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Calcium-regulated fusion of yolk granules is important for yolk degradation during early embryogenesis of *Rhodnius prolixus* Stahl

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Summary

This study examined the process of membrane fusion of yolk granules (YGs) during early embryogenesis of *Rhodnius prolixus*. We show that eggs collected at days 0 and 3 after oviposition contain different populations of YGs, for example day-3 eggs are enriched in large YGs (LYGs). Day-3 eggs also contain the highest free [Ca^{2+}] during early embryogenesis of this insect. *In vitro* incubations of day-0 YGs with [Ca^{2+}] similar to those found in day-3 eggs resulted in the formation of LYGs, as observed *in vivo*. Fractionation of LYGs and small YGs (SYGs) and their subsequent incubation with the fluorescent membrane marker PKH67 showed a calcium-dependent transference of fluorescence from SYGs to LYGs, possibly as the result of membrane fusion. Acid phosphatase and H^+-PPase activities were remarkably increased in day-3 LYGs and in calcium-treated day-0 LYGs. Both fractions were found to contain vitellins as major components, and incubation of YGs with calcium induced yolk proteolysis *in vitro*. Altogether, our results suggest that calcium-induced membrane fusion events take part in yolk degradation, leading to the assembly of the yolk mobilization machinery.

Key words: calcium, embryogenesis, membrane fusion, yolk degradation, yolk granules.

Introduction

Chagas disease is the first cause of cardiac lesions in countries of Latin America, where it is endemic (Moncayo, 2003), and *Rhodnius prolixus* is one of the most important vectors of this disease (World Health Organization, 2002). In this context, this insect is crucial for the propagation of Chagas disease and consequently is an important target for vector control. From this point of view, the understanding of reproductive processes, such as oogenesis and embryogenesis, can offer new insights and targets for population control of this insect.

Oogenesis in insects, as in all oviparous animals, occurs by massive incorporations of yolk protein through receptor-mediated endocytosis (Engelman, 1979; Raikhel and Dadhialla, 1992). Endocytic activities during insect oogenesis involve the incorporation of the lipophosphoglycoprotein vitellogenin (Oliveira et al., 1986; Raikhel and Dadhialla, 1992; Valle, 1993; Sappington and Raikhel, 1998). Once inside the oocyte the vitellogenin, now referred to as vitellin (VT), is stored in organelles known as yolk granules (YGs) (Kunkel and Nordin, 1985; Purcell et al., 1988; Machado et al., 1998). After oviposition, the YGs fill almost the entire volume of the fresh egg and can vary significantly in size and density. During embryogenesis, *R. prolixus* VT represents the major yolk protein, being mobilized as the main amino acid source for embryo development.

Yolk degradation occurs by activation of acidic hydrolases also stored within YGs. The exact origin of these hydrolases is poorly understood but some of these enzymes are also taken into the oocytes during oogenesis, being stored near yolk proteins in an inactive state. *Aedes aegypti* carboxypeptidase (Cho et al., 1991) and cathepsin B-like protease (Cho et al., 1999), *Blatella germanica* vitellin-processing protease and *Xenopus laevis* lysosomal enzymes have been shown to accumulate in the oocytes during oogenesis (Wall and Meleka, 1985; Liu and Nordin, 1998; Yin et al., 2001). To activate hydrolases, YGs undergo a process of acidification mediated by proton pumps, such as proton ATPases (H^+-ATPases)

(Fagotto, 1991; Nordin et al., 1991; Mallya et al., 1992; Fagotto, 1995) and proton pyrophosphatases (H^+-PPases) (Motta et al., 2004). In insects, several hydrolytic enzymes found in YGs such as cathepsins (Takahashi et al., 1996; Cho et al., 1999; Ribolla et al., 2001), acid phosphatases (Nussenzveig et al., 1992; Ribolla et al., 1993; Fialho et al., 2002; Fialho et al., 2005) and glycosidases (Purcell et al., 1988) were shown to be activated by low pH. Acidification of YGs, therefore, results in activation of hydrolases and degradation of VT. This process is widely conserved within oviparous animals, being essential for yolk mobilization during embryo development. Processing and degradation of VT during embryogenesis of *R. prolixus* is also dependent on acidification of YGs (Oliveira et al., 1989). In addition, YG-associated hydrolases such as cathepsin D (CD) and acid phosphatase (AP) have been identified in the oocytes of this insect (Nussenzveig et al., 1992; Fialho et al., 2002).

It is generally believed that the YG population is not homogeneous since the vesicles can vary in their macromolecule content and can be fractionated according to their different size and density (Fagotto, 1991; Chestkov et al., 1998; McNeil et al., 2000; Yamahama et al., 2003). For example, in echinoderms, such as starfish and sea urchin, the egg vesicle population is divided in two main groups: reserve and cortical granules. Each group has distinct functions in fertilization signaling and embryo nourishment (Chestkov et al., 1998). In the silkmoth *Bombyx mori*, the acid enzymes are only present in the small YGs (SYGs), whereas the yolk proteins are localized in the large ones (Yamahama et al., 2003). In the stick insect *Carausius morosus*, small YGs are frequently more acidic than large YGs and are often seen surrounding the large ones (Fausto et al., 2001). For the hard tick *Boophilus microplus*, proteolytic activity has also been correlated with differential acidification of YGs and the presence of small vesicles at the periphery of the egg (Abreu et al., 2004). However, the exact origin of the different YGs remains unknown.

Intracellular fusion machinery is composed of dynamic proteins that are assembled upon a signal and are dismantled immediately after the fusion is over, being reutilized. In general, this process is mediated by proteins of the Rab complex (Rabs), SNARES and SM protein families (Jahn et al., 2003). Many proteins involved in membrane fusion are made active by intracellular signaling, involving phosphorylation and protease cleavage (Rutledge and Whiteheart, 2002; Lilja et al., 2004; Huyng et al., 2004; Hepp et al., 2005). Calcium binding proteins, such as calmodulin, are known to work as calcium sensors, leading to activation of kinases that in turn activate membrane fusion proteins (Hilfiker et al., 1999). Many membrane fusion events are therefore calcium-dependent and $[Ca^{2+}]$ elevation has been described as a fusion signal for several processes (Burgoyne, 1995; Burgoyne and Morgan, 1998). This variation in $[Ca^{2+}]$ could be due to Ca^{2+} mobilization from specialized internal stores (Tse et al., 1997; Petersen et al., 1999) or influx through plasma membrane (Augustine et al., 1991). In addition, free Ca^{2+} concentration can also be regulated by calcium binding proteins (Meldolesi and Pozzan, 1998). In echinoderms, such as the starfish (McNeil et al., 2000; Chestkov et al., 1998) Ca^{2+} was shown to mediate YG fusion, and Ca^{2+}-dependent membrane interactions were recently described in sea urchin eggs (Hayley et al., 2006). In this work, we show that YGs from the blood sucking bug *R. prolixus* undergo a process of membrane fusion. We show that these events might occur *in vivo* in a calcium-dependent manner, being important for yolk processing during early embryogenesis of this insect.

