Change of Ganglioside Accessibility at the Plasma Membrane Surface of Cultured Neurons, Following Protein Kinase C Activation†

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ABSTRACT: While the mechanism of signal transduction across the plasma membrane from the exo- to the endoplasmic side has been extensively investigated, the possible return of messages back to the outer layer is less known. We studied the effect of protein kinase C activation on the ganglioside accessibility at the exoplasmic face of intact rat cerebellar granule cells in culture, using the enzyme sialidase as the probing molecule. Under the experimental conditions (1 milliunit/mL enzyme, 2 min incubation at 37 °C), only GT1b and GD1a gangliosides were partially affected by the enzyme (28.6 and 25.7% hydrolysis, respectively). After cell treatment with phorbol 12-myristate 13-acetate, inducing protein kinase C activation, GT1b and GD1a ganglioside susceptibility to sialidase was strongly decreased (8.6 and 15.9% hydrolysis, respectively). A reduction of ganglioside hydrolysis was also observed when protein kinase C activation was induced by cell treatment for 15 min with 100 μM glutamate. On the contrary, accessibility did not vary when protein kinase C translocation was not effective (either in the absence of Ca^{2+} in the medium or using 1 μM glutamate) or when the kinase activity was inhibited by staurosporine. These data suggest that following PKC activation, a key step of inbound transmembrane signaling, cell may dispatch outbound messages to the plasma membrane outer layer, changing the selective recognition and crypticity of glycolipids at the cell surface, possibly through a modulation of their segregation state.

Signals arriving to the cell surface from the extracellular environment are transduced through a variety of mechanisms inducing molecular and functional changes at the cytosolic side of the plasma membrane bilayer (*1*−*3*). Whether or not these modifications are also followed by signals returning back to the external membrane layer, changing its organization and function, is unknown. To test this possibility, within the frame of a signal transduction process, we induced a stimulus at the cytosolic side of the plasma membrane of a proper cell and monitored the occurrence of changes at the exoplasmic side. The study model was constituted by intact cerebellar granule cells in culture, obtained from 8-day-old rats, in which the consequences of protein kinase C activation—typical event, at the cytosolic side, of a transmembrane signaling process—on the accessibility of gangliosides were investigated, using the membrane-impermeant enzyme *Vibrio cholerae* sialidase (VCS)[1] as the probe. Gangliosides were chosen because they are abundant components of the neurons, are located on the outer layer of the plasma membrane, and expose their oligosaccharide moiety toward the external medium, supplying the cell with a variety of recognition sites. In fact, they have been reported to behave as receptors for toxins, antibodies, viruses, growth factors, and various other ligands, implying their involvement in a series of functions ranging from cell adhesion to autoimmune-related events (*4*−*9*). In particular, a growing body of evidence suggests that modulation of fundamental membrane events, such as signal transmission and lipid/protein sorting, is related to the capability of glycolipids to undergo domain formation (*10*).

MATERIALS AND METHODS

Chemicals. The reagents used (analytical grade), HPTLC plates, and analytical HPLC columns were purchased from Merck GmbH (Darmstadt, Germany). Phorbol 12-myristate 13-acetate (PMA), 4-methylumbelliferone (MUB), 4-methylumbelliferyl-D-*N*-acetylneuraminic acid (MUB-NeuAc), staurosporine, dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (EPC), and glutamate were from Sigma Chemical Co. (St. Louis, MO). (Trimethylamino)diphenylhexatriene (TMA-DPH) was from Molecular Probes (Eugene, OR). Modified Eagle's basal medium (BME) and fetal calf serum (FCS) were purchased from Flow Laboratories (Irvine, U.K.). VCS (1 unit defined as the amount releasing 1 μmol/min of *N*-acetylneuraminic acid from human acid 1-glycoprotein at 37 °C), 40 μg as protein/mL, was from Calbiochem-Behring (La Jolla, CA). [^{3}H]Phorbol-12,13-dibutyrate ([^{3}H]PdBu, specific activity 20 Ci/mmol) was from DuPont NEN (Germany). GD1a ganglioside was extracted and purified from calf brain (*11*). Preparation and purification of native GD1a, tritium labeled at the 3-position of the long chain base, was accomplished as described (*12, 13*).

