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Molecular and functional identification of a mitochondrial ryanodine receptor in neurons

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HIGHLIGHTS

- Dantrolene and ryanodine block mitochondrial Ca^{2+} uptake in striated neurons.
- Ryanodine receptor (RyR) is expressed in the inner mitochondrial membrane in neurons.
- Brain mitochondria bind [^{3}H]ryanodine both in Ca^{2+}- and caffeine-sensitive manner.
- Mitochondrial RyR takes part in the mitochondrial Ca^{2+} influx mechanism in neurons.

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ABSTRACT

Mitochondrial Ca^{2+} controls numerous cell functions, such as energy metabolism, reactive oxygen species generation, spatiotemporal dynamics of Ca^{2+} signaling, cell growth and death in various cell types including neurons. Mitochondrial Ca^{2+} accumulation is mainly mediated by the mitochondrial Ca^{2+} uniporter (MCU), but recent reports also indicate that mitochondrial Ca^{2+}-influx mechanisms are regulated not only by MCU, but also by multiple channels/transporters. We previously reported that ryanodine receptor (RyR), which is a one of the main Ca^{2+}-release channels at endoplasmic/sarcoplasmic reticulum (SR/ER) in excitable cells, is expressed at the mitochondrial inner membrane (IMM) and serves as a part of the Ca^{2+} uptake mechanism in cardiomyocytes. Although RyR is also expressed in neuronal cells and works as a Ca^{2+}-release channel at ER, it has not been well investigated whether neuronal mitochondria possess RyR and, if so, whether this mitochondrial RyR has physiological functions in neuronal cells. Here we show that neuronal mitochondria express RyR at IMM and accumulate Ca^{2+} through this channel in response to cytosolic Ca^{2+} elevation, which is similar to what we observed in another excitable cell-type, cardiomyocytes. In addition, the RyR blockers dantrolene or ryanodine significantly inhibits mitochondrial Ca^{2+} uptake in permeabilized striatal neurons. Taken together, we identify RyR as an additional mitochondrial Ca^{2+} uptake mechanism in response to the elevation of $[Ca^{2+}]_c$ in neurons, suggesting that this channel may play a critical role in mitochondrial Ca^{2+}-mediated functions such as energy metabolism.

Abbreviations: OMM, outer mitochondrial membrane; CS, contact sites; IMM, inner mitochondrial membrane; ER/SR, endo/sarcoplasmic reticulum; SERCA, sarco/endoplasmic Ca^{2+}-ATPase; VDAC, voltage-dependent anion channel; ANT, adenine-nucleotide translocase; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; RyR, ryanodine receptor; MTR, Mitotracker deep Red; Bodyipy, boron-dipyrromethene; Bodipy-Ry, Bodipy-conjugated ryanodine; Bodipy-Thap, Bodipy-conjugated thapsigargin.

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1. Introduction

Mitochondria play an important role in shaping the intracellular Ca^{2+} concentration as they can take up Ca^{2+} in response to physiological changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in various cell-types/tissues including neurons [7,10,24]. Mitochondrial Ca^{2+} accumulation was first recognized as an important mechanism for the acceleration of oxidative phosphorylation and electron transport chain activity, which results in the stimulation of ATP synthesis [12]. In addition, mitochondrial dysfunction and the loss of cellular Ca^{2+} homeostasis are frequently observed together in pathophysiological conditions such as neuronal excitotoxicity, apoptosis and neurodegenerative disorders [8]. However, the detailed mechanisms of how altered mitochondrial Ca^{2+} handling and/or mitochondrial dysfunction affect these neurological pathogenesis are not yet fully understood.

Mitochondrial Ca^{2+} influx was originally considered as a single transport mechanism through mitochondrial Ca^{2+} uniporter (MCU) which can be inhibited by ruthenium red and lanthanides (see reviews [7,24]). However, the molecular identities responsible for mitochondrial Ca^{2+} accumulation have remained an unsolved question until very recently. Recently, several groups have discovered the molecular identity of MCU and its regulatory proteins and confirmed it as the main mitochondrial Ca^{2+} uptake mechanism (see reviews [19,24]). Although in these studies MCU was confirmed as the most dominant Ca^{2+} influx mechanism, previous studies have identified additional Ca^{2+} uptake pathways, which display different physiological and pharmacological characteristics from MCU theory (see reviews [7,24]).