Materials and methods

Chemicals

Arsenazo III, bovine serum albumin, leupeptin, aprotinin, pepstatin A, dithiothreitol (DTT), phenylmethylsulphonyl-fluoride (PMSF), *p*-nitrophenyl phosphate (*p*NPP) and PKH67 Green Fluorescent Cell Linker Kit were purchased from Sigma Chemical Company (St Louis, MO, USA). OCT was obtained from Tissue-Tek (Torrance, CA, USA). All other chemicals were of analytical grade.

Insects

Rhodnius prolixus Stahl were reared in a colony maintained at 28°C and 70–80% relative humidity. The animals were fed with rabbit blood in an artificial apparatus according to Garcia et al. (Garcia et al., 1975). Eggs were collected daily and used promptly or allowed to develop to the required embryogenesis stage.

Isolation of yolk granules

YGs were extracted by gently disrupting the eggs with a plastic pestle in ice cold modified Ringer saline (without addition of $CaCl_2$) containing 130 mmol l^{-1} NaCl, 8.8 mmol l^{-1} KCl, 8.6 mmol l^{-1} $MgCl_2$, 10.2 mmol l^{-1} $NaHCO_3$, 4.4 mmol l^{-1} NaH_2PO_4, 34 mmol l^{-1} glucose, pH 7.2 and supplied with a protease inhibitors cocktail (aprotinin, leupeptin, pepstatin A and PMSF).

Fractionation of yolk granules

Approximately 90 eggs, from day 0 or day 3 of development, were disrupted in 500 μl of Ringer saline in the presence of 10 mmol l^{-1} EGTA and centrifuged at 50 ***g*** for 12 min at 4°C. Pellet and top fractions (enriched in LYGs and SYGs, respectively) were carefully collected, separately resuspended in Ringer saline and centrifuged for 5 min (4°C) at 1000 ***g***, to obtain LYGs, and 17 000 ***g***, for SYGs. The pellets were then resuspended in Ringer saline containing 10 mmol l^{-1} EGTA and used in assays.

Light microscopy

Opercula from freshly laid eggs or eggs that were allowed to develop until day 3 after oviposition were carefully detached using sharp forceps and a histological blade. The eggs were then fixed in 2.5% glutaraldehyde, 4% paraformaldehyde in PBS for 24 h at 4°C. For cryosections, samples were washed

and incubated for 12 h in 20% sucrose in PBS and infiltrated for 96 h in increasing concentrations of OCT (25%, 50%, 75% and pure OCT). After freezing in liquid nitrogen, 7 μm thick transverse sections were obtained, which were adhered to poly-L-lysine-coated glass slides and mounted in glycerol. Alternatively, the fixed material was washed followed by dehydration in an ethanol series (70%, 90% and 2×100%) and embedded in Historesin™ (Leica Historesin Embedding Kit, Nu loch, Heidelberg, Germany). Transverse sections of 7 μm were obtained and stained with 0.1% Toluidine Blue. For LYG counting, 10 different OCT-embedded sections from days 0 and 3 were observed.

Determination of calcium concentrations

Eggs from 0 to 6 days of development were collected and the contents from a pool of four eggs were extracted in 1 ml of modified Ringer saline (pH 7.2). The material was then centrifuged at 10 000 ***g*** for 10 min at 4°C and the supernatants incubated in the presence of 4 μmol l^{-1} Arsenazo III. Measurements were taken using a CINTRA 20 spectrophotometer (GVC Scientific Equipment Pty Ltd, Dandenog, Australia) at the wavelength pair 675/685. In parallel, calcium titers were added to modified Ringer saline and measured in the presence of 4 μmol l^{-1} Arsenazo III (Yingst and Hoffman, 1983).

Calcium-dependent fusion assay

Day 0 eggs were extracted in 50 μl of modified Ringer saline containing 10 mmol l^{-1} EGTA (for chelating of endogenous Ca^{2+}) and incubated in increasing concentrations of $CaCl_2$ (15 mmol l^{-1}, 17 mmol l^{-1} and 27 mmol l^{-1}), at 25°C. Because endogenous [Ca^{2+}] can vary among individuals, the Ca^{2+} concentration for each experimental group was measured and adjusted to achieve final calcium concentrations of 6.6±1.8 mmol l^{-1}, 11.6±3.1 mmol l^{-1} and 23.4±2.2 mmol l^{-1} (mean ± s.d.?). After 1 min, 5 μl of each experimental group were deposited on glass slides and observed using differential interference contrast (DIC) light microscopy. The YGs in one random field of each slide were measured and counted. We considered large YGs (LYGs) as structures >40 μm in diameter and small YGs (SYGs) as structures <10 μm in diameter. Statistical analyses were performed using Graph Pad Instat 4.0 software (P=0.05). Results are representative of at least three experiments.

Calcium-dependent transfer of membrane components from small to large yolk granules

SYGs membranes were labeled with PKH67 Green Fluorescent Cell Linker Kit (Sigma Chemical Co.) according to the manufacturer's protocol. Aliquots of labeled fractions were observed in a fluorescence microscope to assure their purity. For fusion assays, 5 μl of the labeled SYGs were mixed with 20 μl of day 0 total YG fraction and incubated in the presence of 23 mmol l^{-1} $CaCl_2$. The material was observed in a Zeiss Axioplan epifluorescence microscope equipped with a fluorescein filter set and a TK-1270 JVC color video camera.

SDS–PAGE of large and small yolk granules

LYGs and SYGs were obtained and their protein concentration determined by the Lowry method (Lowry et al., 1951). 15 μg of protein of each sample were subjected to SDS–PAGE 7.5% (Laemmli, 1970). The gel was stained with silver nitrate (Merril et al., 1981).