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[1] Abbreviations: PKC, protein kinase C; PMA, phorbol 12-tetradecanoate 13-acetate; MUB-NeuAc, 4-methylumbelliferyl-D-*N*-acetylneuraminic acid; DPPC, dipalmitoylphosphatidylcholine; EPC, egg phosphatidylcholine; TMA-DPH, (trimethylamino)diphenylhexatriene; BME, modified Eagle's basal medium; FCS, fetal calf serum; VCS, *Vibrio cholerae* sialidase; [^{3}H]PdBu, [^{3}H]phorbol-12,13-dibutyrate.

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Cell Cultures. Granule cells, obtained from the cerebella of 8-day-old Sprague−Dawley rats (Charles River, Milano, Italy), were prepared and cultured as described (*14, 15*). The cell culture contained about 90% granule cells, 5% γ-aminobutyric acid−ergic neurons, and 5% glial cells. The replication of nonneuronal cells was prevented by addition of cytosine-β-D-arabinofuranoside (2.5 μg/mL) to the culture medium. Morphological differentiation of granule cells in culture was followed by microscopic examination. The toxic effect of glutamate on culture cells was monitored by assessing cell viability with fluorescein diacetate and propidium iodide as described (*16*). The experiments were performed with cells cultured for 8 days in vitro (DIV), unless otherwise stated.

Ganglioside Extraction and Fractionation. For each experiment, total gangliosides were extracted and purified from a pool of cells prepared from five 60-mm diameter dishes (*17*). The total ganglioside extract was fractionated into the individual components by analytical HPLC using a photodiode-array detector (*18*). Peak identification was accomplished by the use of authentic standard gangliosides and by the analysis of their UV spectra (*19).* Ganglioside quantification was accomplished by peak integration, as described (*18*). As for a control, in some instances the ganglioside pattern was assessed by 2D-HPTLC followed by densitometric scanning of the plate, as described (*20*).

Assessment of Ganglioside Susceptibility to VCS. Cell monolayers were washed three times with Locke's solution (*14*) and, after addition of different amounts of VCS (from 0.75 to 2.5 milliunits in 1 mL of the same solution), previously warmed at 37 °C, incubated for exactly 2 min at the same temperature, under very gentle shaking. After washing (2 mL) with ice-cold physiological saline, cells were scraped from the plates, pelleted by centrifugation (1000*g*, 20 min) and submitted to ganglioside extraction and analysis.

Susceptibility of Gangliosides to VCS, after Treatment with PMA. A series of experiments was performed preincubating cell monolayers with 2 mL of Locke's solution containing 1 μM PMA, a drug able to induce protein kinase C (PKC) activation (*21*), for 5 min at 22 °C, immediately followed by the VCS treatment and ganglioside assay described above. In other experiments, cells were preincubated for 30 min at 37 °C in the presence of 1 μM staurosporine, a PKC activity inhibitor (*22*), followed, in order, by PMA treatment, VCS treatment, and ganglioside analysis. As a control, the ganglioside content of PMA-pretreated cells was assessed before sialidase exposure.

Susceptibility of Gangliosides to VCS, after Glutamate Treatment. To investigate the effects of glutamate upon ganglioside susceptibility to VCS, cells were washed three times with Mg^{2+}-free Locke's solution and, after addition of 2 mL of Mg^{2+}-free Locke's solution containing 100 or 1 μM glutamate, incubated for 15 min at 22 °C (*15*) and then immediately submitted to VCS treatment and to ganglioside analysis, as described above. As a control, the ganglioside content of glutamate-pretreated cells was also assessed before sialidase exposure. In other experiments, cells were preincubated for 30 min at 37 °C in the presence of 1 μM staurosporine followed, in order, by glutamate treatment, VCS treatment, and ganglioside analysis, as described above.

