonged incubation of primary hippocampal cultures with inhibitory ryanodine affected neuronal ER calcium content. Our results indicate that inhibitory ryanodine does not cause Ca^{2+} release from the ER in primary hippocampal neurons, even though ryanodine diffusion should produce initially low intracellular concentrations, within the RyR activation range. Moreover, neurons treated for 1 h with inhibitory ryanodine had comparable Ca^{2+} levels as control neurons. These combined findings imply that prolonged incubation with inhibitory ryanodine, which effectively abolishes RyR-mediated Ca^{2+} release, preserves ER Ca^{2+} levels and thus constitutes a sound strategy to suppress neuronal RyR function.

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1. Introduction

In neuronal cells, activity-dependent elevations in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) have a central role in synaptic plasticity [1,2], and the calcium-dependent gene transcription events [3,4] underlying learning and memory [5]. Activity-dependent neuronal Ca^{2+} signals arise from Ca^{2+} entry mostly via voltage-gated or ligand-gated Ca^{2+} channels, and/or from Ca^{2+} release from intracellular stores via inositol 1,4,5-trisphosphate or ryanodine receptor (RyR) Ca^{2+} channels. Numerous studies support the crucial role of RyR-mediated Ca^{2+} release on cardiac and skeletal muscle function [6]. Mounting evidence also points to a significant role of RyR-mediated Ca^{2+} release on normal [7,8] and pathological neuronal function [5,9].

Ryanodine is a cell permeant plant alkaloid [10] that binds selectively and with high affinity to the RyR channel protein but dissociates very slowly once bound [11]. These binding properties allowed the initial identification of the RyR protein (reviewed in Ref. [12]). Early studies established the presence of RyR channels in avian cerebellar Purkinje neurons [13,14] and rabbit brain [15], where they function as caffeine-sensitive Ca^{2+} release channels [16]. Subsequent studies identified the presence of the three mammalian RyR isoforms in different brain regions (reviewed in Ref. [17]), and described changes in their expression and location during rodent hippocampus development [18]. At the sub-cellular level, RyR channels are present in the soma, dendrites, dendritic spines and synaptic terminals of rodent brain neurons [19].

The contribution of RyR-mediated Ca^{2+} release to synaptic plasticity and memory processes is receiving increasing attention

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(for recent reviews, see Refs. [7,8]). In dendritic spines of hippocampal CA1 neurons, RyR channels amplify Ca^{2+} entry signals elicited by synaptic stimulation [20], albeit this role has been questioned [21]. In hippocampal slices, presynaptic RyR inhibition prevents the induction of long-term depletion (LTD) [22], whereas RyR-mediated Ca^{2+} release contributes to spontaneous and activity-induced transmitter release and induces specific forms of synaptic plasticity in hippocampal and cerebellar neurons [23,24]. Likewise, hippocampal long-term potentiation (LTP) requires postsynaptic RyR-mediated Ca^{2+} release (reviewed in Ref. [8]). Brain-derived neurotrophic factor (BDNF) enhances dendritic growth in an activity dependent manner in rodent hippocampus, and increases dendritic spine density through ERK1/2 pathway activation [25,26]. These changes require functional RyR channels [27], suggesting that RyR-mediated Ca^{2+} release is a key component of the complex neuronal plasticity processes induced by BDNF, which may underlie the formation/storage of long-term memory. In fact, recent reports have implicated RyR-mediated Ca^{2+} release in both normal and defective hippocampal-dependent memory processes (reviewed in Ref. [8]).

