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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).

2.2. Preparation of a crude cellular suspension from rat submandibular gland

The rats were anaesthetized and killed with ether. The submandibular glands were immediately dissected and finely minced. The minced tissue was digested in the presence of 0.4–0.5 U per ml of collagenase P for 20 min at 37 °C under constant shaking in 10 ml HEPES-buffered saline (HBS) medium containing (mM): 24.5 HEPES (pH 7.4), 96 NaCl, 6 KCl, 1 $MgCl_2$, 2.5 NaH_2PO_4, 11.5 glucose, 5 sodium pyruvate, 5 sodium glutamate, 5 sodium fumarate, 1% (v/v) glutamine-free amino acids mixture and 0.125% (w/v) BSA. Ten minutes after the beginning of the digestion, the cells were aspirated five times with 10, 5 and 2 ml glass pipettes. At the end of the digestion the crude suspension was mechanically dispersed by gentle pipetting, filtered and washed in an isotonic NaCl solution. The last pellet was resuspended in HBS medium and kept at 4 °C until use.

2.3. Isolation of raft-like membranes with a method using detergent

After its isolation, the crude cellular suspension was centrifuged at 500×*g* for 1 min. The pellet was resuspended in 500 μl HEPES–saline (HS) medium (25 mM HEPES, 150 mM NaCl, pH 7.4). The cells were homogenized with a sonifier (Soniprep 150, MSE) at an amplitude of 15 μm (5 strokes of 5 s). The extracts were kept at 4 °C for 30 min. Aliquots were taken in this step as "cell lysate". Membranes were solubilized by the addition of an equal volume of Triton X-100 1% (w/v) and agitated for 1 h at 4 °C. In order to remove cell debris, the extracts were centrifuged at 4 °C for 3 min at 500×*g*. The supernatants were mixed with an equal volume of sucrose 90% in HS medium. Gradients were prepared by the successive addition in centrifuge tubes of 1.4 ml of the Triton extract in sucrose, 1.8 ml 35% sucrose and finally 1 ml 5% sucrose. These sucrose solutions were also prepared with HS medium. The tubes were centrifuged for 16 h at 200,000×*g* in a SW60Ti rotor. Four hundred μl fractions were removed from the top to the bottom of the gradient (fraction 1 to 10) and kept on ice. The presence of vesicles in these fractions was assessed by light scattering as previously described [33,34].

2.4. Isolation of raft-like membranes with a detergent-free method

Rafts were isolated by the neutral pH detergent-free method described by Liu et al. with slight modifications [35]. The cellular pellet was resuspended in 1 ml TEEA hypotonic buffer (20 mM Tris, 1 mM EGTA, 1 mM EDTA, and a cocktail of inhibitors of proteases containing 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.0) and passed ten times through a 29G needle. After a 10-min centrifugation at 1000×*g* at 4 °C, the supernatant was saved and the pellet extracted by the same procedure. The process was repeated a total of four times and the pooled supernatants were centrifuged at 100,000×*g* for 30 min at 4 °C in a SW60Ti rotor. The pellets were resuspended in 800 μl of HS buffer containing the cocktail of protease inhibitors and sonicated on ice for 20 s four times at 10–12 μm amplitude (Soniprep 150, MSE). To increase the reproducibility of the results this step was performed most of the times by the same person. The tip of the sonifier (titanium exponential microprobe) was introduced in the middle of the solution. This homogenate was mixed with an equal volume of sucrose 90% in HS medium. The sucrose gradient, the ultracentrifugation and the fractionation were performed similarly to the method described in the previous paragraph.

2.5. Cholesterol determination

Cholesterol was measured as described previously [33]. Fifty μl aliquots of each sucrose gradient fraction were reacted for 60 min at 37 °C in the dark in 1.5 ml of K-phosphate buffer (0.1 M, pH 7.4) containing 2 mM sodium cholate, 0.66 mg/ml *p*-hydroxyphenylacetic acid, 0.1 UI/ml cholesterol oxidase and 1 UI/ml horseradish peroxidase. Parallel samples without cholesterol oxidase were also run as blanks. The final product of the coupled reactions, oxidized *p*-hydroxyphenylacetic acid derivative was measured fluorimetrically (excitation and emission wavelengths, 325 and 415 nm, respectively). Results were expressed as percentage of fluorescence in each fraction when compared to the sum of the signal for all fractions.

2.6. Protein assay

The protein concentration of the samples was measured with the BCA protein assay kit. The assay was performed in duplicate in a microtiter plate (Grenierbio-One, Microlon 600). Twenty-five μl of the sample or of a standard solution of BSA were mixed with 200 μl of the working reagent. The assay was performed at 37 °C for 30 min and the samples were read at 540 nm. Results were expressed as percentage of protein when compared to the sum of protein for all fractions.

