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Use of a photoactivable GM1 ganglioside analogue to assess lipid distribution in caveolae bilayer

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A new photoactivable, radioactive derivative of ganglioside GM1 has been utilized to assess lipid distribution in the caveolae bilayer, taking advantage of the ability of the glycolipid, endogenous or exogenously added, to concentrate within this membrane compartment and to crosslink neighboring molecules upon illumination. After insertion into A431 plasma membrane and photoactivation, a membrane-enriched and a detergent-resistant fraction, enriched in gangliosides, sphingomyelin and cholesterol, were isolated. While a few radioactive proteins were detected in the membrane-enriched fraction, only radioactive caveolin was detected in the detergent-resistant fraction, indicating at the same time the enrichment of this fraction in caveolae and the presence of ganglioside within this compartment. Among lipids, crosslinked phosphatidylcholine, sphingomyelin and cholesterol were detected in the membrane-enriched fraction, while only crosslinked sphingomyelin was detected in the detergent-resistant fraction. These results suggest the enrichment in sphingomyelin—along with ganglioside—within the outer leaflet, and the preferential localization of cholesterol within the endoplasmic leaflet, of the caveolae bilayer.

Keywords: **GM1 ganglioside, caveolae, plasma membrane**

Abbreviations: **MEF, Membrane-enriched fractions; DRF, detergent-resistant membrane fraction; FCS, fetal calf serum; MES, (2[N-Morpholino]ethanesulfonic acid; PBS, phosphate buffered saline; TID-GM1, 9-[[[2-(^{125}I)iodo-4-((trifluoromethyl)-3*H*-diazirin-3-yl)benzyl]oxy]carbonyl]nonanoyl]-GM1 ganglioside; PVDF, polyvinylidene difluoride; PC, phosphatidylcholine; SM, sphingomyelin; Ch, cholesterol.**

Introduction

Caveolae are specialized plasma membrane domains, morphologically defined as small flask-shaped invaginations, lacking the clathrin coating [1]. A striking feature of caveolae is their protein and lipid composition, different from the rest of the plasma membrane [2]. For instance, proteins of the caveolin family and many of the molecular components of trans-membrane signaling are concentrated within these relatively small areas of the plasmamembrane [3,4]. As another peculiar feature, caveolae are enriched—along with sphingomyelin and cholesterol—in GM1 ganglioside, which has been utilized as a lipid marker for these membrane structures [5,6]. However, several features of the postulated or demonstrated role played by lipids in the biogenesis, maintenance and functions of the caveolae assembly, are still debated. For instance, the topology of lipids in caveolae has not yet been definitively established. In particular, neither the lipid distribution within the two leaflets of the caveolae membrane bilayer, nor their lateral distribution within the plane of a single leaflet is clear.

Taking advantage of the ability of exogenously added GM1 to concentrate within caveolae, as the endogenous one, the use of photoactivable, radioactive derivatives of GM1 ganglioside to identify proteins present within these membrane structures has been suggested. Using this approach, in A431 cells it has been shown the preferential interaction of GM1 with caveolin [7]. In the present investigation, the same approach has been utilized, using the same cellular model system, to assess ganglioside-lipid interactions in caveolae.

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Materials and Methods

Reagents

Common reagents (analytical grade), and HPTLC plates (Kieselgel 60), were purchased from Merck (Darmstadt, Germany). ^{125}I (IMS-300, 5 mCi, 2000 Ci/mmol), [^{14}C]-methylated protein standard for electrophoresis, autoradiographic films, secondary antibodies and enhanced chemiluminescence (ECL) detection kit were from Amersham Pharmacia Biotech (Uppsala, Sweden). Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), trypsin, cholesterol, *Bacillus Cereus* sphingomyelinase and phospholipase C, bovine serum albumin (BSA) and digitonin were from Sigma Chemical Co. (St. Louis, MO, USA). Policlonal anti-caveolin-1 antibody (N20) was from Santa Cruz Biotech. (Santa Cruz, CA, USA).