The use of ryanodine at micromolar concentrations represents a common strategy to suppress RyR activity. However, ryanodine exerts complex effects on RyR channel function so that modification of RyR activity with ryanodine requires particular conditions [8]. Low concentrations of ryanodine (5–10 nM) activate single RyR channels from frog skeletal muscle [28]. Higher concentrations (0.25–1 μM) lock RyR channels from frog skeletal muscle [28] and rat brain microsomes [29] in a lower conductance level with a fractional open time near unity and cause substantial Ca^{2+} release from skeletal muscle sarcoplasmic reticulum (SR) vesicles [30]. Ryanodine concentrations ≥10 μM irreversibly inhibit RyR channel activity [30]. Ryanodine binds preferentially to open RyR channels, a condition infrequently found in living cells. Accordingly, effective RyR inhibition with ryanodine requires long pre-incubation (≥1 h) of live cells with concentrations of ryanodine ≥ 10 μM. Following inhibitory ryanodine addition, intracellular ryanodine levels may reach initially low concentrations – within the RyR activating range – that may result in sustained calcium depletion from the endoplasmic reticulum (ER). In fact, inhibitory ryanodine concentrations promote contraction and deplete intracellular Ca^{2+} stores in skeletal muscle cells [31,32].

To our knowledge, however, there are no reports testing directly if addition of inhibitory ryanodine elicits RyR-mediated Ca^{2+} release and depletes ER Ca^{2+} content in neuronal cells. Accordingly, we examined here the effects of inhibitory ryanodine on Ca^{2+} release and neuronal ER calcium content in primary hippocampal neurons.

2. Materials and methods

2.1. Primary rat hippocampal cultures

Primary cultures were prepared from the hippocampus dissected from Sprague–Dawley rats at embryonic day 18 [27,33]. Cells were plated in minimum essential medium plus 10% horse serum for 40 min to allow the adhesion of neurons and minimize glial cell adhesion. The cultures were maintained subsequently at 37 °C under 5% CO_2 in serum-free Neurobasal medium supplemented with GIBCOTM B27 serum-free and 2 mM GlutamaxTM (Invitrogen). Hippocampal cultures were used at 14 days in vitro (DIV). All experiments were carried out following the guidelines provided by National Institutes of Health (USA) and the regulations for the Care and Use of Animals for Scientific Purposes; the Bioethics Committee, F. Medicine, Universidad de Chile approved all protocols used in this work.

2.2. Determination of cytoplasmic Ca^{2+} signals

To detect changes in $[Ca^{2+}]_{cyt}$, we used Fluo-4 (Kd = 345 nM) as fluorescent cytoplasmic Ca^{2+} probe. Cultures in modified Tyrode solution (in mM: 129 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 30 glucose, and 25 HEPES-Tris, pH 7.3) were pre-incubated for 30 min at 37 °C with 5 μM Fluo-4 AM (Molecular Probes). After washing the cultures for 10 min in modified Tyrode solution to allow complete dye de-esterification, they were transferred to Ca^{2+}-free medium just prior to fluorescence recording, unless otherwise indicated. Fluorescence images of intracellular Ca^{2+} signals in primary hippocampal neurons (14 DIV) were obtained every 5 s with an inverted confocal microscope (Carl Zeiss, Axiovert 200, LSM 5 Pa, Oberkochen, Germany, Plan Apochromatic 63× Oil DIC objective, optical slice 1000 μm, excitation 488 nm, argon laser beam). Image data were acquired from different regions of optical interest (ROI) defined with the same area and located in the cell bodies, excluding the nucleus; frame scans were averaged using the equipment data acquisition program. For experiments performed in nominally Ca^{2+}-free Tyrode solution, 0.5 mM EGTA was added and 2 mM $CaCl_2$ was replaced by 2 mM $MgCl_2$. In all cases, the increase in intracellular $[Ca^{2+}]$ caused by 0.5 mM 4-chloro-m-cresol (4-CMC), 5 μM thapsigargin or 2.5 μM ionomycin did not saturate the probe. All experiments were done at room temperature (20–22 °C).

2.3. Determination of endoplasmic reticulum Ca^{2+} signals

To detect Ca^{2+} changes in the ER we used Fluo-5N AM, a low affinity (Kd = 90 μM) fluorescent Ca^{2+} probe. Cells were transferred to modified Tyrode solution (see above) and were loaded for 180 min at 37 °C with 5 μM Fluo-5N AM (Molecular Probes). During the last 30 min, the cultures were treated with 10 uM 1,2-bis(o-amino phenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) AM (Molecular Probes) to eliminate cytoplasmic fluorescence and were washed for 10 min in modified Tyrode solution to allow complete dye de-esterification. Cultures were transferred to Ca^{2+}-free solution just prior to fluorescence recording. Fluorescence images were obtained every 5 s with an optical slice of 1 μm in the inverted confocal microscope described above. Image data were acquired from specific ROI corresponding to fluorescent hot spots located in the soma and excluding the nucleus. Frame scans were averaged using the equipment data acquisition program. The fluorescence decay for control and ryanodine-treated cultures (1 h, 50 μM) followed a single exponential function; decay constants (k) and half-life ($t_{1/2}$) values were calculated with the GraphPad Software (San Diego, CA). All experiments were done at room temperature (20–22 °C).