2.7. Measurement of the membrane fluidity

The fluidity of "rafts" and "non-rafts" was estimated by fluorescence anisotropy as described previously [33]. After centrifugation of the samples prepared by the detergent-free method, the light scattering of each fraction was measured as described previously [33]. The fractions corresponding to the different populations of membranes, termed here as "rafts" (2–4) and "non-rafts" (6–10), were pooled. They were diluted with HS medium containing 2 mM EDTA and centrifuged for 90 min at 200,000×*g*. The pellet was resuspended in the same buffer and centrifuged in the same conditions. The pellet was resuspended in HS medium and sonicated for 5 s at 15 μm amplitude. Membranes were labeled with the fluorescent probe DPH (2-[3-(diphenylhexatriene)]) by adding the probe in tetrahydrofuran at a 1 μg/ml final concentration and incubating at 37 °C for about 15 min. The measurements were performed in a SLM 8000C spectrofluorimeter with Glan-Thompson polarizers placed in T-geometry. Excitation was performed at 360 nm and emission was recorded at 430 nm. For each experiment, 2 ml HS with the labeled vesicles were transferred in a 10×10×45 mm acrylic cuvette (VWR, Brussels) placed in a thermostatic chamber. Polarization measurement was made by simultaneously measuring the vertical and horizontal components of the polarized emission. The results were corrected for background polarization due to turbidity using unlabeled samples. The ratio of the intensities in the vertically and horizontally polarized detectors was measured with vertically and horizontally polarized excitation, giving, respectively, the R_{vert} and R_{horiz} ratios. Polarization (P) was calculated as $P=(R_{corr}-1)/(R_{corr}+1)$ where $R=R_{vert}/R_{horiz}$. Anisotropy was derived from P using $r=2P/(3-P)$.

2.8. Preparation of samples for transmission electronic microscopy

Low density fractions (2–4) prepared with or without detergent were pooled and concentrated by ultracentrifugation as described in the previous paragraph. After centrifugation, the pellets were fixed (glutaraldehyde 2% w/v, tannic acid 0.2% w/v, in cacodylic acid 0.1 M, pH 7.4) for 2 h at 4 °C. They were then washed three times for 15 min with cacodylate buffer (0.1 M, pH 7.4) at 4 °C. Samples were postfixed with osmium tetroxide 1% (w/v) in cacodylate buffer for 1 h at 4 °C. After three washes, they were dehydrated through an ethanol series (30%, 50%, 70%, 90%, 100% v/v, 15 min each one). They were embedded in Epon 812 resin (Polarbead, Bio-

Rad, Hercules, CA) and let polymerize for at least 48 h at 60 °C. Solid blocks were cut in ultrathin sections (50–100 nm) and deposited in copper grids. Finally, they were visualized in a transmission electronic microscope (Philips EM 208S) at 80 kV.

2.9. Determination of the fatty acid composition

Low density fractions (fractions 2–4, termed here as "rafts") prepared with or without detergent were pooled and concentrated by ultracentrifugation as described in a previous paragraph. Heavy fractions from the detergent-free method (fractions 6–10, termed here as "non-rafts") and membranes from the cell lysate were ultracentrifuged in the same conditions. The fatty acids were analyzed as described previously [33]. Briefly, a 250 μl aliquot of each fraction was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl chloroform. Two hundred and fifty μl of water were added. The samples were mixed before the successive additions of 1 ml methanol: dichloromethane (3:1) and 200 μl fresh colourless acetyl chloride. The tubes were incubated for 1 h at 75 °C, cooled and 4 ml 7% potassium carbonate aqueous solution was added. The methyl esters of the fatty acids were extracted with 2 ml hexane and 2 ml acetonitrile. The extract was evaporated to dryness under nitrogen at 50 °C and resuspended in 30 μl n-hexane. Two μl were injected in a Hewlett-Packard HP5890A gas chromatograph coupled to an HP5970B mass-selective detector and a PC-based ChemStation. A polydimethylsiloxane HP I stationary phase with a film thickness of 0.33 μm and chemically bonded on a 25 m × 0.2 mm internal diameter fused-silica capillary column (Hewlett-Packard, Palo Alto, CA) was connected to an open–split interface. Helium was used as the carrier gas at a linear velocity of 30 cm/s and the injector was set to the splitless mode. The injector and interface temperatures were, respectively, 270 °C and 300 °C. The oven temperature program was: initial temperature 140 °C for 1 min, rate rise of 15 °C/min up to a temperature of 230 °C, rate rise of 2.5 °C/min up to a final temperature of 290 °C which was maintained for 4 min. The mass spectrometer operated in the electron-impact mode (70 eV) with data acquisition starting at time 5 min in the scan mode (scan range, 80 to 387 *m*/*z*). Dwell time was adjusted at 2 cycles/s through the run. Quantification of the compounds was based on the total ion abundance signal.

2.10. SDS-PAGE and protein staining

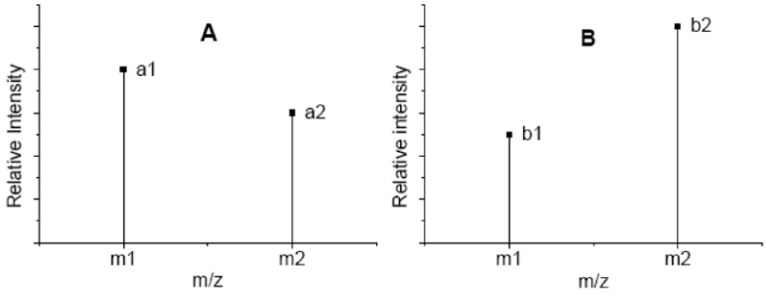
Low density fractions (2–4) prepared with or without detergent were pooled by ultracentrifugation as described in a previous paragraph. Equal amounts of protein (15–30 μg) were vacuum dried (Savant SpeedVac AS290) and resuspended in 20 μl Laemmli Sample Buffer® (Bio-Rad) plus 1 μl of mercaptoethanol. Samples were incubated at 80 °C for 10 min and centrifuged at 10,000×*g* for 10 min to remove any potential aggregate. Proteins were separated by electrophoresis on ReadyGel® 4–20% gradient gels (Bio-Rad) at 200 V for 50 min using Tris–Glycine–SDS Electrophoresis Buffer® (Bio–Rad). Proteins were stained by the Silver Stain Plus® kit (Bio-Rad) following manufacturer's instructions. Development reaction was stopped by acetic acid 5% (v/v) when bands were clearly visualized. Gels were washed with abundant water and incubated overnight in an aqueous solution with glycerol 5% (v/v) and sodium azide 0.02% (w/v) for conservation. Finally, images of the gels were taken in a scanner.