To investigate the effects of glutamate in the absence of Ca^{2+} in the external medium, the same procedure was adopted with the only difference that a Mg^{2+}-and Ca^{2+}-free Locke's solution, containing 5 μM EGTA, was employed (*23*), followed by washings and by VCS treatment in complete Locke's solution, as described above.

In some instances, ganglioside susceptibility to VCS was investigated using cells at 3 or 15 DIV. In such cases, cells cultured for the same time were used as the control.

Effect of Glutamate, PMA, or Staurosporine on VCS Activity. To assess the possible influence of glutamate, PMA, or staurosporine on VCS, the enzyme activity was assayed both on artificial and natural substrates. VCS activity, using the fluorogenic substrate MUB−NeuAc, was fluorometrically determined as described (*24*). Moreover, the enzyme activity was evaluated upon GD1a ganglioside, embedded in DPPC or EPC vesicles, as described (*25*). For this purpose, vesicles were prepared by 10 successive extrusions of aqueous dispersions of the phospholipid through 1000 Å pore-size filters (Nucleopore, Pleasanton, CA), using a N_2-operated extruder (Lipex Biomembranes, Vancouver, Canada) (*26*). Vesicles containing 3% molar GD1a mixed with radiolabeled ganglioside (250 000 dpm, corresponding to 0.15 nmol), asymmetrically embedded in the outer leaflet of the bilayer were prepared as reported (*25*) and used within 1 day of preparation. 1 milliunit of VCS was added to GD1a-containing vesicles (350 nmol of phospholipids) suspended in 154 μM Tris-HCl buffer, pH 6.8, final volume 50 μL. The quantification of the released GM1 ganglioside was performed radiochemically, as described (*27*).

[^{3}H]PdBu Binding to Intact Cells. Granule cells, grown on 30-mm diameter dishes, were tested at 8 DIV for [^{3}H]-PdBu binding (*21*) according to ref (*15*). Briefly, cells were incubated in 1 mL of Locke's solution containing 5 nM [^{3}H]-PdBu (0.1 μCi/mL) for 25 min at room temperature; $MgCl_2$ was omitted when indicated. After incubation, cells were washed three times with ice-cold Locke's solution, harvested, collected after centrifugation, and dissolved in 0.1 N NaOH (0.3 mL). Aliquots were used for protein assay and liquid scintillation counting. Nonspecific binding was assessed in the presence of 10 μM PMA.

Evaluation of Endocytosis in the Presence or Absence of Glutamate. The lipophilic fluorescent probe TMA-DPH was utilized for its marker properties of fluid phase pinocytosis (*28*), as already described (*29*). Briefly, cells were incubated in the presence of the probe solution (2×10^{-6} M) in the presence or in the absence of glutamate. After 15 min of incubation, cells were washed with Locke's solution (10 mL) and the fluorescence at the emission wavelength of 430 nm was recorded, exciting at 366 nm (washed sample). The internalized fraction of the probe was measured by the ratio: fluorescence intensity of washed sample/fluorescence intensity unwashed sample. The fluorescence of unwashed cells was taken after replacing the incubation medium with a TMA-DPH solution at the same concentration (*28, 29*).

Other Analytical Procedures. The ganglioside content was assayed as bound sialic acid by the resorcinol-HCl method (*30*). The protein content was determined by the method of Lowry (*31*).

Results are generally presented as mean ± SD values. ANOVA and *t*-test were used for statistical comparison.