2.4. Statistics

Results are expressed as Mean ± SE. Significance was evaluated with the GraphPad Software (San Diego, CA), using unpaired t-test analysis.

3. Results and discussion

3.1. The RyR agonist 4-CMC elicits ryanodine-sensitive Ca^{2+} release

We used the fluorescent calcium probe Fluo-4 to determine changes in $[Ca^{2+}]_{cyt}$ in primary hippocampal cultures. To validate the determination of RyR-mediated Ca^{2+} signals, we used the RyR agonist 4-CMC, an agent extensively employed to study RyR-mediated Ca^{2+} release in muscle fibers and other cells, including primary hippocampal neurons [27,33–35]. Addition of 0.5 mM 4-CMC to control cultures previously transferred to Ca^{2+}-free

solution increased Fluo-4 fluorescence in the neuronal soma; the fluorescence increase was transient and decayed to basal levels in less than 2 min (Fig. 1A, solid symbols). The representative images (Fig. 1B) show the transient fluorescence increase induced by 4-CMC in control neurons. Hippocampal neurons pre-incubated for 1 h with 50 μM ryanodine did not present a fluorescence increase following addition of 0.5 mM 4-CMC (Fig. 1A, gray symbols). These results confirm and add to our previous reports, describing suppression by inhibitory ryanodine of the Ca^{2+} signals elicited by 4-CMC in primary cultures kept in Ca^{2+}-containing solution [27,33]. The 4-CMC concentration used in this work (0.5 mM) is below the concentrations (>1 mM) that inhibit the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in skeletal muscle [36]. Therefore, the ryanodine-sensitive $[Ca^{2+}]_{cyt}$ increase produced by 4-CMC in control hippocampal neurons likely reflects solely RyR-mediated Ca^{2+} release.

3.2. Ryanodine addition does not generate detectable cytoplasmic Ca^{2+} signals

To evaluate whether addition of inhibitory ryanodine to primary hippocampal neurons induces initially Ca^{2+} release from the ER, we determined Fluo-4 fluorescence before and after addition of 50 μM ryanodine to cultures kept in Ca^{2+}-containing solution. Ryanodine addition did not increase Fluo-4 fluorescence, which remained constant at basal levels for 1 h after ryanodine addition and did not increase after subsequent addition of 4-CMC (0.5 mM), an indication of effective RyR inhibition (Fig. 2). In contrast, ensuing addition of the calcium ionophore ionomycin (2.5 μM) increased probe fluorescence, which decreased to minimal levels after addition of 10 mM EGTA (Fig. 2). These results, which show for the first time that addition of inhibitory ryanodine does not promote RyR-mediated Ca^{2+} release in primary hippocampal neurons, contrast to the effects of inhibitory ryanodine in skeletal muscle cells, where it promotes Ca^{2+} release-mediated contraction [31,32].

3.3. Extended incubation with inhibitory ryanodine does not affect the neuronal Ca^{2+} signals induced by thapsigargin or ionomycin