Immunolocalization of egg vitellin

Day 0 and 3 eggs were fixed in 4% paraformaldehyde in PBS, pH 7.2, for 24 h at 4°C followed by infiltration in OCT and sectioning as described above. Sections were blocked for 30 min in 100 mmol l^{-1} NH_4Cl in TBS, washed and incubated for 30 min in 3% BSA in TBS plus 0.8% Triton X-100 at room temperature. Sections were then incubated for 1 h in polyclonal antibodies raised against *R. prolixus* vitellins diluted 1:100 in TBS 3% BSA, washed and incubated for 1 h at room temperature in Cy3-conjugated anti-rabbit IgG secondary antibodies. Samples were then washed again, mounted in *N*-propyl gallate and observed in a Zeiss Axioplan epifluorescence microscope equipped with a rhodamine filter set and a TK-1270 JVC color video camera.

Hydrolase activities

For the determination of acid phosphatase (AP) specific activity, SYGs and LYGs were subjected to three cycles of freeze and thaw and centrifuged at 20 000 ***g*** for 30 min. Protein concentrations of the supernatants were determined by the Lowry method (Lowry et al., 1951). For each fraction, aliquots containing 30 μg of protein were assayed at 37°C against 4 mmol l^{-1} *p*NPP in the following reaction medium: 20 mmol l^{-1} sodium acetate, pH 4.0, 1 mmol l^{-1} DTT and 1 mmol l^{-1} EDTA. Reactions were stopped after 1 h by the addition of 0.2 mol l^{-1} NaOH (corresponding to 10% of the total reaction volume) and each sample, containing the reaction hydrolysis product (*p*-nitrophenol, *p*NP), had their absorbance measured at 405 nm in a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA). For cathepsin D (CD) specific activity, SYGs and LYGs were submitted to freeze and thaw, centrifuged as described above and assayed using the CD-specific fluorogenic peptide substrate Abz-AIAFFSRQ-EDDnp (Pimenta et al., 2001). For each fraction, 300 μg of protein were incubated with 5 μmol l^{-1} fluorogenic peptide diluted in 20 mmol l^{-1} sodium acetate, pH 4.0, 1 mmol l^{-1} DTT and 1 mmol l^{-1} EDTA at 37°C for 10 min. The fluorescent products were monitored with an F-max fluorometer (Molecular Devices) using a 320 nm excitation filter and 460 nm emission filter.

H^+-PPase and vacuolar H^+-ATPase activities

Membrane fractions of SYGs and LYGs were obtained as follows: samples were resuspended in equal volumes of ice-cold buffer containing 10% (v/v) glycerol, 0.13% (w/v) BSA, 5 mmol l^{-1} EDTA, 150 mmol l^{-1} KCl, 3.3 mmol l^{-1} DTT, 1 mmol l^{-1} PMSF and 100 mmol l^{-1} Tris–HCl, pH 8.0. The YG suspensions were homogenized using a glass Potter-Elvehjem homogenizer and then centrifuged at 10 000 ***g*** for 20 min at

4°C. The supernatants obtained were centrifuged at 100 000 ***g*** for 40 min at 4°C and the pellet was resuspended in 10% glycerol, 10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} DTT, pH 7.5 and recentrifuged at 100 000 ***g*** for 40 min at 4°C. The final pellet was resuspended in a small volume of the last buffer and assayed for H^+-PPase and vacuolar H^+-ATPase activities (Motta et al., 2004). Protein concentration was determined as described above. Reactions were started by the addition of 40 μg of protein in reaction medium containing 50 mmol l^{-1} Mops-Tris, 100 mmol l^{-1} KCl, 0.3 mmol l^{-1} NaPPi (sodium pyrophosphate), 0.6 mmol l^{-1} $MgCl_2$, pH 7.5 for H^+-PPase and 50 mmol l^{-1} Mops-Tris ,100 mmol l^{-1} KCl, 1 mmol l^{-1} ATP, 2 mmol l^{-1} $MgCl_2$, pH 7.5, for vacuolar H^+-ATPase. After 1 h at 28°C, reactions were stopped by the addition of 50 μl of trichloroacetic acid (50% w/v) and colorimetrically measured by determining the rate of Pi release (Fiske and Subbarow, 1925). For vacuolar H^+-ATPase, all samples were also incubated in the presence of 50 mmol l^{-1} nitrate (KNO_3), which is a specific vacuolar H^+-ATPase inhibitor (Moriyama and Nelson, 1989). Only the nitrate inhibited activity was considered. Statistical analyses were performed in Graph Pad Instat 4.0 software (P=0.05). Results are representative of at least three experiments.

Acidification of YG

To investigate the location of proton pumps (H^+-PPase and vacuolar H^+-ATPase), day-0 and day-3 eggs were extracted and incubated in the dark for 10 min in Ringer saline plus 10 mmol l^{-1} EGTA containing 5 μg ml^{-1} Acridine Orange (AO). For experimental groups, PPi and ATP were added for final concentrations of 0.3 mmol l^{-1} and 1 mmol l^{-1}, respectively. After incubation the YGs were deposited on glass slides and observed at an excitation wavelength of 418 nm in a Zeiss Axioplan epifluorescence microscope equipped with a fluorescein filter set and a TK-1270 JVC color video camera.

Calcium-dependent yolk proteolysis assay

YGs were extracted from day-0 and day-3 eggs and incubated in Ringer saline plus 10 mmol l^{-1} EGTA containing 1 mmol l^{-1} ATP and 1 mmol l^{-1} PPi. For experimental groups ~23 mmol l^{-1} Ca^{2+} was added. The samples were incubated for 1 h at 37°C and 13 μg of protein, for each sample, were submitted to 10% SDS–PAGE. The gel was stained with silver nitrate (Merril et al., 1981).

Results

Day-3 eggs contain enlarged yolk granules

Transverse sections revealed differences in the internal morphology between eggs from day 0 and day 3 of development. In day-0 eggs, the YG population filled the entire egg, whereas in day-3 eggs the YG population was variable in size and LYGs were often more abundant (Fig. 1A,B, white arrows). A visible embryo, at the gastrula stage, could be observed only during day 3 of development (Fig. 1B). To differentiate between YGs and lipid droplets, the material was also prepared for Historesin™, a procedure in which the material is submitted to a dehydration series in organic solvents and part of the lipid component is extracted leaving the protein content intact. Fig. 1C,D shows that day-3 eggs had a higher number of LYGs (black arrows), as observed in DIC images of unstained material. Morphometric analysis showed that LYGs are sixfold more frequent in eggs during day 3 than during day 0 of early embryogenesis. The number of LYGs per egg section were: 2±1 on day 0 and 12±2 on day 3 (mean ± s.d.).