Table 1: Ganglioside Content (nmol/mg of Protein) of Cultured Cerebellar Granule Cells before (A) and after (B−G) Treatment with VCS (1 milliunit/mL, 2 min)[a]

	A	B	C	D	E	F	G
GM1	0.96 ± 0.08[a]	2.1 ± 0.15	1.36 ± 0.1[a]	2.00 ± 0.1	1.55 ± 0.1[a]	1.98 ± 0.18	2.13 ± 0.13
GD1a	2.95 ± 0.3[b]	2.19 ± 0.1	2.48 ± 0.17[c]	2.22 ± 0.16	2.58 ± 0.1[b]	2.20 ± 0.1	2.27 ± 0.12
GD1b	1.41 ± 0.2[b]	2.14 ± 0.16	1.90 ± 0.15[c]	2.05 ± 0.14	1.71 ± 0.13[b]	2.12 ± 0.15	2.18 ± 0.2
O-Ac-GT1b	1.33 ± 0.1	1.24 ± 0.05	1.28 ± 0.1	1.29 ± 0.1	1.30 ± 0.09	1.29 ± 0.5	1.30 ± 0.1
GT1b	3.70 ± 0.2[a]	2.64 ± 0.11	3.38 ± 0.2[a]	2.72 ± 0.2	3.29 ± 0.1[a]	2.7 ± 0.1	2.61 ± 0.13
GQ1b + O-Ac-GQ1b	0.45 ± 0.03	0.41 ± 0.06	0.42 ± 0.04	0.45 ± 0.05	0.43 ± 0.05	0.4 ± 0.05	0.38 ± 0.05

[a] B, no pretreatment; C, pretreated with 1 μM PMA; D, pretreated with 1 μM glutamate; E, pretreated with 100 μM glutamate; F, pretreated with 100 μM glutamate in the absence of Ca^{2+}; G, pretreated with 100 μM glutamate and 1 μM staurosporine. The values are the means ± SD of four experiments. For each ganglioside analyzed, the statistical evaluation of the differences between data in columns A, C, D, E, F, and G against data in column B was made ANOVA by and *t*-test. Significance: (a) $p < 0.001$; (b) $p < 0.01$; (c) $p < 0.05$.

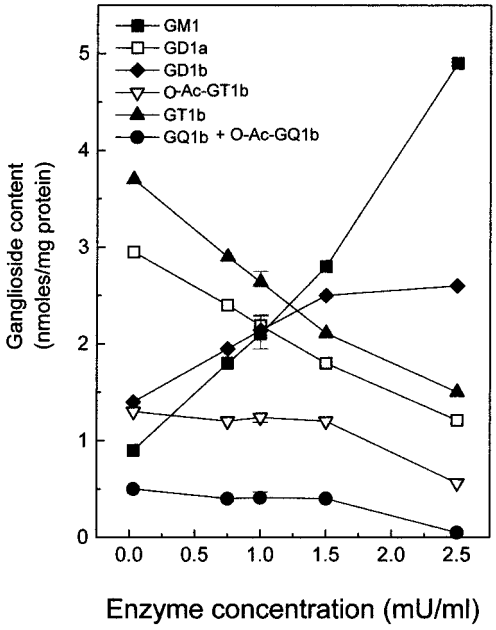

FIGURE 1: Ganglioside content of intact rat cerebellar granule cells in culture, after treatment with different amounts of VCS (2 min at 37 °C). Bars are ± SD.

RESULTS

Susceptibility of Granule Cell Gangliosides to VCS. The total sialic acid content of cells (7.5 μg/mg of protein) and the ganglioside pattern were similar to those already published (*17*, *32*, *33*). In particular, GT1b and GD1a content were 3.7 ± 0.2 and 2.95 ± 0.3 nmol/mg of protein, respectively. After treatment with VCS, the proportion of the major gangliosides GT1b and GD1a decreased, while that of GM1 and GD1b increased (Figure 1). When VCS concentrations up to 1.5 milliunits/mL were used, the amount of ganglioside hydrolyzed/2 min was linear with respect to the enzyme concentration (Figure 1). 2D HPTLC showed that, under these conditions, GT1b and GD1a were the only gangliosides affected by sialidase (data not shown). Using VCS concentrations higher than 1.5 milliunits/mL, *O*-acetyl-GT1b, GQ1b, and *O*-Acetyl-GQ1b gangliosides were also affected (Figure 1). All the successive experiments were therefore carried out using 1.0 milliunit/mL VCS. In a typical experiment under the above said conditions, GT1b and GD1a content were reduced to 2.64 ± 0.11 and 2.19 ± 0.1 nmol/mg protein, respectively, corresponding to 28.6 and 25.7% hydrolysis. From now on, these values will be referred to as "control" (Figure 1, Table 1).

