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QUARTERLY

Ca^{2+}-dependent phosphatidylserine synthesis in immature and mature starfish oocytes✪

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We found that in starfish oocytes two different enzymes, phosphatidylserine synthase-1 (PSS1) and -2 (PSS2), which synthesize phosphatidylserine by a base-exchange reaction, are present. We studied phosphatidylserine synthesis in immature oocytes which still contain the nucleus (germinal vesicles) and in mature cells, in which the re-initiation of the meiotic cycle induced by the hormone 1-methyladenine led to structural changes in the endoplasmic reticulum, to the disappearance of the nuclear envelope and to the intermixing of the nucleoplasm with the cytoplasm. It was found that the levels of PSS1 and PSS2 transcripts were higher in immature and mature oocytes, respectively. The level of the expressed PSS2 protein, higher than that of PSS1, was not influenced by the maturation process, whereas the level of PSS1 protein was higher in immature than in mature oocytes. Serine incorporation into phosphatidylserine was enhanced in immature oocytes. The depletion of calcium stores by thapsigargin resulted in 50% lowering of phosphatidylserine synthesis. We suggest that changes in phosphatidylserine synthesis may be affected by the release of calcium stored in the nuclear envelope and in the endoplasmic reticulum, the membranes that undergo disintegration and fragmentation during meiosis. The reason for the greater synthesis of PS may be the higher level of expression of PSS1 in immature oocytes.

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Abbreviations: CFSW, calcium free sea water; DAG, 1,2-diacylglycerol; ER, endoplasmic reticulum; $InsP_3$, 1,4,5-inositol trisphosphate; 1-MA, 1-methyladenine; Me_2SO, dimethylsulfoxide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C; PS, phosphatidylserine; PSS1, phosphatidylserine synthase-1; PSS2, phosphatidylserine synthase-2; SW, sea water.

In animal tissues, phosphatidylserine (PS) is synthesized *via* a Ca^{2+}-dependent serine base-exchange reaction, which requires high concentration of Ca^{2+} and takes place mainly in the endoplasmic reticulum (ER) (Kanfer, 1980; Barańska, 1982). The synthesis depends strongly on the high level of Ca^{2+} within the ER lumen (Barańska 1982; Czarny & Barańska, 1993); agonists that promote its release, e.g., thapsigargin, a selective inhibitor of the ER Ca^{2+}-ATPase, inhibits this process (Czarny *et al.*, 1992; Pelassy *et al.*, 1992; Czarny & Barańska, 1993; Rakowska & Wojtczak, 1995). PS is synthesized by at least two enzymes, phosphatidylserine synthase-1 (PSS1) and phosphatidylserine synthase-2 (PSS2) (Kuge *et al.*, 1991; Saito *et al.*, 1996; Kuge & Nishijima, 1997; Kuge *et al.*, 1997). They catalyse the exchange reaction between free serine and the base moiety of pre-existing phospholipids: phosphatidylcholine (PC) for PSS1 and phosphatidylethanolamine (PE) for PSS2 (Kuge *et al.*, 1986). The reaction occurs not only in mammals but also in Protozoa and plants as well (Barańska, 1989; Vincent *et al.*, 1999; 2001).

PS synthesis in the nucleus has also been well documented (van Golde *et al.*, 1974; Jelsema & Morré, 1978; Dygas *et al.*, 2000). We have recently shown that PS synthesis occurs in rat liver nuclei depleted of the outer membrane of the nuclear envelope (Dygas *et al.*, 2000). However, the role of PS in the nucleus is still obscure. It is generally accepted that PS is required for the association of protein kinase C (PKC) isoenzymes with membranes, and thus for full PKC activation. During the cell cycle, PKC isoenzymes are translocated from the cytoplasm to the nuclei (Martelli *et al.*, 1999). The differential activation and subcellular translocation of specific PKC isoenzymes could be thus linked to the generation of endogenous PKC activators in specific subcellular compartments. In the nucleus, activation of phospholipase Cβ results in the generation of 1,2-diacylglycerol (DAG), an activator of PKC (Nishizuka, 1995; D'Santos *et al.*, 1998; Martelli *et al.*, 1999; Chi & Crabtree, 2000; Dygas & Barańska, 2001). All conventional isoforms of PKC (α, β and γ) that have been detected in the nucleus are Ca^{2+}, DAG and PS dependent (Martelli *et al.*, 1999; 2002).

