Zygote **8** (May), pp 159–169. © 2000 Cambridge University Press Printed in the United Kingdom

Inhibition of the synthesis of glycosphingolipid by a ceramide analogue (PPMP) in the gastrulation of *Bufo arenarum*

Manuel J. Aybar, Analía Fuentes and Sara S. Sánchez
Departamento de Biología del Desarrollo, Instituto Superior de Investigaciones Biológicas y Técnicas (CONICET) y Universidad Nacional de Tucumán (UNT), Chacabuco 461, 4000–San Miguel de Tucumán, Argentina

Date submitted: 28.10.99. Date accepted: 20.12.99

Summary

In the present study the role of glycosphingolipids (GSL) in amphibian development was investigated. We analysed the *de novo* synthesis of neutral GSL and gangliosides through the initial stages of *Bufo arenarum* embryo development and their participation during gastrulation using 1-phenyl-2-palmitoyl-3-morpholino-1-propanol (PPMP), a potent inhibitor of glucosylceramide synthase. Ganglioside synthesis began at the blastula stage and reached a maximum during gastrulation (stages 10-12) while neutral GSL synthesis showed a slight gradual increase, the former being quantitatively more significant than the latter. Ganglioside synthesis was reduced by 90% while neutral GSL synthesis was inhibited by 65% when embryos at blastula stage were cultured for 24 h in 20 µM PPMP. The depletion of GSL from amphibian embryos induced an abnormal gastrulation in a dose-dependent manner. We found that PPMP had a pronounced effect on development since no embryos exhibited normal gastrulation; their developmental rate either slowed down or, more often, became totally arrested. Morphological analysis of arrested embryos revealed inhibition of the gastrulation morphogenetic movements. Analysis of mesodermal cell morphology in those embryos showed a severe decrease in the number and complexity of cellular extensions such as filopodia and lamellipodia. Mesodermal cells isolated from PPMP-treated embryos had very low adhesion percentages. Our results suggest that glycosphingolipids participate in *Bufo arenarum* gastrulation, probably through their involvement in cell adhesion events.

Keywords: Amphibian, Gastrulation, Glycosphingolipids, Inhibitor, PPMP

Introduction

Early amphibian development is characterised by precisely coordinated cellular interactions in which cell surface and extracellular molecules are thought to play an important role (Hay, 1991). Amphibian gastrulation, the most profound change in embryo morphology, is accomplished by active cell movements such as the migration of mesodermal cells, the epibolic movement of the ectodermal wall and the convergence-extension of the marginal zone (Keller, 1986). In all these events, cell morphology, attachment, migration and differentiation may require cell–cell and cell–environment interaction.

All correspondence to: S.S. Sánchez, Depto. de Biología del Dessarrollo, INSIBIO (CONICET-UNT), Chacabuco 461, 4000 – San Miguel de Tucumán, Argentina. Fax: +54 381 4248025. e-mail: ssanch@unt.edu.ar

Cell surface glycoconjugates have long been known to play a role in cell adhesion and recognition in amphibian embryos. Early studies in this laboratory have shown that changes in cell surface glycoconjugates occur during primary induction in *Bufo* embryos (Barbieri *et al.*, 1980). Moreover, amphibian embryonic development is affected by the presence of tunicamycin (Romanosky & Nosek, 1980; Sánchez & Barbieri, 1983) and Ara-C (Peralta *et al.*, 1995), both of which are inhibitors inducing abnormal gastrulation. These findings show that glycoconjugates (glycoproteins and/or glycolipids) are necessary for normal gastrulation, even though their role is not completely understood.

Glycosphingolipids (GSL), a group of cell surface glycoconjugates, are ubiquitous components of the eukaryotic plasma membrane. These amphipathic molecules consist of a hydrophobic residue, ceramide, embedded in the outer leaflet of the lipid bilayer, and a hydrophilic oligosaccharide chain that extends into the

aqueous environment at the cell surface (Kopitz, 1997). GSL are known to change during various cellular processes that take place during growth and differentiation (for review see Hakomoro, 1981; Kopitz, 1997). There is increasing evidence that GSL breakdown products regulate a variety of developmental processes, including cell proliferation, recognition and differentiation (Hakomori, 1990; Hannun *et al*., 1996). A pharmacological agent termed 1-phenyl-2-palmitoyl-3-morpholino-1-propanol (PPMP) has been developed and characterised as an important inhibitor of GSL biosynthesis (Radin *et al*., 1993), and is thus a useful tool for the study of the involvement of GSL in many cellular processes. PPMP is able competitively to inhibit UDP-glucose:ceramide β1→glucosyltransferase (glucosylceramide synthase) (Abe *et al*., 1992, 1959). Since GSL normally undergo catabolic degradation, the blockage of glucosylceramide (GlcCer) biosynthesis should ultimately lead to the depletion of endogenous GSL, including gangliosides, all of which are biosynthesised from GlcCer.

In 1991, Hidari *et al*. determined the composition of acidic glycolipids in the oocyte of the amphibian *Xenopus*. More recently, Gornati *et al*. (1995) established the pattern of both neutral glycosphingolipids and gangliosides during the first 6 days of *Xenopus laevis* development. Moreover, a neutral glycolipid was found to be involved in a novel cellular adhesion mechanism. This glycolipid antigen was developmentally regulated and differentially expressed in the animal hemisphere during the blastula stage (Turner *et al*., 1992). The above-mentioned studies suggest that glycosphingolipids may be biologically involved in early amphibian development. However, the participation of such molecules in the various well-defined processes of gastrulation has not been fully elucidated.

Amphibian oocytes contain and/or synthesise the molecular components necessary for the complex series of morphogenetic events that take place during early embryonic development. It is a well-known fact that yolk platelets, the most abundant organelles in oocytes, provide the organic components, precursors, templates, structural molecules and tissue-inducing substances (Carson & Lennarz, 1979; Eckelbarger, 1986; Aybar *et al*., 1996) required by the oocyte after its activation. As regards the yolk platelet precursors, in a previous paper (Villecco *et al*., 1999) we described the presence of abundant lipid droplets in the multivesicular body, where lipoproteins condense to form the yolk during oogenesis. GSL might either be synthesised during oogenesis and remain stored in the egg yolk prior to fertilisation; or they could be synthesised during the early stages of embryonic development, constituting a fraction of the total amount of glycolipids present in the embryo. In order to examine the latter possibility, in the present paper we report our investigation of the *de novo* synthesis of GSL during the early stages of *Bufo arenarum* development, placing special emphasis on the effects of the inhibition of such synthesis by PPMP during gastrulation.

Materials and methods

Embryos

Bufo arenarum embryos were obtained by artificial fertilisation and cultured, dejellied and staged as previously described (Genta *et al*., 1997).