To determine if 1 h pre-incubation with 50 μM ryanodine led to a reduction in neuronal ER Ca^{2+} content, cultures were treated with thapsigargin, a SERCA inhibitor that in the absence of extracellular Ca^{2+} causes net Ca^{2+} efflux from this compartment. Addition of 5 μM thapsigargin induced a transient increase in Fluo-4 fluorescence in the soma of control neurons, which returned to basal levels about one minute after the maximum increase (Fig. 3A, solid symbols). In cultures pre-incubated with ryanodine, thapsigargin addition produced a comparable increase in Fluo-4 fluorescence (Fig. 3A, gray symbols). The areas under the curve (≥7 records, four independent cultures) show no differences between both conditions (Fig. 3A, inset), with values (in arbitrary units) of 16.7 ± 2.4 and 21.8 ± 4.2, for control and ryanodine-treated neurons, respectively. In Ca^{2+}-free solution, addition of 2.5 μM ionomycin also increased Fluo-4 fluorescence to comparable levels in control neurons and in neurons of cultures pre-incubated for 1 h with inhibitory ryanodine (Fig. 3B). The areas under the curve (≥9 records, three independent cultures) show no differences between both conditions (Fig. 3B, inset), with values (in arbitrary units) of 112.4 ± 13.83 and 113.9 ± 10.3 for control and ryanodine-treated neurons, respectively. The Fluo-4 fluorescence increase induced by ionomycin was larger than that elicited by thapsigargin. This difference may reflect the contribution of thapsigargin-insensitive intracellular stores, such as the mitochondria, to the calcium increase induced by ionomycin.

3.4. Extended incubation with ryanodine does not reduce the ER Ca^{2+} content

To explore in further detail if prolonged treatment with inhibitory ryanodine affected ER Ca^{2+} content, we added 5 μM thapsigargin to control and ryanodine-treated cultures loaded with Fluo-5N AM and transferred to Ca^{2+} free solution just before starting the

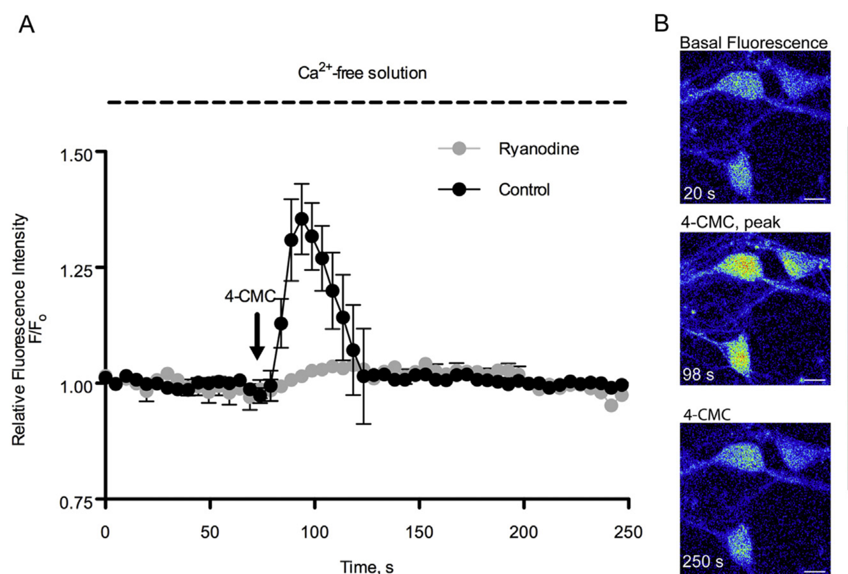

Fig. 1. The RyR agonist 4-chloro-methyl-cresol (4-CMC) elicits RyR-mediated Ca^{2+}-signals in primary hippocampal neurons. Representative time course of Fluo-4 fluorescence changes collected from ROI recorded in the soma before and after addition of 0.5 mM 4-CMC. Fluorescence values are expressed as (F/F_0), where F_0 corresponds to the basal fluorescence recorded in the soma before 4-CMC addition. Control cultures (black symbols) displayed a transient increase in fluorescence after 4-CMC addition. Pre-incubation with ryanodine (1 h, 50 μM) prevented the fluorescence increase induced by 4-CMC (gray symbols). Data (Mean ± SE) correspond to the fluorescence of 9 ROI recorded in control neurons and 6 ROI in ryanodine-treated neurons, from three independent primary cultures. Scale bars: 20 μm.