The aim of this study was to characterize PS synthesis in starfish oocytes, in which this process had not been examined earlier. We studied the incorporation of [^{14}C]serine into PS in oocytes arrested at the prophase of the first meiotic division, which still contain a large nucleus, called germinal vesicle, and in oocytes matured after addition of the hormone 1-methyladenine in which the nuclear envelope no longer exists. Our results indicate that PS synthesis is much higher in immature oocytes that contain the nucleus and is a calcium dependent process. We also show using RT-PCR analysis that in immature oocytes the expression level of the PSS1 enzyme mRNA is higher than that of PSS2.

MATERIALS AND METHODS

Preparation of gametes. *Astropecten auranciacus* starfish were collected in the Bay of Naples (Italy) during the breeding season. Starfish oocytes were extracted as described by Nusco *et al.* (2002). Maturation was promoted by adding 10 μM hormone 1-methyladenine (1-MA) (Sigma) for 30 min following oocytes washing in 470 mM NaCl, 10.1 mM KCl, 35.9 mM $MgCl_2$, 17.5 mM $MgSO_4$, (Merck, Darmstadt, Germany) and 20.0 mM Hepes, 2 mM EGTA (Sigma Chemical Company, St. Louis, MO, U.S.A.) adjusted to pH 8.0. This procedure rendered the oocytes free of the jelly coat and follicle cells. After washing several times in the same medium without EGTA the oocytes were sedimented by centrifugation at 100 × ***g*** for 0.5 min. The pellet was immediately used for serine base-exchange measurements.

Serine base-exchange activity. The serine base-exchange reaction was performed ac-

cording to Dygas *et al.* (2000) with some modifications. The incubation medium contained 1–1.2 mg protein in calcium free sea water (CFSW), 470 mM NaCl, 10.1 mM KCl, 35.9 mM $MgCl_2$, 17.5 mM $MgSO_4$, 20.0 mM Hepes, pH 8.0, or in sea water (SW), 470 mM NaCl, 10.1 mM KCl, 35.9 mM $MgCl_2$, 17.5 mM $MgSO_4$, 9.2 mM $CaCl_2$, 20.0 mM Hepes, pH 8.0, and 25.6 μM (2 μCi/ml) of L-[U-^{14}C]serine (spec. radioact. 151 mCi/mmol, Amersham International, Little Chalfont, Buckinghamshire, U.K.). Cells were incubated at room temperature for various periods of time, after which lipids were extracted using chloroform/methanol (1:2, v/v). Phospholipids were analysed as described by Czarny *et al.* (1992). Protein was determined according to Lowry *et al.* (1951) with the modification of Markwell *et al.* (1978).

Microinjections, photolysis of caged $InsP_3$ and Ca^{2+} imaging. The calcium fluorescent dye Oregon Green 488 BAPTA-1 coupled to a 70-kDa dextran (OGBD, Molecular Probes, Eugene, Oregon, U.S.A.), was injected into the cytoplasm or into the nucleus of immature oocytes and in the cytoplasm of mature cells as described by Santella & Kyozuka (1994). The concentration of the dye in the pipette (tip diameter 1 μm) was adjusted to 5 mg/ml with injection buffer (450 mM KCl, 10 mM Hepes, pH 7.0, with KOH). The volume of injected dye corresponded to 1–2% of the total cell volume so that the final concentration of the injected substances in the cellular environment was 50–100 times lower than in the micropipette. For the experiments with caged compound, the solution in the pipette contained 200 μM caged $InsP_3$ in injection buffer (Calbiochem, La Jolla, CA, U.S.A.). Cytoplasmic Ca^{2+} changes during thapsigargin treatment (Tocris, Co., U.K.) were measured every 1.3 s using a confocal laser scanning microscope (FVX-ZM-IL, Olympus, Japan). Caged $InsP_3$ was photoactivated by irradiating the oocyte with UV light (330 nm) emitted by a shutter-controlled (Lambda 10-2, Sutter Instruments Co., Novato, CA, U.S.A.) mercury lamp. Ca^{2+} changes following $InsP_3$ uncaging was measured with a cooled CCD camera (MicroMax, Princeton Instruments Inc., Trenton, NJ, U.S.A.). Fluorescence images were acquired with a Fluoview Personal Confocal Microscope System, and processed with the MetaMorph Imaging System (Universal Imaging Corporation, West Chester, PA., U.S.A.). To exclude variations in fluorescent intensity, the signals were corrected for changes in dye concentration by displaying the images in terms of the relative fluorescence $F_{rel} = [(f_t - f_0)/f_0]$, where f_t is the recorded fluorescence and f_0 is the mean of between five and eight sequential frames before UV irradiation with no apparent Ca^{2+} activity.