Metabolic labelling of gangliosides

Metabolic labelling of glycosphingolipids was performed using *N*-[^{3}H]acetyl glucosamine (NEN, UK), specific activity 35.4 mCi/mmol. In order to analyse the incorporation of the radioactive precursor into glycosphingolipids and gangliosides during early amphibian development, batches of embryos were cultured in 10% Ringer's solution containing 15 μCi/ml *N*-[^{3}H]acetyl glucosamine. After fertilisation, embryos were cultured until different developmental stages were reached (7, 8, 9, 12, 13, 14 and 15). After labelling, embryos were washed three times with Ringer's solution and then homogenised by sonication in ice-cold distilled water. An aliquot was removed to determine the uptake of the radioactive precursor. In the remaining homogenate, the macromolecules were precipitated with cold 10% trichloroacetic acid (TCA) to determine the incorporation of the precursor into glycoproteins. The suspension was centrifuged at 400 *g* for 20 min and the precipitates passed through Whatman GF/C glass fibre filters. After washing the precipitates on the filters with cold 5% TCA, filters were dried and placed in a scintillation solution containing 0.2% PPO and 10% naphthalene in dioxane, and radioactivity was estimated in a Beckman liquid scintillation spectrometer. The incorporation of the radioactive precursor into both the neutral glycolipid fraction and the ganglioside fraction was determined by the extraction and purification of each. A neutral glycolipid fraction was obtained as described by Spiegel *et al*. (1988). The ganglioside fraction was purified from embryo homogenates as described by Daniotti *et al*. (1990). The respective fractions were dried, and samples measured for radioactivity by liquid scintillation spectrometry as described above.

Inhibitor

To monitor the various effects of the inhibitors of glycosphingolipids biosynthesis on embryonic development and glycolipid synthesis, blastulae, early gastrula

and midgastrula embryos were cultured for 24 h in 10% Ringer's solution with or without the PPMP inhibitor (D-threo-1-phenyl-2-palmitoyl-3-morpholino-1-propanol, PDMP; Matreya, PA) at different concentrations. To facilitate the penetration of the inhibitor into the blastocoele, the vitelline envelope was removed and small slits made on the animal pole of the blastulae with a tungsten needle and a hair loop. The same procedure was carried out in the control embryos in a saline medium. Sterile procedures were employed in all embryo cultures. At various times, embryos were observed under a stereo microscope. After incubation with inhibitors, embryos were fixed and processed for scanning electron microscopy (SEM) as previously described (Peralta *et al*., 1995). Ganglioside and glycoprotein labelling in inhibitor-treated and control embryos was performed by adding 15 µCi/ml *N*-[^{3}H]acetyl glucosamine to the culture medium as described above.

The degree of gastrulation was determined by the extent of pigmentation and by the size of the yolk plug in the vegetal view of embryos (external criteria according to Del Conte & Sirlin, 1951) up to the period when control embryos reached the final gastrula stage.

Whole-mount immunocytochemistry

Gangliosides were located by whole-mount immunocytochemistry in *Bufo arenarum* embryos at the blastula and gastrula stages. Selected areas from different regions of the embryos were excised and fixed in 4% formaldehyde in Barth's medium, pH 7.4, for 1 h at room temperature. All fragments excised were assayed for cell breakage and viability as previously described (Genta *et al*., 1997). No cytolysis ascribable to the microdissection procedure was detected (viability > 99%). Fixed specimens were then carefully rinsed in Barth's medium containing 3% bovine serum albumin (BSA) for 2 h. For immunofluorescence staining, specimens were incubated in 0.5 mg/ml cholera toxin B subunit (CTB) for 2 h at room temperature, rinsed and incubated again with rabbit anti-CTB antibodies (1:200 dilution) for 2 h at room temperature. After washing, specimens were treated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibodies (1:200 dilution) for 2 h at room temperature. After blocking, specimens were incubated with human anti-GM1 antibodies prepared as described above (dilution 1:200) for 2 h at room temperature. After washing, specimens were treated with FITC-conjugated anti-human antibodies (1:200 dilution) for 2 h at room temperature. After immunofluorescent staining, specimens were washed in Barth's medium and mounted in Nowiol (Hoechst, Germany). Fluorescence was visualised using a Nikon microscope with appropriate filters for fluorescein.

Control specimens were treated with anti-GM1 antibodies preadsorbed with an excess amount of pure GM1 or CTB preincubated with an excess amount of GM1, with a non-immune rabbit IgG fraction or without the primary antibody; they were then incubated with the corresponding FITC-conjugated antibodies. To distinguish between a glycolipid and a possible glycoprotein receptor for CTB, the following procedure proposed by Wu *et al.* (1995) was used. Specimens were immersed in chloroform:methanol (1:1, v/v) at room temperature for 15 min and then staining was performed as described above. This treatment produced no fluorescence, thus indicating the existence of a lipid receptor for CTB. Another indication of CTB or anti-GM1 binding to a lipid receptor was the fact that CTB and anti-GM1 antibody did not show specific binding to embryo glycoproteins separated by SDS-PAGE and transferred to nitrocellulose filters (data not shown). In agreement with these results, glycoproteins showed no immunofluorescent staining with CTB or anti-GM1 in chloroform:methanol-delipidated explants.

Cell adhesion assay

The adhesion assay was performed as described previously (Peralta *et al*., 1995). Mesodermal cells were removed from control and inhibitor-treated embryos with a hair loop and disaggregated in a dissociation medium (Ca^{2+}- and Mg^{2+}-free double 'A' Niu-Twitty solution containing 2 mM EDTA) (Barth & Barth, 1959) for 30–60 min at room temperature. Adhesive substrata were conditioned by deposition of native extracellular matrix from fragments of the blastocoele roof of early gastrula stage on glass coverslips, according to Nakatsuji & Johnson (1983). Between 250 and 300 mesodermal cells were obtained from individual embryos at the early gastrula stage and transferred onto conditioned substrata. After 90 min of incubation to allow cell adhesion, cultures were scored for percentage of attached cells. Control slides were coated with 100 µg/ml BSA in Barth's solution.

Results

Glycosphingolipid biosynthesis during development: effect of PPMP

To determine whether glycosphingolipid synthesis could be an early developmental event we assessed the *in vivo* uptake and incorporation of the radioactive precursor *N*-[^{3}H]acetyl glucosamine by the embryo into the neutral glycolipids as well as into the ganglioside fraction. Results (Fig. 1) indicate that the rate of *N*-[^{3}H]acetyl glucosamine uptake by the embryos reached a maximum at stage 10, just at the onset of the

