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Selective localization of phosphatidylcholine-derived signaling in detergent-resistant membranes from synaptic endings

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ABSTRACT

Detergent-resistant membranes (DRMs) are a class of specialized microdomains that compartmentalize several signal transduction processes. In this work, DRMs were isolated from cerebral cortex synaptic endings (Syn) on the basis of their relative insolubility in cold Triton X-100 (1%). The lipid composition and marker protein content were analyzed in DRMs obtained from adult and aged animals. Both DRM preparations were enriched in Caveolin, Flotillin-1 and c-Src and also presented significantly higher sphingomyelin (SM) and cholesterol content than purified Syn. Total phospholipid-fatty acid composition presented an increase in 16:0 (35%), and a decrease in 20:4n-6 (67%) and 22:6n-3 (68%) content in DRM from adults when compared to entire synaptic endings. A more dramatic decrease was observed in the 20:4n-6 and 22:6n-3 content in DRMs from aged animals (80%) with respect to the results found in adults. The coexistence of phosphatidylcholine-specific-phospholipase C (PC-PLC) and phospholipase D (PLD) in Syn was previously reported. The presence of these signaling pathways was also investigated in DRMs isolated from adult and aged rats. Both PC-PLC and PLD pathways generate the lipid messenger diacylglycerol (DAG) by catalyzing PC hydrolysis. PC-PLC and PLD1 localization were increased in the DRM fraction. The increase in DAG generation (60%) in the presence of ethanol, confirmed that PC-PLC was also activated when compartmentalized in DRMs. Conversely, PLD2 was excluded from the DRM fraction. Our results show an age-related differential fatty acid composition and a selective localization of PC-derived signaling in synaptic DRMs obtained from adult and aged rats.

1. Introduction

Membrane rafts are small (10–200 nm), heterogeneous, sterol- and sphingolipid-enriched domains. Although enrichment in cholesterol (Chol) and sphingolipids is the main characteristic of membrane rafts, they also contain specific subpopulations of membrane proteins often being enriched in glycophosphoinositol-linked proteins (GPI-proteins), specific tyrosine kinases, heterotrimeric G-proteins, cholesterol-linked and palmitoylated proteins [1–3]. Membrane rafts compartmentalize important cellular processes such as cell adhesion and endocytosis and have been proposed as signal transduction platforms and as key regulators of several signaling pathways [3–5].

One of the first methods originally used for the study of these domains was based on their insolubility in non-ionic detergent such as Triton X-100 [6]. These detergent-resistant membranes (DRMs) can be isolated from most mammalian cells. DRMs, like membrane rafts, are also rich in Chol and sphingolipids and are in the liquid-ordered phase. Moreover, the tight acyl chain packing and the enrichment in sphingomyelin (SM) and Chol are probably responsible for their detergent insolubility [7,8]. In spite of the existence of detergent-free methods and modern microscopy techniques, isolation of DRMs is still one of the most widely used methods for studying certain properties of membrane rafts. Certainly there remains a large degree of controversy surrounding the purity, the physiological importance, and even the existence of DRM in intact cells [9]. It is also possible that DRM preparations do not reflect the exact composition of the membrane rafts in intact cells, however, the purification of such domains has led to significant progress in understanding the functional architecture of biological membranes and in the dissection of signal transduction pathways [10].

DRM localization of phosphatidylinositol 4,5-bisphosphate (PIP_2)-metabolizing enzymes, several isoforms of PIP_2 specific phospholipase C (PLC), as well as phospholipase D (PLD) suggests a specific role of these specialized microdomains in lipid signaling [11–16].

