Inhibition of glycosphingolipid synthesis induces p34^{cdc2} activation in *Xenopus* oocyte

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Abstract In *Xenopus* prophase-blocked oocytes, it is assumed that progesterone interacts with the plasma membrane to initiate a signalling cascade that ultimately leads to MPF activation. Progesterone regulates negatively the cAMP pathway through an inhibition of adenylate cyclase. However, the mechanisms linking the initial action of the hormone with adenylate cyclase activity remain to be elucidated. Here, we demonstrate that PDMP, an inhibitor of glucosphingolipid synthesis, triggers oocyte meiotic maturation in a cAMP- and cycloheximide-dependent manner, whereas exogenous ceramide is unefficient. We propose that sphingolipid metabolism and targeting represent an important regulatory process of oocyte meiosis.

Key words: p34^{cdc2} kinase; *Xenopus* oocyte; Ceramide; Glucosylceramide synthase; Sphingomyelin

1. Introduction

Sphingolipids were recently identified as potential second messengers in the regulation of cell proliferation, cell division, as well as mediators of cell death [1–4]. Varnold and Smith [5,6] reported that sphingomyelinase (SMase) from *Staphylococcus aureus* is a potent inducer of germinal vesicle breakdown (GVBD) in full-grown prophase-blocked oocytes of *Xenopus*. This result suggests a role for the sphingomyelin pathway (Fig. 1) in *Xenopus* oocytes in the activation of M-phase promoting factor (MPF), a complex between the p34^{cdc2} kinase and cyclin B responsible for entry into M-phase of the eukaryotic cell cycle [7]. Very recently, while this work was under progress, Strum et al [8] confirmed that exogenous SMase induces activation of p34^{cdc2} and GVBD in *Xenopus* oocytes, in the absence of the physiological inducer progesterone. Since exogenous SMase would be expected to catalyze the hydrolysis of membrane sphingomyelin into ceramide and phosphocholine [9], it may be assumed that a transient increase in ceramide or in ceramide metabolites stimulates a signalling pathway leading to the activation of MPF. The effect of exogenous SMase raises important questions, among them: are sphingolipids involved in the poorly understood initial action of progesterone, the natural inducer of MPF and meiotic maturation [10]? Which cellular targets are stimulated by sphingolipids and how are they connected with the p34^{cdc2} activating pathway?

In a first approach to answer these questions, we investigated the effects of different agents that interfer with the metabolism of sphingolipids. After having confirmed that addition of various SMases to oocyte culture medium induces maturation, we now report that DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), an inhibitor of glucosylceramide synthase [11] (Fig. 1), an enzyme localized in the pre-Golgi/Golgi apparatus, induces in a dose-dependent manner an apparent normal meiotic maturation. Interestingly, at low doses inefficient to cause GVBD, PDMP potentializes progesterone-induced maturation.

These data strengthen the importance of the sphingomyelin pathway in the mechanism of oocyte maturation. They also indicate that the metabolism of sphingolipids is highly regulated in the *Xenopus* prophase-arrested oocyte.

2. Materials and methods

2.1. Materials

Xenopus laevis adult females (Centre de Recherche de Biochimie Macromoléculaire, CNRS, Montpellier, France) were bred and maintained under laboratory conditions. Sphyngomyelinases (SMases) from *Staphylococcus aureus* and from *Streptomyces sp.* are references 8633 and 8889 from Sigma, respectively. DL-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is from Biomol (reference SL-210). Reagents, unless otherwise specified, were from Sigma.

2.2. Oocyte treatments

Isolated oocytes were prepared as described in ref. [12]. Germinal vesicle breakdown (GVBD) was ascertained by the absence of the germinal vesicle determined by oocyte dissection after fixation in 10% trichloroacetic acid. For some experiments, prophase oocytes were pretreated for 1 h in the presence of 100 μg/ml cycloheximide, or 1 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.1 μg/ml cholera toxin. For kinetic analysis throughout post-GVBD maturation, prophase oocytes from a single female were treated with progesterone or PDMP. Oocytes just undergoing GVBD (first pigment rearrangement) were collected within a window of 5 min and these 'synchronized' oocytes were pooled. At regular intervals, oocytes were removed from the pooled oocytes and analyzed for histone H1 kinase assay.