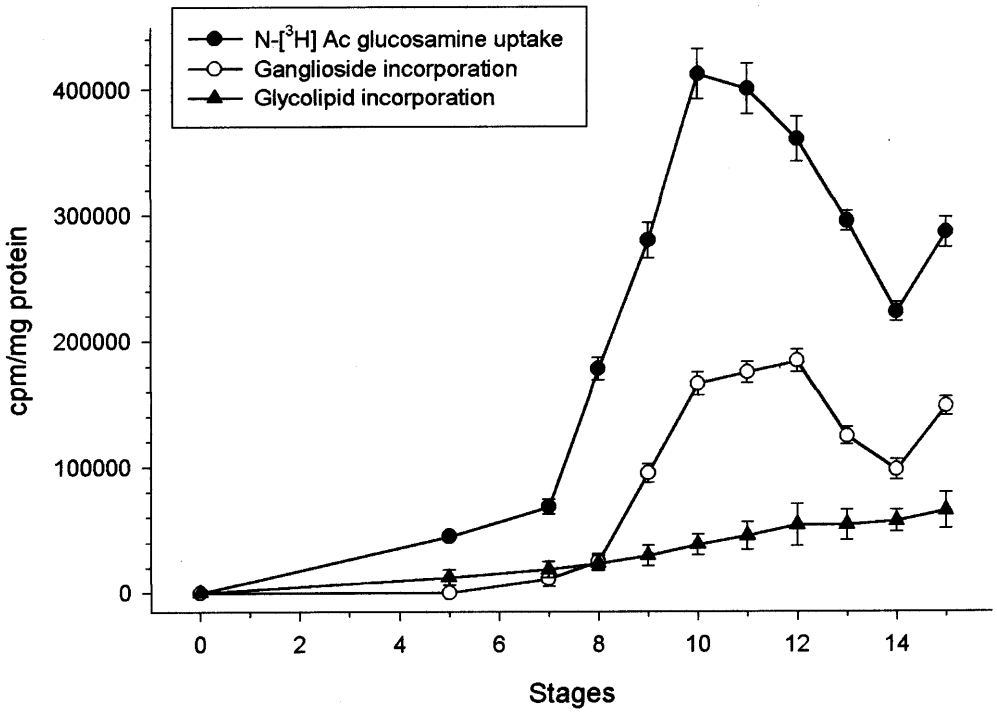

Figure 1 Biosynthesis of neutral glycosphingolipids and gangliosides during early development. Batches of embryos were cultured with 15 µCi/ml using *N*-[^{3}H]acetyl glucosamine in 10% Ringer's solution. Neutral glycosphingolipid and ganglioside fractions were isolated and the incorporation of a radioactive precursor was measured as indicated under Materials and Methods.

gastrulation process. Ganglioside synthesis began at the blastula stage (stage 8) and increased gradually, reaching a maximum during gastrulation (stages 10–12). Then, a transient decrease in ganglioside synthesis was observed at the beginning of neurulation (stages 13–14), followed by another increase in the incorporation of the radioactive precursor. As regards neutral glycosphingolipid biosynthesis, a slight gradual increase was observed throughout the developmental period analysed in this study. The results of the synthesis of gangliosides and neutral glycosphingolipids seem to indicate that there is a stronger requirement for the former during gastrulation.

To demonstrate the effect of PPMP on GSL synthesis, control and PPMP-treated embryos were pulsed with *N*-[^{3}H]actyl glucosamine. The biosynthesis of glycosphingolipid was inhibited by PPMP as shown in Table 1. The incorporation of the radioactive precursor into the ganglioside fraction was reduced in a dose-dependent manner in those embryos cultured with the inhibitor. Ganglioside synthesis showed a 90% decrease when embryos were cultured for 24 h in 20 µM PPMP while neutral glycolipid synthesis underwent 65% inhibition under the same experimental conditions. Consequently, the inhibition of glycolipid synthesis might lead to a significant decrease in the content of glycolipids during development. The maximum concentration of the inhibitor (20 µM) used in this study produced only a slight effect on glycoprotein synthesis, since only a 12% decrease in the incorporation of the precursor was observed.

Effect PPMP on embryonic development

Table 2 summarises the results from a series of three experiments with embryos from different females. Treated embryos cultured from the blastula stage in the presence of different concentrations of PPMP exhibited a significant slowing down in their developmental rate and an almost immediate arrest of development in a dose-dependent manner.

In control experiments, all embryos completed gastrulation during the same experimental period. The increase in the number of arrested embryos was concomitant with the increase in the concentration of PPMP (Table 2). When 20 µM PPMP was incorporated into the culture medium, nearly 35% and 65% delayed and arrested embryos were produced, respectively. Although the proportion of affected embryos varied when different concentrations of the inhibitor were tested, the same effects were consistently induced by the antimetabolite in all experiments. Similar results were obtained when embryos were cultured with PPMP from early and midgastrula stages. No toxicity of the inhibitor was observed under the assay conditions since cell viability was not reduced and the bio-

Table 1 Effect of PPMP on the synthesis of neutral glycolipids, gangliosides and glycoproteins[a]

PPMP	Neutral glycolipid synthesis		Ganglioside synthesis[b]		Glycoprotein synthesis[c]	
	c.p.m. 10^{-3}/mg protein	Inhibition (%)	c.p.m. 10^{-3}/mg protein	Inhibition (%)	c.p.m. 10^{-3}/mg protein	Inhibition (%)
–	126.36 ± 16.25	0	221.59 ± 18.81	0	316.23 ± 21.63	0
1 µM	64.52 ± 11.23	48.9	132.23 ± 21.44	40.3	291.22 ± 17.16	7.9
5 µM	54.17 ± 14.77	57.1	54.19 ± 11.51	75.5	288.81 ± 15.17	8.6
10 µM	51.89 ± 12.54	58.9	36.56 ± 8.22	83.5	280.02 ± 14.36	11.4
20 µM	43.31 ± 13.09	65.7	20.10 ± 5.91	90.9	276.16 ± 17.08	12.6

[a]The values represent the mean ± SD of three different experiments. Blastula embryos (stage 9) were cultured for 24 h with or without PPMP in 10% Ringer's solution containing 15 µCi/ml *N*-[^{3}H]acetyl glucosamine.
[b]Ganglioside labelling was analysed by isolation of the total ganglioside fraction as described in Materials and Methods.
[c]Glycoprotein labelling was analysed in the precipitated material by 10% TCA.

Table 2 Effect of PPMP on gastrulation of *Bufo arenarum* embryos

PPMP	No. of embryos[a]	Effect on development	
		Delay[b] (%)	Arrest[c] (%)
–	210	0	0
1 µM	210	71.90 ± 2.18	28.10 ± 2.18
5 µM	210	64.28 ± 2.86	35.72 ± 2.86
10 µM	210	44.75 ± 7.86	55.25 ± 7.86
20 µM	210	34.76 ± 2.97	65.24 ± 2.97
20 µM + 20 µM GlcCer	210	12.33 ± 2.51	0

GlcCer, glycosylceramide.
[a]Total number of embryos in three experiments made with embryos from different females.
[b]The progression of gastrulation was assessed by observing the external criteria for gastrulation defined by Del Conte & Sirlin (1951). Embryos with development delay of 1 or 2 stages with respect to control embryos were considered.
[c]Embryos arrested at the beginning of treatment.

logical effects of the treatment could be reverted by elimination of PPMP from the culture medium up to 12–15 h after the beginning of the experiment. Additional evidence of the lack of toxicity of PPMP was provided when PPMP-treated embryos were cultured in the presence of GlcCer in order to bypass the inhibition, since when using this combined treatment most embryos were found to develop normally, with only 12% delayed embryos and no arrested ones.

Taking into account the fact that the inhibition of UDP-glucose:β1-glucostyltransferase by PPMP may promote the accumulation of ceramide, we investigated whether the effect of PPMP on embryonic development was a result of high levels of ceramide rather than of the inhibition of the synthesis of complex glycosphingolipid. Embryos incubated with 20 µM ceramide showed no alteration in their development. This result would support the idea that PPMP not only blocks normal gastrulation but also inhibits glycosphingolipid biosynthesis. This fact led us to think that complex GSL, which could be blocked by PPMP, might be required in certain gastrulation events.

Effect of PPMP on gastrulation processes

To determine the effect of GSL depletion on gastrulation processes we analysed the morphological alterations observed in PPMP-treated embryos. The general external appearance of the arrested embryos treated with the different doses of PPMP was not markedly different form that of blastula embryos except for a slight tendency to cell dissociation. Midsagittal sections of arrested embryos showed a single rounded, enlarged cavity formed by a slight expansion of the blastocoele cavity into the endodermal mass (Fig. 2*A*). This cavity appeared to be completely depleted of extracellular matrix (ECM) and no deposition of materials on cell surfaces could be detected (Fig. 2*A*, *C*, *D*). No involution was observed and no migrating mesodermal cells were found in arrested embryos.