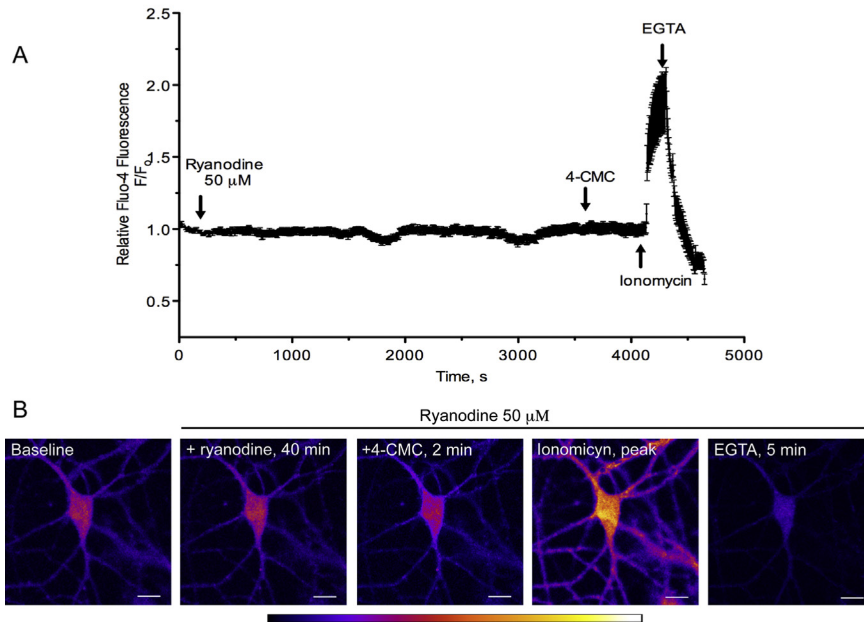

Fig. 2. Addition of inhibitory ryanodine does not elicit intracellular Ca^{2+} signals in primary hippocampal neurons. A) Representative time course of Fluo-4 fluorescence, recorded in neuronal soma before and after addition of 50 μM ryanodine (Rya, arrow). The RyR agonist 4-CMC, added 1 h after ryanodine addition (second arrow) did not increase Fluo-4 fluorescence, an indication of complete RyR inhibition. In contrast, subsequent addition (10 min later) of the Ca^{2+} ionophore ionomycin (third arrow) induced a significant fluorescence increase. Minimal fluorescence values were recorded at the end of the experiment following addition of 10 mM EGTA. Fluorescence changes (Mean ± SE) are expressed as (F/F_0), where F_0 corresponds to the basal fluorescence recorded in the soma before ryanodine addition. The graph illustrates average values from 6 ROI registered at the soma of neurons from two different cultures. Similar findings were obtained in four different cultures. B) Representative fluorescent images captured at different times from the fluorescence record illustrated in A. Scale bars: 20 μm.

fluorescence determination. The existence of fluorescent well-defined hot spots in neurons indicated successful ER loading with Fluo-5N (Fig. 4A). Addition of 5 μM thapsigargin in Ca^{2+}-free solution significantly decreased Fluo-5N fluorescence collected from hot spots, both in control neurons (Fig. 4B, black symbols) and in neurons from ryanodine-treated cultures (Fig. 4B, gray symbols), which in both cases reached minimal values 30–35 min after thapsigargin addition, as illustrated for control neurons in Fig. 4A. The fluorescence decreases followed a single exponential decay function with half-life values (s) of 439.8 ± 13.2 for control and 634.9 ± 11.4 for ryanodine-treated neurons (Fig. 4B). These half-life values correspond to decay constants (min^{-1}) of 0.0945 ± 0.012 and 0.0654 ± 0.012 for control and ryanodine treated neurons, respectively (Fig. 4C). These combined results suggest first that incubation with inhibitory ryanodine does not affect ER Ca^{2+} content, in contrast to the significant Ca^{2+} depletion from the sarcoplasmic reticulum produced by inhibitory ryanodine in skeletal muscle cells [31,32]. In addition, these results indicate that RyR inhibition slows down but does not suppress Ca^{2+} release from the ER, which presumably occurs via other pathways [37].