2.3. Extract preparation

3 oocytes were washed in 1 ml MPF extraction buffer (EB: 80 mM β-glycerophosphate pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors: 2 mM phenylmethanesulfonylfluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 10 μg/ml pepstatin, 0.1 mg/ml soybean trypsin inhibitor and 1 mM benzamidine), homogenized in 50 μl EB at 4°C, and centrifuged at 100,000 rpm at 4°C for 10 min in a TL-100 Beckman centrifuge (TLA-100 rotor). The supernatant was collected for p13 binding and histone-H1 kinase assays as described in [13]. For Western blot analysis, 15 oocytes were homoge-

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Abbreviations: ER, endoplasmic reticulum; GVBD, germinal vesicle breakdown; IBMX, 3-isobutyl-1-methylxantine; MAP kinase, mitogen-activated protein kinase; MPF, M-phase promoting factor; PDMP, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; SMase, sphingomyelinase.

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nized in 40 μl of EB, centrifuged as previously described, and 15 μl of Laemmli buffer [14] was added to the supernatant. Proteins were electrophoresed and transferred to nitrocellulose filters (Schleicher and Schull) as described in ref. [13]. The anti-phosphotyrosine antibody is described in ref. [13] and the anti-MAP kinase antibody provided by Dr. J. Pouyssegur recognizes *Xenopus* oocyte p42 MAP kinase [15]. Anti-phosphotyrosine and anti-MAP kinase antibodies were revealed by autoradiography after [^{125}I]protein-A incubation (Dupont NEN).

2.4. Immunocytochemical procedures

Oocytes were fixed in 1% acetic acid in 95° ethanol. Microtubular structures were revealed on cryostat serial sections (10 μm) by immunofluorescence with an anti-*Xenopus* tubulin antibody [16] and a FITC-labelled anti-guinea pig IgG (Biosys BI3105).

3. Results

3.1. Exogenous ceramide does not promote GVBD in oocytes from unstimulated females

It has been recently reported that oocyte external treatment in the presence of *Staphylococcus aureus* SMase induces meiotic maturation of *Xenopus* oocytes [5,6,8]. We confirmed and extended these results with two SMases, extracted from *Staphylococcus aureus* and *Streptomyces* (see further). These results suggest that an accumulation of ceramide could in vivo promote MPF activation and meiotic maturation. However, in contrast with the results of Strum et al. [8], incubation or microinjection of oocytes with C2- or C8-ceramide, cell permeable short chain ceramide, failed to induce GVBD at all tested concentrations (20 to 500 μM). The lack of effect of ceramide led us to test other metabolites known to act on sphingolipid metabolism. Fumonisin b_1, a natural inhibitor of ceramide synthase [17,18] (Fig. 1), neither induces maturation nor inhibits progesterone-induced maturation up to 50 μM, indicating that the biosynthetic pathway of ceramide synthesis is not critical for release of the prophase block. The possibility that metabolism between sphingolipids and glycosphingolipids (Fig. 1), which have been characterized in *Xenopus* oocyte membranes [19], is involved in MPF activation was then investigated.

3.2. PDMP, an inhibitor of glucosylceramide synthase, induces GVBD in vivo

DL-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is an inhibitor of glucosylceramide synthase [11] (Fig. 1). When applied to cultured cells, PDMP carries a time- dependent depletion of glycosphingolipids and a secondary accumulation of sphingolipids [4,11]. PDMP is therefore an useful molecular tool to investigate the role of sphingolipids in regulation of specific cellular functions, including cell cycle [4,20]. Full-grown *Xenopus* oocytes were incubated in the presence of 10 to 300 μM PDMP. In at least 6 different females, 50% germinal vesicle breakdown (GVBD) was obtained with PDMP concentrations ranging from 50 to 150 μM. The appearance of the maturation white spot was slightly delayed when compared to control progesterone-induced GVBD (Fig. 2A). As progesterone-induced maturation, PDMP induced-maturation is in-

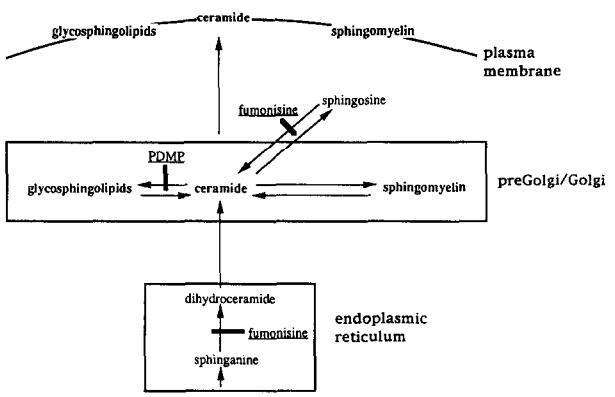

Fig. 1. Metabolic pathway of ceramide. This simplified scheme illustrates the role of ceramide in sphingolipid biosynthesis and catabolism, as well as the action level of the inhibitors used in this study.