During epiboly, the ectodermal layer of normal embryos turned into a thin smooth layer of epithelial cells (2 to 3 cells thick, Fig 2*B*) after their local inward or outward migration along the radii of the embryo (radial interdigitation). In addition, a spreading of the outer ectodermal layer occurred. In PPMP-treated embryos normal epiboly never occurred and no radial interdigitation was observed since the blastocoelic roof layers remained unchanged (Fig. 2*A*).

Effect of PPMP on cell morphology and adhesion

Cellular morphology was analysed by scanning electron microscopy in those embryos treated with 20 µM PPMP that exhibited a 1 or 2 stage delay in their development. The morphology of both ectodermal and

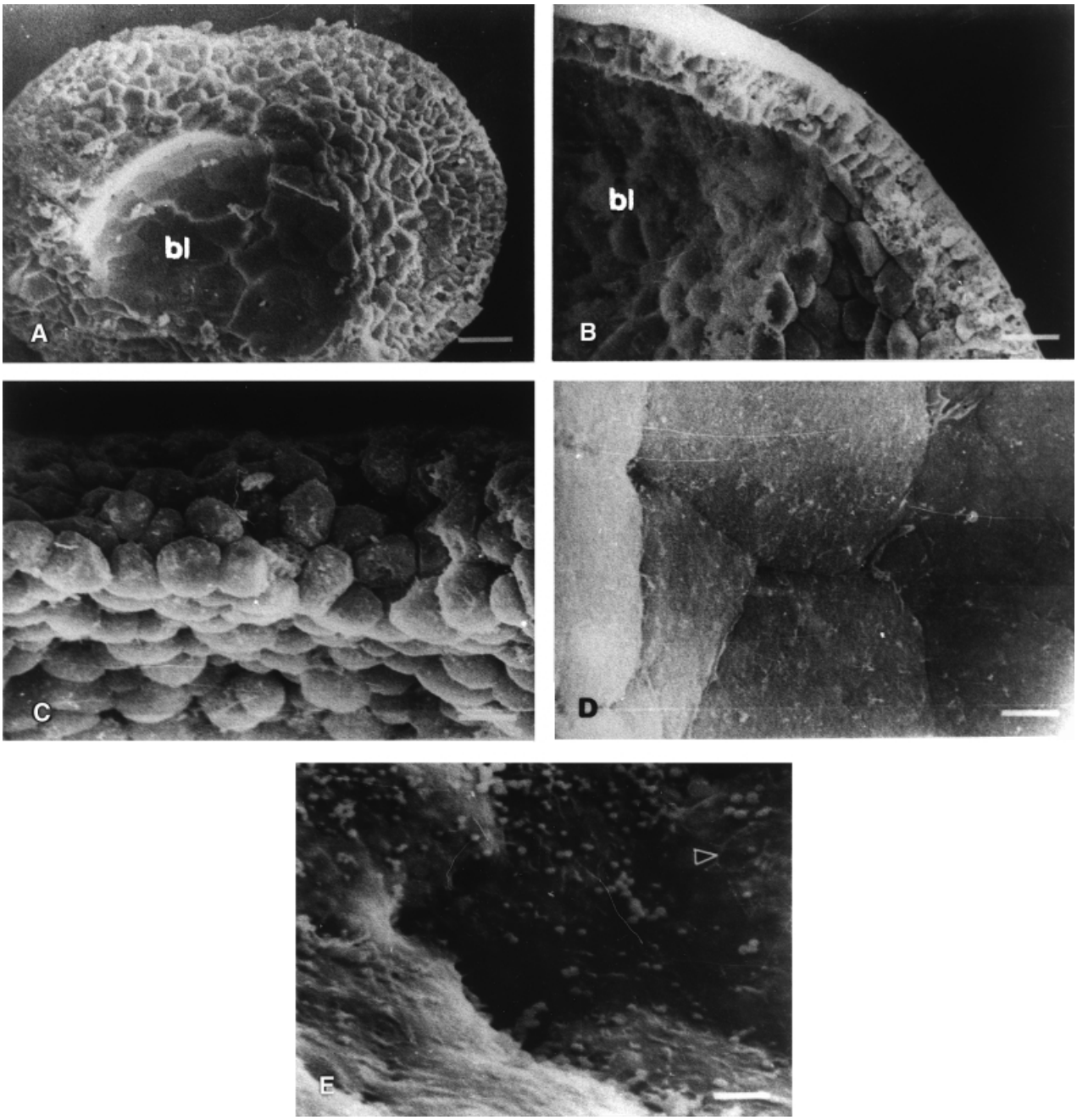

Figure 2 Morphological analysis of the effect of PPMP on the gastrulation of *Bufo arenarum* embryos. (*A*) Inner view of a mid-sagittal fracture of a PPMP-treated embryo. Embryos were fixed when controls reached the gastrula stage (stage 12). bl, blastocoele. (*B*) Blastocoelic cavity and blastocoelic roof of a normal embryo at the gastrula stage. Note the presence of extracellular materials in the blastocoelic cavity (bl). (*C*) Blastocoelic roof of a PPMP-treated embryo fixed when control embryos reached stage 12. No radial interdigitation of ectodermal cells and no extracellular materials can be seen in the treated embryos. (*D*) Blastocoelic floor of a PPMP-treated embryo. (*E*) Blastocoelic floor of a control embryo. Note the presence of numerous cellular projections and extracellular matrix deposition (arrowhead). Scale bars represent: *A*, 150 µM; *B*, 50 µm; *C*, 20 µm; *D*, 15 µm; *E*, 15 µm.

mesodermal cell populations was affected by treatment with PPMP. The main effect observed was a severe decrease in the number and complexity of the cellular processes of mesodermal cells, particularly those involved in cell-cell and cell-ECM interactions, such as lamellipodia and filopodia (Fig. 3*B*, *D*). Changes in the intercellular contacts were also observed. The ectodermal cells of the blastocoelic roof lost their epithelial morphology and acquired a rounded shape (Fig. 2*C*).