Lipid messengers are generated by the concerted action of phospholipases and lipid kinases on membrane phospholipids. Phosphatidylcholine (PC), the most abundant glycerophospholipid in the membrane, plays a key role in cellular signaling since it is the main substrate for several signaling enzymes such as: PLD, phospholipase C (PC-PLC) and PLA^2 [17–19]. Phosphatidic acid (PA) generated by PLD

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can be further hydrolyzed by lipid phosphate phosphatases (LPPs) to generate the lipid second messenger diacylglycerol (DAG). Besides, DAG derived from PC can be generated by PC-PLC action. The wave of DAG elicited from PC hydrolysis is generated more slowly than that reported for PIP_2-PLC activity and it occurs without elevation of intracellular Ca^{2+}.

The coexistence of PLD and PC-PLC pathways has already been reported in cerebral cortex synaptic endings specific, sites heavily concentrated in signaling molecules [20]. Moreover, the synaptic endings appear to be the first target of neuronal damage in several neurodegenerative disorders. In this sense, the identification and characterization of signaling pathways have gained priority in order to understand how synaptic signaling operates both under normal and/or pathological conditions. We have also reported the activation of synaptic PLD and PC-PLC pathways under oxidative stress conditions and a differential regulation of PLD during aging [21–23].

In this work, we characterized the lipid and protein composition of synaptic endings and of DRMs obtained from cerebral cortex synaptic endings isolated from adult (4 month old) and aged (28 month old) rats. We also described for the first time the presence and the differential localization of PC-derived signaling pathways (PC-PLC and PLD) in these specialized microdomains.

2. Materials and methods

Wistar-strain adult (4 months old) and aged (28 months old) rats were kept under constant environmental conditions and fed on a standard pellet diet ad libitum until decapitation.

1-[^{14}C]palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]-DPPC) (111 mCi/mmol), was purchased from New England Nuclear-Dupont, Boston, MA, USA. Preblended dry fluor 2a70 (98% PPO and 2% bis–MSB) was obtained from Research Products International Corp., USA. Triton X-100 (octyl phenoxy polyethoxyethanol), GTPγS (Guanosine 5′-[*gamma*-thio]triphosphate) and DL-propranolol were obtained from Sigma-Aldrich St. Louis, MO, USA. The kit (Colestat enzimático AA) for measuring cholesterol was from Wiener laboratory, Rosario, Argentina. The kit for measuring protein content (DC protein assay) was from Bio-Rad Life Science group. Rabbit polyclonal anti-Caveolin 1 antibody was from BD Biosciences, rabbit polyclonal anti-PLD1 antibody was from Cell Signaling, mouse monoclonal anti-c-Src antibody, polyclonal anti-Flotillin-1 antibody, polyclonal horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG and polyclonal HRP-conjugated goat anti-mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-PLD2 was a kind gift from Dr. Raben, Johns Hopkins University School of Medicine, Baltimore, USA. Rabbit polyclonal anti-PC-PLC antibody raised against *Bacillus cereus* PC-PLC was generously provided by Dr. Goldfine, University of Pennsylvania, Philadelphia, USA. All other chemicals were of the highest purity available.

2.1. Preparation of synaptosomal fraction

Total homogenates were prepared from the cerebral cortex (CC) of 4 month old (adults) and 28 month old (aged) rats. Rats were killed by decapitation and CC was immediately dissected (2–4 min after decapitation). All proceedings were in accordance with *Principles of Use of Animals* and *Guide for the Care and Use of Laboratory Animals* (NIH regulation).

