Dynamics of glycolipid domains in the plasma membrane of living cultured neurons, following protein kinase C activation: a study performed by excimer-formation imaging

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Dynamic changes of glycolipid domains within the plasma membranes of cultured rat cerebellar granule cells have been investigated. For this purpose, a pyrene-labelled derivative of G_{M1} ganglioside has been incorporated in the cell plasma membrane, and the rate of excimer formation, directly related to the formation of domains, has been studied by a fluorescence imaging technique (excimer-formation imaging). Fluorescence imaging showed that upon addition of 100 μ M glutamate, indirectly inducing the activation of protein kinase C (PKC), glycolipid concentration within domains increases in cell bodies. Comparable effects were exerted by the addition of PMA, directly

inducing the activation of PKC. On the contrary, the phorbol ester was not effective in the presence of the specific PKC inhibitor, bisindolylmaleimide. These results suggest that glycolipid-enriched domains are dynamic supramolecular structures affected by membrane-associated events, such as PKC activation. Dynamic changes of domains could be important in modulating their postulated participation in a series of functions, including signal transduction and lipid/protein sorting.

Key words: fluorescence imaging, G_{M1} ganglioside, glutamate, pyrene, signal transduction.

INTRODUCTION

A growing body of evidence suggests that modulation of fundamental membrane events, including signal transmission and lipid/protein sorting, is related to the capability of glycosphingolipids to segregate and to undergo domain formation [1–3]. In particular, domains isolated from a variety of cells expressing G_{M1} ganglioside are typically enriched in this glycolipid [4–9], which has been proposed as a marker of these structures. Whether or not the dynamics of domains are subjected to modulation by specific signals at the level of the plasma membrane is not completely understood. Some experimental clues suggest this possibility [10–12]. In particular, recent studies [13,14] suggest that activation of protein kinase C (PKC), a key enzyme of inbound transmembrane signalling, can affect the segregation state of glycolipids within the membrane. In order to investigate this problem, we decided to study the changes of ganglioside domains following a specific membrane event, namely the addition of phorbol ester (PMA) or glutamate, in cultured rat cerebellar granule cells. Ganglioside segregation was monitored utilizing a fluorescence microscopy technique previously set up in the same model system [15], evaluating the rate of excimer formation of a fluorescent analogue of G_{M1} ganglioside [4-(1-pyrene) butyryl-G_{M1} ganglioside, or pyrene-G_{M1}], carrying a pyrene-butyryl residue in place of the native fatty acid.

EXPERIMENTAL

Chemicals

Solvents and silica-gel HPTLC plates were from Merck (Darmstadt, Germany); 4-(1-pyrene)butyric acid succinimidyl ester and trimethylamino-diphenylhexatriene (TMA-DPH) were from

Molecular Probes (Eugene, OR, U.S.A.). Basal modified Eagle's medium (BME) and fetal calf serum (FCS; heat-inactivated before use) were from Irvine (Santa Ana, CA, U.S.A.). PMA, bisindolylmaleimide (BIM), poly-L-lysine, 1- α -D-arabinofuranosylcytosine, NeuAc, gentamycin, dimyristoyl phosphatidylcholine (DMPC) and crystalline BSA were from Sigma (St. Louis, MO, U.S.A.).

Synthesis and characterization of pyrene-G_{M1}

G_{M1} ganglioside carrying a pyrene-butyryl residue in place of the native fatty acid moiety was synthesized following a procedure already described, with minor modifications [15,16]. Briefly, 6 μ mol of lyso-G_{M1} were mixed in tetrahydrofuran with 20 μ mol of pyrene-butyryl succinimidyl ester. Water (20 μ l) and 5 μ l of trimethylamine were added and the mixture was maintained overnight under continuous stirring at 40 °C. The products were column-chromatographed on silica gel-100 and eluted with chloroform/methanol/water (60:35:5, by vol.). The purity and integrity of fluorescent ganglioside were checked as described in [16]. The concentration of pyrene-G_{M1} was estimated by sialic acid assay [17] or by spectrophotometry [15].