GM1 ganglioside was extracted and purified as described [8]. GM1 tritium labeled at the 3-position of the long chain base, was prepared as described [9,10].

Chemical synthesis of photoactivable, ^{125}I-labeled, GM1 ganglioside (TID-GM1)

The photoactivable radioactive GM1 ganglioside analog, TID-GM1, carrying a 9-[[[2-(^{125}I)iodo-4-((trifluoromethyl)-3*H*-diazirin-3-yl)benzyl]oxy]carbonyl]nonanoyl-fatty acyl residue in substitution of the native fatty acid present in the ceramide moiety, was synthesized relying on procedures previously described [11,12] adapted for the synthesis of this particular glycolipid, and has been described in details elsewhere [13]. Briefly, the carboxylic acid function of 9-[[[2-tributylstannyl-4-(trifluoromethyl-3*H*-diazirin-3-yl)-benzyl]oxy]carbonyl]nonanoic acid was activated by formation of the N-hydroxysuccinimide ester [11]. Next, 6 µmoles lyso-GM1, prepared as described in [12] were N-acylated using an excess (21 µmoles) of the hydroxysuccinimide ester in 250 µl THF, 6 µl N-methylmorpholine and 5 µl H_2O, with formation of the stannylated photoactivable ganglioside. TID-GM1 was obtained from stannylated photoactivable GM1 by radioiododestannylation, following the protocol reported for various lipid analogues [11]. The specific radioactivity of TID-GM1 was estimated to be >100 Ci/mmole. Its radiochemical purity was assessed by TLC, using different solvent systems followed by autoradiography, and was >98%. Samples were photolyzed using a UV lamp (300 W, Jelosyl, Milano, Italy). In order to define the time required for photolysis of TID-GM1, a 10^{-5} M solution of the stannylated, unlabeled photoactivable ganglioside (see above), was irradiated for different periods of time. Photolysis of the diazirine group was monitored spectroscopically, by measuring the disappearance of the characteristic diazirine band at 350 nm (molar extinction coefficient at 350 nm = 300).

Preparation of standard lipid/TID-GM1 complexes

Lipids were extracted and partitioned from A431 cells, an aqueous and an organic phase being obtained [8]. The organic phase was submitted to silicic acid column chromatography, according to [14]. Three fractions—neutral lipids, glycolipids and phospholipids—were separated. The phospholipid fraction was further fractionated on a Silica gel column eluted with chloroform/methanol/acetic acid/water 60/45/4/2, v/v/v/v [15] to obtain phosphatidylcholine (PC) and sphingomyelin (SM). The final purity of PC and SM was over 99%, as assessed by HPTLC using the same solvent system. PC and SM, purified as above described, and commercial cholesterol (Ch) were crosslinked with TID-GM1 to be used as standards. For this purpose, binary 1:1 (molar) mixtures of TID-GM1 (6 pmoles) with each of the above said lipid were prepared in THF, dried, resuspended in 10 µl water and illuminated with U.V. light (5 min on ice). After photoactivation of the mixtures, 150 µl THF/water (4/1 v:v) were added, and an aliquot submitted to 2D-TLC, as further described, followed by autoradiography.

Treatment of cells with TID-GM1

A-431 cells were cultured in DMEM as described [7], and used at 80–90% confluence. The morphology of cultured cells was examined by using a phase contrast microscope. TID-GM1 was dissolved in 1 ml DMEM containing 10 mM Hepes (DMEM-H). After removal of the medium, followed by rapid washing, the cells were incubated for 4 h at 4°C with 2.5 ml of the same solution containing 10^{-6} M TID-GM1. At the end of incubation, the cells were washed three times with 3 ml (each time) of DMEM and then incubated (4 times, 5 min each time) with DMEM containing 0.2% BSA at 37°C, in order to minimize nonspecific binding [7]. After washing, cell-associated TID-GM1 was photoactivated by irradiation with UV light for 5 min at 4°C. Cells were collected and subjected to subfractionation for preparation of the membrane-enriched fraction and of the detergent-resistant fraction. An aliquot of the homogenate was treated for determination of associated radioactivity [16].