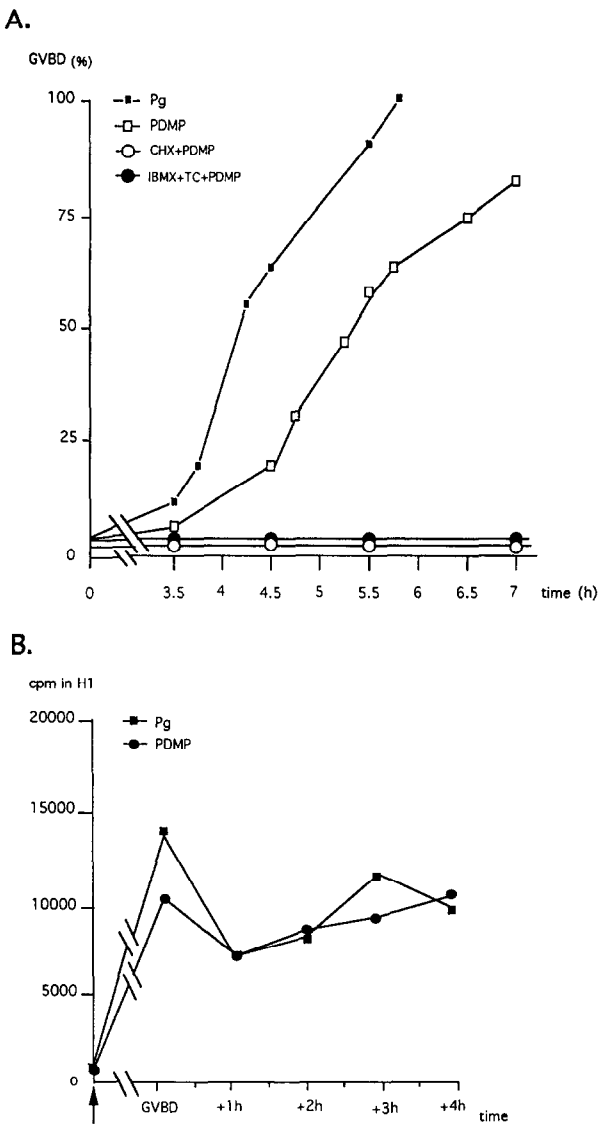

Fig. 2. PDMP induces GVBD in vivo. (A) Time course analysis of GVBD. (■—■): at time 0, prophase oocytes were incubated in the presence of 1 μM progesterone; (□—□): at time 0, prophase oocytes were incubated in the presence of 150 μM PDMP; (○—○): prophase oocytes were pretreated for 2 h in the presence of 100 μg/ml cycloheximide before 150 μM PDMP addition at time 0; (●—●): prophase oocytes were pretreated for 2 h in the presence of 1 mM IBMX and 0.1 μg/ml cholera toxin before 150 μM PDMP addition at time 0. (B) PDMP induces in vivo $p34^{cdc2}$ kinase activation. At time 0 (arrow), oocytes were incubated in the presence of either 150 μM PDMP (●—●) or 1 μM progesterone (■—■). H1 kinase activity of $p34^{cdc2}$ was estimated at various times after GVBD.