RT-PCR analysis. Total RNA from immature and mature oocytes was isolated initially in TRIZOL reagent (Sigma) to remove fats from the lipid-rich cells. For final RNA purification the Nucleospin RNA II kit (Marchery-Nagel, Düren, Germany) was used. First-strand cDNA was synthesized from 1 to 2 μg total oocyte RNA according to manufacturer's instruction enclosed to the Enhanced Avian First Strand Synthesis Kit (Sigma). Briefly, RNA was incubated in a 15 μl sample containing 1 μl of oligo $(dT)_{23}$ primer at 70°C for 10 min. Then the buffer, the RNase inhibitor and M-MuLV-Reverse Transcriptase (Stratagene, Cedar Creek, U.S.A.) were added and the samples were incubated 15 min at 25°C followed by 50 min incubation at 42°C. The cDNA was stored at −20°C.

Amplification of the cDNA products was performed exactly to the procedure described by Sturbois-Balcerzak *et al.* (2001) using the taq PCR Core kit (Quiagen, Hilden, Germany). β-Actin was used as an internal control. The amplification of β-actin was performed with sense 5′-TTGTAACCAACTGGGACGATATGG-3′ and antisense 5′-GATCTTGATCTTCATGGTGCT-3′ primers (all primers were synthesized by ARK Scientific GmbH Biosystems, Darmstadt, Germany). β-Actin was amplified under PCR conditions as for PSS. The PCR products were separated on 0.6%

agarose gels (Merck) stained with ethidium bromide (Sigma). DNA markers, from pUC19 (65–2364 base pairs) were from BTL (Łódź, Poland).

Immunoblotting. For immunoblotting, cellular membranes were obtained by lysis of oocytes performed in 20 mM Tris/HCl, 2 mM EDTA, 5 mM EGTA, pH 7.4, 100 mM DTT (BIO-RAD, Munchen, Germany), with 40 μM leupeptin, 1 mM PMSF, 1 mM aprotinin (all inhibitors were from Sigma). The lysate was centrifuged at 100 000 × ***g***. The pelleted membranes were then dissolved in a sample buffer consisting of 62.5 mM Tris/HCl, pH 6.7, 1% SDS, 2% mercaptoethanol (v/v), (Sigma), 10% glycerol and bromophenol blue (POCh, Gliwice, Poland) and frozen at –20°C. Just before electrophoresis, an appropriate amount of membrane material was incubated at 37°C for 30 min, and separated in a 10% polyacrylamide gel containing 0.1% SDS as described by Stone & Vance (2000) and electroblotted by the semidry method for 70 min to a HyBond CL membrane (Amersham Pharmacia Biotech., Little Chalfont, Buckinghamshire, U.K.). The bound antibody was detected by enhanced chemiluminescence according to the manufacturer's instruction in the ECL kit (Amersham Pharmacia Biotech.). Anti-PSS1 and anti PSS-2 antibodies were a generous gift of Dr. M. Nishijima and Dr. O. Kuge (National Institute of Infectious Diseases, Tokyo, Japan) and were used: anti-PSS1 at 1:2500 dilution and anti-PSS2 at 1:5000.

Annexin V-FITC binding. The annexin V-FITC kit (Bender MedSystem, Vienna, Austria) was used for the study of annexin V-FITC binding. Nuclei were manually isolated from starfish oocytes in 140 mM KCl and 10 mM Hepes pH 7.4. A few nuclei (1–5) were placed in 40 μl of 140 mM KCl, 10 mM Hepes, pH 7.4, on a coverslip at room temperature and 1 μl of annexin V-FITC solution was added. Optical analysis of the samples was performed using a digital video imaging system and MetaMorph software. One microliter of 100 mM $CaCl_2$ was then added and the annexin V-FITC fluorescence was monitored with an inverted confocal microscope (Olympus Fluoview).

RESULTS

Phosphatidylserine synthesis in immature and mature oocytes

Figure 1 shows L-[U-^{14}C]serine incorporation into PS in immature oocytes, not treated with the hormone and still containing the nucleus (Fig. 1A), and in mature oocytes 1 h after 1-MA treatment (Fig. 1B). Both oocyte types, deprived of follicle cells and of the jelly coat, were incubated in SW (filled squares) or in CFSW (filled circles) at room temperature. The figure shows that serine incorporation

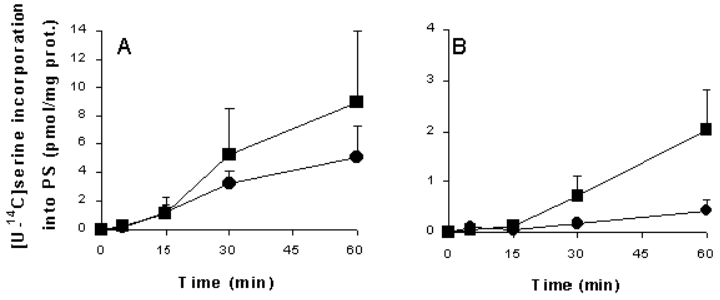

Figure 1. Time-dependence of L-[U-^{14}C]serine incorporation into phosphatidylserine in starfish oocytes.

