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Characterization and comparison of raft-like membranes isolated by two different methods from rat submandibular gland cells

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Abstract

Lipid rafts are defined as cholesterol and sphingolipid enriched domains in biological membranes. Their role in signalling and other cellular processes is widely accepted but the methodology used for their biochemical isolation and characterization remains controversial. Raft-like membranes from rat submandibular glands were isolated by two different protocols commonly described in the literature; one protocol was based on selective solubilization by Triton X-100 at low temperature and the other protocol consisted in extensive sonication. In both cases a low density vesicular fraction was obtained after ultracentrifugation in a sucrose density gradient. These fractions contained about 20% of total cholesterol but less than 8% of total proteins, and were more rigid than bulk membranes. Fatty acid analyses revealed a similar composition of raft-like membranes isolated by the two different methods, which was characterized by an enrichment in saturated fatty acids in detriment of polyunsaturated acids when compared with the whole cell membranes. Protein profile of detergent resistant membranes or raft-like membranes prepared by sonication was assessed by silver staining after SDS-PAGE and by MALDI-TOF. Both analyses provided evidence of a different protein composition of the Triton X-100 and sonication preparations. Immunoblot experiments revealed that raft-like membranes prepared by detergent extraction or sonication were free of Golgi apparatus or endoplasmic reticulum protein markers (β-COP and calnexin, respectively) and that they were not substantially contaminated by transferrin receptor (a non-raft protein). While caveolin-1 was highly enriched in raft-like membranes prepared by the two methods, the $P2X_7$ receptor was enriched in raft-like membrane fractions prepared by sonication, but almost undetectable in the detergent resistant membranes. It can be concluded that both methods can be used to obtain raft-like membranes, but that detergent may affect protein interactions responsible for their association with different membrane domains.

Keywords: Membrane microdomains; Triton X-100; purinergic receptors; mass spectrometry; $P2X_7$

1. Introduction

For many years, biological membranes were believed to be a "fluid mosaic": lipids would be homogeneously dispersed in the plane of the membrane and proteins would float in the membrane with a relative freedom of lateral movement [1]. However, several works from the early 1990s led to the proposal of a new model for understanding the nature of biological membranes [2,3]. This model implies the lateral segregation of biological membranes in discrete domains with different physical states:

Abbreviations: MALDI-TOF, matrix assisted laser desorption ionization-time of flight; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PUFA, polyunsaturated fatty acid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*- piperazine-*N*′-[2-ethanesulfonic acid]; HBS, HEPES-buffered saline; HS, HEPES-saline; DPH, 2-[3-(diphenylhexatriene)]; PBS, phosphate-buffered saline; GC-MS, gas chromatography-mass spectrometry; TfR, transferrin receptor; CNX, calnexin; COP, coatomer protein

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the bulk of membranes would be disorganized in a liquid-disordered state while specific domains called "lipid rafts" would form rigid platforms in a liquid-ordered state, floating on the more fluid rest of the membrane [4]. This lateral organization in two distinct states has already been described in model membranes with compositions close to that observed in native membranes [5], as well as in living cell membranes, including epithelial cells [6–9]. The physical state of lipid rafts is probably a consequence of their lipid composition. These domains are enriched in cholesterol and (glyco)sphingolipids as well as glycerophospholipids with a high degree of saturation of their fatty acid chains [10,11]. The tight interactions between these components provide the basis of their higher packing and rigidity, which are likely to provoke the phase separation [4,10,12]. At the same time, microdomain formation is accompanied by lateral segregation of proteins. The presence in lipid rafts of many proteins implicated in cell signalling has supported the currently widely accepted idea that these domains play a major role in signal transduction [11]. If we take into account (1) that signalling processes are very fast, (2) that the components involved in these processes are normally expressed at low overall abundance and (3) that the interactions must be very specific and strictly regulated, compartmentalization, as observed in lipid rafts, should be necessary to explain these properties of signal transduction [13–15].