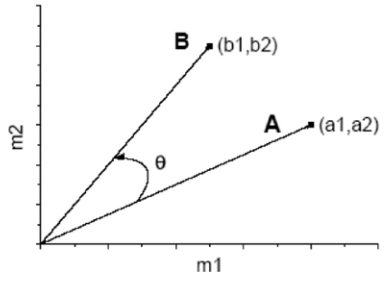
2.11. Protein analysis by mass spectrometry (MALDI-TOF)

Low density fractions (2–4) prepared with or without detergent were pooled by ultracentrifugation as described in a previous paragraph. The pellets were resuspended in a small volume of ultrapure water. Protein concentration was typically 0.5–2 mg/ml. For MALDI-TOF analysis, proteins samples were mixed with a saturated solution of sinapinic acid in a 1:1 ratio. One or two μl of the mixture were spotted in each well of the stainless steel target plate and co-crystallized by evaporation. Spectra were acquired in positive linear mode with the aid of a Bruker Reflex IV time-of-flight mass spectrometer, employing 20 and 16.9 kV in the extraction and acceleration plates and 9.2 kV in the focusing lens. Each spectrum is an average of 30 shots.

2.12. Mathematical analysis of mass spectrometry spectra

The similarities between mass-spectra were analyzed using the *Spectral Contrast Angle computation method* [36,37]. Briefly let A and B be two spectra in which only two peaks appear at *m*/*z* m_1 and m_2, respectively, in both spectra, and with relative intensities a_1 and a_2 in A and b_1 and b_2 in B. We can consider m_1 and m_2 as a pair of variables in the bidimensional space that adopt the values (a_1, a_2) in spectrum A and (b_1, b_2) in spectrum B, forming therefore vectors **A** and **B**. The spectral contrast angle is therefore defined by the angle formed by those two vectors:





$$\cos\theta = \frac{\sum_i a_i b_i}{\sqrt{\sum_i a_i^2 \sum_i b_i^2}} \quad (1)$$

The more similar the spectra are the smaller the angle θ is. In the case of real spectra with n mass-peaks (m_1, m_2,..., m_n) the space becomes n-dimensional and the vectors will have n components $\mathbf{A}=(a_1, a_2,..., a_n)$ determined by the intensity of each peak. The spectral contrast angle θ between two spectra is again defined by the scalar product of the two n-dimensional (normalized) vectors scaled by the arccos function, so as if the angle between the two vectors is 0, both vectors point to the same point of the n-hypersphere of radius 1. Following the above procedure, the cosθ value was calculated between all possible pairs of spectra using a homemade algorithm implemented in Mathematica 5.0. The spectra to be compared form a matrix in which the rows are the spectra and the columns the mass-channels. Using this algorithm, the matrix of the cosine of the angles is obtained in which the element (i,j) is the cosine of the spectral contrast angle between spectra i and j (one must keep in mind that after applying the algorithm each spectrum is transformed in a single vector). In this way, two-dimension representations are obtained, in which the similarities are expressed in a grey scale, the white colour meaning a value of cos$\theta=1$.

2.13. Immunoblotting

Twenty μl 4× NuPage® LDS sample buffer and 4 μl mercaptoethanol were added to 60 μl of each sucrose density fraction and incubated at 80 °C for 10 min. In some experiments, the fractions corresponding to the different populations of membranes obtained by sonication, termed here as "rafts" (2–4) and "non-rafts" (6–10), were pooled as described in a previous paragraph and their concentration equalized to 1 μg per lane. The samples were centrifuged at 10,000×g for 10 min to remove potential aggregates. Proteins were separated by electrophoresis on NuPage® Bis–Tris 4–12% gels (200 V for 50 min). Proteins were then electrophoretically transferred to a 0.2 μm nitrocellulose membrane (30 V for 60 min). The membranes were blocked for 90 min at room temperature in phosphate-buffered saline with Tween 20 (PBS-T, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl and 0.1% Tween 20) containing 5% non-fat dried milk powder. After 2 washes with PBS-T, the membranes were exposed overnight at 4 °C to the primary antibody in PBS-T with 2.5% non-fat dried milk. The antibody dilutions from manufacturer's stock and final concentrations were: $P2X_7$, 1: 666 (0.45 μg/ml); caveolin-1, 1:1,250; transferrin receptor, 1: 250 (4 μg/ml); β-COP, 1: 750 (4 μg/ml); calnexin, 1: 1,000. After 5 washes with PBS-T, the membranes were incubated with peroxidase-conjugated donkey anti-rabbit IgG (1:10,000 dilution) or goat anti-mouse IgG (1: 2,000 dilution) for 1 h in PBS-T with 2.5% non-fat dried milk. After another run of 5 washes, the immunoreactive proteins were visualized on X-ray films (BioMax MR films, Kodak) with a chemiluminescent horseradish peroxidase substrate (ECL+).

2.14. Statistical analysis

Results are expressed as means±S.E.M. of the number of experiments indicated. Statistical significance between various conditions was assessed with Student's t test.