Cell subfractionation

Preparation of membrane-enriched fraction (MEF). All the following operations were carried out at 4°C. Cells were harvested and then homogenised by sonication in 500 µl Tris-HCl 10 mM, pH 7.4. After flotation for 18 h at 250 000 *g*, 4°C, membranes at the 0.8/1.2 M sucrose interface fraction were diluted two-fold and pelleted for 1 h at 230 000 *g* to yield the membrane-enriched fraction [7].

Preparation of detergent-resistant membrane fractions (*DRF*). Cells were harvested and incubated for 30 min on ice in 2 ml of 1% (v/v) Triton X-100 in 25 mM MES buffer, pH 6.5, containing 150 mM NaCl, 1 mM PMSF and 75 units/ml aprotinin. A mild homogenization was carried out

with 10 strokes of a loose-fitting Dounce homogenizer. The resulting suspensions were submitted to discontinuous sucrose density gradient centrifugation for the separation of low density, detergent-resistant membrane fractions, DRF, as described [17]. For this purpose, the suspensions were adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose in MBS and placed at the bottom of an ultracentrifuge tube. Two 4-ml layers of 30 and 5% sucrose in MBS were overlaid atop the suspensions and the resulting gradient centrifuged at 39 000 rpm for 20 h in a SW41 rotor (Beckman Instruments). The band at the interface between 30 and 5% sucrose layers (DRF) was withdrawn and centrifuged at 230 000 *g* for 1 hour. The resulting pellet was submitted to further analysis.

Lipid and protein composition of the membrane-enriched and of the detergent-resistant fraction

Lipids. Lipids were extracted and partitioned from MEF and DRF [8]. Gangliosides, present in the aqueous phase, were analysed by HPTLC (solvent system: chloroform/methanol/0.2% $CaCl_2$, 50:42:11, v/v/v) and revealed by an Ehrlich spray reagent. The organic phase was further fractionated by silicic acid column chromatography [14] and the neutral lipid fraction, containing cholesterol, was submitted to HPTLC (solvent system: hexane/diethylether/acetic acid, 20:35:1, v/v/v), the sterol being visualized by spraying with anisaldehyde reagent. Phospholipids, recovered in a separate fraction after silicic acid column chromatography, were analysed by HPTLC (solvent system: chloroform/methanol/acetic acid/water, 60:45:4:2, v/v/v/v) and revealed with I_2. Quantification of PC, SM, Ch and gangliosides was performed by densitometric scanning of the plates and comparison with known amounts of standard lipids.

Protein. The delipidized pellets of MEF and DRF, resulting after lipid extraction, were analyzed by 2D SDS-PAGE, as described [7], and stained with Comassie Blue. The identity of caveolin was assessed, after transfer to PDVF membranes, by western blotting with anti-caveolin antibody (1,000-fold diluted) and detection by chemiluminescence using the ECL detection kit (Amersham).

Detection of proteins crosslinked by TID-GM1 after photoactivation

Mono-dimensional electrophoresis of MEF and of the DRF proteins was performed using the BioRad Mini-protean II system according to the manufacturer's recommendations, using 15% gels. Immunoprecipitation of DRF with anti-caveolin antibodies was performed as already described [7], and immunoprecipitate was then subjected to electrophoresis. The gels were submitted to autoradiography or analyzed using a phosphorus imaging system (Biorad).