The serine base-exchange reaction was measured in immature (**A**) and mature (**B**) oocytes incubated in SW (■) and CFSW (●). Data represent measurement from three experiments for both oocyte stages ± S.D.

was higher in immature (Fig. 1A) than in mature (Fig. 1B) oocytes. As shown, after 30 min of incubation, 3 pmol of L-[U-^{14}C]serine/mg protein was incorporated into PS in immature oocytes suspended in CFSW as compared to 5 pmol/mg protein in SW, whereas in mature oocytes this values amounted to 0.2 and 0.7 pmol/mg protein, respectively. The oocytes used in the study were obtained from non-cultured starfish, collected in the sea. The absolute level of the serine base-exchange activity thus differed from animal to animal, giving relatively high standard deviation values, although in all cases the activity was much higher in immature than in mature cells (Fig. 1).

Effect of Ca^{2+} store depletion on PS synthesis

To establish whether Ca^{2+} that stimulates PS synthesis could come from intracellular stores, we studied the effect of the SERCA pump inhibitor thapsigargin on oocytes injected with the fluorescent indicator calcium green coupled to 70-kDa dextran and incubated in CFSW. Surprisingly, the nanomolar concentration of thapsigargin routinely used to deplete the intracellular Ca^{2+} stores in other cell types failed to produce a rapid and sustained increase in the cytosolic Ca^{2+} concentration. Figure 2A shows the effect of a 30 min treatment of an oocyte with 10 μM thapsigargin. The Ca^{2+} leakage from the ER store was very modest, leading to a value of 0.2 of relative fluorescence in the cytoplasm. The thapsigargin concentration was evidently insufficient to completely deplete the store since the uncaging of 1,4,5-inositol trisphosphate ($InsP_3$) previously injected into the cytoplasm 25 min after the thapsigargin treatment still induced a Ca^{2+} transient whose amplitude and kinetics were similar to those observed in control oocytes. Thapsigargin is a highly lipophilic compound, which could easily become adsorbed to the vesicles. We found that as much as 200 μM thapsigargin was necessary to induce intracellular calcium depletion in the Neapolitan starfish species used for this contribution. Evidently, the sensitivity to thapsigargin varies with starfish species: recent data from our laboratory have shown it to be significantly higher in the Japanese starfish species (*Asterina pectinifera*) we have frequently used (Moccia *et al.*, 2003). Therefore, in all other experiments the cytoplasmic and nuclear Ca^{2+} pools were explored using 200 μM thapsigargin, and calcium green dextran 70 kDa injected into the cytoplasm or the nucleus of immature cells, and in

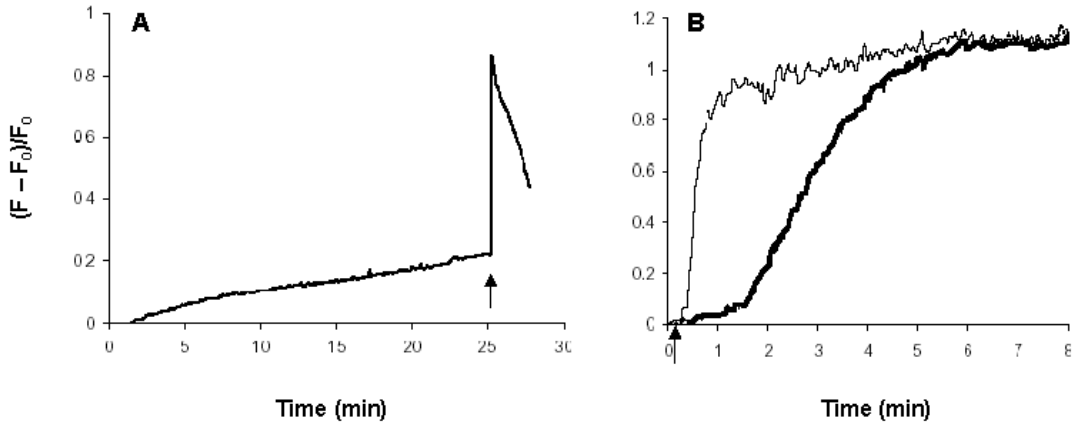

Figure 2. Changes in the cytoplasmic and nucleic Ca^{2+} level evoked by thapsigargin.

