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Caveolin-rich lipid rafts of the plasma membrane of mature cerebellar granule neurons are microcompartments for calcium/reactive oxygen and nitrogen species cross-talk signaling

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ABSTRACT

In previous works, we have shown that L-type voltage-operated calcium channels, N-methyl-D-aspartate receptors (NMDAr), neuronal nitric oxide synthase (nNOS) and cytochrome b_5 reductase (Cb_5R) co-localize within the same lipid rafts-associated nanodomains in mature cerebellar granule neurons (CGN). In this work, we show that the calcium transport systems of the plasma membrane extruding calcium from the cytosol, plasma membrane calcium pumps (PMCA) and sodium–calcium exchangers (NCX), are also associated with these nanodomains. All these proteins were found to co-immunoprecipitate with caveolin-1 after treatment with 25 mM methyl-β-cyclodextrin, a lipid rafts solubilizing agent. However, the treatment of CGN with methyl-β-cyclodextrin largely attenuated the rise of cytosolic calcium induced by L-glutamate through NMDAr. Fluorescence energy transfer imaging revealed that all of them are present in sub-microdomains of a size smaller than 200 nm, with a peripheral distribution of the calcium extrusion systems PMCA and NCX. Fluorescence microscopy images analysis revealed high calcium dynamic sub-microcompartments near the plasma membrane in fura-2-loaded CGN at short times after addition of L-glutamate. In addition, the close proximity between sources of nitric oxide (nNOS) and superoxide anion (Cb_5R) suggests that these nanodomains are involved in the fast and efficient cross-talk between calcium and redox signaling in neurons.

1. Introduction

A highly efficient and rapid functional coupling is particularly relevant for neuronal activity, as neurons have to deliver fast responses to many repetitive and simultaneous extracellular stimuli coming from different neighbor cells. Protein compartmentation within microdomains allows for a more efficient and rapid functional coupling, and studies on calcium signaling in neurons have played a pioneer role to demonstrate the outstanding role of subcellular compartmentation in the control of neuronal activity [1–3]. In addition, the efficient functional coupling of several of the major plasma membrane calcium transport systems allows for a fine control of cytosolic calcium homeostasis within the narrow range required for neuronal survival [4–7]. Deregulation of their functional coupling can lead to a sustained alteration of intracellular calcium homeostasis in neurons, a common feature in oxidative stress-mediated neurodegeneration, and plasma membrane calcium transport systems have been shown to be molecular targets for reactive oxygen species generated in neurodegenerative insults and diseases, reviewed in [8,9]. In primary cultures of mature cerebellar granule neurons (CGN) the entry of calcium through L-type voltage-operated calcium channels (L-VOCC) plays a major role to keep cytosolic calcium within the optimal 70–200 nM concentration range needed for survival of these neurons in vitro [4–7]. Indeed, calcium entry through L-VOCC accounts for more than 75% of the steady state cytosolic calcium increase in the soma of mature

Abbreviations: BF, bright field microscopy images; Cb_5R, cytochrome b5 reductase; CGN, cerebellar granule neurons; DMEM, Dulbecco's-modified Eagle's medium; EC_{50}, concentration needed to afford 50% activation; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; FRET, fluorescence or Förster resonance energy transfer; GF, green fluorescence; HEPES, N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]; IgG, immunoglobulin G; L-VOCC, L-type voltage-operated calcium channels; MβCD, methyl-β-cyclodextrin; NCX, sodium–calcium exchanger; NMDAr, N-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; PBS, phosphate-buffered saline; PMCA, plasma membrane calcium pump; RF, red fluorescence; ROI, region of interest for quantitative microscopy images analysis; ROS, reactive oxygen species; TBS, tris-buffered saline; Tris–HCl, tris–(hydroxymethyl)aminomethane hydrochloride; Tween-20, polyoxyethylenesorbitan monolaurate.

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CGN in culture after the partial depolarization of the plasma membrane elicited by an increase of the extracellular K^+ concentration from 5 to 25 mM [6], consistent with the fact that low K^+-induced neuronal apoptosis can be blocked by increasing the extracellular concentration of potassium [4,5,10]. Calcium entry through L-VOCC and N-methyl-D-aspartate receptors (NMDAr) play a major role in the maintenance of cytosolic calcium needed for CGN survival and excitability [4,6,11,12], and alterations of their functional response by reactive oxygen species (ROS) or redox modulation can lead to CGN death in culture either by apoptosis or by excitotoxicity [6,7,9,13,14]. NMDA receptors are found in synaptic and in extra-synaptic locations [15–17]. As activation of extra-synaptic NMDA receptors can lead to a less focalized increase of cytosolic calcium, the extra-synaptic NMDA receptors are likely to play a role more relevant than synaptic NMDA receptors in the control of cytosolic calcium homeostasis in the neuronal soma.

Lipid rafts of the plasma membrane are dynamic nanodomains of a dimension between 10 and 200 nm, characterized by their heterogeneity and by their high content in cholesterol and esphingolipids [18]. These lipid rafts nanodomains define cellular sub-microdomains of the plasma membrane anchoring caveolins, flotillin and also actin microfilaments [19], and the presence of caveolins associated with neuronal plasma membrane in microdomains without the morphological appearance of "caveola invaginations" has been well documented during last decade, revised in [20]. Furthermore, it has been suggested that these caveolin-rich nanodomains can serve to focalize cell signaling transduction in neurons [20–23].