Identification of lipids crosslinked by TID-GM1 after photoactivation

After cell treatment with TID-GM1 and photoactivation, the MEF and the DRF were prepared and submitted to lipid extraction [8] in 250 µl of THF/water (4:1 v/v). The unpartioned extracted lipids were separated by two-dimensional HPTLC (2D-TLC) as follows. For detection of phospholipid/TID-GM1 crosslinking products, the plate was developed with chloroform/methanol/0.2% $CaCl_2$ (50:42:11, v/v/v) for the first run, and chloroform/methanol (2:8, v/v) for the second run. For detection of cholesterol-ganglioside complexes, the solvent system chloroform/methanol/$CaCl_2$ 0.2%, 50:42:11, v/v/v, for the first run and 20:35:1 hexane/diethylether/acetic acid v/v/v for the second run was utilized. After chromatography, detection of radioactive spots was performed by autoradiography or using a radioactivity imaging system (Biorad). The identification of radioactive lipid/TID-GM1 complexes was performed by comparison with the standard radioactive lipid/TID-GM1 complexes, prepared as above described. In addition, their identity was also confirmed on the base of their susceptibility to: a) enzymatic treatment with phospholipase C and sphingomyelinase in the case of phospholipid/TID-GM1 complexes, or b) treatment with digitonin in the case of cholesterol/TID-GM1 complex, as below described.

Enzymatic treatments

The conditions for enzymatic treatment of samples were set up either on PC and SM or on the standard lipid/TID-GM1 complexes, in order to obtain exhaustive (but still selective) lipid hydrolysis. The final conditions adopted were the following. For treatment with phospholipase C (active on glycerophospholipids), aliquots of MEF or DRF lipid extracts (0.2 µCi) were dried and suspended in 25 µl of 0.1% Na-deoxycholate in 100 mM Tris-HCl buffer, pH 7.4. 10 mU of enzyme were added and mixtures were incubated for 30 min at 37°C.

For treatment with sphingomyelinase (acting on SM) aliquots of MEF or DRF lipid extracts (0.2 µCi) were dried and suspended in 25 µl of 0.2% Na-deoxycholate in 50 mM Tris-HCI buffer, pH 7.4. 25 mU of enzyme were added and mixtures were incubated for 30 min at 37°C. At the end of the incubations, THF (100 µl) was added to samples, and the mixtures were vortexed and centrifuged (12 000 × *g*, 5 min); aliquots of the supernatants were taken and submitted to 2D-TLC, followed by autoradiography.

Treatment with digitonin

Lipids extracts obtained from MEF and from DRF were subjected to digitonin precipitation in order to remove cholesterol, according to [18] with slight modifications. Treatment with digitonin was previously set up either on cholesterol or on standard cholesterol/TID-GM1 complex in

order to obtain complete cholesterol removal and full recovery of other lipids. Briefly, 50 µl of lipid extract in THF/water were acidified with 1 µl of 10% acetic acid, then 50 µl of a 0.5% digitonin solution (in ethanol/water, 1/1 v/v) were added and precipitates were allowed to stay overnight at 4°C. After centrifugation (12 000 × *g*, 5 min), aliquots of the supernatant were analyzed as described.

Other assays.

Protein content was determined by the method of Lowry [19].

Results

Preparation of standard lipid/TID-GM1 complexes

TID-GM1 or mixtures of TID-GM1 and lipids were UV-irradiated and submitted to 2D-TLC. In the case of SM/TID-GM1 mixture, two evident additional radioactive spots were present in the TLC besides a major large radioactive spot corresponding to TID-GM1 (Fig. 1B, arrows), and likely due to SM molecular species [20]. In the case of PC/TID-GM1 mixture, only an additional radioactive spot was present (Fig. 1, C, arrow). In the case of cholesterol/TID-GM1 mixtures, a different solvent system was utilized, and an additional radioactive band was detected in the TLC besides that of TID-GM1 (Fig. 1, E, arrow).

Association of TID-GM1 with cells

The viability of cells after incubation with 10^{-6} M TID-GM1 at 4°C, followed up to 6 h, was good and identical to that of control cells. After 3 h at the same temperature, 42.4 pmoles/mg protein TID-GM1 were associated to A-431 cells. A parallel experiment using [^{3}H]-GM1 gave very similar figures (38.1 pmoles/mg protein), indicating that association of photoreactive ganglioside was comparable to that of isotopically labeled GM1 ganglioside.