Fluorescence spectroscopy of pyrene-G_{M1} dispersions

Different amounts of pyrene-G_{M1} were dried from a stock solution in chloroform/methanol (2:1, v/v) and the residue dissolved in a proper amount of BME (without FCS) to give final ganglioside concentrations ranging between 10⁻⁷ and 10⁻⁴ M. In order to assess the critical micellar concentration of pyrene-G_{M1}, a procedure described previously was utilized [18].

Abbreviations used: BIM, bisindolylmaleimide; PKC, protein kinase C; pyrene-G_{M1}, 4-(1-pyrene)butyryl-G_{M1} ganglioside; TMA-DPH, trimethylamino-diphenylhexatriene; FCS, fetal calf serum; DMPC, dimyristoyl phosphatidylcholine; BME, basal modified Eagle's medium.

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Cell cultures

Cerebellar granule cells, obtained from 8-day-old Sprague–Dawley rats (Charles River, Milano, Italy), were prepared and cultured according to [19]. Glial proliferation was prevented by adding cytosine arabinofuranoside (final concentration, $10\ \mu\text{M}$) 18–20 h after plating. Primary cultures were grown for 8 days in 35-mm plastic culture dishes, containing 30-mm-diameter glass coverslips for fluorescence microscopy, coated with poly-L-lysine.

Binding to cells of fluorescent ganglioside

For binding studies, the procedure already described was followed [12,15]. Briefly, cells were incubated in the culture dishes for different times with pyrene- G_{M1} in 1 ml of BME. At a given time cells were harvested, collected as a pellet after centrifugation (800 g for 10 min), submitted to lipid extraction, and pyrene- G_{M1} assayed. The occurrence of metabolic processing was assessed by submitting the ganglioside extract to TLC separation. Fluorescent spots were detected by fluorescence scanning of the plate [15]. In some experiments, after incubation with pyrene- G_{M1} , cells were washed with BME and treated with trypsin [15] for 5 min at $37\ ^\circ\text{C}$. At the end of the treatment, the trypsin solution was removed and pyrene- G_{M1} assayed, as described above.

Fluorescence imaging

The excimer-imaging technique was followed essentially as described previously [15]. Microscopic fluorescence images were recorded by an Argus 100VIM photon-counting processor (Hamamatsu, Herrsching, Germany) equipped with a Hamamatsu C-2400-09 ISIT camera coupled to a Leitz (Wetzlar, Germany) Orthoplan fluorescence microscope operating under epi-illumination conditions. The video camera was operated at medium gain and in the linear portion of the sensitivity range. The fluorescence input video images of pyrene- G_{M1} -loaded cells were digitalized and integrated for 64 frames at 1-frame interval using an Hamamatsu imaging integration function. The time required for image collection did not exceed 2 s. All measurements were performed with a Leitz NPL Fluotar $100\times$ diaphragmed objective. Taking into account both the optical resolution of the microscope at the wavelength considered and the characteristics of the video camera, a spatial resolution of the image of about $0.2\ \mu\text{m}$ was obtained with our experimental apparatus.

Treatment of cells with pyrene- G_{M1} for imaging experiments

The glass coverslips contained in the culture dishes were removed and used as the upper wall of a water-tight chamber equipped with a flow system for additions and washings [15]. Pyrene- G_{M1} was dissolved in an appropriate amount of BME (without FCS) in order to obtain a $10^{-6}\ \text{M}$ solution, and injected into the chamber. After a given incubation time at $10\ ^\circ\text{C}$ the chamber was submerged in water at $37\ ^\circ\text{C}$ for 30 s and then immediately transferred on the heated stage of the microscope at $37\ ^\circ\text{C}$. The first image was taken 2 min after the end of incubation at $10\ ^\circ\text{C}$ (time 0).