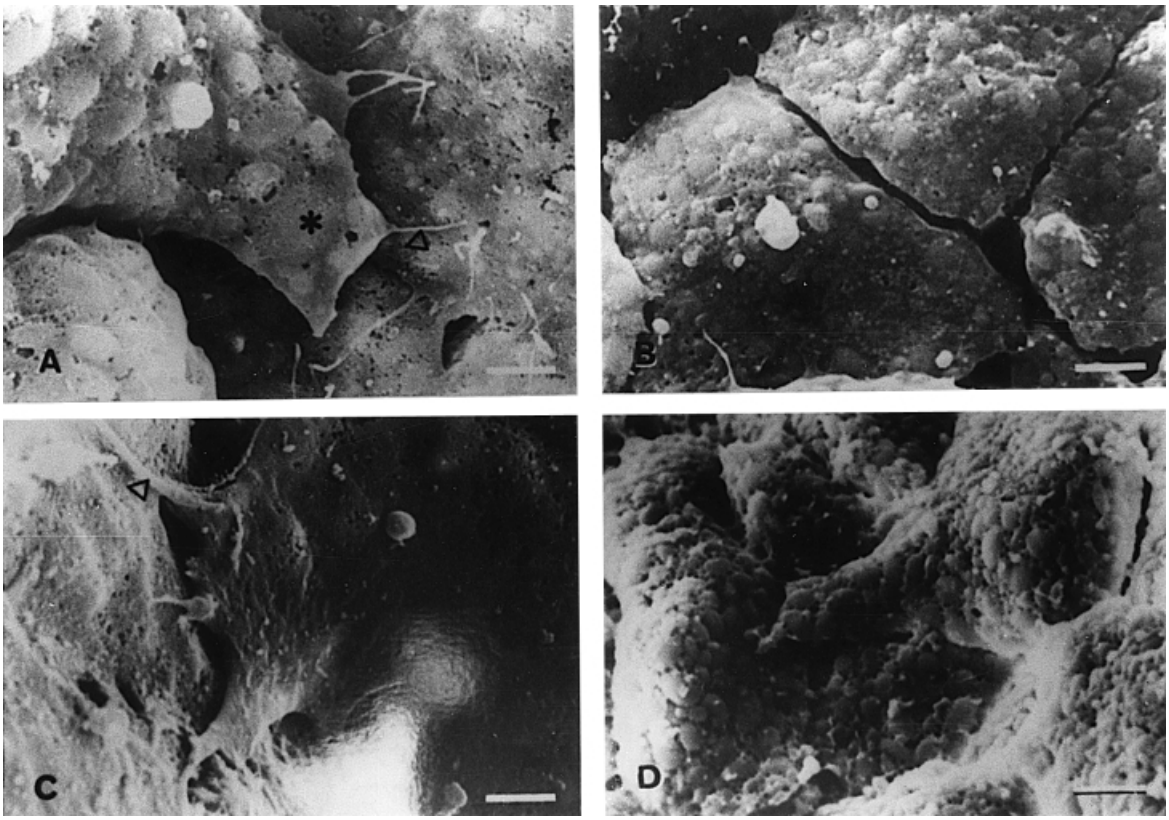

Figure 3 Effect of PPMP on mesodermal cell morphology. (*A*), (*C*) Cellular projections of mesodermal cells of stage 10.5 control embryos. Migrating mesodermal cells show lamellipodia and filopodia contacting between the cells and the extracellular matrix. (*B*), (*D*) Mesodermal cells of PPMP-treated embryos that showed a 1 to 1.5 stage delay in their development (fixed when control embryos reached stage 11). Note the decrease in the number and complexity of cellular processes. Scale bars represent: *A*, 3.5 µm; *B*, 10 µm; *C*, 5 µm; *D*, 10 µm.

Table 3 Effect of PPMP in the development of *Bufo arenarum* embryos and in cellular adhesion to native extracellular matrix substrate[a]

Inhibitor	No. of embryos	Normal embryos	Abnormal embryos[b]	Cell adhesion[c]
–	300	93.67 ± 3.51	6.33 ± 3.51	94.33 ± 1.52
PPMP (20 µM)	300	–	100	5.33 ± 2.50
PPMP (20 µM) + GlcCer (20 µM)	300	90.66 ± 1.52	9.34 ± 1.52	85.33 ± 3.51
Ceramide (20 µM)	300	92.67 ± 2.51	7.33 ± 2.51	92.00 ± 3.60

GlcCer, glucosylceramide.

[a]The values represent the mean ± SD of three different experiments. Blastula embryos (stage 9) were cultured for 24 h with or without PPMP, GlcCer and ceramide in 10% Ringer's solution.

[b]The progression of gastrulation was assessed by observing the external criteria for gastrulation defined by Del Conte & Sirlin (1951).

[c]Expressed as the percentage of cells firmly adhered to the substratum. Treated and control embryos were dissociated and mesodermal cells cultured on native extracellular matrix substratum as described under Materials and Methods.

From the above, we thought it a matter of interest to determine whether PPMP induced changes in the adhesiveness of mesodermal cells. Changes in cell adhesion were analysed in dissociated mesodermal cells isolated from the controls and from PPMP-treated embryos (Table 3). After a 90 min incubation on a native extracellular matrix substrate, mesodermal cells from control embryos adhered and spread normally, becoming flattened and polygonal. In contrast, PPMP-treated mesodermal cells remained spherical and showed neither attachment to the substratum nor spreading. PPMP induced a strong inhibition in the

ability of mesodermal cells to adhere to the native ECM substrate. The addition of GlcCer to the PPMP-containing medium in order to bypass the inhibition restored to normality the diminished adhesion of mesodermal cells. Moreover, treatment of embryos with 20 μM ceramide produced no effect on the adhesion properties of isolated mesodermal cells.

Effect of PPMP on the expression of the GM1 ganglioside

Whole-mount immunocytochemistry was used to study the expression of GM1 on the cell surfaces of embryo regions that actively participate in morphogenetic gastrulation movements. Embryos cultured in the presence of 20 μM PPMP exhibited a loss of expression of the GM1 ganglioside on the inner surface of the blastocoelic roof (Fig. 4*A*). In contrast, control embryos showed positive GM1 staining that became more strongly marked at the zones of intercellular contacts and around the cell periphery (Fig. 4*B*).

Discussion

Glycosphingolipids have been suggested to play various important roles during nervous system development in many biological systems (see Rösner, 1993) but little information is available as regards the presence and participation of glycosphingolipids in early embryonic development. Previous studies in amphibians suggested that cell surface N-linked glyconjungates (Sanchez & Barbieri, 1983; Romanosky & Nosek, 1980) and glycolipids (Peralta *et al.*, 1995) are important recognition factors during embryonic development. There is increasing evidence that GSL and especially gangliosides are expressed during amphibian development and may be involved in the cellular events that take place during development (Turner *et al.*, 1992; Gornati *et al.*, 1995; Kubo *et al.*, 1995).

In 1974, Stearns suggested that yolk platelets provide the organic components used during segmentation and in cell–cell recognition during gastrulation (Stearns, 1974). More recently, Kuksis (1992) proposed that glycosphingolipid could be synthesised or stored in amphibian eggs during vitellogenesis. However, the nature of the cell surface molecules that influence the progression of morphogenesis remains only partially understood. To better understand the role of GSL in amphibian development, we analysed the *de novo* synthesis of neutral glycosphingolipids and gangliosides through the initial developmental stages of *Bufo arenarum* embryos. Ganglioside synthesis began at the blastula stage and reached a maximum during gastrulation (stages 10–12). These changes suggest that gangliosides could be developmentally regulated. Although neutral glycosphingolipid synthesis increased gradually during the analysed period, ganglioside synthesis appeared to be quantitatively more significant than neutral glycolipid synthesis. These results are in agreement with those of Gornati *et al.* (1995, 1997) who, working on the development of *Xenopus laevis*, reported very low amounts of neutral glycolipids together with low activity of the various enzymes involved in the biosynthetic pathway of these glycolipids.