Experimental evidences supporting the possible implication of lipid rafts in cytosolic calcium homeostasis and calcium signaling are merging only during last years, see, e.g. [24]. The most ubiquitous and relevant calcium transport systems involved in the neuronal cytosolic calcium homeostasis are: NMDAr and L-VOCC as calcium entry systems, and plasma membrane Ca^{2+}-pumps (PMCA) and Na^+/Ca^{2+} exchanger (NCX) as calcium extrusion systems [1,2,9,25].

L-VOCC have been shown to be associated with lipid rafts in cardiomyocytes [19,26], and in a previous work we have shown that in mature CGN in culture L-VOCC is associated with lipid rafts prepared from these neurons [27]. Since the two major subtypes of L-VOCC present in the brain, namely $Ca_V1.2$ and $Ca_V1.3$, directly interact with many proteins having the PDZ binding domain [28,29], proteins that also bind to the NMDA receptor [30], the association of these receptors with lipid rafts nanodomains is not an unexpected finding. The presence of NMDA receptors in isolated lipid rafts has been shown by different investigators [31,32], and using fluorescence resonance energy transfer (FRET) microscopy imaging their association with L-VOCC-containing lipid rafts nanodomains in mature primary cultures of cerebellar granule neurons has been demonstrated in a recent work of our laboratory [33]. However, the association of the calcium extrusion systems PMCA and NCX with lipid rafts nanodomains containing L-VOCC and NMDAr has not been experimentally assessed yet, despite that PMCA has been shown to be associated with lipid rafts of synaptic plasma membranes [34] and of primary cortical and hippocampal rat neurons in culture [35] and NCX binding to lipid rafts has been demonstrated in coronary artery smooth muscle preparations [36]. Owing to the strong toxicity of sustained and widespread cytosolic calcium concentrations above 0.4 μM, see, e.g. [4,6,7], a close spatial location of these calcium entry and calcium extrusion transport proteins in the neuronal plasma membrane can provide obvious advantages for long-term survival of the highly interconnected brain neurons.

On the other hand, two enzymes that can produce ROS that modulate the activity of these calcium transport systems have also been shown to associate with the neuronal plasma membrane, namely, nNOS producing nitric oxide [37] and cytochrome b_5 reductase (Cb_5R), which generates a peak of superoxide anion near the plasma membrane of CGN that plays a critical role in the progress of apoptosis in low K^+ medium [38,39]. In previous works, we have shown that nNOS and Cb_5R are associated with caveolin-rich lipid rafts nanodomains of the plasma membrane of mature CGN in culture, close to NMDAr and L-VOCC, respectively [27,33,38]. Indeed, both nNOS and Cb_5R have been shown to contain protein domains that can form stable complexes with caveolin-1 [39,40]. The presence of Cb_5R within L-VOCC and NMDAr-rich nanodomains suggests that they could be derived from endoplasmic reticulum/plasma membrane junctions which are also known to be associated with lipid rafts [41]. The possibility of co-localization of nNOS and Cb_5R within the same nanodomains deserved to be experimentally assessed, because $NO^\bullet$ rapidly reacts with superoxide anion to produce peroxynitrite [42], a harmful and strongly neurotoxic agent.

In this work, using preparations of lipid rafts-enriched membrane fragments from mature CGN in culture, co-immunoprecipitation and FRET imaging of CGN we show that the calcium extrusion systems (PMCA and NCX) co-localize with the major calcium entry systems (L-type calcium channels and NMDA receptors) and with the ROS/RNS enzyme sources (Cb_5R and nNOS) within lipid rafts-associated sub-microdomains of a size lower than 200 nm. Our results strongly suggest that in neurons lipid rafts can be seen as markers of nanodomains of the plasma membrane of particular relevance in the cross-talk between redox and calcium signaling.

2. Materials and methods

2.1. Preparation of rat cerebellar granule neurons (CGN)

CGN were obtained from dissociated cerebella of 7 days old Wistar rats as described in previous works of this laboratory [6,10,27,38,43]. Briefly, cells were plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 5 mM glucose, 19.63 mM KCl, 3.7 ng/mL insulin, 7 μM 4-aminobenzoic acid, 50 U/mL penicillin, 25 U/mL streptomycin, 0.91 mM pyruvate and 2 mM glutamine on 35-mm dishes (Corning, NY, USA) and plates with 24 wells coated with poly-D-lysine, at a density of 2.5 × 106 cells/dish. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Cytosine arabinofuranoside (10 μM) was added to fresh culture medium 48 h after plating to prevent replication of non-neuronal cells. Seven days after plating, the culture medium was replaced with the following serum-free DMEM:F12 medium (1:1) supplemented with 12.5 mM glucose, 20.82 mM KCl, 5 μg/mL insulin, 0.1 mg/mL apo-transferrin, 20 nM progesterone, 50 U/mL penicillin, 25 U/mL streptomycin, 0.1 mg/mL pyruvate and 2 mM L-glutamine. Cell viability was experimentally assessed measuring the amount of colored formazan by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as in previous works [6,7,10,43].

The composition of the Locke's K25 buffer (pH 7.4 at 37 °C) used in this work is as follows: 4 mM $NaHCO_3$, 10 mM N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES), 5 mM glucose, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, and 134 mM NaCl and 25 mM KCl.

2.2. Isolation of lipid rafts

Lipid rafts were isolated running sucrose gradients as in a previous work of this laboratory [27,33,39], following a method adapted from the protocols described in references [44,45]. CGN cultured for 8 days in vitro were washed with Locke's K25 buffer. The lysates