Pyrene- G_{M1} excimer imaging

The original procedure is described in [15]. The technique relies on the fact that the emission wavelength of molecules interacting with each other (excimers, E) is different from that of non-

interacting ones (monomers, M) [18,20,21]. The visualization of the E/M ratio, whose value increases on increasing the extent of segregation, allows monitoring of the presence and dynamics of domains. Briefly, two sample images were acquired under excitation at 366 nm in the spectral ranges 400–450 nm, by means of a N.36 Kodak Wratten filter, and 470–490 nm, by means of a 480 interference filter, corresponding to monomer and excimer emission, respectively. The imaging of the E/M intensity ratio, directly related to the segregation state of the probe, was obtained by normalizing the intensity of each pixel element of the excimer fluorescence image to the intensity of the corresponding pixel element of the monomer fluorescence image. Before normalizing, the background contribution was subtracted from the intensity of the pixel elements of each image. The background was evaluated on samples before pyrene- G_{M1} loading and turned out to be almost homogeneously distributed all over the samples, the cell autofluorescence being negligible with respect to the fluorescence of the poly-L-lysine coating. The background contribution in pyrene- G_{M1} -treated samples was therefore calculated as the mean signal recorded on a wide area surrounding the cells under examination.

TMA-DPH fluorescence anisotropy imaging

For anisotropy imaging of TMA-DPH, the technique described previously [15] was followed. TMA-DPH ($10^{-6}\ \text{M}$ final concentration) in BME without FCS was added to the chamber containing the cells [15,22], maintained at $37\ ^\circ\text{C}$ on the microscope stage. Fluorescence was recorded through a TK400 dichroic mirror combined with a 430 nm barrier filter and a rotatable polarizer (analyser) inserted in the Leitz FSA 50/30R polarizer tube. Fluorescence images pairs of cells were obtained by aligning the analyser first in parallel ($\text{F.I.}_{\parallel}$) and then perpendicular ($\text{F.I.}_{\perp}$) to the polarization plane of the excitation beam. After subtracting background, the anisotropy imaging was performed by processing each corresponding pixel element of both parallel and perpendicular fluorescence images according to the expression [15,22,23]:

$$\text{Fluorescence anisotropy} = (\text{F.I.}_{\parallel} - \text{F.I.}_{\perp}) / (\text{F.I.}_{\parallel} + \text{F.I.}_{\perp})$$

The polarization-dependent light-transmission properties of the microscope emission optics as well as the depolarization of the excitation beam by the objective were tested. No corrections were made because the undesirable effects were negligible ($< 10\%$).

The actual fluorescence-intensity measurements (parallel and perpendicular to the excitation polarization direction) were carried out within 2 min of the addition of the probe (time 0).

Endocytosis

The amount of fluorescent ganglioside present at the cell surface under different experimental conditions was measured by extraction ('back-exchange') on to BSA in the medium [24]. For this purpose, the medium was replaced by BSA (1% in Locke's solution), three times at $10\ ^\circ\text{C}$, for 20 min each time. After each incubation the BSA solution was recovered and the pyrene- G_{M1} content assessed.

Effect of glutamate and PMA

Cells were treated with pyrene- G_{M1} or with TMA-DPH, as described above, and a first image obtained (time 0). The medium in the chamber was replaced with Locke's solution containing

glutamate (1 or 100 μM , without Mg^{2+}) or, in different experiments, containing 1 μM PMA, and further images were obtained after 7 min at 37 °C. In additional experiments, BIM (2 μM) was also added to the solution containing PMA [25].

Viability experiments

The viability of cells enriched or not with fluorescent glycolipids was evaluated as described in [26].

Electron microscopy

Cells were fixed in glutaraldehyde (4% in PBS for 2 h at 4 °C) and post-fixed in 1% osmium tetroxide in Veronal acetate buffer, pH 7.4, for 2 h at 4 °C. All samples were stained with uranyl acetate (5 mg/ml), dehydrated in acetone, and embedded in Epon 812. Ultrathin sections were observed with a Philips CM100 electron microscope.

Fluorescence spectroscopy of pyrene- G_{M1} /DMPC dispersions

The E/M values of pyrene- G_{M1} in DMPC unilamellar vesicles were measured at different temperatures and at different ganglioside proportions. For this purpose, vesicles were prepared as described in [13], by 10 successive extrusions of dispersions of the lipid mixtures in BME (without FCS) through 100 nm pore-size filters (Nucleopore, Milan, Italy), using an N_2 -operated extruder (Lipex Biomembranes, Vancouver, BC, Canada) at 30 °C. The excitation and emission spectra of each dispersion were recorded with a spectrofluorometer (Jasco, Tokyo, Japan).