Eggs at day 3 of development contain the highest [Ca^{2+}] during early embryogenesis

Calcium concentration was determined in eggs from 0 to 6 days of development. Fig. 2 illustrates an increase in free calcium concentration in eggs from 0 to 3 days of development. [Ca^{2+}] remained at ~7 mmol l^{-1} during days 0 and 1, beginning to increase on day 2 (~12 mmol l^{-1}). The highest free [Ca^{2+}] was found to be 23±2.3 mmol l^{-1} on the third day of embryogenesis. This period is coincident with the beginning of

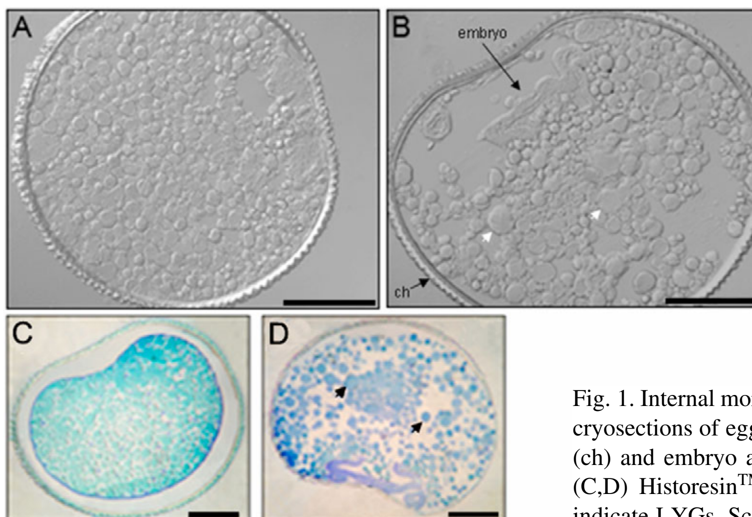


Fig. 1. Internal morphology of *R. prolixus* eggs. (A,B) DIC micrographs of transverse cryosections of eggs at days 0 and 3 after oviposition, respectively. In B, the chorion (ch) and embryo are visible and white arrows indicate large yolk granules (LYG). (C,D) Historesin™ sections of day 0 and day 3 eggs, respectively. Black arrows indicate LYGs. Scale bars, 200 μm.

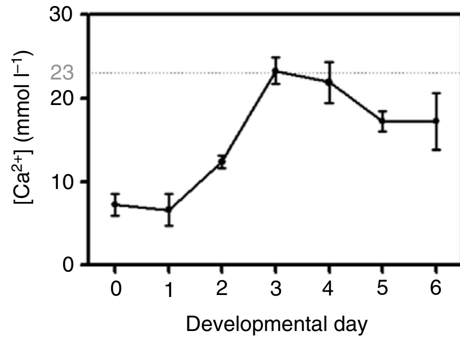
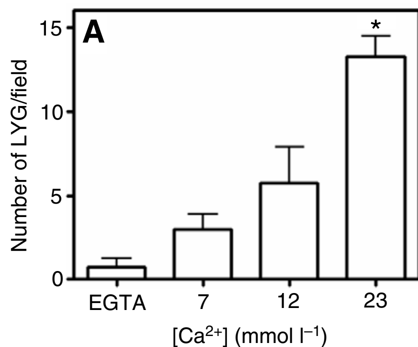


Fig. 2. [Ca^{2+}] during *R. prolixus* early embryogenesis. Ca^{2+} was measured in eggs using the sensor dye Arsenazo III. The highest [Ca^{2+}] (23±2.3 mmol l^{-1}) was found on the third day of embryogenesis. Values are means ± s.e.m. of 5 different experiments.

the yolk mobilization in *Rhodnius prolixus* embryogenesis (Fialho et al., 2005). After that, [Ca^{2+}] continuously decreased until day 5 and remained at similar levels (~18 mmol l^{-1}) until the sixth day of embryogenesis.

Calcium-induced formation of large yolk granules

Fig. 3A shows that incubation of YGs obtained from day 0 eggs with ~7 mmol l^{-1} Ca^{2+} (the amount of Ca^{2+} found in day-0 and -1 eggs) led to the appearance of threefold more LYGs in comparison with those incubated with EGTA. Incubation of day 0 YGs with the [Ca^{2+}] found in day-2 eggs (~12 mmol l^{-1}) increased the number of LYGs sevenfold and, ~23 mmol l^{-1} Ca^{2+}, found in day-3 eggs, induced the formation of 13-fold more LYGs in day 0 eggs. LYG formation showed a dose–response profile to Ca^{2+} addition. Treatment of day 0 YGs with [Ca^{2+}] over 23 mmol l^{-1} did not result in any significant LYG increase (data not shown). In Fig. 3B a general view of day-0 YGs in the presence of 10 mmol l^{-1} EGTA (calcium-free conditions) is illustrated, and in Fig. 3C day-0 YGs in the presence of ~23 mmol l^{-1} Ca^{2+} is shown. In the latter, the appearance of LYGs is clearly evident. Because day-3 eggs contained the highest [Ca^{2+}] found during embryogenesis and this concentration (~23 mmol l^{-1}) is the most effective in inducing LYG formation, all subsequent experiments were performed in the presence of ~23 mmol l^{-1} Ca^{2+}, and day-0 and day-3 eggs were compared.



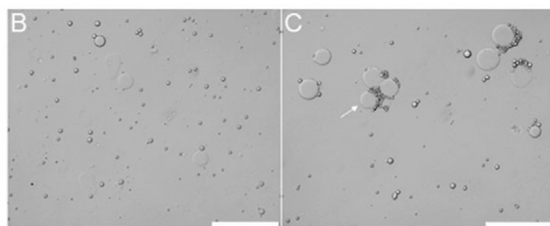


Fig. 3. Induction of large yolk granule (LYG) formation by *in vitro* calcium treatment. (A) Formation of LYGs (>40 μm) as a function of calcium concentration. Incubation of day 0 SYGs with increasing concentrations of calcium lead to the formation LYGs (a 13-fold increase of LYGs can be observed in 23 mmol l^{-1} Ca^{2+}). Values are means ± s.e.m. of 4 different experiments. *Statistically significant difference (P<0.05, one-way ANOVA). (B,C) DIC microscopy showing the general views of YGs incubated with 10 mmol l^{-1} EGTA (B) and ~23 mmol l^{-1} Ca^{2+} (C). Note the presence of a higher number of LYGs (arrow) after incubation with calcium. Scale bars, 200 μm.