MEF and DRF composition

The two membrane fractions were characterized for their lipid composition. The results are reported in Table I. The DRF was enriched in gangliosides, SM and Ch, while depleted in PC and PE. DRF were also enriched in caveolin (data not shown), being this protein about 1% of total proteins present in the MEF and about 15% in DRF.

Detection of proteins crosslinked by TID-GM1 after photoactivation

After cell incubation with TID-GM1 (3 h at 4°C) and photoactivation, the electrophoresis of MEF (Fig. 2), showed a remarkably simple pattern of radiolabeled proteins. A broad radioactive band was visible in the 21–24 and two others in the 55–60 KDa range. A big radioactive band migrating at the gel front was also present, corresponding to self-quenching ganglioside as already reported [7].

The electrophoresis of the DRF (Fig. 2), showed almost exclusively the presence of a radioactive band in the range 21–24 KDa. The identity of the band with caveolin was assessed by immunoprecipitation, electrophoresis and autoradiography (Fig. 2).

Detection of lipids crosslinked by TID-GM1 after photoactivation

The lipid extracts of MEF and of DRF were submitted to 2D-TLC. In the TLC of MEF, the radioactive spots displaying the same chromatographic behavior of standard SM/TID-GM1 and of PC/TID-GM1 complexes were present (Fig. 3 arrows).

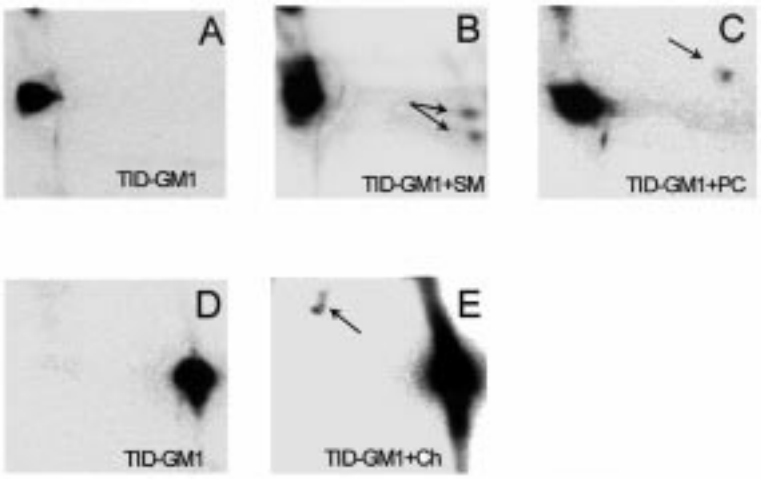

Figure 1. Characterization of standard lipids crosslinked with TID-GM1. TID-GM1 alone, or mixtures of TID-GM1 with PC, or SM or cholesterol were illuminated with U.V. light and subjected to 2D-TLC (see text for details), followed by autoradiography. Panel A, D = TID-GM1, analyzed by 2D-TLC in the solvent system for phospholipids (A) and cholesterol (D); Panel B = TID-GM1/SM mixture; panel C = PC/TID-GM1 mixture; panel E = Ch/TID-GM1 mixture.

The identity of the products was further confirmed on the base of their susceptibility to enzymatic treatment with sphingomyelinase or phospholipase C. In fact, the putative SM/TID-GM1 complex was sphingomyelinase-sensitive but phospholipase-resistant, while the putative PC/TID-GM1 complex was sphingomyelinase-resistant but phospholipase-sensitive (Fig. 3).

In the case of DRF, only a radioactive product displaying the same chromatographic behavior of standard SM/TID-GM1 was detected (Fig. 3, arrows). The identity of the product was further confirmed on the base of its susceptibility to enzymatic treatment with sphingomyelinase. In fact, after treatment of DRF with sphingomyelinase, the putative SM/TID-GM1 complex was not any more present in the TLC (Fig. 3). No change was exerted by treatment with phospholipase C (data not shown).