3. Results

3.1. Lipid characterization

Rat submandibular gland cells were subjected to the two protocols described in Materials and methods, based either on Triton X-100 extraction or on extensive sonication. In both cases the extracts were centrifuged in a sucrose density gradient in order to obtain a buoyant low density fraction of vesicles. The presence of vesicles in the low density 5–35% sucrose interface (fractions 2–4) was assessed by light scattering (as shown in [33,34]) and transmission electron microscopy (see Fig. 1). Fractions from the gradient were analyzed in terms of protein and cholesterol content. As shown in Table 1, the light fractions (2–4) contained a low quantity of proteins, (around 7% of total proteins), while cholesterol content represented about 20% of the total. This indicates a relatively high enrichment of cholesterol with respect to proteins for the buoyant fractions pooled after the two different protocols (relative ratios 3.5±0.1 and 3.7±0.9 for sonication and Triton X-100, respectively) when compared to the high density fractions (fractions 6–10) (relative ratios 0.74±0.05 and 0.81±0.07 for sonication and

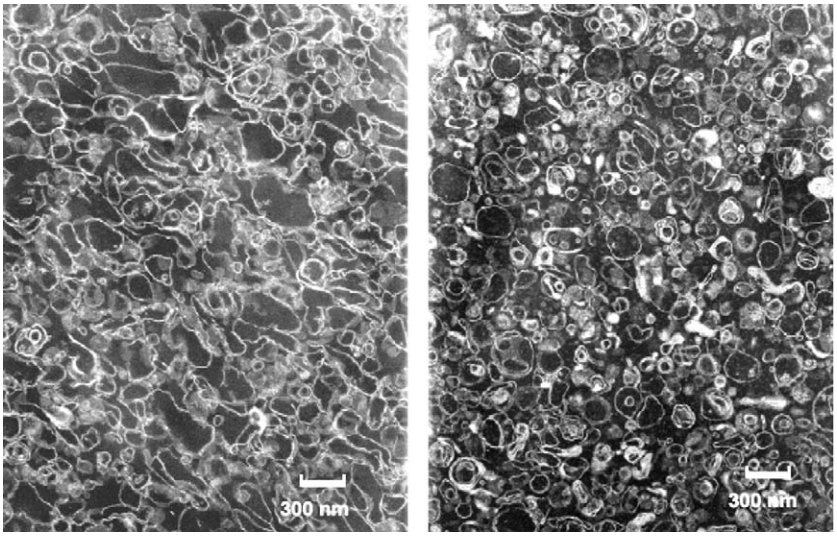


Fig. 1. Morphology of the light fractions prepared by detergent extraction or by sonication. Raft-like membranes were prepared either with an extraction using Triton X-100 (left panel) or by sonication (right panel) followed in both cases by ultracentrifugation in a discontinuous sucrose gradient. Fractions 2, 3 and 4 of the gradient were pooled and examined by electron microscopy as described in Materials and methods. Representative pictures from two different preparations are shown.

Table 1
Comparison of protein and cholesterol content of the different fractions obtained by the two different protocols

Fractions	Low density fractions (2–4)			High density fractions (6–10)		
	Cholesterol (% total)	Protein (% total)	Cholesterol/ Protein	Cholesterol (% total)	Protein (% total)	Cholesterol/ Protein
Detergent ($n=5$)	20.1±4.8	7.2±3.3	3.7±0.86	73.7±6.6	90.5±4.4	0.81±0.07
Sonication ($n=3$)	22.9±3.6	6.5±1.1	3.5±0.14	68.4±5.3	92.1±1.4	0.74±0.05

Cells were fractionated as described in Materials and methods by the protocol based either on solubilization with 0.5% Triton X-100 (Detergent) or sonication. Cholesterol and proteins were determined in the fractions collected from the gradient as described in Materials and methods. Results were expressed as percentage of total cholesterol or proteins present in fractions 2–4 (low density fractions) or in fractions 6–10 (high density fractions). Cholesterol/protein was calculated as the ratio between the relative amount (percentage) of each component in the low or high density fractions. Ratio equal to 1 represents the cholesterol/protein ratio for the homogenate (sum of all fractions). Results are expressed as average±S.E.M. of n independent experiments.

Triton X-100, respectively). The lipid content of the fractions was further characterized by analyzing their fatty acid composition. Low density fractions (2–4) from the two different methods were pooled and their phospholipid fatty acid methyl esters analyzed by gas chromatography-mass spectrometry (GC-MS). As shown in Table 2, comparison of light fractions with the cell lysate revealed significant differences in terms of fatty acid composition. The main differences for light fractions of both methods were a higher content in saturated fatty acids (+ 33%), mainly due to the contribution of 16:0 species (approximately + 30%). They also had a lower content in polyunsaturated fatty acids (− 50%). Very long chain fatty acids (C>20) were significantly enriched in light fractions prepared by Triton X-100 solubilization when compared to the cell lysate or to light fractions obtained by sonication (Table 2). Importantly, the composition of the heavy fractions obtained by sonication was different from the light fractions from both methods, but similar to the cell lysate (Table 2). The higher degree of saturation of phospholipid acyl chains and the higher content of cholesterol are characteristics of rafts and are generally associated with their higher rigidity [10]. In fact, we have previously reported that the raft-like membranes prepared by the method using Triton X-100 are more rigid than bulk membranes [33]. As shown in Fig. 2, raft-like membranes prepared by sonication were also more rigid than heavier membranes, as assessed by higher values of anisotropy (0.117±0.008 for rafts versus 0.072±0.013 for non-rafts at 37 °C). All these properties of the light fractions prepared by any of the two methods are characteristics of lipid rafts.