In the present study, we investigated the consequences of a blockage in the synthesis of GSL on embryonic development using D-threo-PPMP (PPMP), a potent inhibitor of UDP-glucose:ceramide glucosyltransferase (Radin *et al.*, 1993; Abe *et al.*, 1992, 1995). This inhibitor provides a new method for reducing gly-

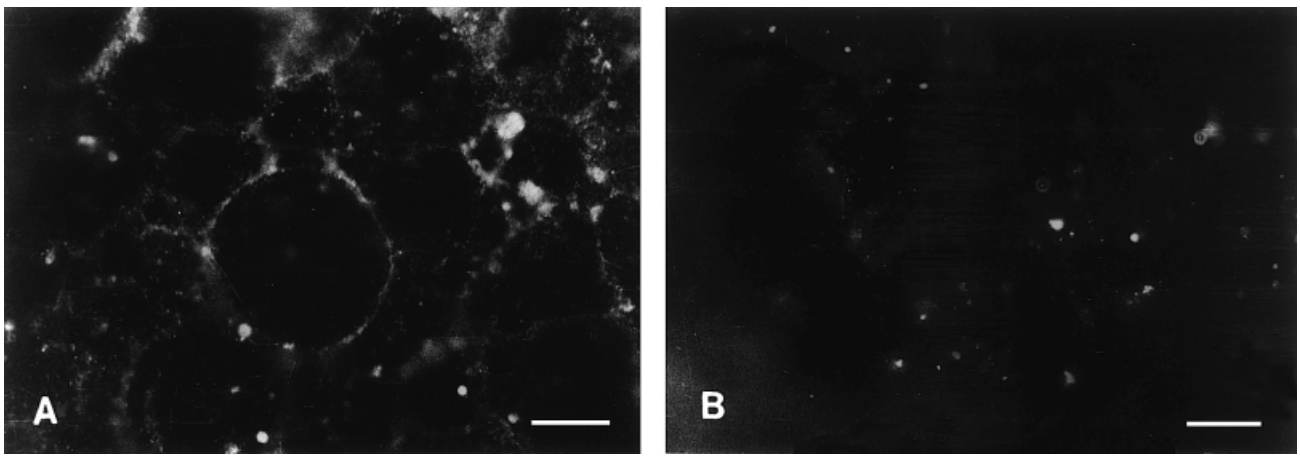

Figure 4 Effect of PPMP on GM1 ganglioside expression. (*A*) *In situ* distribution of GM1 in whole mounts of the inner surface blastocoelic roofs of stage 11 control embryos. (*B*) *In situ* distribution of GM1 on the inner surface of the blastocoelic roof of PPMP-treated embryos (fixed when controls reached stage 11). PPMP caused depletion of glycosphingolipids from cellular membranes and no specific fluorescence can be seen. Scale bars represent 20 μm.

cosphingolipid biosynthesis and decreasing the amount of cellular glycosphingolipids. Thus, PPMP and its congeners have been shown to effectively deplete GSL from the cellular membranes of 3T3 cells (Inokuchi & Radin, 1987), Madin-Darby canine kidney cells (Abe *et al*., 1992), neuroblastoma cell lines (Uemura *et al*., 1991), A431 cells (Barbour *et al*., 1992) and many other cellular systems.

We found that PPMP had a pronounced effect on development since no embryos exhibited normal gastrulation; their developmental rate either slowed down or, more often, became totally arrested. To investigate the possibility of a direct correlation between PPMP-induced inhibition of GSL synthesis and arrested gastrulation, we determined the amount of radioactive glucosamine in both neutral GSL and gangliosides as a measure of the biosynthesis of the molecules synthesised from GlcCer. The incorporation of a radioactive precursor into gangliosides that are synthesised distally from the PPMP blocking site was lower than that found in neutral GSL. Consequently, the most strongly affected glycolipid synthesis was that of gangliosides. This fact suggests that embryonic cells might use GlcCer to make complex gangliosides. In this context, results showed that complex GSL are required for normal gastrulation. In agreement with this fact, when the PPMP-containing medium was supplemented with GlcCer, mesodermal cell adhesiveness and complete gastrulation returned to normal. In contrast with our results, in the living medaka fish PDMP produced embryos that developed almost normally even though glycolipid synthesis and antigen cell surface expression were reduced (Fenderson *et al*., 1992).

The morphological analysis of arrested embryos evidenced the inhibition of morphogenetic movements since no radial interdigitation, epiboly of the ectodermal wall, mesodermal cell migration or convergence-extension of marginal zones could be observed. The analysis of mesodermal cell morphology in those embryos showed a severe decrease in the number and complexity of cellular extensions such as filopodia and lamellipodia. These cells also lost their cell-cell and cell-contacts. This observation was confirmed by studying *in vitro* cellular adhesiveness to the native ECM substrate. In *in vitro* culture mesodermal cells normally attach, spread and migrate on ECM protein substrates (Alfandari *et al*., 1995; Ramos *et al*., 1996). However, mesodermal cells isolated from PPMP-treated embryos evidenced very low adhesion percentages. Recently, various gangliosides have been proposed as mediators of neuronal (Hakomori, 1990; Zeller & Marchese, 1992) and embryonic chick retinal neural cell adhesion (Blackburn *et al*., 1986). In non-neuronal cells, such as melanoma cells (Inokuchi *et al*., 1989), human leukaemia cells (Kan & Kolesnick, 1992) and GSL-deficient mutant cell lines (Hidari *et al*., 1996), adhesion was reduced when GSL were depleted from the plasma membrane by PDMP. Three is also considerable evidence demonstrating that some GSL are located at the focal contact and interact with integrins (Cheresh *et al*., 1984, 1987; Zheng *et al*., 1992, 1993; Barletta *et al*., 1993; Merzak *et al*., 1995), which are already known to play a role in amphibian development (Alfandari *et al*., 1995; Ramos *et al*., 1996; Lallier *et al*., 1996). It has been suggested that the adhesion of the cells to ECM proteins could be mediated by complex glycosphingolipids and integrins at the focal contact. Consequently, the absence of complex glycosphingolipids may disrupt the adhesive function of integrins, thus leading to altered development.

A signal transduction pathway initiated by sphingomyelin hydrolysis and leading to the generation of the second messenger ceramide has been proposed (Hannun *et al*., 1996; Kolesnick & Krönke, 1998). Although there is increasing information as regards the ceramide pathway, its involvement during early development has not been explored. It has been suggested that PPMP treatment might raise ceramide levels (Schwarz *et al*., 1995; Posse de Chaves *et al*., 1997). Consequently, when using PPMP, it is essential to distinguish between effects caused by changes in the levels of this bioactive intermediate and the effects caused by the depletion of complex glycosphingolipids. In our experiments, the lack of effect on embryonic development of incubation with ceramide and the ability of GlcCer to reverse the effects of PPMP even in presence of the inhibitor, strongly suggest that the alterations found in gastrulation are due to the depletion of complex GSL. A similar explanation has been suggested for the changes in cell morphology and growth produced by PPMP in hippocampal neurons (Schwarz *et al*., 1995) and Swiss 3T3 fibroblasts (Meivar-Levy *et al*., 1997).

There is increasing evidence that GSL are not randomly distributed at the cell membrane but rather are self-assembled to form clusters or glycosphingolipid-enriched microdomains. These structures were found participating in both cell adhesion and signal transduction through a coupled mechanism (Iwabuchi *et al*., 1998*a, b*; Hakomori *et al*., 1998). It is probable that GSL depletion by PPMP treatment causes a failure in such functional domains, but whether or not a similar process occurs in amphibian cells during development certainly deserves additional investigation.