Synaptosomal fraction (Syn) was obtained as previously described by Cotman with slight modifications [22,24]. Briefly, CC homogenate (20%, w/v) was prepared in a medium containing 0.32 M sucrose, 1 mM EDTA, 10 mM HEPES buffer (pH 7.4) in the presence of 1 mM DTT, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.1 mM PMSF. The cerebral cortex was homogenized by 10 strokes with a Thomas tissue homogenizer. The homogenate was centrifuged at 1800×*g* for 7.5 min at 4 °C using a JA-21 rotor in a Beckman J2-21 centrifuge. The pellet was discarded, and the supernatant was retained and centrifuged at 14,000×*g* for 20 min at 4 °C. The resulting pellet was washed and resuspended in 3 ml of 0.32 M sucrose isolation buffer, layered over a discontinuous ficoll gradient (8.5% pH 7.4, 13% pH 7.4 ficoll solutions, each prepared in isolation buffer) and spun at 85,500 *g* for 30 min at 4 °C using a SW 28.1 rotor in a Beckman Optima LK-90 ultracentrifuge. Synaptosomes in the 8.5%–13% ficoll interface were removed, resuspended in isolation buffer, and centrifuged at 33,000×*g* for 20 min at 4 °C using a JA-21 rotor in a Beckman J2-21 centrifuge. For the experiments, synaptosomes were diluted in Tris base buffer medium (TBM) containing: 120 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 10 mM glucose, 20 mM Tris (pH 7.2) and protein content was determined by a previously published method [25] using the DC protein assay kit from Bio-Rad.

2.2. Isolation of detergent-resistant membranes (DRMs)

The isolation of the DRM fraction from entire synaptosomes was based on the procedure previously described by Brown and Rose and by Molander-Melin et al. [6,26], with slight modifications to our experimental system. Briefly, the synaptosomal pellet obtained from 3 CC was resuspended in 5 ml of iced-cold (0–4 °C) lysis buffer consisting of 1% Triton X-100 in DRM buffer (10 mM Tris–HCl (pH 7.4), 70 mM NaCl, 2 mM $MgCl_2$ and 0.5 mM EDTA) with protease inhibitors added (1 mM DTT, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.1 mM PMSF). The suspension was homogenized by passing it through a 21 ga×1.5 in. needle 4–5 times and it was incubated at 4 °C for 30 min. The homogenization was repeated after 15 min of incubation. Then, the homogenate was centrifuged at 1000×*g* for 15 min at 4 °C (2500 rpm, Beckman JA-21 rotor, Beckman J2-21 centrifuge). The supernatant was diluted 1:1 in 80% (w/v) sucrose, divided into two samples and placed at the bottom of ultracentrifuge tubes. On top of this, 20 ml of 30% (w/v) sucrose were layered, followed of 8 ml 5% (w/v) sucrose. All the sucrose solutions were handled at 0–4 °C and diluted in the DRM buffer (without Triton X-100) containing protease inhibitors (1 mM DTT, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.1 mM PMSF). Samples were centrifuged for 20 h at 120,000×*g* (27,000 rpm, Beckman SW 28 rotor in a Beckman Optima LK-90 ultracentrifuge) at 4 °C. A visible and floating band (present in the 5–30% sucrose interface) was collected and transferred to a new centrifuge tube, which was filled up with DRM buffer and centrifuged for 1 h at 120,000×*g*. The pellet containing DRMs was resuspended in TBM buffer and protein content was determined by a previously published method [25] using the DC protein assay kit from Bio-Rad.

2.3. Determination of DAG generation from PC

PC hydrolysis was determined using lipid vesicles containing [^{14}C]-DPPC and cold DPPC to yield 100,000 dpm and 0.125 mM per assay in a buffer containing 0.2% Triton X-100 and 0.1 M Tris (pH 7.2). 100 μl of these lipid vesicles were added to 100 μl of Syn or DRMs (150 μg of protein) in a final volume of 200 μl. The reaction was carried out at 37 °C for 20 min and stopped by the addition of 5 ml of chloroform: methanol (2:1, v/v). Blanks were prepared identically, except that membranes were boiled for 5 min before use. Lipids were extracted and separated as described below [20,21]. To evaluate the contribution of the PC-PLC and PLD pathways to total DAG formation, the enzyme reaction was carried out under control conditions (vehicle) and in the presence of 2% ethanol or 1.5 mM DL-propranolol [21,22,27].

2.4. Lipid separation

After the enzyme reaction lipids were extracted according to Folch [28]. Briefly, the lipid extract was washed with 0.2 volumes of 0.05%