In addition, fluorescence anisotropy of TMA-DPH in DMPC was measured at different temperatures. For this purpose, TMA-DPH (200:1, molar ratio) was added to DMPC dispersions prepared as described above (in the absence of pyrene- G_{M1}). The fluorescence emission at 430 nm (excitation wavelength 360 nm), parallel and perpendicular to the polarization plane of the excitation beam was measured at different temperatures and fluorescence anisotropy calculated using the formula described above.

RESULTS

Pyrene- G_{M1} dispersions at different concentrations, ranging from 10^{-7} to 10^{-4} M, were prepared and the fluorescence emission spectra recorded. The E/M intensity ratio (Figure 1) shows a steep increase from 10^{-5} M G_{M1} , indicating strong pyrene-pyrene interactions and suggesting the presence of ganglioside micelles over this concentration [18].

In order to avoid the binding of whole micelles to the cell surface, the experiments were carried out at a pyrene- G_{M1} concentration of 10^{-6} M, corresponding to a monomeric or oligomeric dispersion of the glycolipid. The time course of 10^{-6} M pyrene- G_{M1} binding to cells was followed at 10 °C and the results are reported in Figure 2. Concomitantly to its association with cells, a strong increase of the fluorescence emission intensity was observed. The amount of pyrene- G_{M1} associated after 6 min of incubation was 0.5 nmol/mg of protein. The amount of fluorescent ganglioside released from the cells by trypsin treatment was almost negligible (< 1%). The endogenous ganglioside content of cells was 10.9 nmol/mg of protein [15].

Imaging experiments

After incubation for 6 min at 10 °C with the ganglioside solution (10^{-6} M), the chamber containing the cells was brought to 37 °C.

Figure 1 E/M ratio of pyrene- G_{M1} as a function of the ganglioside concentration

Excimer fluorescence emission intensity was measured at 480 nm and monomer intensity at 398 nm using an excitation wavelength of 343 nm. The data are from one of two experiments performed, giving almost overlapping results.

Figure 2 Time course of the association of pyrene- G_{M1} with rat cerebellar granule cells

Cells were incubated at 10 °C with 10^{-6} M pyrene- G_{M1} and the amount of fluorescent ganglioside associated was assessed by assaying the lipid extract. The data are one of two experiments performed, giving almost overlapping results. ○, Pyrene- G_{M1} bound; ■, fluorescence emission intensity at 398 nm.

After 2 min, images at the wavelengths corresponding to the excimer and monomer, respectively, were taken and an E/M ratio image was produced (image at time 0). The average surface density of pyrene- G_{M1} (as judged by the fluorescence intensity/ μm^2) was higher in cell bodies than in neurites, confirming previous results [15]. In fact, the intensity in cell bodies was 1.5–2.1-fold higher than in neurites when measured at the monomer-emission wavelength, and was 1.1–1.9-fold higher in cell bodies than in neurites when measured at the excimer wavelength. The E/M ratio, directly related to the ganglioside concentration, was scattered in patches of different size, value

and shape, reaching some local maxima. In particular, the means $\pm$ S.D. recorded at the level of neuritic processes were higher than those observed in cell bodies (Table 1), confirming previous evidence [15]. One of the images obtained is shown in Figure 3(a).

Treatment with PMA or glutamate

Successive experiments were carried out in order to evaluate the effect of PMA addition on the ganglioside segregation state. For this purpose, immediately after the acquisition of the images at time 0, the phorbol ester was added and a second image of the same field taken after 7 min. The treatment with PMA caused the mean E/M to increase in cell bodies, but not in neuritic processes, while the E/M ratio was always scattered in patches of different shape, value and size (Table 1, Figure 3). In order to observe more details, three-dimensional images of the E/M distribution in one of the cell bodies are also shown in Figure 3. In a further experiment, cells were treated with PMA in the presence of the specific PKC inhibitor, BIM. After this treatment the E/M ratio was always distributed in patches, but the mean $\pm$ S.D. values in cell bodies and neurites did not change (Table 1).

Table 1 Excimer/monomer emission intensity ratio of pyrene-G_{M1} within the plasma membranes of cultured rat cerebellar granule cells

Cells were incubated with pyrene-G_{M1} (control) and then submitted to various treatments. The values are the means $\pm$ S.D. (with *n* shown in parentheses). Statistical significance (Student's *t* test): † versus *, *P* < 0.01; ‡ and § versus †, *P* < 0.02.