When the lipid extract of MEF was subjected to TLC using the solvent system utilized for the detection of cholesterol, a radioactive spot displaying the same chromatographic behavior of standard Chol/TID-GM1 was present (Fig. 4, arrow).

Table 1. Lipid composition (% as nmol/mg of protein) of MEF and DRF. Values are the mean of three determinations. Standard deviation never exceeded 8%.

	MEF	*DRF*
PC	39.2	34.1
SM	9.52	19.8
PE	26.1	11.2
Ch	24	31.8
Gangliosides	1.18	3.1

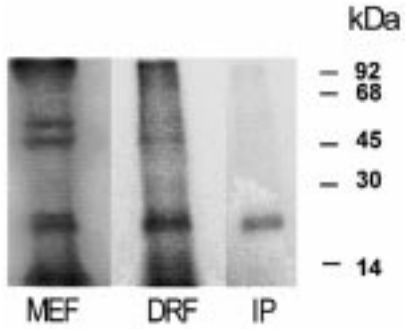

Figure 2. Detection of proteins crosslinked by the photoactivable radioactive GM1 ganglioside analogue TID-GM1. A431 cells were incubated with TID-GM1. After crosslinking induced by illumination with UV light, a membrane-enriched and a detergent-resistant fraction were prepared and electrophoresis carried out. An aliquot of the detergent-resistant fraction was submitted to immunoprecipitation using anti-caveolin antibody. Lane MEF = autoradiography of the electrophoresis of the membrane-enriched fraction. Lane DRF = autoradiography of the electrophoresis of the detergent-resistant fraction. Lane IP = autoradiography of the electrophoresis of the immunoprecipitate obtained from detergent-resistant fraction with anti-caveolin antibody.

The identity of the product was further confirmed on the base of its susceptibility to treatment with digitonin. In fact, the spot was not any more present in the supernatant analyzed by TLC after digitonin precipitation.

Accordingly, also the lipid extract of DRF was analyzed for the detection of cholesterol/TID-GM1 complex, by 2D-TLC and autoradiography. In this case, no radioactive band was present in the area corresponding to cholesterol/TID-GM1 complex (fig 4). As a further evidence of the lack of the cholesterol/TID-GM1 complex in DRF, after digitonin treatment of the DRF lipid extract, no change was observed in the 2D-TLC (Fig. 4).

Discussion

This investigation took advantage of the ability of GM1 ganglioside, either endogenous or exogenously added, to concentrate in caveolae [21], to gain information on the distribution and topology of lipids within these membrane structures. This information was inferred through the identification of lipids crosslinked by the photoactivable, radioactive GM1 ganglioside analogue, TID-GM1. A431 cultured cells were used, previously characterized with respect to the association with exogenous photoactivable gangliosides, enrichment of GM1 within caveolae and interaction of GM1 with caveolin [7,21–23]. Following the experimental protocol of previous investigations [7], incubation of cells in the presence of ganglioside and crosslinking experiments were performed at a low temperature in order to minimize the possibility of intracellular labeling [7,13,21]. The characteristics of association of TID-GM1 with cells were similar to those previously reported for other cell types and for isotopically tritium-labeled GM1 [13]. After incorporation into plasma membranes, crosslinking of TID-GM1 with neighboring molecules was induced by illumination of intact living cells and then MEF and DRF were prepared. A few radioactive proteins, including caveolin, were detected in MEF, mimicking previous results [7], while the radioactive protein within DRF was almost exclusively caveolin. DRF was also enriched in caveolin as protein, gangliosides, cholesterol and sphingomyelin, suggesting its enrichment in molecules of the caveolae membrane compartment. At the same time, these results suggest that TID-GM1 was localized in caveolae prior to the isolation of DRF, as indicated by its exclusive crosslinking with caveolin. It is worth to pointing out that isolation of DRF and MEF was performed only after crosslinking of TID-GM1 with neighboring molecules, in order to avoid artifacts deriving from remixing of molecules occurring during cell subfractionation. Therefore, the molecules crosslinked—and radioactive—should represent the actual environment of the ganglioside in caveolae of living cells.

