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L-type voltage-operated calcium channels, *N*-methyl-D-aspartate receptors and neuronal nitric-oxide synthase form a calcium/redox nano-transducer within lipid rafts

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ABSTRACT

Cytosolic calcium plays a leading role in the control of neuronal excitability, plasticity and survival. This work aims to experimentally assess the possibility that lipid rafts of the plasma membrane can provide a structural platform for a faster and tighter functional coupling between calcium and nitric-oxide signaling in neurons. Using primary cerebellar granule neurons (CGN) in culture this hypothesis has been experimentally assessed with fluorescence resonance energy transfer imaging, preparations of lipid rafts-enriched membrane fragments and western blotting. The results obtained in this work demonstrated that major calcium entry systems of the plasma membrane of CGN (L-type calcium channels and *N*-methyl-D-aspartate receptors) and nitric-oxide synthase are separated by less than 80 nm from each other within lipid rafts-associated sub-microdomains, suggesting a new role of lipid rafts as neuronal calcium/redox nano-transducers.

1. Introduction

A highly efficient and rapid functional coupling is particularly relevant for neuronal activity, and studies on calcium signaling in neurons have played a pioneer role to demonstrate the outstanding role of sub-cellular compartmentation in the control of neuronal activity [1–3].

Calcium entry through L-VOCC and NMDAr play a major role in the maintenance of cytosolic calcium needed for mature cerebellar granule neurons (CGN) survival and excitability [4–6], and alterations of their functional response by ROS or redox modulation can lead to CGN death in culture either by apoptosis or by excitotoxicity [6–9]. Moreover, a sustained alteration of intracellular calcium homeostasis in neurons is a common feature in oxidative stress-mediated neurodegeneration, and plasma membrane calcium transport systems have been shown to be molecular targets for ROS generated in neurodegenerative insults and diseases [10,11]. In primary cultures of mature CGN the entry of calcium through 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* [5,6,9].

Calcium entry through opening of L-VOCC can generate transients of micromolar calcium concentration up to distances close to 100 nm from the L-VOCC pore depending on the calcium buffering capacity of its microenvironment [3]. L-VOCC have been shown to be associated with lipid rafts in cardiomyocytes [12,13], the L-VOCC subunit α22 has been shown to partition within lipid rafts in cerebellum bound to caveolin [14], and we have shown that in mature CGN in culture L-VOCC is associated with lipid rafts [15]. Lipid rafts define cellular sub-microdomains of the plasma membrane anchoring caveolins, flotillin and also actin microfilaments [12]. The presence of caveolins associated with neuronal plasma membrane in microdomains without the morphological appearance of "caveola invaginations" has been documented during last decade, revised in [16]. Furthermore, it has been suggested that these caveolin-rich nanodomains can serve to focalize cell signaling transduction in neurons [16–18]. Indeed, it has been shown that caveolin-1 interacts with nNOS [19] and that the actin cytoskeleton modulates the activity of NMDAr [20].

Lipid rafts of the plasma membrane are dynamic nanodomains of a size between 10 and 200 nm [21]. FRET is a spectroscopic ruler that allows to measure distances in the nanometer scale range [22,23]. Quantitative FRET-imaging is a powerful tool for the analysis of cellular sub-microdomains, because intensity readings of

Abbreviations: CGN, cerebellar granule neurons; CTB, cholera toxin subunit B; FRET, fluorescence or Förster resonance energy transfer; IgG, immunoglobulin G; L-VOCC, L-type voltage-operated calcium channels; NMDAr, *N*-methyl D-aspartate receptor; nNOS, neuronal nitric-oxide synthase; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

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volume elements of sub-micrometers section size can yield information on their structure in the nanometer scale range. FRET between membrane-bound proteins labeled with fluorescent antibodies is a case of FRET from one donor to multiple acceptors which can be used to identify proteins separated less than 100 nm within lipid rafts sub-microdomains [15,24,25].

In this work, we have extended our studies using FRET-imaging approaches, preparations of lipid rafts-enriched membrane fragments and western blotting to show that L-VOCC, NMDAr and nNOS co-localize within lipid rafts-associated sub-microdomains of a size lower than 200 nm.

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 previously [6,15,24,26,27].

2.2. Isolation of lipid rafts

Lipid rafts were isolated running sucrose gradients as in previous works [15,25], following a method adapted from the protocols described in [28,29]. Samples were analyzed by SDS–PAGE followed by Western blotting.

2.3. Western blotting

SDS–PAGE were run at a concentration of 7.5%, 10.4% or 12.5% acrylamide depending upon the molecular weights of the target proteins, using 2 μg protein of CGN lysates in each lane. Gels were transferred to nitrocellulose membranes of 0.2 μm average pore size (Trans-BloT Transfer Medium, BioRad), as in [15,25].