Among these studies, we reported that ryanodine receptor (RyR) is one of the mitochondrial Ca^{2+} influx mechanisms in another excitable cell-type, cardiomyocytes, termed mRyR (mitochondrial RyR) [2,3] (see also reviews [24,25]). Our group first identified that a low level of RyR is expressed in the mitochondrial inner membrane (IMM) in cardiomyocytes through a combination of biochemical, cell biological and electrophysiological experiments. Since cardiac mRyR exhibits a bell-shaped Ca^{2+}-dependent activation (bimodal activation) in the physiological range of $[Ca^{2+}]_c$, this unique property places mRyR as an ideal candidate for sequestering Ca^{2+} quickly and transiently during physiological $[Ca^{2+}]_c$ oscillation in excitable cells. In addition, using not only native cardiomyocytes, but also RyR overexpression/knock-out in cultured cardiac myoblasts and knock-out mouse hearts, we showed that the molecular identity of mRyR is possibly a skeletal-muscle type-isoform RyR type 1 (RyR1) and is required for Ca^{2+}-dependent acceleration of ATP production in cardiomyocytes even though the expression level is much lower than RyR2 which is the main RyR isoform expressed in cardiac sarcoplasmic reticulum (SR)/endoplasmic reticulum (ER) [3,23].

Although RyR is expressed [9,11,31] in the brain and serves as a Ca^{2+}-release channel of the intracellular Ca^{2+} store (ER) in addition to inositol 1,4,5-trisphosphate (IP_3) receptors [15,17,18], the interaction of between the RyR expression and mitochondrial functions under physiological and pathophysiological conditions in brain has been completely unknown. Therefore, the objective of this study was to investigate the possibility whether neuronal mitochondria possess mRyR similar to cardiomyocytes and to assess if mRyR takes part in the mitochondrial Ca^{2+} influx mechanism. Our finding suggests that RyR is expressed at IMM and takes up Ca^{2+} into mitochondria in response to $[Ca^{2+}]_c$ elevations.

2. Materials and methods

An expanded Section 2 is available in the online Supplementary file.

2.1. Reagents and antibodies

All reagents and antibodies were purchased from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise indicated.

2.2. Cells

All procedures involving animal use were in accordance with *the NIH Guide for the Care and Use of Laboratory Animals*, and were approved by the University Committee on Animal Resources. Primary striatal neurons were prepared from Sprague-Dawley rat embryos (embryonic day 18) [1].

2.3. Preparation of rat brain mitochondria and mitochondrial subfractions

Mitochondria-enriched protein fractions and mitochondrial subfractions were prepared using differential centrifugation [3,23].

2.4. Fluorescence microscopy

Localization of RyR was observed in live or fixed cultured striatal neurons using confocal microscopy (Leica, Heidelberg Germany). For live cells, RyR, SR/ER and mitochondria were labeled with boron-dipyrromethene (Bodipy)-conjugated ryanodine (Bodipy-Ry), Bodipy-conjugated sarco/endoplasmic Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (Bodipy-Thap) and Mitotracker Deep Red (MTR) (Molecular Probes, Eugene, OR), respectively. The pretreatment of unlabeled ryanodine completely abolished the Bodipy-Ry staining, confirmed that Bodipy-Ry specifically binds to RyRs in live striatal neurons (Supplementary Fig. 1). For time-lapse studies, live cell images were collected by TILL system (TILL Photonics, München, Germany). For detection of RyR in fixed striatal neurons, cells were probed with primary antibodies against RyR (Santa Cruz biotechnology, Santa Cruz, CA) and cytochrome *c* oxidase (Cox) (for labeling mitochondria) (Molecular Probes) followed by fluorescence-conjugated secondary antibodies. Scatter 2D plots of pixel intensities and Pearson's correlation coefficient were obtained by ImageJ software (NIH) [23].

2.5. Measurements of cytosolic and mitochondrial Ca^{2+} concentration

$[Ca^{2+}]_c$ in permeabilized striatal neurons was measured by Ca^{2+} indicator Fura-2 with a fluorescent microscope (TILL Photonics) [26]. For mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) measurements, cells were skinned by saponin and then stained by an acetoxymethyl (AM) ester form of Fura-2 or Rhod-2 [26]. Fura-2 calibration was performed as previously reported [1].

2.6. [^{3}H]ryanodine binding

[^{3}H]ryanodine binding assays were performed as we previously described [3].

2.7. Data and statistical analysis

All results are shown as mean standard error or otherwise indicated. Unpaired Student's *t*-test was performed. Statistical significance was set as a p value of <0.05.