(**A**) An oocyte pre-injected with caged $InsP_3$ was incubated for 30 min with 10 μM thapsigargin and then irradiated (arrow) to release $InsP_3$, followed by Ca^{2+} release from subcellular stores into the cytoplasm. (**B**) A typical pattern of Ca^{2+} increase in the cytoplasm (thin line) and in the nucleus (thick line) in an immature oocyte following the addition of 200 μM thapsigargin (arrow).

the cytoplasm of mature cells. The observed increase in Ca^{2+} level started from the periphery of the oocyte and propagated across the entire cytoplasm (not shown). Figure 2B shows that the addition of thapsigargin to CFSW in which immature oocytes were kept induced a Ca^{2+} increase in the cytoplasmic compartment (thin line). The graph of the relative fluorescence shows that the Ca^{2+} increase reached a maximum of 1.0 ± 0.09 (n = 5) in several minutes. In the nuclear compartment the onset of the Ca^{2+} increase (Fig. 2B, thick line) following thapsigargin addition (arrow) was consistently delayed, reaching the peak value several minutes after the cytosolic Ca^{2+} increase. As in the case of the cytoplasm, Ca^{2+} remained elevated for at least 30 min (not shown). The different dynamics of Ca^{2+} changes in the two compartments indicates that thapsigargin emptied both the ER lumen and the nuclear envelope stores.

The effect of increasing the concentration of thapsigargin on PS synthesis is presented in Fig. 3. As shown, PS synthesis was decreased in thapsigargin treated immature oocytes. The inhibition reached about 50% at 20 μM thapsigargin and did not increase further at higher concentrations. Since in the oocytes treated with 20 μM thapsigargin Ca^{2+} was still present inside the ER store (Fig. 2A), the synthesis of PS might still occur in this subcellular structure (Fig. 3). On the other hand, 200 μM thaspigargin resulted in the complete Ca^{2+} emptying of the ER lumen, and caused the appearance of Ca^{2+} in the nucleoplasm (Fig. 2B). These Ca^{2+} fluctuations might decrease the PS synthesis in the ER and increase it inside the nucleus, explaining the nearly identical effect of 20 and 200 μM thapsigargin on PS synthesis in immature oocytes. In mature oocytes the inhibition of PS synthesis reached 50% of controls at 200 μM thapsigargin. Me_2SO, the solvent used to dissolve thapsigargin, did not affect the base-exchange activity (not shown).

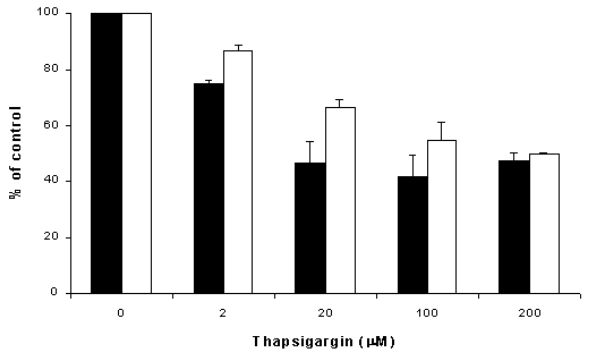

Figure 3. Effect of thapsigargin on PS synthesis in immature and mature starfish oocytes.

Cells were incubated with L-[U-^{14}C]serine for 30 min in CFSW in the absence (control) and presence of thapsigargin. Closed bars represent immature oocytes, open bars mature oocytes. Values are expressed as percentage of control. An appropriate amount of Me_2SO was added to control samples taken as 100%. Data represent measurements from three experiments for both oocyte stages ± S.D.

Phosphatidylserine synthesis by PSS1 and PSS2

The next set of experiments was performed to show whether PSS1, PSS2, or both are expressed in starfish oocytes. To this aim, we analysed PSS1 and PSS2 mRNAs using RT-PCR. In immature oocytes (Fig. 4A) the PSS1 transcript was higher, that of PSS2 being barely detectable. In contrast, in mature oocytes (Fig. 4B) both PSS1 and PSS2 mRNAs were detected, however, the level of the latter was higher. The RT-PCR products were about 300 base pairs for PSS1 and about 500 base pairs for PSS2, as expected.