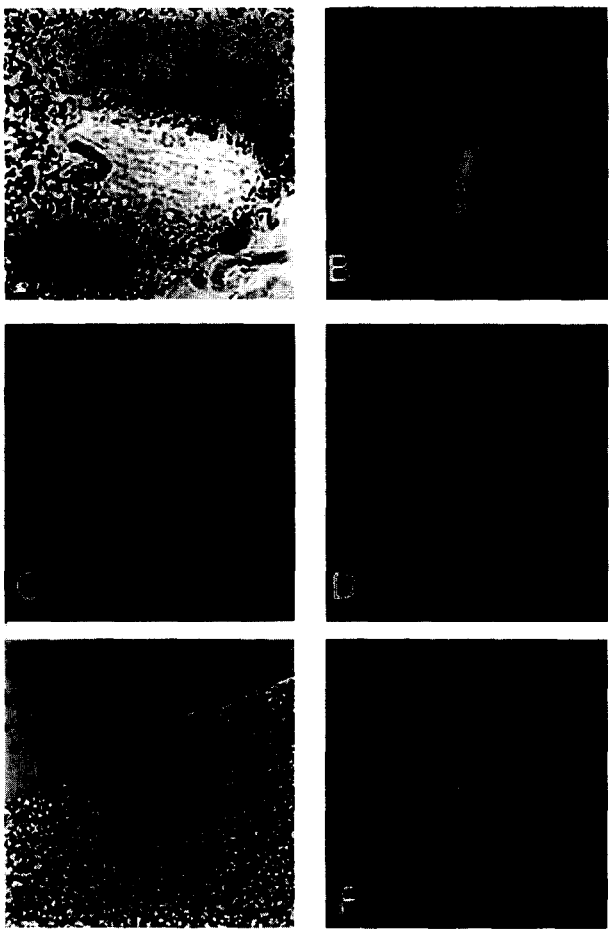

Fig. 3. Cytological analysis of oocytes treated with progesterone, PDMP and *Streptomyces* SMase. (A,B) Prophase-blocked oocytes were treated with 1 μM progesterone and fixed 3 h after GVBD (300 ×): (A) phase contrast; (B) Hoëchst dye staining; (C,D) Prophase oocytes were treated with 150 μM PDMP and fixed 3 h after GVBD (1500 ×): (C) anti-tubulin antibody staining; (D) Hoëchst dye staining; (E,F) Prophase oocytes were treated with 0.7 U/ml *Streptomyces* SMase and fixed 3 h after GVBD (1500 ×): (E) Phase contrast; (F) Hoëchst dye staining.

nibited either by cycloheximide or by an increase in the intracellular cAMP level (Fig. 2A). When PDMP-treated oocytes were cytologically analyzed 3 h after GVBD, a typical metaphase II-spindle was found anchored in the oocyte cortex, as after progesterone induction (Fig. 3A–D). Similarly, oocytes matured by external addition of *Streptomyces* SMase also exhibit a typical metaphase II-spindle (Fig. 3E,F).

3.3. PDMP induces in vivo $p34^{cdc2}$ kinase and MAP kinase activation

The histone H1 kinase activity of $p34^{cdc2}$ was measured at different times after PDMP addition. The activation of the kinase as well as fluctuations of its activity after GVBD were indistinguishable from those induced by progesterone (Fig. 2B). Immunoblots performed with an anti-phosphotyrosine antibody show that $p34^{cdc2}$ activation induced by PDMP or *Streptomyces* SMase correlates with tyrosine dephosphorylation of the protein at GVBD time (Fig. 4); interestingly, activation of MAP kinase, as judged by its retarded electrophoretic mobility, occurs at the same period (Fig. 4). As previously reported, tyrosine phosphorylation of MAP kinase was shown to correlate with its mobility shift at GVBD time during progesterone-induced maturation [15]. Similarly, it was ascertained that PDMP and SMase lead to tyrosine phosphorylation of MAP kinase at GVBD (not shown).

3.4. PDMP potentializes progesterone action

Since PDMP is able to trigger a physiological meiotic maturation, we investigated the possibility that ceramide metabolism interferes in vivo in the signalling cascade initiated by the physiological inducer of maturation, progesterone. Oocytes were pretreated for 18 h by a subthreshold concentration of PDMP (10 μM), a dose which is not able to promote maturation, and then incubated in the presence of 1 μM progesterone (Fig. 5A). Time course of the progesterone-induced maturation process was accelerated (Fig. 5A). Conversely, pretreatment of oocytes with 10^{-8} M progesterone, which is unable to elicit maturation, accelerates the action of 150 μM PDMP (Fig. 5B), demonstrating again that both signalling pathways of progesterone and sphingolipid metabolism in vivo are connected.