Treatment	E/M ratio	
	Neuritic processes	Cell bodies
Control cells	0.142 $\pm$ 0.03 (19)*	0.11 $\pm$ 0.02 (15)†
7 min at 37 °C	0.141 $\pm$ 0.03 (19)	0.11 $\pm$ 0.02 (15)
+1 μ M PMA	0.141 $\pm$ 0.02 (14)	0.129 $\pm$ 0.015 (10)‡
+1 μ M PMA + 2 μ M BIM	0.139 $\pm$ 0.02 (8)	0.10 $\pm$ 0.01 (8)
+100 μ M Glutamate	0.143 $\pm$ 0.03 (13)	0.13 $\pm$ 0.013 (10)§
+1 μ M Glutamate	0.139 $\pm$ 0.03 (10)	0.11 $\pm$ 0.02 (13)

Another series of experiments was carried out to assess the effects exerted by glutamate addition. Also in this case, immediately after the acquisition of the images at time 0, glutamate (100 or 1 μ M) was added and a second image of the same field

Figure 3 Fluorescence E/M ratio imaging microscopy of pyrene-G_{M1} within the plasma membrane of rat cerebellar granule cells in culture: effect of PMA

Cells were incubated with pyrene-G_{M1}, images at the excimer and monomer emission wavelengths taken and the E/M ratio displayed as a pseudocolour image. Images of the same field were taken before (a, a') and after (b, b') the addition of 1 μ M PMA, respectively. (a, b) Two-dimensional imaging microscopy. (a', b') Three-dimensional imaging of one of the cell bodies displayed in (a) and (b), respectively. Scale bars, 10 μ m. The scales relate the calculated E/M ratio values to the colour displayed. Due to constraints of the software utilized, the scale for (a') and (b') is different from that of panels (a) and (b).

Figure 4 Fluorescence E/M ratio imaging microscopy of pyrene- G_{M1} within the plasma membrane of rat cerebellar granule cells in culture: effect of glutamate

Cells were treated with pyrene- G_{M1} , images at the excimer and monomer emission wavelengths taken, and the E/M ratio displayed as a pseudocolour image. Images of the same field were taken before (a) and after (b) addition of 100 μ M glutamate. Scale bar, 10 μ m. The scale relates the calculated E/M ratio values to the colour displayed.

taken after 7 min. After addition of 100 μ M glutamate the mean E/M values increased in cell bodies, while remaining constant in neuritic processes, mimicking the results obtained with PMA (Table 1, Figure 4). On the contrary, after addition of 1 μ M glutamate the E/M ratio was always distributed in patches, the patterns of which changed with time, but the mean $\pm$ S.D. values of cell bodies and neurites remained constant (Table 1).

As a control, the E/M ratio was also followed in untreated cells. For this purpose, a first image was produced at the end of the incubation with pyrene- G_{M1} and a second one after 7 min at 37 °C. The comparison between the two images showed that the E/M ratio was always distributed in patches, with patterns that changed over time, while the mean E/M values in cell bodies and neuritic processes did not vary (Table 1).

Endocytosis experiments

A series of experiments was carried out in order to assess the extent of endocytosis of the probe during the imaging experiments. For this purpose, the back-exchange of pyrene- G_{M1} on to BSA, and the metabolism of pyrene- G_{M1} , were evaluated under the experimental conditions utilized for imaging experiments. At the end of cell incubation with pyrene- G_{M1} at 10 °C, 98.7 % of fluorescent ganglioside could be re-extracted by back-exchange. After additional 7 min at 37 °C, 96.6 % of pyrene- G_{M1} was re-extractable. After these treatments no metabolite of pyrene- G_{M1} was detected in the TLC of the ganglioside extract of the cells, indicating no lysosomal degradation [27]; moreover, cell treatment with glutamate or PMA did not affect the above values. After 14 min at 37 °C, 94 % pyrene- G_{M1} was re-extractable by back-exchange; the TLC of the ganglioside extract showed the presence of a new fluorescent band (about 2 % of the total fluorescence), migrating above pyrene- G_{M1} and likely corresponding to a pyrene derivative of G_{M2} (results not shown).