Table 2
Analysis of fatty acid composition of phospholipid fractions obtained by different protocols and comparison with the cell lysate

Fatty acid	Lysate	Non-rafts (Sonication)	Rafts (Sonication)	Rafts (Detergent)
14:0	1.13±0.03	1.13±0.12	1.14±0.14 †	1.96±0.07 **
15:0	0.55±0.15	0.18±0.10	0.59±0.36	1.14±0.08
16:1	2.42±0.09	2.70±0.30 $^{\#}$	1.82±0.10 **	2.04±0.07 **
16:0	28.68±0.37	31.87±2.93 $^{\#}$	43.14±0.82 ***	36.70±3.45
17:0	1.08±0.21	0.62±0.21	0.44±0.44	1.37±0.19 *
18:2	12.07±0.40	11.25±0.44 $^{\#\#}$	6.89±0.32 ***	7.61±1.55 *
18:1	14.30±0.47	15.75±0.78	15.45±1.10	13.68±0.58
18:0	17.80±0.43	18.04±0.38 $^{\#}$	21.26±0.46 **	19.82±0.37
20:4	12.09±0.17	10.61±0.72 $^{\#}$	4.63±0.95 ***	4.80±1.79 *
20:3	4.96±0.16	4.97±0.43 $^{\#}$	2.37±0.75 *	2.47±0.66
20:2	0.92±0.13	0.55±0.22	0.15±0.14 **	0.57±0.20
20:1	0.69±0.14	0.66±0.24	0.00±0.00 *	0.44±0.18
20:0	0.37±0.03	0.31±0.10	0.00±0.00 **, ††	0.43±0.07
22:0	0.47±0.09	0.25±0.09	0.29±0.29	0.87±0.13
23:0	0.16±0.02	0.03±0.03	0.00±0.00 **, ††	0.40±0.04 **
24:1	1.04±0.07	0.42±0.26	0.64±0.35 †	2.63±0.12 **
24:0	1.27±0.27	0.66±0.15	1.18±0.61	3.08±0.46 **
Saturated	51.51±0.34	54.58±2.76 $^{\#}$	67.50±0.75 ***	65.76±3.65 *
Monouns.	18.45±0.61	18.54±1.40	18.80±1.28	18.79±0.58
PUFAs	30.04±0.38	26.89±1.42 $^{\#}$	13.71±1.48 ***	15.44±4.19 *
C>20	2.94±0.41	1.37±0.49	2.11±0.84 ††	6.98±0.51 **

Cell lysate and different fractions prepared by the two methods ("Sonication" and "Detergent") were prepared as described in Materials and methods. The fatty acid content was measured by GC-MS. The results are expressed as the percentage area for each fatty acid when compared to the total area of the fatty acids. They are the means±S.E.M. of 4 independent experiments.

* $P<0.05$ when compared to the cell lysate.
** $P<0.01$ when compared to the cell lysate.
*** $P<0.001$ when compared to the cell lysate.
† $P<0.05$ when compared to "Rafts (Detergent)".
†† $P<0.01$ when compared to "Rafts (Detergent)".
$^{\#}$ $P<0.05$ when compared to "Rafts (Sonication)".
$^{\#\#}$ $P<0.01$ when compared to "Rafts (Sonication)".

3.2. Protein characterization

In the next experiments we characterized the protein composition of raft-like membranes isolated by the two different methods. Light fractions (2–4) pooled from gradients performed after the Triton X-100 extraction or the sonication protocol were developed by electrophoresis and silver stained (Fig. 3A). Protein band profile was apparently different for both preparations. Protein analysis by mass spectrometry (MALDI) has been previously used to successfully analyze protein profiles of complex samples such as cell homogenates or tissue slices [38–40]. Similarly to SDS-PAGE results, MALDI-TOF spectra showed a different profile for each type of light membranes (Fig. 3B). In order to avoid subjective interpretations and to provide a statistical significance to these results, similarity of the different mass spectra obtained by MALDI-TOF were mathematically analyzed (Fig. 3C). They were analyzed in an "all-against-all" fashion, taking into account the position (m/z) and the intensity of the peaks present in 40–50 different spectra for each type of raft-like membranes (see Materials and methods). As shown in Fig. 3C, a certain grade of similarity ($\cos\theta$ mean values 0.74 and 0.55 for T vs. T and S vs. S, respectively) was observed when spectra from the same type of preparation (either Triton X-100 or sonication, corresponding to quadrants 1 and 2,

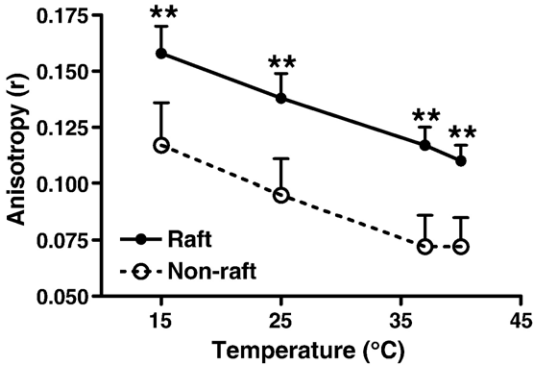


Fig. 2. Anisotropy of light and heavy membranes prepared by sonication from rat submandibular glands. Fractions 2–4, termed here as "rafts", and fractions 6–10, termed here as "non-raft", obtained by sonication were pooled as described in Materials and methods. The fluorescent probe DPH was incorporated into the membranes of the different samples. Anisotropy was determined for the different indicated temperatures. Results are averages±S.E.M. of 5 independent preparations. $^{**}P<0.01$.

respectively) were compared. On the other hand, there was no similarity ($\cos\theta<0.0005$) between raft-like membranes from the two types of preparation (T vs. S, corresponding to quadrants 3 and 4).