2.4. Fluorescence microscopy imaging and FRET analysis

Fluorescence microscopy images of CGN were acquired with a Hamamatsu Orca-R2 CCD camera (binning mode 2×2) attached to a Nikon Diaphot 300 epifluorescence microscope (objective NCF Plan ELWD 20×), and quantitative analysis of the average fluorescence intensity of selected neuronal soma was done with the HCImage software, as described previously [15,24,25]. Images were acquired with an excitation filter of 470 nm, and 510 nm dichroic mirror/520 nm emission filter (donor-green fluorescence) and 580 nm dichroic mirror/590 nm emission filter (acceptor-red fluorescence). Acquired images were exported as TIFF pseudo-color images for further processing using the Image J software. Direct-fluorescence intensity images are presented in gray scale (black: very low or no-signal, and white: saturated signal).

FRET measurements were performed with mature CGN fixed and stained with primary and fluorescence-labeled secondary antibodies as in [15,24,25]. The basic criteria used to confirm the occurrence of FRET and calculation of FRET-efficiency was the simultaneous occurrence of quenching of the green fluorescence (GF) and an increase of the ratio between red (acceptor fluorescence) and green fluorescence intensities (ratio red/green) in CGN stained with Alexa488- and Cy3-secondary antibodies, as discussed in more detail in [15]. To quantitate the effects of the treatment for labeling with the second primary antibody/IgG-Cy3 complex in the green and red fluorescence intensities of CGN stained with the first primary antibody plus IgG-Alexa488 we have carried out control experiments performing these treatments without the second primary antibody. These control experiments showed that on average this treatment resulted in 12 ± 3% quenching of the green fluorescence of the IgG-Alexa488 antibody, and no statistically significant change of the red fluorescence intensity. This has been taken into account in all the calculations of the quenching of green fluorescence afforded by CGN double labeling with antibodies shown in this work.

2.5. Chemicals and reagents

Primary antibodies: goat anti-NMDAr (sc-1468), rabbit anti-L-VOCC (sc-25686), mouse anti-nNOS (sc-5302), goat anti-H-Ras (sc-32026), rabbit anti-caveolin-1 (sc-894), rabbit anti-caveolin-2 (sc-7942), goat anti-caveolin-2 (sc-1858), goat anti-flotillin-1 (sc-16640) and rabbit anti-flotillin-1 (sc-25506) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluorescence-labeled secondary antibodies used to label the primary antibodies listed above: anti-rabbit IgG-Alexa488 (cat. no. A11008), anti-goat IgG-Alexa488 (cat. no. A11055) and anti-mouse IgG-Alexa488 (cat. no. A11001) from Invitrogen (Molecular Probes, Eugene, OR, USA), and anti-rabbit IgG-Cy3 (cat. no. C2306) and anti-goat IgG-Cy3 (cat. no. C2821) from Sigma (St. Louis, MO, USA). Anti-goat, anti-rabbit and anti-mouse IgG horseradish peroxidase and Super-Signal West Dura Extended Duration Substrate used in Western blotting were supplied by Pierce (Rockford, IL, USA).

DM-bodipy dihydropyridine (cat. no. D7443), ST-bodipy dihydropyridine (cat. no. S7445) and cholera toxin subunit B conjugated with Alexa555 (cat. no. C34776) were supplied by Invitrogen (Molecular Probes, Eugene, OR, USA). All other reagents and chemicals were of analytical grade from Sigma–Aldrich or Roche-Merck (Darmstadt, Germany).

2.6. Statistical analysis

Results are expressed as mean ± standard error (s.e.). Statistical analysis was carried out by Mann–Whitney non-parametric test. Significant difference was accepted at the $p < 0.05$ level. All the results were confirmed with duplicate measurements of at least three different CGN preparations.

3. Results

3.1. Lipid rafts membrane fractions are enriched in transport systems relevant for cytosolic calcium homeostasis and also in ROS-producing redox systems

Membrane fragments prepared from CGN lysates were fractionated in sucrose density gradients as indicated in the Methods. Western Blot analysis of the fractions showed that typical lipid rafts markers, H-Ras, flotillin, caveolin-1 and caveolin-2 were largely enriched in fractions 1–5 (Fi. 1). NMDAr, L-VOCC and nNOS were found to be highly enriched in the fractions 1–5 (Fig. 1), as it was also shown earlier for L-VOCC [15]. On these grounds, we have experimentally ascertained this conclusion by FRET-imaging in fixed mature CGN.

3.2. L-VOCC and NMDAr co-localize within cholera toxin B-binding sites and caveolin-rich sub-microdomains in the neuronal plasma membrane of mature C

Fluorescence microscopy images of CGN double stained with anti-L-VOCC/IgG-Alexa488 and anti-NMDAr/IgG-Cy3 were obtained with an excitation filter of 470 nm, i.e. with a negligible direct excitation of the Cy3-dye. The images showed an extensive co-localization of both antibodies as shown by merge images displayed in Fig. 2. The results obtained for a total number of 1400 neuronal somas of, at least, three different CGN preparations ($n \geqslant 6$) were accumulated and subjected to detailed pixel-analysis