Fluidity measurements

In order to exclude the possibility that changes of membrane fluidity could affect the E/M differences remarked upon above, the steady-state fluorescence anisotropy of TMA-DPH inserted into the plasma membrane was measured before and after addition of PMA or glutamate. Images of the parallel and perpendicular polarized emission intensities were taken and images of the fluorescence anisotropy were created. The mean $\pm$ S.D. values of the fluorescence anisotropy, inversely related to fluidity [15,22,23], were significantly lower in cell bodies (0.238 ± 0.06 , $n = 11$; $P < 0.01$), than in neuritic processes (0.331 ± 0.1 , $n = 15$), confirming previous results [15], and for this reason the corresponding images are not reported. The above-reported values did not vary after 7 min and were not affected by addition of PMA or glutamate (results not shown).

Microscopy

The cell viability, evaluated after the addition of glutamate, PMA or pyrene- G_{M1} , was 99 %, similar to that of control, untreated cells. The morphology of the cells, observed in transmitted light, did not change under the experimental conditions utilized for imaging experiments.

Electron microscopy observations showed that cells treated for 6 min at 10 °C with pyrene- G_{M1} appeared aggregated and rounded with scarce cytoplasm, similarly to untreated cells. No apparent differences at the level of the plasma membrane were seen on comparing control cells, cells treated with pyrene- G_{M1} and cells treated with pyrene- G_{M1} and PMA (results not shown).

Fluorescence spectroscopy of pyrene- G_{M1} /DMPC dispersions

The temperature dependence of the E/M ratio of pyrene- G_{M1} (10 % molar) in DMPC is reported in Figure 5(A). The plot exhibits no apparent discontinuity in the region of the phospholipid phase transition, about 23 °C [21], indicating a nearly ideal mixing of the two components [21] (no segregation or preference for the fluid or gel state).

In Figure 5(B), the E/M ratio of pyrene- G_{M1} in DMPC is reported as a function of ganglioside concentration. In particular, from the plot it can be estimated that an E/M value of 0.21 (the maximum value detected in cells) corresponds to a pyrene- G_{M1}

Figure 5 E/M fluorescence intensity ratio of pyrene-G_{M1}

E/M fluorescence intensity ratio of pyrene-G_{M1} (10% molar) in small unilamellar DMPC vesicles as a function of: (A) temperature; (B) pyrene-G_{M1} concentration in small unilamellar DMPC vesicles, at 30 °C; and (C) TMA-DPH anisotropy (inversely related to membrane fluidity) in small unilamellar DMPC vesicles. To obtain this plot, first, E/M values of 10% molar pyrene-G_{M1} in DMPC were measured at different temperatures. Successively, the fluorescence anisotropy of TMA-DPH in DMPC was measured at the same temperatures. Finally, the E/M values at a given temperature were plotted against the anisotropy values at the same temperature.

concentration of 34%. Moreover, it can be estimated that an E/M shift from 0.11 to 0.13 (average E/M values in cell bodies before and after glutamate treatment; see Table 1) corresponds to a change of pyrene-G_{M1} concentration from 9.8 to 13.8%.

Finally, the dependence of the E/M ratio of pyrene-G_{M1} on the membrane fluidity was investigated. For this purpose, the E/M ratio of fluorescent ganglioside (10% molar) in DMPC is reported in Figure 5(C), as a function of TMA-DPH fluorescence anisotropy, whose value is inversely related to membrane fluidity. The plot shows biphasic behaviour, with a break in correspondence with the gel-to-liquid-crystalline temperature transition of the phospholipid. In particular, anisotropy values of 0.331 and 0.238 (detected in cell bodies and neuritic processes, respectively), correspond to E/M values of 0.075 and 0.115, respectively.