$P2X_7$ is a receptor with unique properties among purinergic receptors. Apart from forming a non-specific cation channel, it has several properties implicated in the regulation of cell death in many cellular types [41,42]. We have recently observed that $P2X_7$ receptors could couple to different signalling pathways depending on their localization in cholesterol-rich domains [34]. Here, we compared the distribution of this receptor in light and heavy fractions obtained either by the Triton X-100 or by the sonication method (Fig. 4). In both cases $P2X_7$ receptor subunits were detected in light and heavy fractions, but the distribution was very different depending on the method. Only a small population of these receptors was found in raft-like membranes prepared by Triton X-100 (<5%, $n=4$), while 52±5% ($n=8$) of these receptors were found in light fractions prepared by sonication as estimated by semi-quantitative densitometry. Caveolin-1, a marker of lipid rafts, was highly enriched in light fractions prepared by the two methods (Fig. 4A and B), ruling out the possibility of a different yield of $P2X_7$ recovery in light fractions related to a different yield in the isolation of rafts. Importantly, light fractions isolated by both methods were free of Golgi apparatus or endoplasmic reticulum, as assessed by the presence of specific markers (β-COP and CNX, respectively [26,43]) only in heavy fractions (Fig. 4A and B). This result argues against the presence in low density fractions of a $P2X_7$ precursor instead of the plasmalemmal mature protein. Transferrin receptor (TfR), a marker of the non-raft fraction of the plasma membrane [26,43,44], was mainly found in heavy fractions. Only a small fraction of this protein (~10%) was present in the "non-detergent" light membranes. This fraction is much lower than the enrichment in caveolin-1 and $P2X_7$. Considering that low amounts of proteins are found in light fractions (Table 1), the enrichment of $P2X_7$ receptor in "non-detergent" raft-like membranes was obvious, as demonstrated by immunodetection of $P2X_7$ subunits in lanes loaded with equal amounts of protein from "rafts" and "non-rafts" samples prepared by sonication (Fig. 4C).

4. Discussion

4.1. Raft-like properties

We have isolated by a detergent or a non-detergent method a membrane fraction from rat submandibular glands with properties analogous to that described for lipid rafts: these fractions had a high cholesterol/protein ratio (Table 1), were enriched in saturated fatty acids (Table 2) and in a protein marker of membrane microdomains like caveolin-1 (Fig. 4). These fractions excluded non-raft, Golgi apparatus and endoplasmic reticulum protein markers (TfR, β-COP and CNX, respectively, Fig. 4). We previously described that raft-like membranes prepared by Triton X-100 extraction [33] were more rigid than bulk membranes. Here we provide similar evidence for light fractions obtained by sonication (Fig. 2), thus confirming the main physical feature of lipid rafts for membranes prepared by both methods. Morphologically, they were vesicles of 50–400 nm of diameter, but it should be noted that preparations obtained by sonication had a more heterogeneous aspect. Similar morphologies have been observed for raft-like fractions prepared by Triton X-100 [45,46] or detergent-free protocol [47]. It has also been reported that rafts might be a heterogeneous population [19]. Gaus et al. observed that rafts can reach a few micrometers of area [6] but it is usually claimed that the size of the vesicles from Triton X-100 resistant membranes vesicles is too large to reflect the size of rafts in intact membranes of living cells; the treatment with the detergent might promote membrane fusion and mixing [48]. In our hands vesicles prepared with or without detergent showed similar size. Thus, we cannot discard lipid mixing or membrane fusion during other steps of the preparation by the two methods such as high speed ultracentrifugation or sonication. In fact, extensive sonication is a widely used protocol to prepare liposomes. In spite of this drawback, other results discussed below argue in favour of the raft-like nature of the membrane fraction obtained by sonication.

The relative enrichment in cholesterol was very similar for both preparations of light membranes (about 4-fold) when compared to heavy fractions (Table 1). This result is in agreement with previous results showing a similar cholesterol/protein ratio between "detergent" and "non-detergent rafts" obtained from synaptosomes [49]. The fatty acid composition was also similar for both preparations, rich in saturated and poor in polyunsaturated fatty acids (Table 2). The higher saturation of phospholipid acyl chains is in agreement with previous results obtained with a Triton X-100 based method [50–52]. This higher degree of saturation, as well as the cholesterol enrichment, allows a tighter packing of the phospholipid bilayer and is probably responsible for the higher rigidity of these microdomains (Fig. 2, [33]). These