In this study we have shown developmentally associated changes in glycosphingolipid biosynthesis. Evidence has been presented that glycosphingolipids participate in *Bufo arenarum* gastrulation, probably through their involvement in cell adhesion events. However, further studies will be necessary to provide deeper insights into the involvement of glycosphingolipids in the complex molecular mechanisms of gastrulation.

Acknowledgements

This research was supported by CONICET and CIUNT grants to S.S.S. and by a FONCYT PICT98 grant to M.J.A. We wish to thank Mr E. Dozetos for his photographic assistance and Mrs Virginia Méndez for her proofreading.

References

Abe, A., Inouchi, J., Jimbo, M., Shimeno, N., Nagamatsu, A., Shayman, J.A. & Radin, N.S. (1992). Improved inhibitors of glycosylceramide synthase. *J. Biochem.* **111**, 191–96.

Abe, A., Radin, N.S., Shayman, J.A., Wotring, L.L., Zipkin, R.E., Sivakumar, R., Ruggieri, J.M., Carson, K.G. & Ganem, B. (1995). Structural and stereochemical studies of potent inhibitors of glucosylceramide synthase and tumor cell growth. *J. Lipid Res.* **36**, 611–21.

Alfrandari, D., Whittaker, C.A., DeSimone, D.W. & Darribere, T. (1995). Integrin alpha V subunit is expressed on mesodermal cell surfaces during amphibian gastrulation. *Dev. Biol.* **170**, 249–61.

Aybar, M.J., Genta, S.B., Villecco, E.I., Sánchez Riera, A.N. & Sánchez, S.S. (1996). Amphibian *Bufo arenarum* vitronectin-like protein: its localization during oogensis. *Int. J. Dev. Biol.* **40**, 997–1008.

Barbieri, F.D., Sánchez, S.S. & Del Pino, E. (1980). Changes in lectin-mediated agglutinability during primary embryonic induction in the amphibian *Bufo arenarum*. *J. Embryol. Exp. Morphol.* **57**, 95–106.

Barbour, S., Edidin, M., Felding-Habermann, B., Taylor-Norton, J., Radin, N.S. & Fenderson, B.A. (1992). Glycolipid depletion using a ceramide analogue (PDMP) alters growth, adhesion, and membrane lipid organization in human A431 cells. *J. Cell Physiol.* **150**, 610–19.

Barletta, E., Mugnai, G. & Ruggieri, S. (1993). Complex gangliosides modulate the integrin-mediated adhesion in a rat hepatoma cell line. *Biochem. Biophys. Res. Commun.* **192**, 214–22.

Barth, L.G. & Barth, L.J. (1959). Differentiation of cells of *Rana pipiens* gastrula in unconditional medium. *J. Embryol. Exp. Morphol.* **7**, 210–20.

Blackburn, C.C., Swank-Hill, P. & Schaar, R.L. (1986). Gangliosides support neural retina cell adhesion. *J. Biol. Chem.* **261**, 2873–81.

Carson, D.D. & Lennarz, W.J. (1979). Inhibition of polyisoprenoid and glycoprotein synthesis causes abnormal embryonic development. *Proc. Acad. Natl. Sci. USA* **76**, 5709–13.

Cheresh, D.A., Harper, J.R., Schulz, G., Reisfeld, R.A. (1984). Localisation of the gangliosides GD2 and GD3 in adhesion plaques and on the surface of human melonomal cells. *Proc. Natl. Acad. Sci. USA* **81**, 5767–71.

Cheresh, D.A., Pytela, R., Piershbacher, M.D., Kilier, F.G., Ruoslahti, E. & Reisfeld, R.A. (1987). An Arg-Gly-Asp-directed receptor on the surface of human melanoma cells exists in a divalent cation-dependent functional complex with the disialonganglioside GD2. *J. Cell Biol.* **105**, 1163–73.

Daniotti, J.L., Landa, C.A., Gravotta, D. & Maccioni, H.J.F. (1990). GD3 ganglioside is prevalent in fully differentiated neurons from rat retina. *J. Neurosci. Res.* **26**, 436–46.

Del Conte, E. & Sirlin, J.L. (1951). Serie tipo de los primeros estadios embrionarios en *Bufo arenarum*. *Zata Zool. Lilloana* **12**, 495–9.

Eckelbarger, K.J. (1986). Vitellogenic mechanisms and the allocation of energy to offspring in polychaeks. *Bull. Marine Sci.* **39**, 426–43.

Fenderson, B.A., Ostrander, G.K., Hausken, Z., Radin, N.S. & Hakomori, S.I. (1992). A ceramide analogue (PDMP) inhibits glycolipid synthesis in fish embryos. *Exp. Cell Res.* **198**, 362–6.

Genta, S.B., Aybar, M.J., Peralta, M.A. & Sánchez, S.S. (1997). Evidence for the presence and participation of 85–75 kDa extracellular matrix components in cell interactions of *Bufo arenarum* gastrulation. *J. Exp. Zool.* **177**, 181–97.

Gornati, R., Rizzo, A.M., Tong, X.W., Berra, B. & Bernardini, G. (1995). Glycolipid patterns during *Xenopus* embryo development. *Cell Biol. Int.* **19**, 183–9.

Gornati, R., Basu, S., Bernardini, G., Rizzo, A.M., Rossi, F. & Berra, B. (1997). Activities of glycolipid glycosyltransferases and sialidases during the early development of *Xenopus laevis*. *Mol. Cell Biochem.* **166**, 117–24.

Hakomori, S.I. (1981). Glycosphingolipids in cellular interaction, differentiation and oncogenesis. *Annu. Rev. Biochem.* **50**, 733–64.

Hakomori, S.I. (1990). Bifunctional role of glycosphingolipids: modulators for transmembrane signaling and mediators for cellular interactions. *J. Biol. Chem.* **265**, 18713–16.

Hakomori, S.I., Handa, K., Iwabuchi, K., Yamamura, S. & Prinetti, A. (1998). New insights in glycosphingolipid function: 'glycosignaling domain', a cell surface assembly of glycophingolipids with signal transducer molecules, involved in cell adhesion coupled with signaling. *Glycobiology* **8**, XI–XIX.

Hannun, Y.A., Obeid, L.M. & Dbaibo, G.S. (1996). ceramide: a novel second messenger and lipid mediator. In *Handbook of Lipid Research*, vol. 8, *Lipid Second Messengers*, ed. R. Bell, pp. 177–203. New York: Plenum Press.

Hay, E.D. (1991). *Cell Biology of Extracellular Matrix*, 2nd edn. New York: Plenum Press.

Hidaro, K., Itonori, S., Sania, Y., Ohashi, M., Kasama, T. & Nagai, Y. (1991). Isolation and characterization of a monosialogangliopentaosyl ceramide from *Xenopus* oocytes. *J. Biol. Chem. (Tokyo)* **110**, 412–16.

Hidaro, K.I.P.J., Ichikawa, S., Fujita, T., Sakiyama, H. & Hirabayashi, Y. (1996). Complete removal of spingholipids from the plasma membrane disrupts cell to substratum adhesion of mouse melanoma cells. *J. Biol. Chem.* **271**, 14636–41.

Inokuchi, J. & Radin, N.S. (1987). Preparation of the active isomer of 1-phenyl-2-decanoylamino-3-morpholino-l-propanol, inhibitor of murine glucocerebroside synthase. *J. Lipid. Res.* **28**, 565–71.