DISCUSSION

The most interesting result obtained in this investigation was the indication that glycolipid-enriched domains are dynamic structures that can be modulated by signals occurring at the level of the plasma membrane. The importance of glycolipid-domain modulation lies in its potentiality to affect membrane-associated events such as signal transduction, enzymic recognition, interaction with toxins, conformation, protein sorting and biogenesis of caveolae and caveolae-like domains [6,28–32]. In order to investigate this issue, the excimer-formation imaging technique, previously set up with cerebellar granule cells [15], has been

utilized in the same model system, coupled to the fluorescent analogue of G_{M1} ganglioside, pyrene-G_{M1}. Fluorescence spectroscopy experiments indicate that at the concentrations utilized here for incubation with cells, pyrene-G_{M1} is present in monomeric form. This feature increases the probability that ganglioside molecules insert correctly into the membrane, i.e. with their ceramide moiety embedded into the lipid core of the bilayer and with the saccharide moiety exposed towards the extracellular medium. As a first proof of the correct insertion, upon incubation, fluorescence was strongly enhanced, a behaviour typical of the pyrene probe in a hydrophobic environment [20,21,23]. In order to further substantiate the correctness of this assumption, cells were submitted to trypsin treatment after incubation with pyrene-G_{M1}. According to the known features of association of exogenously administered gangliosides with cells [33–35], the resistance of the fluorescent glycolipid to such treatment suggests its correct insertion in the plasma membrane. Moreover, under the conditions utilized for imaging experiments, endocytosis of the probe was extremely limited and no lysosomal degradation occurred [27]. All these observations and the presence of a bulk, negatively charged, hydrophilic portion, preventing the possibility of transmembrane movement, imply that the fluorescent ganglioside is located in the plasma-membrane outer layer, together with its endogenous counterpart [33–35].

Images of the E/M distribution showed that pyrene-G_{M1} is scattered in patches of differing sizes and shapes, those present in neurites displaying higher average values. Since the E/M ratio is directly related to the probe concentration, these results indicate

that the average pyrene- G_{M1} concentration is higher in the patches of neuritic processes than in those of cell bodies, definitively confirming previous data [15].

Since the excimer formation is both a concentration- and diffusion-controlled event [20], E/M differences can arise also from differences in membrane fluidity, whose increase enhances the diffusion of the probe. In the present case, the fluidity in cell bodies is higher than in neurites. Therefore, the outcome of the fluidity component on E/M is more substantial in cell bodies than in neurites. Experiments carried out with artificial membranes suggest that the E/M values in cell bodies should be decreased by about 35% in order to compensate for the fluidity difference. This correction, to be taken with cautiousness, causes a further amplification of the difference in ganglioside concentration between cell bodies and neurites, as already reported [15].

The glycolipid concentration within the patches is maximal in a central nucleus, which most probably is the actual domain, and steeply decreases all around. This feature suggests that a small number of molecules, possibly through more specific interaction, could behave as a nucleation point around which other molecules gather through less specific interaction. This hypothesis is supported by the evidence that gangliosides interact with receptors [30], proteins of caveolae [6] and glycoproteins [36], and are able to self-associate [3], particularly within sphingomyelin environments [37].

The distribution of ganglioside herein detected suggests that membrane patches of different sizes, even bigger than the actual domain, could be cut out from the membrane, depending on the isolation technique employed [2,5,8,9] and possibly following different contours of membrane fluidity, since this latter feature depends on the ganglioside concentration [1]. Moreover, it is interesting to observe that detergent-insoluble complexes isolated from brain and cerebellum revealed the presence of a heterogeneous population of vesicles ranging from 0.05 to 1.5 μm in diameter [38,39].

Further experiments showed that domains are subject to spontaneous time evolution. It is likely that lateral diffusion of ganglioside, along with other molecules, is responsible for the changes, by continuously varying the composition of a given membrane zone. On the other hand, more specific ganglioside-lipid or ganglioside-protein interactions, discussed above, can counteract this trend, rendering domains more stable [40].

Successively, we evaluated the effects exerted by PMA or glutamate on pyrene- G_{M1} domains. Experimental conditions were adopted under which the bulk of pyrene- G_{M1} was still at the plasma-membrane level, as herein inferred, and under which the effects of PMA and glutamate could be exerted on cerebellar granule cells [13,41]. The treatment with PMA, a drug able to induce PKC activation in many cell types, cerebellar granule cells included [13,41,42], induces an increase of glycolipid segregation in cell bodies, suggesting that activation of the kinase can modulate ganglioside domains. In order to substantiate the involvement of PKC in the observed phenomenon, we carried out experiments with PMA in the presence of BIM and experiments with glutamate.