Starting from these experimental evidences, we sought lipids crosslinked by TID-GM1 in the cell subfractions. A first series of analyses showed the presence in MEF of crosslinking

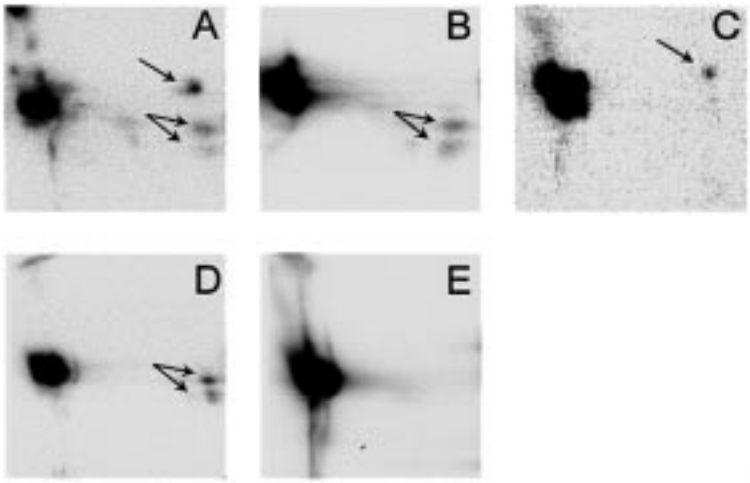

Figure 3. Detection of phospholipids crosslinked by the photoactivable radioactive GM1 ganglioside analogue, TID-GM1. A431 cells were incubated with TID-GM1. After crosslinking induced by illumination with UV light, a membrane-enriched fraction and a detergent-resistant fraction were prepared. Lipids were extracted from the fraction and submitted to 2D-TLC and autoradiography (see text for details) for detection of PC/TID-GM1 and SM/TID-GM1 complexes. Panel A, B, C = lipid extract of MEF; panel D, E = lipid extract of DRF. Panel B, E = lipid extracts after treatment with sphingomyelinase; panel C = lipid extract after treatment with phospholipase C.

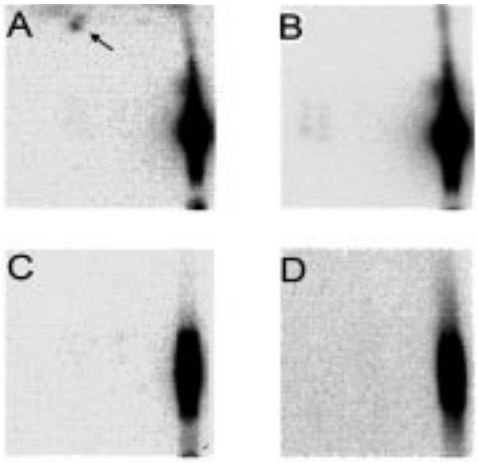

Figure 4. Detection of cholesterol crosslinked by the photo-activable radioactive GM1 ganglioside analog, TID-GM1, in the membrane-enriched fraction and in the detergent-resistant fraction. A431 cells were incubated with TID-GM1. After crosslinking induced by illumination with UV light, MEF and DRF were prepared. Lipids were extracted from the fractions and submitted to 2D-TLC and autoradiography (see text for details) for detection of cholesterol complexes. Panel A, B = lipid extract of MEF; panel C, D = lipid extract of DRF. Panel B, D = lipid extracts after treatment with digitonin.

products with PC and with SM, carrying comparable radioactivity. In DRF, on the contrary, only the product of crosslinking between TID-GM1 and SM was detectable. These results indicate that the ability of TID-GM1 to cross-react with SM is greatly enhanced when it interacts almost exclusively with caveolin, and suggest that the amount of sphingomyelin in the environment of the ganglioside is much higher in the caveolae domain than in the bulk membrane.