Calcium-induced transfer of membrane components from small to large yolk granules

After centrifugation, two fractions of YGs, enriched in either LYGs or SYGs, were obtained (Fig. 4) and they were then labeled with the lipophilic fluorescent membrane marker PKH67 and incubated with calcium. Incubation of SYGs with PKH67 resulted in labeling of the membranes (Fig. 5A,B). To investigate if the increase in the amount of LYGs found in day-0 eggs incubated in the presence of calcium was the result of calcium-induced membrane fusion, PKH67-labeled SYGs was added to day 0 total YG fraction (without labeling) in the presence of 23 mmol l^{-1} Ca^{2+} (the calcium concentration found in day-3 eggs). Fig. 5C,D shows that incubation with calcium induces the transfer of the dye from SYGs to LYGs. When the labeled SYGs were incubated with day 0 total YG fraction in the presence of 10 mmol l^{-1} EGTA (no free calcium), no transfer of the dye from SYGs to LYGs could be observed (Fig 5E,F).

Small and large yolk granules express different levels of proton-pump activity

Membrane fractions of SYGs and LYGs were separated as described in the Materials and methods and assayed for H^+-PPase and vacuolar H^+-ATPase-specific activities. The results showed that SYGs from days 0 and 3 contain similar levels of H^+-PPase activity but higher levels than LYGs (Fig. 6A). By contrast, LYG H^+-PPase activities showed differences between day 0 and day 3 with a 90% increase in H^+-PPase activity on day 3 (Fig. 6A). Because egg homogenate activity levels did not change, i.e. it seems that there is no H^+-PPase synthesis between days 0 and 3, this suggests that the increase in LYG H^+-PPase activity could be the result of transfer of the enzyme from SYGs to LYGs. To investigate whether this increase in H^+-PPase activity was the result of calcium-mediated events, day-0 eggs were incubated in the presence of 23 mmol l^{-1} Ca^{2+} and the H^+-PPase activity was measured. Results showed that incubation with calcium increased the H^+-PPase activity in day-0 LYGs (Fig. 6B), suggesting that the calcium-induced events observed

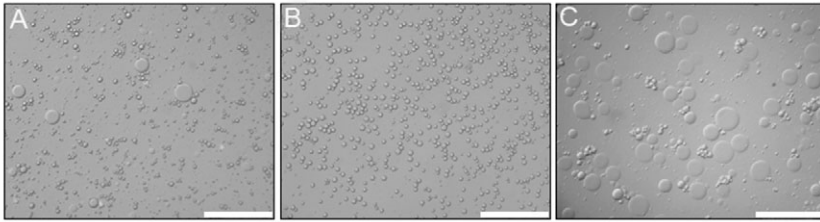


Fig. 4. Fractionation of yolk granules (YGs). (A) DIC light microscopy showing the day 0 total yolk granules before fractionation by differential centrifugation. (B,C) YG fractions after differential centrifugation. Note that the top (B) and pellet (C) fractions are enriched in small YGs (SYGs) and large YGs (LYGs), respectively. Scale bars, 200 μm.

in vitro (Figs 3 and 5) are also able to cause increase in H^+-PPase activity in LYGs. To further investigate whether the H^+-PPase could be relocated during embryogenesis, YGs were incubated with PPi in the presence of AO, which has been proved to be a reliable marker of intracellular acidic compartments (Anderson and Orci, 1988), and observed with a fluorescence microscope. Results showed that addition of PPi preferentially induced the acidification of SYGs on day 0 (Fig. 6C,D) whereas on day 3 it also induced the acidification of large ones (Fig. 6E,F). During embryogenesis the YGs undergo endogenous acidification, thus incubation of day 3 YGs with AO in the absence of PPi showed SYGs and LYGs already acidified (not shown). To confirm if PPi addition was able to induce further acidification of LYGs, acidified LYGs from day-3 eggs incubated, or not, with PPi were counted and compared.

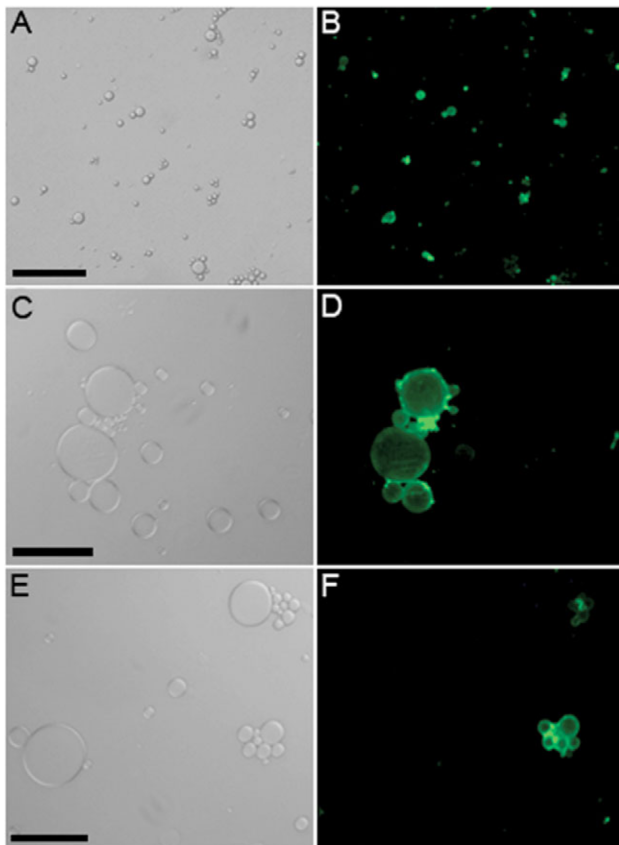
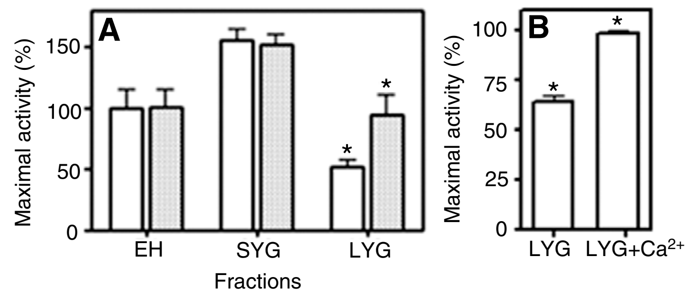


Fig. 5. Transfer of the fluorescent membrane dye, PKH67 from labeled small yolk granules (SYGs) to larger YGs after calcium treatment. (A,C,E) DIC images; (B,D,F) corresponding fluorescence images. (A,B) SYGs. Scale bar, 50 μm. (C,D) Dye transfer from SYGs to large ones. Note the appearance of large stained YGs as the result of incubation with Ca^{2+}. Scale bar, 25 μm. (E,F) Incubation of labeled SYGs with day 0 YGs in the presence of 10 mmol l^{-1} EGTA (calcium-free medium). Note that dye transfer did not occur. Scale bar, 25 μm.