The first series of experiments showed that in the presence of BIM, a specific inhibitor of PKC [43], PMA was no longer able to exert the effect detected previously. The second series of experiments was performed with glutamate. Glutamate, added to cerebellar granule cells in culture at high concentration (100 μM), binds to plasma-membrane ionotropic receptors, opening Ca^{2+} channels. Extracellular Ca^{2+} influx induces PKC activation. Moreover, glutamate stimulates metabotropic receptors, starting the phosphoinositide cycle and also contributing

to PKC activation [41,42]. The results with 100 μM glutamate were comparable with those obtained with PMA; on the contrary, the addition of 1 μM glutamate, unable to activate PKC [13], did not exert any effect, indicating that PKC activation is a step required for the increase of glycolipid segregation. Both PMA and glutamate exert their effects only in cell bodies and not in neuritic processes: this observation argues in favour of a high specificity of this response and excludes the possibility that the change is due to the spontaneous time evolution of glycolipid domains. It is also possible that the present technique fails to detect an increase of E/M in processes, given the state of glycolipid clustering already existing. However, high E/M values are present in neurites, indicating that the average values can be exceeded, and suggesting that this cell compartment is not affected by PKC activation.

Finally, fluorescence anisotropy and electron-microscopy experiments showed that the average fluidity and the overall morphological features of the plasma membrane were not affected under the experimental conditions, also ruling out such interference in the imaging experiments.

Taken all together, these data suggest that PKC activation affects the dynamics of ganglioside domains within the plasma membrane of cerebellar granule cells. Thus, glycolipid segregation can be subjected to a modulation dependent on specific events occurring at the membrane level. This interpretation fits also with the results observed in the same model system, showing that PKC activation changes either ganglioside exposure at the plasma-membrane surface [13] or the amount of ganglioside associated with the tyrosine kinase receptor TrkB [14], both probably due to a change of glycolipid membrane distribution. The average G_{M1} concentration in patches, estimated by comparison with data obtained on artificial membranes, increases by about 40% after PKC activation. It is worth saying that this value must be taken with some cautiousness, since it was obtained with a model system, whereas *in vivo* the segregative properties of the ganglioside could be different. Moreover, *in vivo*, the pyrenyl group could affect the segregative properties of G_{M1} , even though in artificial membranes it does not, as assessed in the present investigation and in [21].

What is the physiological significance of the change in G_{M1} concentration induced by PKC activation? Dynamic changes in domains could be important for modulating their participation in a series of membrane-associated events, primarily ganglioside-protein interactions. In fact, it is known that ganglioside segregation affects their recognition by enzymes, conformation of integral membrane proteins, the mechanisms of lipid and protein sorting and regulation of receptors [13,14,30–32,36]. In particular, concerning the latter issue, a change in G_{M1} concentration of 33% in the environment of TrkB leads to a dramatic modification of the receptor activity [14].

It is puzzling that an event occurring at the cytoplasmic side of the membrane (PKC activation) can induce a change of ganglioside local concentration within the outer leaflet of the bilayer. Anyway, it is worth noting that: (i) G_{M1} interacts with integral membrane proteins (even those facing the cytosolic side, by its hydrophobic tail) undergoing segregation [6,30,36]; and (ii) PKC activation induces redistribution of the reaction components (lipids and proteins, PKC included) in mixed membrane domains [44]. Our hypothesis is that the phosphorylation of integral membrane proteins within a specialized domain can modulate ganglioside segregation at the exoplasmic membrane face. This hypothesis includes also the possibility of a PKC-dependent cytoskeletal modification that could induce a molecular reorganization of plasma-membrane-associated molecules. Such speculation is prompted by the observation that proteins of

the MARCKS (myristoylated alanine-rich C-kinase substrate) family, associated with membranes and the actin cytoskeleton, are well-known substrates for PKC [45]. This possibility would imply the involvement of ganglioside domains in a sort of outward-directed mechanism of signalling across the plasma membrane. Of course, the evaluation of all these hypotheses deserves and requires further investigative work.

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