Susceptibility of Granule Cell Gangliosides to VCS after Pretreatment with PMA. After pretreatment with PMA followed by incubation with VCS, the content of GT1b and GD1a were markedly higher than in control cells (3.38 ± 0.2 and 2.48 ± 0.17 nmol/mg protein), corresponding to reduced exposure (8.6 and 15.9% hydrolysis, respectively) and indicating that the phorbol ester treatment renders the glycolipids less susceptible to the enzyme (Table 1). 2D HPTLC showed that after pretreatment with PMA only GT1b and GD1a gangliosides were affected by sialidase (data not shown). Control experiments showed that the mere pretreatment with PMA (before incubation with VCS) was not able to affect the ganglioside content and pattern of cells.

Susceptibility of Granule Cell Gangliosides to VCS after Pretreatment with Glutamate. When cells were pretreated with 100 μM glutamate for 15 min and then with VCS, GT1b and GD1a ganglioside exposure was markedly reduced with respect to the control (12.9 and 12.5% hydrolysis, respectively). These data indicate that the pulse with the amino acid renders these glycolipids less susceptible to the enzyme (Table 1). 2D HPTLC showed that, also in the presence of glutamate, GT1b and GD1a were the only gangliosides affected by sialidase (data not shown).

On the contrary, the susceptibility of gangliosides to VCS was comparable with the control under different experimental conditions (a) when a low glutamate concentration (1 μM) was utilized (Table 1), (b) when the experiment with 100 μM glutamate was preceded by treatment with staurosporine (Table 1), and (c) also when the experiment with PMA described above was preceded by treatment with staurosporine (data not shown). Control experiments showed that ganglioside content and pattern of cells pretreated or not with glutamate or with staurosporine (before incubation with VCS) was comparable.

Additional experiments were carried out to evaluate the effects of 100 μM glutamate in the absence of Ca^{2+} in the medium. Under these conditions, the susceptibility of GT1b and GD1a to VCS was comparable with the control (Table 1), contrarily to the results observed in the presence of the cation. All the data are summarized in Table 1.

Other experiments were performed with cells cultured for different times. The decrease of ganglioside susceptibility to VCS, exerted by the addition of 100 μM glutamate, was detected on cells at 8 and 15 but not at 3 DIV (Table 2). An exception was displayed by GD1a, whose susceptibility to VCS strongly decreased at 15 DIV, suggesting that its insensitiveness to glutamate at this time in culture was linked to this feature.

Table 2: Ganglioside Content (nmol/mg of Protein) of Cerebellar Granule Cells Cultured for Different Days in Vitro (DIV), before (A) or after (B, C) Treatment with VCS (1 milliunit/mL, 2 min) (B, No Pretreatment; C, Pretreated with 100 μM Glutamate)[a]