To check whether two PSS proteins are present in both oocyte meiotic stages we used Western blot analysis and applied polyclonal antibodies against PSS1 and PSS2 that were previously used in CHO cells (Saito *et al.*, 1996), McArdle hepatoma, and murine cells (Stone & Vance, 2000). The results shown in Fig. 4C and D indicate that the amount of PSS2 protein was higher than that of PSS1 in both stages of oocyte maturation. However, the amount of PSS1 protein was higher in immature oocytes (Fig. 4C).

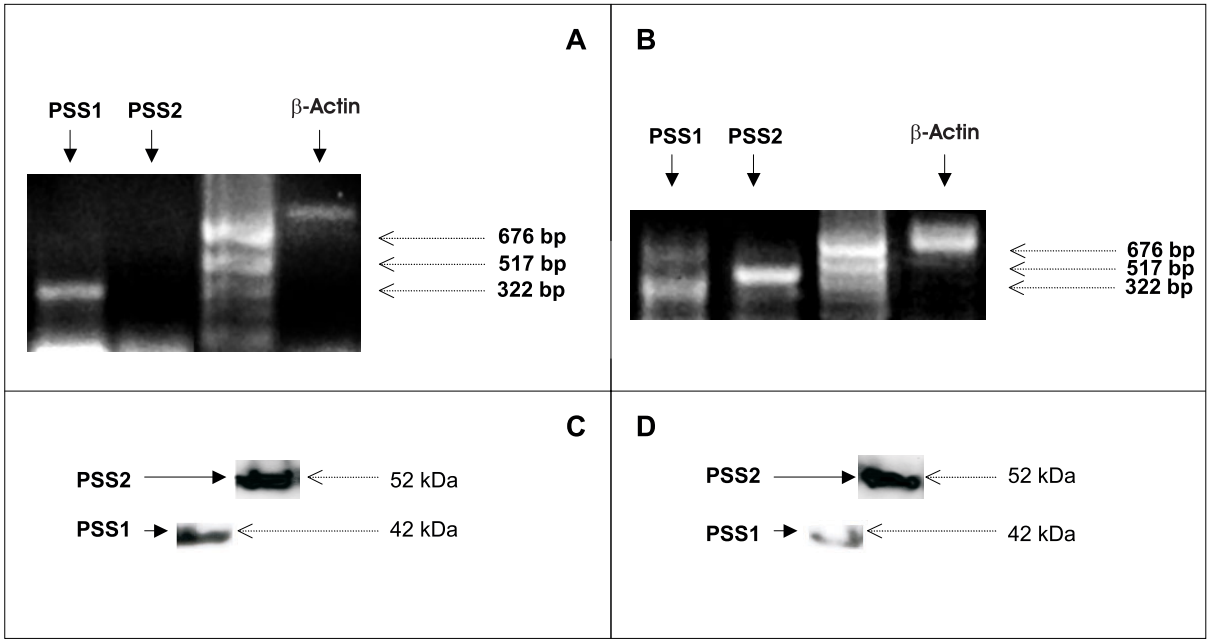

Figure 4. Expression of PSS1 and PSS2 in starfish oocytes.

Panels A and B show RT-PCR products, panels C and D Western blot products. Total RNA from (**A**) immature and (**B**) mature oocytes was reverse-transcribed using an oligo(dT_{23}) primer, and the resulting cDNA was used as a template for PCR using the sense primer 5′-AGAAGAACTATGGCGTCCTGCG-3′ and the antisense primer 5′-CATAAGGCTGGATGAGGTCGAG-3′ specific for PSS1, sense 5′-TGGAGTCACACAAGCCAAAGAC-3′ and an antisense 5′-GTAGGTTGGAATGTTCCAGAGG-3′ primers specific for PSS2, and sense 5′-TTGTAACCAACTGGGACGATATGG-3′ and antisense 5′-GATCTTGATCTTCATGGTGCT-3′ primers for the internal control β-actin. The resulting PCR products were about 300 base pairs for PSS1, 500 base pairs for PSS2 and 700 base pairs for β-actin. Data are representative of three independent experiments with similar results. To identify PSS isoforms for immature (**C**) and mature (**D**) oocytes 50 μg of oocyte membrane proteins were analysed by the Western blot method. Data are representative of three independent experiments with identical results.