To summarize, the present results show conclusively that inhibitory ryanodine addition to primary hippocampal neurons

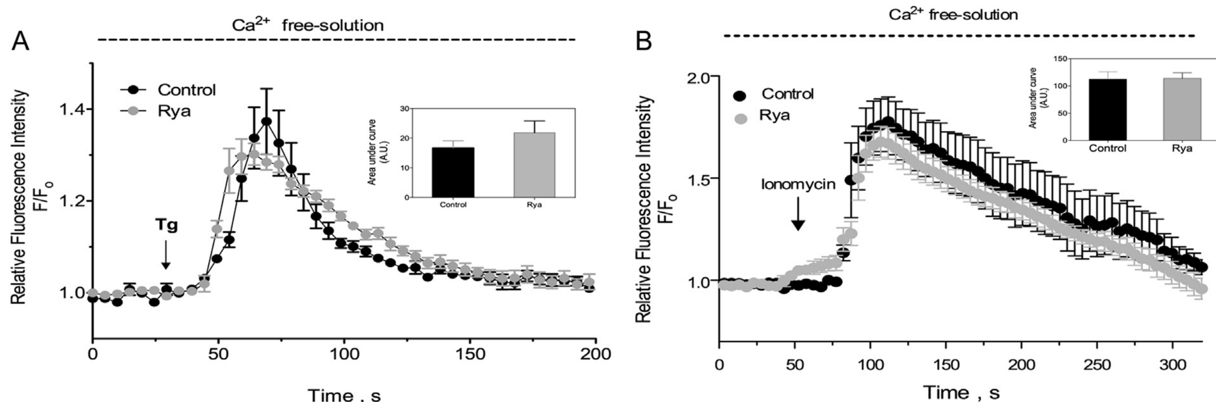

Fig. 3. Thapsigargin addition elicits similar Ca^{2+} signals in control and ryanodine-treated neurons. Time course of Fluo-4 fluorescence recorded from the neuronal soma before and after addition of thapsigargin or ionomycin to cultures loaded with Fluo-4 AM and transferred to Ca^{2+}-free solution just before starting the record. Fluorescence values are expressed as (F/F_0), where F_0 represents the basal fluorescence recorded before addition of thapsigargin or ionomycin. A) Addition of 5 μM thapsigargin (Tg, arrow) elicited similar Ca^{2+} signals in control neurons (black symbols) and in neurons pre-incubated with 50 μM ryanodine for 1 h (gray symbols). The figure illustrates average results (Mean ± SE) from 7 ROI in control and 5 ROI from ryanodine-treated neurons, taken from four different primary cultures. B) Addition of 5 μM ionomycin (arrow) induced similar Ca^{2+} signals in both control (black symbols) and ryanodine-treated neurons (50 μM, 1 h, gray symbols). The figure illustrates average results (Mean ± SE) from 13 ROI in control and 10 ROI in ryanodine-treated neurons, recorded from three independent primary cultures. The graphs in the insets to A and B show the quantification of the areas under the curve.