	3 DIV			15 DIV		
	A	B	C	A	B	C
GM1	0.57 ± 0.07^a	1.45 ± 0.07	$1.42 \pm 0.0.7$	1.10 ± 0.12^b	1.58 ± 0.11	1.32 ± 0.11^c
GD1a	1.55 ± 0.07^a	0.75 ± 0.1	0.81 ± 0.11	3.97 ± 0.18	3.57 ± 0.18	3.66 ± 0.2
GD1b	0.81 ± 0.05^a	1.37 ± 0.06	1.32 ± 0.07	1.12 ± 0.08^a	2.2 ± 0.15	1.58 ± 0.12^b
O−Ac-GT1b	0.39 ± 0.02	0.37 ± 0.03	0.38 ± 0.02	1.84 ± 0.07	1.66 ± 0.08	1.69 ± 0.1
GT1b	1.88 ± 0.1^a	0.75 ± 0.03	0.74 ± 0.03	3.68 ± 0.17^a	2.65 ± 0.14	3.28 ± 0.2^b
GQ1b + O−Ac-GQ1b	0.24 ± 0.01	0.22 ± 0.01	0.22 ± 0.02	0.51 ± 0.03	0.51 ± 0.04	0.51 ± 0.04

[a] The values are the means ± SD of three experiments. For each ganglioside analyzed, the statistical evaluation of the differences between data in columns A and C against data in column B was made by ANOVA and *t*-test. Significance: (a) $p < 0.001$; (b) $p < 0.01$; (c) $p < 0.05$.

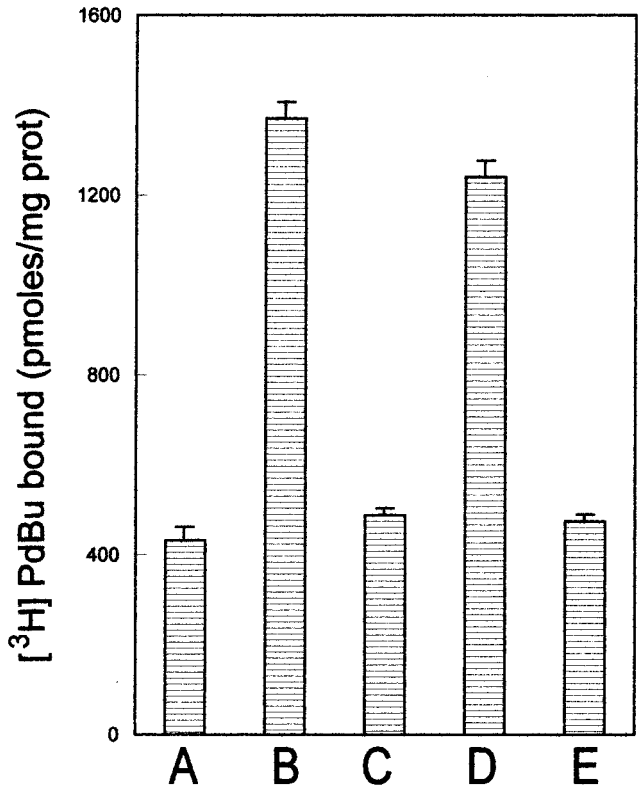

FIGURE 2: Binding of [^{3}H]PdBu to intact rat cerebellar granule cells in culture not pretreated (A) or pretreated with 100 μM glutamate (B), 1 μM glutamate (C), 100 μM glutamate and 1 μM staurosporine (D), and 100 μM glutamate in the absence of Ca^{2+} (E). The values are the mean ± SD of four experiments.

[^{3}H]PdBu Binding. Treatment of cells (8 DIV) with 100 μM glutamate elicited an increase of [^{3}H]PdBu binding with respect to untreated cells (Figure 2). Similar results were observed when cells were pretreated with staurosporine and then with 100 μM glutamate. On the contrary, when 100 μM glutamate was added in a Ca^{2+}-free medium, or when 1 μM glutamate was added, no increase of [^{3}H]PdBu binding was observed (Figure 2).

Endocytosis. Fluorescence intensity after 15 min incubation with TMA-DPH was taken before and after washing of the cells. Comparison of the values showed that the fraction of internalized probe, under the experimental conditions, was about 10%. The same figure was obtained in the presence of glutamate.

Cell Viability. The percentage of surviving cells treated with 100 μM glutamate was 20.5% after 24 h. The survival of control cells was 97%.