PS in the nuclear compartments of the oocytes

In addition to studying the synthesis of PS that occurred in intact oocytes we also examined the synthesis of this phospholipid in the nuclei. Unfortunately, the methods used in other laboratories to isolate the nuclei (Dygas *et al.,* 2000; Nemoto *et al.,* 1992) appeared inadequate to measure the base-exchange reaction, as they destroyed the nuclear structure in starfish oocytes. The methods involving the use of detergents were also useless, because detergents inhibit PS synthesis (Saito & Kanfer, 1973; Rakowska *et al.,* 1997). Therefore, to explore PS in the nucleus and in the nuclear subcompartments we used annexin V, a protein with high affinity for PS. Our results showed that the incubation of annexin V-FITC with manually isolated nuclei labelled the nuclear envelope (panel A in Fig. 5). In agreement with the annexin V-FITC binding kit protocol (see Materials and Methods) the binding of the protein to the nuclear phospholipids was only detected in the presence of calcium. In the absence of calcium no binding was observed (not shown). In manually disrupted nuclei, i.e., when the nucleoplasm became exposed, various components of the nucleoplasm also became labelled, with the highest fluorescence being detected near the nucleolus (panel C and E in Fig. 5). These data indicate clearly that PS is present in the oocyte nucleus not only in the envelope, but also in the internal nuclear subcompartments around the nucleolus.

DISCUSSION

The study demonstrates that immature and mature starfish oocytes incorporate serine into PS using the two enzymes, PSS1 and PSS2, which are active in mammalian cells. Thus, PS can be produced by the base-exchange reaction from both PC and PE. The results show that the level of PSS1 transcript was higher in immature, and that of PSS2 in mature oocytes, but the level of protein expression was instead much higher for PSS2 than for PSS1. In addition, it did not depend on the maturation stage.

Taken together, the results indicate that changes in expression level and enzymes activities regulate PS synthesis in starfish oocytes during the sequential steps of meiosis. Serine incorporation into PS was much higher in immature oocytes, which still contained the nucleus, than in mature oocytes, in which meiosis had been reinitiated. The matu-