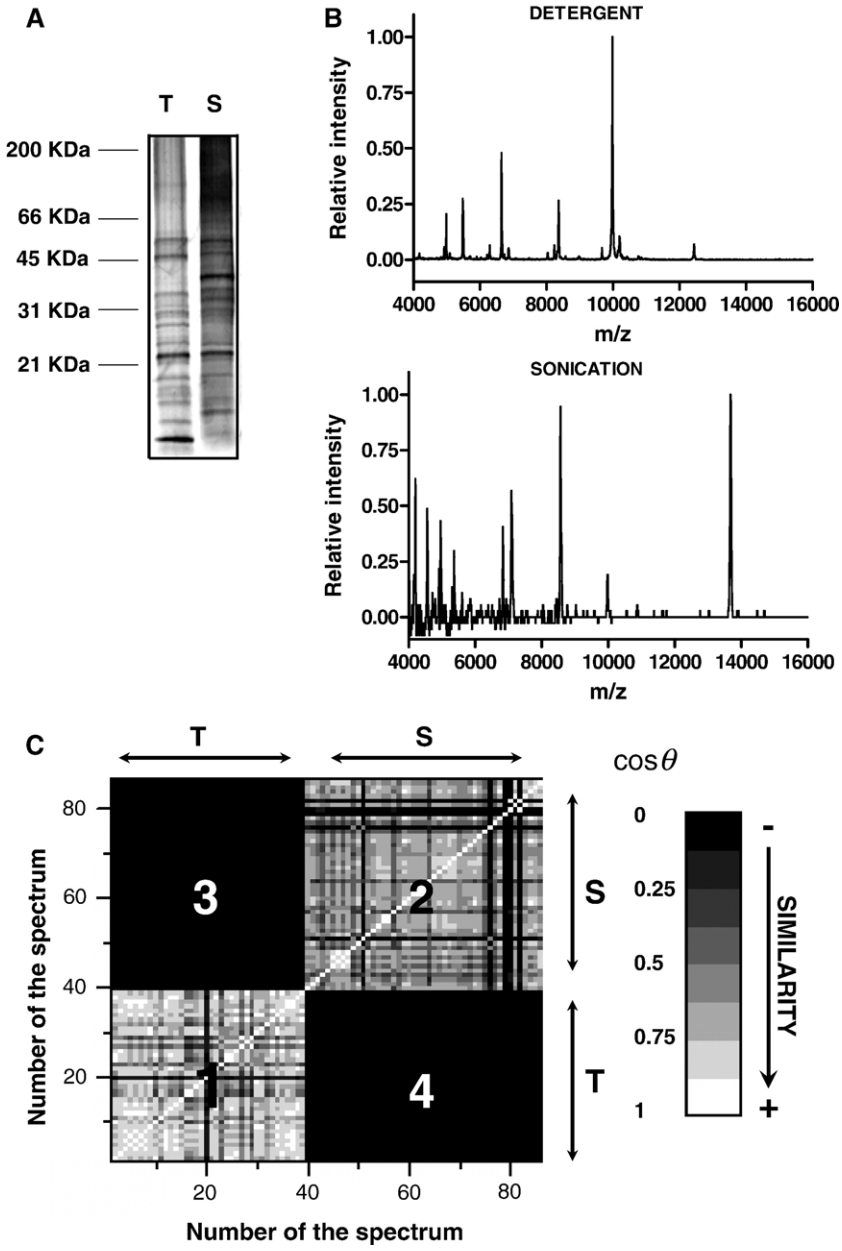


Fig. 3. Protein profiles of the raft-like membranes prepared by detergent extraction or by sonication. Raft-like membranes were prepared as described in the Legend to Fig. 1. Fractions 2, 3 and 4 of the gradient were pooled and analyzed in terms of protein composition. (A) SDS-PAGE followed by silver staining was performed in parallel for light membranes obtained by both methods. T: Triton X-100; S: sonication. The results are from one experiment representative of 4 independent preparations. (B) Proteins from the two preparations were co-crystallized with sinapinic acid and analyzed by MALDI-TOF. A representative spectrum from 4 independent experiments is shown. (C) MALDI-TOF spectra of Triton X-100 (spectra from 1 to 39) or sonication (spectra from 40 to 86) preparations were mathematically analyzed as described in Materials and methods. The similarity between different spectra was defined by cosθ and represented in a grey scale, from black (lower similarity) to white (higher similarity). T: Triton X-100; S: sonication.

results are at variance with Pike et al. who reported that the cholesterol/protein ratio is higher for raft-like membranes prepared by Triton X-100 extraction than by sonication [43,53] but that the latter were specifically enriched with arachidonic acid [53]. These discrepancies could be attributed to different cell types or methodologies. For instance, we have used 0.5% (w/v) Triton X-100, while Pike et al. used a higher (1%) concentration. In fact, Gaus et al. observed that the cholesterol content of raft-like membranes isolated from macrophages by Triton X-100 was highly dependent on detergent concentration [44]. These authors reported that a high (1%) concentration of detergent depletes cholesterol from rafts, while a low (0.2%) concentration keeps cholesterol content close to that observed in detergent-free preparations.

4.2. "Non-detergent rafts"

Methods for raft-like membranes isolation based on fine disruption of the membranes by sonication and subsequent

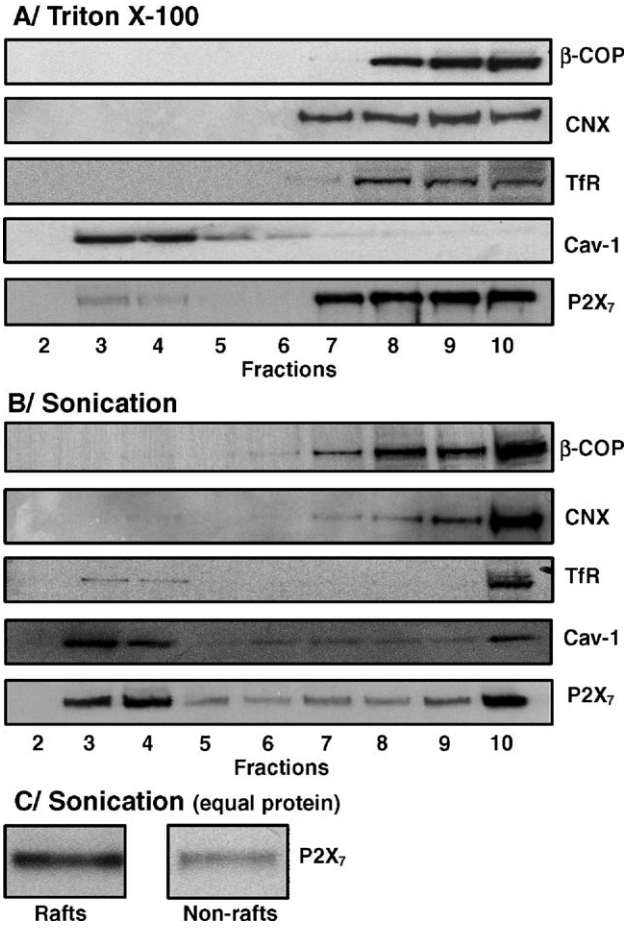


Fig. 4. Localization of $P2X_7$ receptors in membrane fractions prepared by Triton X-100 extraction or by sonication. Membrane fractions were prepared either with an extraction with Triton X-100 (A) or by sonication (B, C) followed by centrifugation in a discontinuous sucrose gradient. Equal volumes of each fraction of the gradient were used for β-COP, CNX, TfR, caveolin-1 or $P2X_7$ immunodetection as described in Materials and methods (A, B). For panel C, fractions 2–4 and fractions 6–10 were pooled as described in Materials and methods. Equal quantity of protein (1 μg) of each fraction (termed here as "rafts" or "non-rafts") was used for $P2X_7$ immunodetection. Pictures shown are representative of at least 3 independent experiments with very similar results.