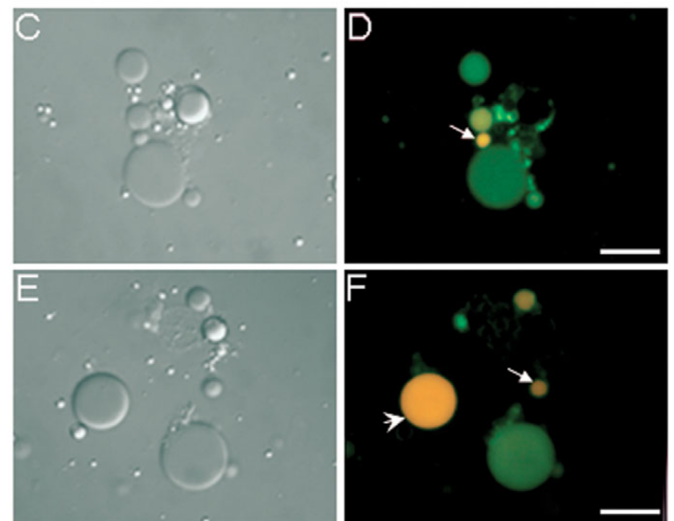


Fig. 6. H^+-PPase specific activity levels in different yolk granule (YG) fractions at days 0 and 3 of development. Membrane fractions were separated from LYGs and SYGs and assayed for H^+-PPase activity. (A) H^+-PPase activity levels during days 0 and 3 of embryogenesis. White bars, day 0 activity; gray bars day 3 activity. EH, total egg homogenate activity. Values are means ± s.e.m. of 3 different experiments. (B) H^+-PPase specific activity levels in day 0 LYGs with and without Ca^{2+}. *Statistically significant difference ($P<0.05$, *t*-test). (C–F) To determine the location of the H^+-PPase, YGs were also incubated with Acridine Orange in the presence of PPi during day 0 (C,D) and day 3 (E,F) of embryogenesis. Arrows indicate acidic SYGs and arrowheads indicate acidic LYGs. (C,E) DIC images; (D,F) corresponding fluorescence images. Scale bars, 25 μm.

Results showed that PPi addition increased by 18% the number of acidic LYGs during day 3 of embryogenesis, indicating that PPi addition also induces LYG acidification during day 3.

In contrast to the previous finding with the vacuolar H^+-PPase, the vacuolar H^+-ATPase activity did not significantly alter between days 0 and 3 of development (Fig. 7A). Incubation of YGs with ATP in the presence of AO induced acidification of both SYGs and LYGs at both stages of development (Fig. 7B–E).

Small and large yolk granules express different levels of hydrolase activity

SYGs and LYGs were assayed for determination of acid phosphatase (AP) and cathepsin D (CD)-specific activities. It has been shown that day-0 egg homogenates contain very low levels of CD (Fialho et al., 2005) and AP (Fialho et al., 2002) activities. Thus, to better understand the data, both samples were normalized and expressed as a percentage of the total activity of the egg homogenate (relative activity) for each day. Results showed that day-0 SYGs contained the highest levels of AP activity, twofold higher than LYGs (Fig. 8A). However, on day 3 this profile was strongly altered and LYGs showed 30% more activity than SYGs. As observed for H^+-PPase, this data suggests that LYG AP activity increases could possibly be achieved by enzyme relocation mediated by transfer from SYGs to LYGs. By contrast, as observed for vacuolar H^+-ATPase, no significant differences in the levels of CD activity between day 0 and 3 of embryogenesis or between SYGs and LYGs were observed (Fig. 8B).

Calcium induced events are important for yolk proteolysis but do not interfere in vitellin location

To analyze the protein profile of different YG fractions, LYGs and SYGs were submitted to SDS–PAGE. Fig. 9A reveals that vitellin apoproteins are equally present in egg homogenate, SYGs and LYGs. Arrows indicate the four apovitellins, as described elsewhere (Masuda and Oliveira, 1985). Immunofluorescence analysis using antibodies against these proteins showed the presence of vitellins in both LYGs and SYGs on days 0 (Fig. 9B,C) and 3 (Fig. 9D,E) of embryogenesis, confirming the previous results with SDS–PAGE. To investigate whether incubation with calcium could have an effect on the VT apoprotein degradation in the eggs from different days, day-0 and

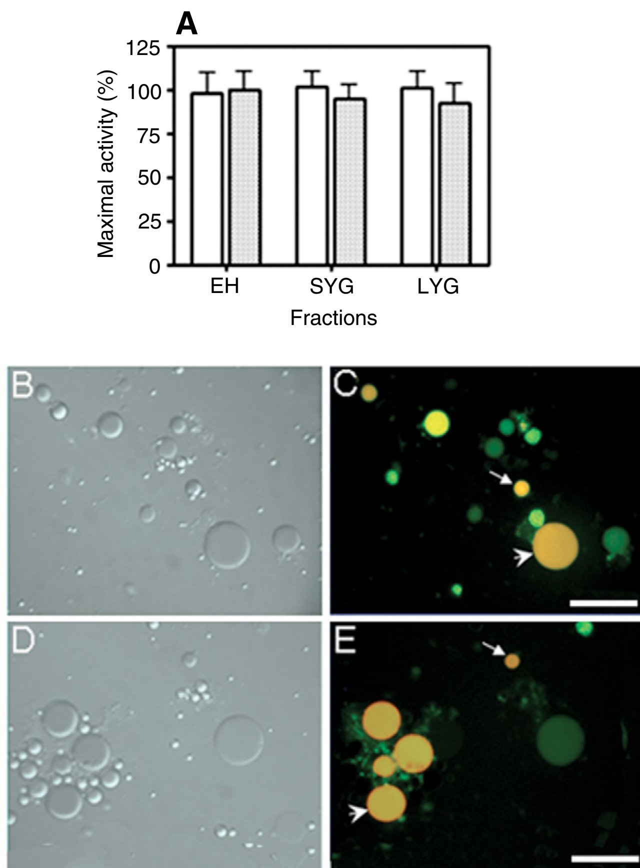


Fig. 7. Vacuolar H^+-ATPase specific activity levels in different yolk granule (YG) fractions during days 0 and 3 of development. LYGs and SYGs were submitted to membrane fraction separation and assayed for H^+-ATPase activity. (A) Vacuolar H^+-ATPase activity levels during days 0 and 3 of embryogenesis. White bars represent day 0 activities and gray bars represent day 3 activities. Values are means ± s.e.m. of 3 different experiments. To investigate the location of the H^+-ATPase, YGs were incubated with AO in the presence of ATP during day 0 (B,C) and day 3 (D,E) of embryogenesis. Arrows indicate acidic SYGs and arrowheads indicate acidic LYGs. (B,D) DIC images; (C,E) corresponding fluorescence images. Scale bars, 25 μm.