A second series of experiments was carried out in order to detect the product of crosslinking between cholesterol and TID-GM1. This product was present in MEF, while its presence was not detectable in caveolae-enriched DRF, at least under the experimental conditions adopted. This finding is somehow surprising since, as already reported [13, and present investigation], this fraction is enriched in cholesterol. Therefore, the most likely explanation of this result is that ganglioside and cholesterol cannot interact with each other since they are localized within two different zones of the caveolae bilayer. Since gangliosides are localized within the exoplasmic leaflet of the membrane [24], a likely hypothesis is that the sterol is more concentrated within the endoplasmic side of the caveolae bilayer.

The distibution of GM1 ganglioside and cholesterol across the caveolae bilayer could also contribute to explain the peculiar morphological features of caveolae. In fact, it is conceivable that an unbalanced distribution of molecules

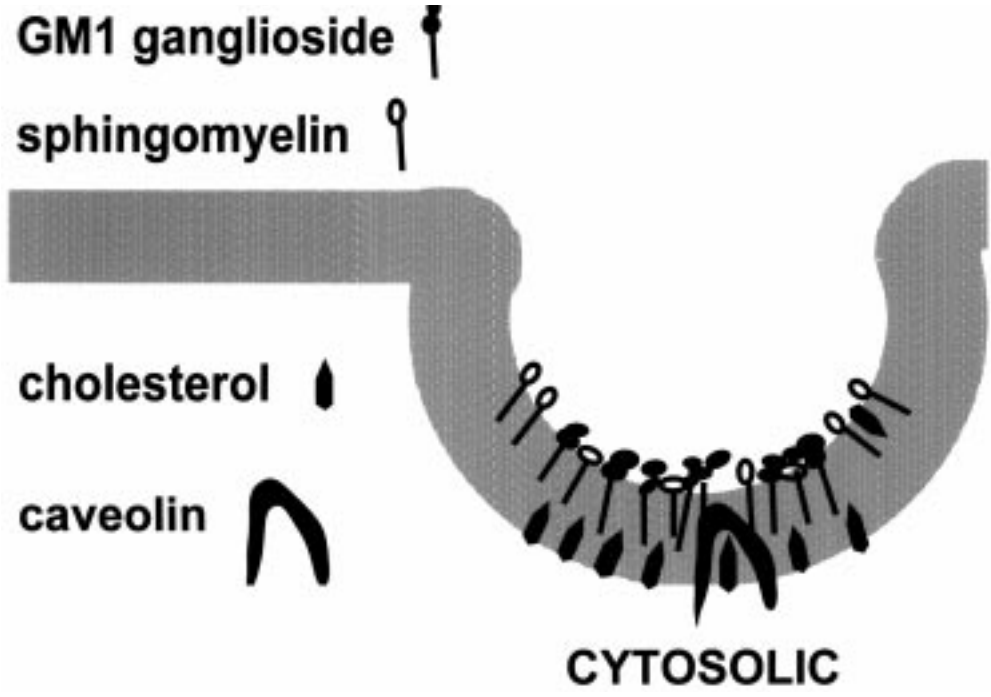

Figure 5. A possible model for the distribution of sphingolipids and cholesterol in caveolae, as inferred from the data obtained in the present investigation. Only lipids enriched within one of the caveolae bilayer leaflets, with respect to the rest of the plasma membrane, are shown.

within the two bilayer leaflets is necessary, in order to maintain the characteristic concave flask-shape of caveolae plasma membrane invaginations. This unbalance could be maintained by cholesterol and caveolin in the cytoplasmic leaflet.

The question could be asked, what is the driving force leading to the asymmetrical distribution of cholesterol in caveolae? Since it is known that cholesterol interacts with VIP21/caveolin [25], the most abundant protein of caveolae, a possible hypothesis is that the binding site for the sterol is located in a zone within the endoplasmic leaflet of the protein. The evaluation of such hypothesis deserves further investigation. A model for the distribution of sphingolipids and cholesterol in caveolae is presented in Fig. 5.

Acknowledgments

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