4. Discussion

Xenopus oocytes undergo normal meiotic maturation when treated with an inhibitor of glucosylceramide synthase. All criteria of PDMP-induced maturation which were analyzed (GVBD, $p34^{cdc2}$ and MAP kinase activation, formation of a metaphase II spindle) were found to be comparable to progesterone-induced maturation. Glucosylceramide synthase catalyzes the first step in the ganglioside biosynthetic pathway (Fig. 1); as other ganglioside glucosyltransferases, this enzyme is associated with the ER/Golgi membranes in *Xenopus* oocytes [19]. Therefore, the main effect of its inhibition by PDMP in whole oocyte should be an increase in the level of ceramide and sphingomyelin in the inner leaflet of membrane vesicles trafficking from the ER through the Golgi apparatus to the plasma membrane. Since fumonisine b_1, that inhibits ceramide synthesis [17,18] (Fig. 1), does not influence PDMP or progesterone-induced maturation, it may be assumed that PDMP modifies

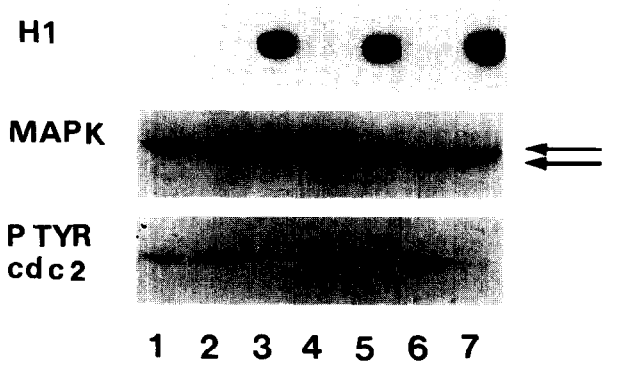

Fig. 4. PDMP and *Streptomyces* SMase induce activation of $p34^{cdc2}$ kinase and MAP kinase at GVBD time. Prophase oocytes were incubated in the presence of either 1 μM progesterone (lanes 2–3), or 0.7 U/ml *Streptomyces* SMase (lanes 4–5) or 150 μM PDMP (lanes 6–7). Oocytes were analyzed at two different times: 2 h after various treatments (lanes 2, 4, 6) and at GVBD time (lanes 3, 5, 7). Lane 1, control not treated prophase oocytes. Upper line: H1 kinase activity (autoradiograph); middle line: Western blot with an anti-MAP kinase; lower line: tyrosine phosphorylation level of $p34^{cdc2}$ revealed with an anti-phosphotyrosine antibody.