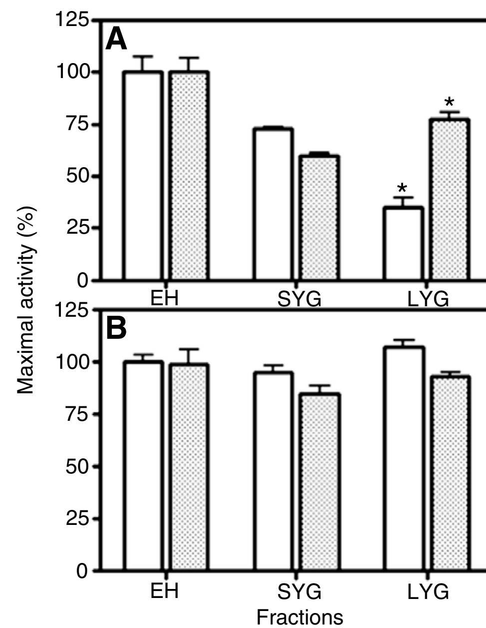


Fig. 8. Specific activity levels of hydrolases in different yolk granule (YG) fractions during days 0 and 3 of development. (A) Acid phosphatase activity levels. (B) Cathepsin D activity levels. White bars, activity on day 0; gray bars, activity on day 3. Values are means ± s.e.m. of 3 different experiments. EH, total egg homogenate. *Statistically significant difference ($P<0.05$, t-test).

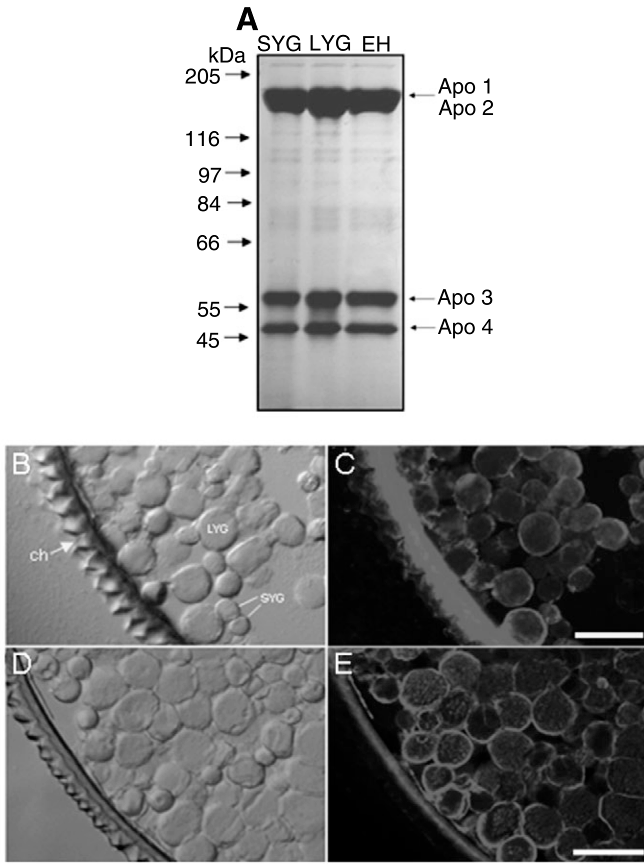


Fig. 9. SDS–PAGE of yolk granule (YG) fractions and vitellin (VT) immunolocalization. (A) SDS–PAGE of the different YG fractions. Arrows indicate the four apoproteins in SYGs, LYGs and total egg homogenate (EH); molecular mass is given on the left. (B–E) Vitellin immunolocalization in *R. prolixus* eggs. Vitellins were present in both LYGs and SYGs during day 0 (B,C) and day 3 (D,E) of embryogenesis. The chorion (ch) was shown to be auto-fluorescent in control groups (data not shown). (B,D) DIC images; (C,E) corresponding fluorescence images. Scale bars, 50 μm.

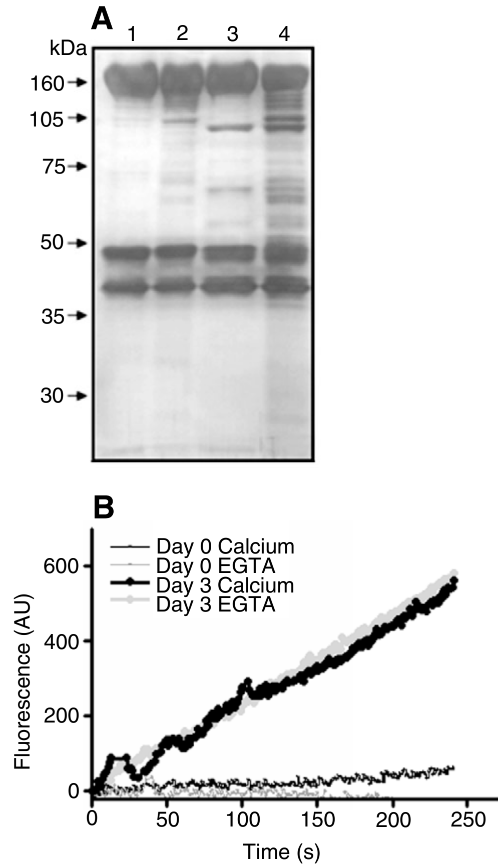


Fig. 10. Vitellin (VT) proteolysis after calcium incubation. (A) SDS–PAGE showing VT proteolysis after calcium incubation. Yolk granules (YGs) were extracted from day 0 and day 3 eggs and incubated in Ringer saline containing 1 mmol l^{-1} ATP and 1 mmol l^{-1} PPi. For experimental groups ~23 mmol l^{-1} Ca^{2+} was added. (Lane 1) Day-0 YGs after incubation with EGTA; (lane 2) day-0 YGs after incubation with calcium; (lane 3) day-3 YGs after incubation with EGTA; (lane 4) day-3 YGs after incubation with calcium. (B) Cathepsin D activities tested in all samples used in A. AU, arbitrary units.