Assessment of the Influence of Glutamate, PMA, or Staurosporine on VCS Activity. The hydrolysis of the artificial substrate MUB-NeuAc was not affected by the presence of 1 μM PMA, 1 μM staurosporine, or glutamate in the 1−100 μM range of concentration (about 10 nmol MUB released/h/milliunit). Also the activity of VCS acting on GD1a ganglioside inserted in liposomes having different phospholipid composition (DPPC or EPC) was not affected by the presence of PMA, staurosporine, or glutamate (about 3.0 and 4.2 nmol of GM1 released /h/milliunit in EPC and DPPC liposomes, respectively).

DISCUSSION

Previous reports indicate that not all membrane gangliosides are equally accessible, when analyzed for their reactivity with various ligands (*34*−*36*). Their accessibility appears to be modulated by factors such as the structure (oligosaccharide and ceramide composition) and membrane concentration of glycolipids, the environment and the nature of the ligand (*25*, *26*, *37*−*39*). Enzymes, sialidase included, have been previously used to monitor glycolipid exposure and accessibility at the membrane surface (*34*−*36*, *39*−*41*). In particular, it has been shown that VCS is sensitive to the surface density of the ganglioside substrate within the membrane (*25*, *42*, *43*). In more detail, it has been shown that upon increasing the extent of lateral phase separation of GD1a with formation of domains, that is, of areas with higher local glycolipid concentration, its susceptibility to sialidase decreases.

Under the standardized conditions that we set up, VCS partially affected gangliosides of intact cerebellar granule cells, whose pattern was assessed by an HPLC technique previously utilized to accurately ascertain changes in various tissues and cell types, cerebellar granular cells included (*17*, *18*, *43*−*45*). By this technique and by 2D TLC we established that the treatment with VCS modified the pattern but not the total ganglioside content of the cells, as expected (*42*, *43*), namely, GT1b and GD1a hydrolyzed to form the respective products, GD1b and GM1. The hydrophilic and macromolecular features of VCS, the very short incubation times utilized, and the use of intact cells imply that, under the experimental conditions, the enzyme did not penetrate through the plasma membrane and was acting upon GT1b and GD1a gangliosides exposed toward the extracellular environment.

A series of experiments was first performed using PMA, a drug known to induce activation of PKC in many cell types, and previously used for this purpose also upon cerebellar

granule cells (*15, 21, 22*). Ganglioside susceptibility to VCS in cells treated with the phorbol ester was markedly reduced, suggesting a relationship with PKC activation. To substantiate the participation of PKC to the phenomenon observed, PKC activation was induced with 100 μM glutamate. In fact, it is known that after 7−9 DIV cerebellar granule cells become mature neurons and acquire sensitiveness to the neurotransmitter (*46*). It has been described that binding of glutamate to ionotropic receptors evokes a series of intracellular events starting with the opening of Ca^{2+} channels. Extracellular Ca^{2+} influx induces PKC activation. On the other hand, the stimulation of metabotropic receptors starts the phosphoinositide cycle and may also contribute to PKC activation (*47*).

Addition of 100 μM glutamate resulted in a massive PKC translocation, as determined by [^{3}H]PdBu binding, confirming data previously obtained upon cerebellar granule cells (*15, 21, 22*), and was followed by cell death 24 h later. The susceptibility of gangliosides to VCS strongly decreased, mimicking the results obtained with PMA. To rule out the possibility that the binding of glutamate to its receptor could affect this result, experiments were carried out with glutamate in the absence of Ca^{2+} in the culture medium. Under these conditions PKC in granule cells is not translocated and activated, as already reported (*15*) and herein confirmed, and the susceptibility of gangliosides to VCS was not modified with respect to untreated cells. All these data imply that the mere glutamate binding to its receptor was not directly affecting ganglioside accessibility at the cell surface, if not occurring together with PKC activation. This deduction was also confirmed by experiments carried out with 1 μM glutamate in the presence of Ca^{2+}. Under these conditions cell death was not observed, activation of PKC was kept to a minimum, and the susceptibility of gangliosides to VCS did not change with respect to the control.