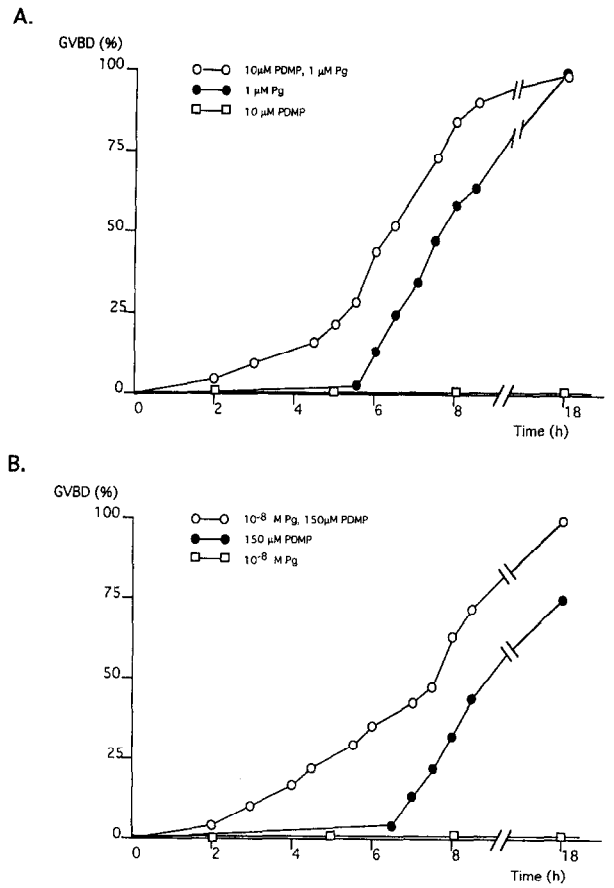

Fig. 5. Reciprocal facilitation of PDMP and progesterone action on meiotic maturation. GVBD was scored at various times after different treatments of prophase oocytes. (A) Prophase oocytes pretreated with 10 μM PDMP for 18 h and then incubated with 1 μM progesterone (○—○); prophase oocytes treated with 1 μM progesterone (●—●); prophase oocytes treated with 10 μM PDMP (□—□). (B) Prophase oocytes pretreated with 10^{-8} M progesterone for 18 h and then incubated with 150 μM PDMP (○—○); prophase oocytes treated with 150 μM PDMP (●—●); prophase oocytes treated with 10^{-8} M progesterone (□—□).

the interconversion between glycospingolipids, ceramide and sphingomyelin.

Our unexpected findings show that the metabolism of sphingolipids needs to be highly regulated in the prophase-blocked oocyte; they furthermore suggest the intriguing possibility that progesterone acts at the level of the membrane of the ER/Golgi vesicles. This hypothesis is reinforced by the recent finding that brefeldin A, that inhibits protein traffic in *Xenopus* oocytes, promotes meiotic maturation [21]. How does a local increase in the concentration of ceramide and sphingomyelin and the concomitant decrease of glycosphingolipids induce $p34^{cdc2}$ activation? $p34^{cdc2}$ is activated in vivo by tyrosine dephosphorylation, that is catalyzed by the cdc25 phosphatase [22]. Cdc25 is activated by phosphorylation by a not yet identified kinase, although it has been hypothetized that MPF itself and raf kinase could participate in ovo to this activating phosphorylation [23,24]. Protein phosphatase 2A, that is in vitro stimulated by ceramide [25,26], negatively regulates directly or indirectly this step [27–30]. The molecular mechanisms linking progesterone action and cdc25 and $p34^{cdc2}$ activation remain poorly understood. Among numerous possibilities, two non exclusive attractive hypotheses could now be envisaged: (1) A local modification in the sphingolipid composition of membrane vesicles changes their targeting towards the plasma membrane or other intracellular membrane compartments. A major consequence of these modifications could be that a key protein implicated in $p34^{cdc2}$ activation and interacting with sphingolipids changes its spatial localization, leading to its accessibility to the activation pathway of MPF. (2) A local release of ceramide from the vesicles could activate a key cytosolic enzyme involved upstream of the pathway leading to MPF activation.

The recent investigation of Strum et al. [8] favors this hypothesis, since they showed that in oocytes from *Xenopus* females primed with PMSG, ceramide microinjection induces maturation and that progesterone increases ceramide levels. These interesting results are, however, intriguing when basal and stimulated concentrations of ceramide are compared. As reported in [8], ceramide levels increase continuously during the maturation process, from 0.2–0.4 mM to reach 0.7 mM at the end of maturation. It is difficult to envisage that such high amounts can regulate enzymes that are known to be regulated by ceramide in the micromolar range [25,26,31]. Strum et al. [8] further reported that microinjection of 40 pmol (40 μM in the oocyte) of C2 or C6 ceramide induces a very slow maturation process. We repeated these microinjection experiments in oocytes from unstimulated *Xenopus* females; whereas PDMP and two different SMases induce a normal maturation in these oocytes, microinjection as well as external incubation with C2 or C6 ceramide were totally unefficient to promote entry into meiosis under our experimental conditions. It has to be noticed that in *Xenopus* oocytes from stimulated females, GVBD in response to progesterone occurs much earlier than in oocytes from unstimulated females. Thus, oocytes from stimulated females could be downstream in the sequence of events leading to GVBD [32]. Therefore, dependent on the actual physiological stage reached by the oocytes, ceramide could have different or opposite effects. Whatever the explanation of the contradiction between our results and data published in ref. [8], to further clarify a possible role of ceramide as second messenger, it would be important to understand the physiological meaning of the basal and stimulated levels of ceramide in whole oocytes.

In conclusion, both induction of maturation following sphingomyelin hydrolysis by SMase ([8]; these results) as well as by inhibiting glucosylceramide synthase by PDMP and the potentializing effect of PDMP on progesterone action indicate that sphingolipid metabolism is an important regulating process in the physiology of the oocyte. In the future, it will be important to determine whether ceramide acts on the sorting of an essential protein at the level of the Golgi apparatus or as a second messenger.

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