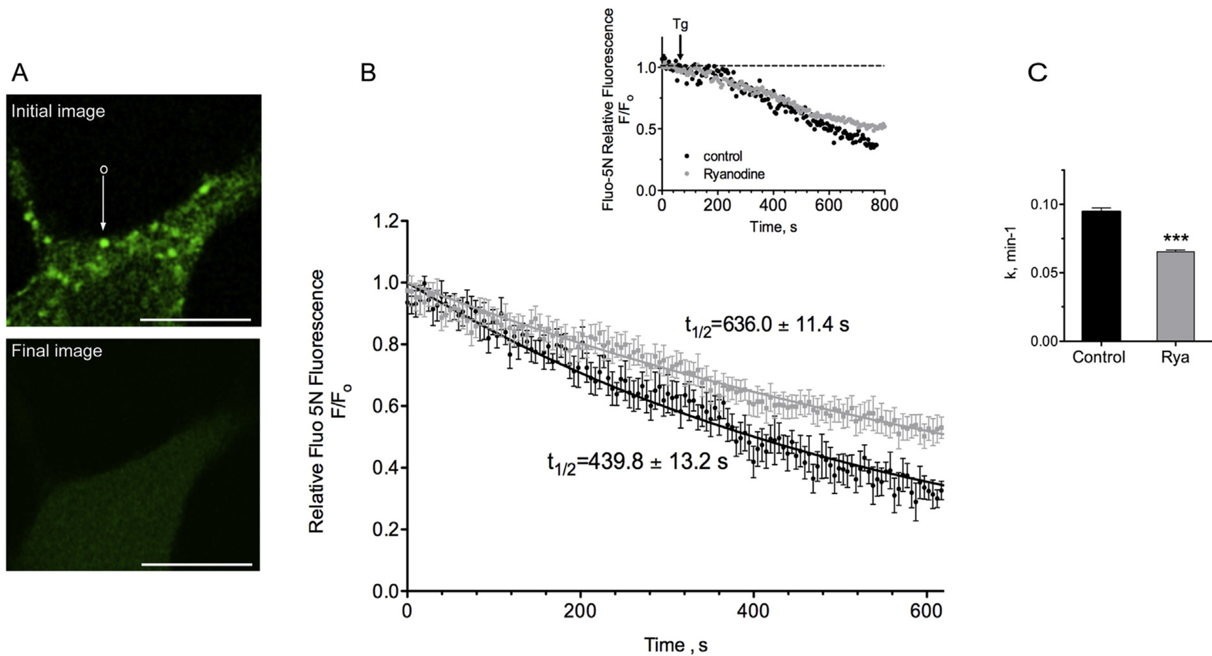

Fig. 4. Neurons incubated with inhibitory concentrations of ryanodine have the same ER Ca^{2+} content as control neurons. Primary hippocampal cultures were loaded with Fluo-5N and transferred to Ca^{2+}-free solution; fluorescence signals were recorded from ROI corresponding to hot spots of the neuronal soma before and after addition of 5 μM thapsigargin. A) Representative images of Fluo-5N fluorescence recorded before (top image) and 30 min after thapsigargin addition (lower image), showing an example of the ROI used for acquisition of fluorescence data. Scale bars: 10 μm. B) Following thapsigargin addition, values of Fluo-5N fluorescence (Mean ± SE) were recorded from 6 ROI in controls (black symbols) and 10 ROI in ryanodine-treated neurons (gray symbols), corresponding to two independent cultures. Fluorescence values are expressed as (F/F_0), where F_0 represents the basal fluorescence recorded in hot spots before thapsigargin addition. Records are representative of three independent cultures with similar experimental results. Fluorescence decayed as a single exponential function in both cases, with $t_{1/2}$ values shown in the graph. The inset shown at the top illustrates the complete record. C) Decay constants (k) calculated from three independent experiments, including those shown in B; ***: $p < 0.001$.

does not cause Ca^{2+} release from the ER, in spite of the fact that by diffusion ryanodine should reach initially intracellular low concentrations that are within the RyR activation range. This is a novel and important observation, because it rules out ryanodine-induced transient increments in $[Ca^{2+}]_{cyt}$ that via activation of Ca^{2+} signaling cascades might impinge on neuronal function. In addition, the results presented in this work indicate that neurons in primary hippocampal cultures treated for 1 h with inhibitory ryanodine contain comparable ER Ca^{2+} levels as neurons in control cultures. These combined findings imply that prolonged incubation with inhibitory ryanodine does not elicit early Ca^{2+} signals but effectively abolishes RyR-mediated Ca^{2+} release, preserves ER Ca^{2+} levels and thus constitutes a sound strategy to suppress neuronal RyR function.

Conflict of interest

None.

Acknowledgments

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Abbreviations

BAPTA 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BDNF brain-derived neurotrophic factor
$[Ca^{2+}]_{cyt}$ cytoplasmic free Ca^{2+} concentration
4-CMC 4-chloro-m-cresol
DIV days in vitro
EGTA ethylene glycol tetraacetic acid
ER endoplasmic reticulum
k exponential decay constant
LTD long-term depletion
LTP long-term potentiation
ROI regions of optical interest
Rya ryanodine
RyR ryanodine receptor
SERCA sarco-endoplasmic reticulum Ca^{2+}-ATPase
$t_{1/2}$ half-life time
Tg thapsigargin

Transparency document

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