Additional experiments were carried out in order to establish whether PKC activation or only its translocation are responsible of the results observed. Conditions already shown to induce PKC translocation concomitantly with its inhibition were set up (*22*), that is, cell treatment with staurosporine, an inhibitor of PKC activity (*22, 48−50*), followed by PMA or 100 μM glutamate. Ganglioside susceptibility was not modified with respect to the control, indicating that PKC translocation is not sufficient to exert changes of the glycolipid exposure, if its activity is not expressed.

Noteworthy, the glutamate-induced change of ganglioside exposure was displayed only by cells sensitive to the toxic action of glutamate (8 or 15 DIV), while this feature was not present in cells at 3 DIV, which are not sensitive to the amino acid (*46*).

Taken all together, these results suggest that PKC activation is involved in the change of ganglioside exposure detected by VCS. In the line of principle, it would be possible that another enzyme, besides PKC, is affected under a given experimental condition (*51*). However, the comparison between different experiments, all able to induce or prevent PKC activation and exerting opposite effects on ganglioside exposure, circumscribes the pivotal role to PKC.

It can be argued that the pretreatments utilized could modify the content of GD1a and GT1b in the plasma membrane, and, if this occurred, the observed changes of ganglioside exposure would be consequent. The assessment of the precise amount of each ganglioside in the plasma membrane would be extremely difficult. However, (a) the rate of fluid-phase endocytosis was very low, and small changes could not account for the strong changes of ganglioside exposure observed. Nevertheless, we observed that the pretreatments did not influence the rate of endocytosis, suggesting that the ganglioside content within the plasma membrane did not change with respect to untreated cells. As a further consideration, the current knowledge seems to exclude the possibility of other internalization pathways, such as translocation, for gangliosides, given the presence of their bulk, negatively charged, oligosaccharide chain (*52*). (b) Control experiments showed that PMA or glutamate pretreatment did not modify the total ganglioside content and pattern of the cells. This latter observation seems also to exclude that pretreatments can affect ectoenzymes such as sialidase or sialyltransferase. All this body of evidence suggests that the pretreatments utilized did not modify the content of gangliosides at the exoplasmic surface of the cell, further suggesting that PKC activation—increasing the apparent crypticity of GD1a and GT1b—is responsible for the change of ganglioside accessibility.

Considering that PKC activity is required to induce the change, a definite possibility is that phosphorylation of membrane proteins is involved in this event. The modification of membrane-associated macromolecules could trigger either a physical shielding of the glycolipid headgroups (*35, 41*) or a change in ganglioside lateral distribution, since this feature is overwhelmed by the interaction with integral membrane proteins (*53−55*). This latter hypothesis is also substantiated by previous investigations (*25, 42, 43*), showing that VCS activity decreases on increasing the extent of ganglioside segregation (in particular of GD1a) within artificial and cell membranes. All these premises suggest that PKC activation increases ganglioside domains formation. In turn, also the segregation may lead to a reduction of the ganglioside motional freedom, modifying the accessibility of sialic acid moiety to VCS, as already postulated to explain changes of glycolipid recognition by antibodies (*56*).

While transduction of a message across the plasma membrane is a well-taken piece of evidence, these results suggest that following PKC activation, a central event in inward-directed signal transduction, cell may dispatch outward-directed messages to glycolipids of the plasma membrane outer layer, changing their segregation state. A growing body of evidence suggests that fundamental membrane events such as signal transmission and lipid/protein sorting are related to the organization of glycolipid in domains (*10, 57−61*). The results obtained herein suggest the possibility of their modulation. The study of this outward-directed path as an alternative way of realizing signal transduction deserves and requires further investigation.

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