Methodologies for lipid rafts studies are controversial. The dynamic nature of lipid rafts and their estimated size (20 nm–2 μm, under spatial resolution of light microscopy in most of the cases) [6,11], has made them difficult to be visualized in living cells, providing a reasonable doubt of their existence [16]. However, phase separation has already been visualized in some cell types related to specific structures such as filopodia or T lymphocytes activation sites [6–8]. It is increasingly accepted that lipid rafts in resting conditions are small regions of the lipid membranes, which tend to cluster after certain stimulus to form larger structures [15,17,18]. Studies supporting this view are based on sophisticated fluorimetry and other biophysical methodologies. On the other hand, several biochemical methods have been described for the isolation of membrane fractions with raft-like properties. Although these methods of isolation imply cell disruption and artefactual reorganization of the membrane fractions, they are widely used to obtain membranous raft-like fractions representative of the native microdomains [19]. A classical biochemical method for raft-like membranes isolation is based on the hypothesis that these domains, contrary to the bulk membranes in liquid-disordered state, are resistant to solubilization at low temperature by non-ionic detergents, such as Triton X-100 [20]. These detergent-resistant membranes can be isolated as a low density fraction after centrifugation in a density gradient. Detergent-based methods have been criticized for several reasons. Conditions used for detergent extraction can lead by themselves to domain formation or induce lipid mixing between different membrane domains and affect protein interactions with these domains [21–23]. Moreover, when different detergents are used, different protein and lipid compositions of detergent-resistant membranes are obtained [22], which have been interpreted as a direct effect of the detergent on native molecular interactions into rafts [16] or a possible isolation of different subsets of lipid rafts [19]. Several detergent-free methods have also been described in the literature. The most widely used methods are based on fine disruption of the membrane by sonication followed by the isolation of raft-like membranes in the light fractions of a density gradient [24,25]. It is likely that these methods have less negative effects on lipid–lipid and lipid–protein interactions, so probably reflecting rafts properties closer to those present in native membranes. Some problems are also associated to this methodology. Rafts isolated after sonication are more often contaminated by other low density membranes. They are also more variable between different preparations or cell types [11,19,26,27]. Thus, different raft-like preparations can be obtained depending on the biochemical method used for their isolation.

These methodological complexities make necessary a consistent characterization of isolated membrane fractions in terms of lipids and proteins to confirm that they are similar to the lipid rafts and to better understand their implication in signalling processes. The aim of this work was to characterize raft-like membranes isolated by two different methods from freshly isolated epithelial cells of the rat submandibular glands, a well established model for signal transduction studies [28–32]. We have isolated a low density fraction with properties analogous to those described in the literature for lipid rafts, using either a Triton X-100 extraction or a sonication protocol. While the general properties of these two preparations were similar, some differences were found, especially in terms of protein composition. Particularly, the distribution in "raft" and "non-raft" fractions of the proapoptotic $P2X_7$ purinergic receptor was considerably different depending on the method used to prepare them. A possible implication of the detergent on the observed differences is discussed.

2. Materials and methods

2.1. Materials

Male Wistar rats (150–200 g) were purchased from Charles River Laboratories (Brussels, Belgium). The housing and care of the animals were in agreement with the regulations of the European Union. The animals were fed ad libitum with free access to water. 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Molecular Probes (Eugene, OR). Collagenase P and bovine serum albumin (BSA) (fraction V) were from Roche (Mannheim, Germany). The glutamine-free amino acids mixture was from Gibco BRL (Paisley, Scotland). *N*-piperazine-*N*′′-(HEPES), cholesterol oxidase, peroxidase, sodium cholate, p-hydroxyphenylacetic acid, sinapinic acid and the anti-β-COP mouse monoclonal antibody (clone maD) were obtained from Sigma (St. Louis, MO). Acetyl chloride (reagent grade) was from Merck (Darmstadt, Germany). The anti-caveolin-1 antibody was purchased from BD Biosciences Pharmingen (San Diego, CA) and the anti-$P2X_7$ polyclonal antibody was from Alomone (Jerusalem, Israël). The anti-transferrin receptor mouse monoclonal antibody (clone OX-26) was from Biogenesis (Poole, England) and the anti-calnexin rabbit polyclonal antibody from Stressgen (Victoria, Canada). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG and chemiluminescence reagents (ECL+) were from Amersham Biosciences (Piscataway, NJ). The BCA protein assay reagent was from Perbio Science (Erembodegem, Belgium).