separation in density gradient have been criticized for their higher susceptibility to contamination by other low density membranous materials [11,19,26,27]. The relatively low content of proteins in light fractions, similar to that observed for the Triton X-100 resistant membranes (Table 1) argues against this criticism. These "non-detergent rafts" were devoid of intracellular membrane markers for Golgi apparatus (β-COP) and endoplasmic reticulum (CNX) but contained a small percentage of the non-raft marker TfR. The different protein composition of light and heavy fractions prepared by sonication was assessed by a mathematical analysis of the similarity degree of MALDI-TOF spectra ($\cos\theta < 0.0005$, data not shown). This result favoured the hypothesis of the isolation from the whole population of membranes of a raft-like membrane fraction with a different composition. The fatty acid composition of the light membranes prepared by sonication further strengthened this hypothesis: it was similar to the light membranes prepared by the detergent based method; it differed from the cell lysate and also from the heavy fractions obtained by sonication (Table 2). The low density membranes prepared by sonication had also higher values of anisotropy probably because they were more rigid than high density membranes ("non-rafts") (Fig. 2), indicating again a separation of membranes of distinct nature and strengthening the identity of these light membranes as raft-like membranes.

4.3. Differences between raft-like membranes isolated by both methods

Major differences were observed in the proteins present in the raft-like membranes prepared by the two methods. The band profile and MALDI-TOF spectra were qualitatively different (Fig. 3). These results which are in agreement with observations reported in the literature [54] were analyzed quantitatively. The mathematical analysis confirmed the difference in the protein composition between "detergent" and "non-detergent rafts" (Fig. 3). Two major explanations might account for these results. It has been suggested that rafts are heterogeneous and that detergents and sonication isolate different populations of raft-like membranes [19]. This is unlikely since the general properties for both preparations were similar. The second hypothesis would be that exposure of the membranes to detergent disrupted interactions between proteins and components of the rafts [15,22,55]. This detergent action would be especially relevant for proteins that interact weakly with rafts. This explains why "raft-resident" proteins such as caveolin-1, which interacts tightly with lipid rafts through cholesterol binding [56,57], is not removed by Triton X-100 extraction (Fig. 4, [33]). This is not the case for the $P2X_7$ receptor. The presence of $P2X_7$ receptors in the isolated raft-like membranes was demonstrated by Western blotting, especially when they were prepared with the detergent-free method. Only a small fraction of the receptors were present in the light fractions prepared with Triton X-100 while nearly 50% of these receptors could be observed in raft-like membranes prepared by sonication, resulting in a significant enrichment of receptors in this membrane fraction (Fig. 4). It is likely that this latter distribution reflects the localization in physiological conditions, since we have recently observed that lipid rafts disruption displaces the $P2X_7$ protein from "raft" to "non-raft" fractions and blocks some responses coupled to this receptor [34].

Our results are fully consistent with those of Bannas et al. [58]. Using mouse lymphoma cells they suggested that the ADP-ribosylated $P2X_7$ receptors were distributed among "raft" and "non-rafts" domains of the plasma membrane. Contrary to GM1 or GPI-anchored proteins, these $P2X_7$ receptors present in raft-like membranes could be extracted by treatment with 1% ice-cold Triton X-100. The presence of $P2X_7$ receptors in the "raft" fraction could only be observed after a treatment of the membrane with a very low concentration (0.05%) of ice-cold Triton X-100 followed by sonication. These results together with our own observations suggest that the $P2X_7$ receptor is not firmly embedded in rafts and that this receptor could interact with an unknown component of the rafts. This interaction is probably not specific to $P2X_7$ receptors since two other groups have already reported on the presence of other P2X receptors in rafts. Vacca et

al. [59] reported that $P2X_3$ receptors are located in raft-like membranes isolated by detergent and non-detergent methods from cerebellar granule neurons, but only present in "detergent-free rafts" of dorsal root ganglia or SH-SY5Y neuroblastoma cells exogenously expressing $P2X_3$ receptors. More recently Vial and Evans reported that $P2X_1$ receptors localized in raft-like membranes prepared by a detergent-free method [60]. Interestingly, when Triton X-100 was included in the process, $P2X_1$ receptors were solubilized, appearing in "non-raft" fractions.

In conclusion, a membrane fraction with properties analogous to those described for lipid rafts has been isolated from rat submandibular gland cells using either a detergent-based or a detergent-free method. While the hydrophobic core of the raft-like membranes prepared by both methods is similar (cholesterol, fatty acids, anisotropy), significant differences are found in protein profiles, indicating a possible effect of detergent in molecular interactions responsible for protein-rafts associations. This is reflected in the behaviour of $P2X_7$ receptor, which is present in "non-detergent rafts" but is solubilized when Triton X-100 is used. These results reflect the importance of choosing a proper method for biochemical isolation of raft-like membranes and support the idea of the necessity of caution in the interpretation of results regarding lipid rafts compartmentalization of signalling molecules.

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