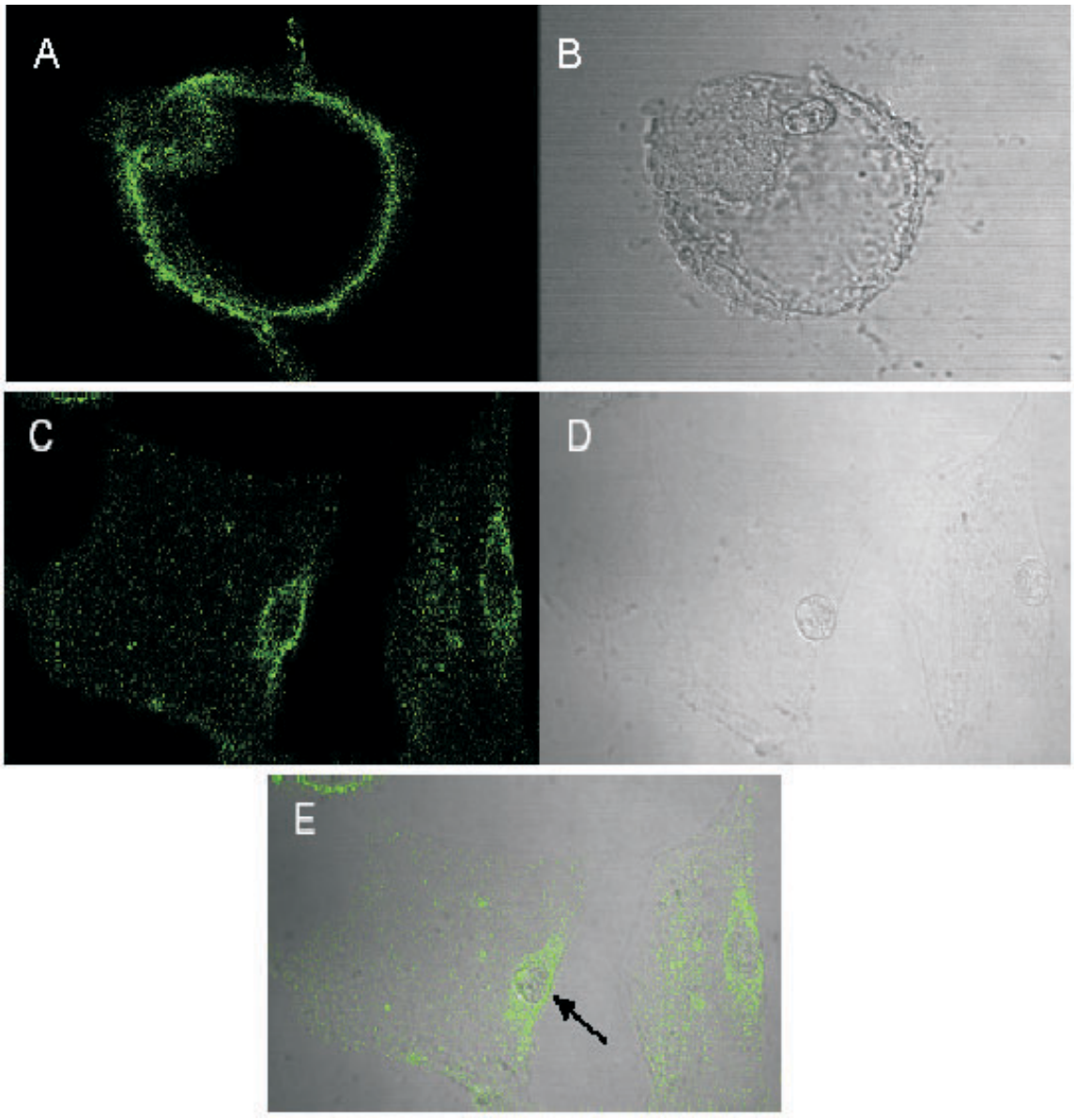

Figure 5. Localisation of annexin V-FITC fluorescence in intact nuclei isolated from immature starfish oocytes.

Confocal (A and C) and corresponding phase contrast images (B and D). A manually isolated nucleus (A and B) and disrupted nuclei (C and D) with exposed nucleoplasm were incubated with annexin V-FITC and 2.5 mM Ca^{2+}. Panel E shows an overlay of the fluorescence image and phase contrast images shown as C and D, respectively.

ration-inducing hormone 1-MA, which initiates the meiosis process with the breakdown of the nucleus and the intermixing of the nucleoplasm with the cytoplasm, also induces profound structural changes in the ER of starfish oocytes (Jaffe & Terasaki, 1994) that are linked to the release of Ca^{2+} from these intracellular stores (Stricker *et al.*, 1998). Thus, we suggest that the disintegration of the nuclear envelope and the fragmentation of the ER, which cause the release of Ca^{2+} to the cytoplasm may contribute to the changes in PS synthesis during oocyte maturation. The experiments described herein have confirmed that the depletion of the ER Ca^{2+} store by thapsigargin resulted in the lowering of PS synthesis in immature oocytes. The phenomenon has already been observed in various mammalian cells (Czarny *et al.*, 1992; Pelassy *et al.*, 1992; Rakowska & Wojtczak, 1995). However, at variance with the cell types where the treatment with thapsigargin and 2,5-di-*tert*-butylhydroquinone (DBHQ) resulted in a 70% inhibition of serine incorporation into PS (Czarny *et al.*, 1992, Wiktorek *et al.*, 1996), in starfish oocytes thapsigargin still allowed 50% of the PS synthesis to proceed. It is worth adding in this context that in *Xenopus* oocytes Ca^{2+} leakage was caused by thapsigargin only after 2 h of incubation (Thomas *et al.*, 1996).

The nuclear envelope contains a Ca^{2+}-ATPase which is identical to that of the ER (the SERCA pump) (Lanini *et al.*, 1992; Malviya & Rogue, 1998). Its inhibition was expected to promote a leak of Ca^{2+} from the nuclear envelope into the nucleoplasm (Santella & Kyozuka, 1994). In our experiments thapsigargin indeed induced a gradual and long lasting Ca^{2+} increase in the nucleoplasm. Evidently, in starfish oocytes thapsigargin depleted the calcium stores of both the ER and the nuclear envelope.

We have recently shown that PS synthesis occurs in rat liver nuclei depleted of the outer membrane of the envelope (Dygas *et al.*, 2000). The experiments with annexin V-FITC described above have shown that anionic lipids, among them PS, could be detected in the nuclear envelope and in the nucleoplasm, mainly in the neighbourhood of the nucleolus. Thus, one can speculate that the strongly decreased, but not completely abolished level of PS synthesis in the presence of thapsigargin on the one hand was a result of the depletion of the ER calcium store, and on the other of the increase of the calcium level in the nucleoplasm. In many organisms the proper development of the embryo depends on the asymmetrical distribution of maternal RNAs and proteins in the eggs (Kloc *et al.*, 2001). Nevertheless, in thapsigargin-treated mature oocytes PS synthesis was only reduced by 50%, suggesting that PS could be partly synthesized thanks to a thapsigargin- and $InsP_3$-insensitive Ca^{2+} store. Perhaps this could occur in the cortically-located compartment sensitive to the newly described Ca^{2+}-linked messenger nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) (Santella *et al.*, 2000; Lim *et al.*, 2001).

The data presented here are the first description of PS synthesis during the meiotic cycle. They indicate that PS synthesis requires unimpaired, integral subcellular structures able to store sufficiently high amounts of calcium, which is necessary for the process.

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