Inokuchi, J., Momosaki, K., Shimeno, H., Nagamatsu, A. & Radin, N.S. (1989). Effects of D-threo-PDMP, an inhibitor of glucosylceramide synthetase, on expression of cell surface glycolipid antigen and binding to adhesive proteins by B16 melanoma cells. *J. Cell Physiol.* **141**, 573–83.

Iwabuchi, K., Yamamura, S., Prinetti, A., Handa, K. & Hokomori, S.I. (1998*a*). GM3 enriched microdomain involved in

cell adhesion and signal transduction through carbohydrate-carbohydrate interaction in mouse melanoma B16 cells. *J. Biol. Chem.* **273**, 9130–8.

Iwabuchi, K., Handa, K., Hakomori, S.I. (1998*b*). Separation of 'Glycosphingolipid signaling domain' from caveolin-containing membrane fraction in mouse melanoma B16 cells and its role in cell adhesion coupled with signaling. *J. Biol. Chem.* **273**, 33766–73.

Kan, C.C. & Kolesnick, R.N. (1992). A synthetic ceramide analog, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, selectively inhibits adherence during macrophage differentiation of human luekemia cells. *J. Biol. Chem.* **267**, 9663–7.

Keller, R.E. (1986). The cellular basis of amphibian gastrulation. In *Developmental Biology*, vol. 2, *The Cellular Basis of Morphogenesis*, ed. L.W. Browder, pp. 241–327. New York: Plenum Press.

Kolesnick, R.N. & Krönke, M. (1998). Regulation of ceramide production and apoptosis. *Annu. Rev. Physiol.* **60**, 643–65.

Kopitz, J. (1997). Glycolipids: structure and function. In *Glycosciences*, ed. H.J. Gabius & S. Gabius, pp. 163–89. London: Chapman & Hall.

Kubo, H., Kotani, M., Ozawa, H., Kawashima, I., Tai, T. & Suzuki, A. (1995). Differential distribution of ganglioside GM1 and sulfatide during the development of *Xenopus* embryos. *Dev. Growth Differ.* **37**, 243–55.

Kuksis, A. (1992). Yolk lipids. *Biochim. Biophys. Acta* **1124**, 205–22.

Lallier, T.E., Whittaker, C.A. & Desimone, D.W. (1996). Integrin α6 expression is required for early nervous system development. *Development* **122**, 2539–54.

Meivar-Levy, I., Sabany, H., Bershadsky, A.D. & Futerman, A.H. (1997). The role of sphingolipids in the maintenance of fibroblast morphology. *J. Biol. Chem.* **272**, 1558–64.

Merzak, A., Koochekpour, S. & Pilkington, G.J. (1995). Adhesion of human glioma cell lines to fibronectin, laminin, vitronectin and collagen is modulated by angliosides *in vitro*. *Cell Adh. Commun.* **3**, 27–43.

Milone, M., Iela, L., Esposito, V., Rastogi, R.K. & Chieffi, G. (1978). Annual variation in the total lipid and protein content of the liver, fat, body, ovary and plasma of female frog (*Rana esculenta* L.). *J. Endocrinol.* **78**, 165–.

Nakatsuji, N. & Johnson, K.E. (1983). Comparative study of extracellular fibrils on the ectodermal layer in gastrulae of five amphibian species. *J. Cell Sci.* **59**, 61–70.

Peralta, M.A., Aybar, M.J. & Sánchez, S.S. (1995). Alteration of morphogenetic cell interactions by 1-β-D-arabinofuranosylcytosine in *Bufo arenarum* embryo. *Biocell* **19**, 159–73.

Posse de Chaves, E.I., Bussière, M., Vance, D.E., Campenot, R.B. & Vance, J.E. (1997). Elevation of ceramide within distal neurites inhibits neurite growth in cultured rat sympathetic neurons. *J. Biol. Chem.* **272**, 3028–35.

Radin, N.S., Shayman, J.A. & Inokuchi, J. (1993). Metabolic and functional effects of inhibiting glucosylceramide synthesis with PDMP and other substances. *Adv. Lipid Res.* **26**, 183–213.

Ramos, J.W., Whittaker, C.A. & Desimone, D.W. (1996). Integrin-dependent adhesive activity is spatially controlled by inductive signals at gastrulation. *Development* **122**, 2873–83.

Romanosky, A. & Nosek, J. (1980). The effects of tunicamycin and 2-deoxy-D-glucose on the development of *Xenopus laevis* embryos. *Wilhelm Rouxs Arch. Dev. Biol.* **189**, 81–2.

Rösner, H. (1993). Developmental expression of gangliosides *in vivo* and *in vitro*. In *Polysialic Acid*, ed. J. Roth, U. Rutishauser & F.A. Troy II, pp. 279–297. Basel: Birkhäuser.

Sánchez, S.S. & Barbieri, F.D. (1983). Developmental and biochemical alteration caused by tunicamycin in *Bufo arenarum* embryos. *Wilhelm Rouxs Arch. Dev. Biol.* **192**, 37–41.

Schwarz, A., Rapaport, E., Hirschberg, K. & Futerman, A.H. (1995). A regulatory role for sphingolipids in neuronal growth. *J. Biol. Chem.* **270**, 10990–8.

Spiegel, S., Matyas, G.R., Cheng, L. & Sacktor, B. (1988). Asymmetric distribution of gangliosides in rat renal brush-border and basolateral membranes. *Biochim. Biophys. Acta.* **938**, 270–8.

Stearns, L.W. (1974). *Sea Urchin Development*, pp. 35–8. Dowden, Hutchinson and Ross, Stroudsburg, PA.

Turner, A.P., Brown, D., Heasman, J., Cook, G.M.W., Evans, J., Vickers, L. & Wylie, C.C. (1992). Involvement of a neutral glycolipid in differential cell adhesion in the *Xenopus* blastula. *EMBO J.* **11**, 3845–55.

Uemura, K.I., Sugiyama, E. & Taketomi, T. (1991). Effects of an inhibitor of glucosylceramide synthase and neurite outgrowth in murine neuroblastoma cell lines. *J. Biochem.* **110**, 96–192.

Vilecco, E.I., Aybar, M.J., Sánchez Riera, A.N. & Sánchez, S.S. (1999). Comparative study of vitellogenesis in the anuran amphibians *Ceratophrys cranwelli* (Leptodactilidae) and *Bufo arenarum* (Bufonidae). *Zygote* **7**, 11–19.

Wu, G., Lu, Z.H. & Leeden, R.W. (1995). Induced and spontaneous neuritogenesis are associated with enhanced expression of ganglioside GM1 in the nuclear membrane. *J. Neurosci.* **15**, 3739–46.

Zeller, C.B. & Marchese, R.B. (1992). Gangliosides as modulators of cell function. *Am. J. Physiol.* **262**, 1341–55.

Zheng, M., Tsuruoka, T., Tsuji, T. & Hakomori, S.I. (1992). Regulatory role of GM3 ganglioside in integrin function, as evidenced by its effect on function of α5β1-liposomes: a preliminary note. *Biochem. Biophys. Res. Commun.* **186**, 1397–402.

Zheng, M., Fang, H., Tsuruoka, T., Tsuji, T., Sasaki, T. & Hakomori, S.I. (1993). Regulatory role of GM3 ganglioside in alpha5beta1 integrin receptor for fibronectin-mediated adhesion of FUA169 cells. *J. Biol. Chem.* **268**, 2217–22.