-3 YGs were submitted to degradation assay in the presence, or absence, of 23 mmol l^{-1} calcium. EGTA-treated day-0 YGs showed no degradation whereas incubation with 23 mmol l^{-1} Ca^{2+} led to mild VT proteolysis (Fig. 10A, lanes 1 and 2, respectively). Because day 3 of embryogenesis is the period when *R. prolixus* VT proteolysis starts (Fialho et al., 2005), incubation of day-3 YGs in the presence of EGTA presented a typical endogenous VT proteolysis profile (lane 3), which shows more proteolysis-derived fragments than day-0 eggs incubated in the presence of EGTA or calcium (lanes 1 and 2). Even so, incubation of day-3 YGs in the presence of calcium still resulted in an increase of VT proteolysis (lane 4). Because both ATP and PPi were added to all samples (EGTA and calcium), this data suggests that acidification of YGs *per se* is not sufficient to activate all yolk degradation machinery, indicating that the observed calcium-induced events take part in this process, probably allowing the assembly of the yolk degradation machinery. To exclude the possibility that VT degradation was the result of calcium activation of CD activity, EGTA and Ca^{2+} samples were assayed for CD activities as described before (Fig. 10B). Results showed that calcium addition did not have any significant effect on increasing CD activity, which suggests that the induction of VT proteolysis was the result of the previously observed calcium-induced events, probably the YG fusions.

Discussion

It is generally accepted that, in insects, yolk degradation occurs at the onset of embryonic development. In this process VT undergoes proteolysis, triggered by activation of latent pro-proteases (Fagotto, 1990; Nordin et al., 1991). Activation of these enzymes is achieved by luminal acidification of YGs, a process that is mediated by proton pumps (Fagotto, 1991; Nordin et al., 1991; Mallya et al., 1992; Fagotto, 1995). VT and

hydrolases, such as cathepsin B and carboxypeptidase, are known to accumulate in the oocytes during oogenesis and to be stored in YGs (Wall and Meleka, 1985; Oliveira et al., 1986; Raikhel and Dadhialla, 1992; Valle, 1993; Sappington and Raikhel, 1998; Cho et al., 1991; Cho et al., 1999; Liu and Nordin, 1998; Yin et al., 2001). Newly laid eggs are therefore able to initiate VT degradation. *In vitro*, measurements of hydrolase activities and yolk degradation using fresh eggs have been investigated by many groups (Nussenzveig et al., 1992; Logullo et al., 1998; Sorgine et al., 2000; Fialho et al., 2002). However, even though freshly laid eggs carry all the yolk mobilization machinery, the massive yolk proteolysis does not occur *in vivo* immediately after the egg is laid, but starts at a certain time later, during early embryogenesis. In *R. prolixus*, massive yolk proteolysis starts at day 3 of embryogenesis (Fialho et al., 2005).

Fusion of YGs during oviparous embryogenesis has been suggested and described by several groups (Nordin et al., 1991; Chestkov et al., 1998; McNeil et al., 2000; Yamahama et al., 2003) and the impressive capacity of YGs to form large structures by homotypic fusion is well illustrated by formation of large membrane barriers in sea urchin eggs (McNeil et al., 2000). Calcium has been shown to mediate YG fusion, *in vitro,* in the starfish *Asterina miniata*, the sea urchin *Lytechinus pictus* and the insect *Periplaneta americana* (Chestkov et al., 1998; McNeil et al., 2000; Ramos et al., 2006). Numerous egg responses, such as production of the fertilization envelope, utilize Ca^{2+} as a messenger (Steinhardt et al., 1977).

In this work, we suggest that YGs undergo membrane fusion *in vivo*, in a calcium-dependent manner, during the third day of embryogenesis in the blood sucking insect *Rhodnius prolixus.* This process is followed by mobilization of vacuolar H^+-PPase, acid phosphatase activation and VT proteolysis. Because of the large amounts of calcium ingested in the *R. prolixus* diet*,* it is known that several metabolic processes operate with high calcium concentrations in this insect. *Rhodnius* hemolymph contains 1–8 mmol l^{-1} of calcium, whereas the Malpighian tubules can store calcium at concentrations close to 1 mol l^{-1} (Maddrell et al., 1991). The origin of calcium inside the eggs is unknown, but a calcium-binding protein has been described for *R. prolixus* and shown to play some role on its transport to the oocytes during oogenesis (Silva-Neto et al., 1996). The finding of such high amounts of calcium inside the egg could be explained by the existence of calcium stores. Since the egg is filled with vesicles, it would be relatively easy for this type of system to operate with calcium pumps, channels and/or exchangers, modulating the amount of free calcium remaining in the ooplasma or inside compartments. Variation of $[Ca^{2+}]$ during *R. prolixus* embryogenesis could also be explained by the possible presence of high amounts of calcium-binding proteins, such as calmodulin, modulating its availability. The presence of calmodulin has been reported in *B. germanica* eggs, corresponding to 1.5% of the total volume of its soluble proteins, which can be reduced to almost undetectable levels at the beginning of yolk protein cleavage (Zhang and Kunkel, 1992), suggesting a connection between Ca^{2+} availability and yolk degradation. This is in agreement with our observations that the highest $[Ca^{2+}]$ is found on the third day of development, the time when yolk degradation starts in *R. prolixus* eggs (Fialho et al., 2005). Moreover, elevation of $[Ca^{2+}]$ represents a controlled way to regulate the YG fusion events. Calcium signaling during embryonic development has been elegantly reviewed (Webb and Miller, 2003), and shown to take part in fertilization, embryonic cleavage, blastula formation and other embryogenesis events. Yolk degradation during embryogenesis is probably modulated by the embryo demands. In this regard, Ca^{2+} signaling inside the egg could also be regulated by the growing embryo.

Whether or not $[Ca^{2+}]$ varies between different egg locations (by local release from internal stores and/or association with calcium binding proteins) is still uncertain. Unfortunately *R. prolixus* eggs contain an opaque and impermeable eggshell, precluding efficient clarification or permeability increase. Thus, experiments involving direct calcium imaging in the ooplasm *in vivo* are still not possible in this model. However, even though we cannot access the organization of global or local calcium signaling in eggs of *R. prolixus*, the presence of calcium wave pacemakers and local calcium signaling (as calcium puffs) in eggs (Dumollard et al., 2002) and oocytes (Parker and Yao, 1995) from other models support our findings that Ca^{2+} elevation during early embryogenesis can trigger the fusion of YGs as a slow calcium release on the ooplasm.

To start yolk degradation, the YGs must contain at least three components: yolk proteins, proton pumps and hydrolases. If one of these three components is not found in a common vesicle, yolk degradation does not proceed. As highlighted in this paper, the YG population is not homogeneous and it has been extensively shown that YGs can vary in size, density, macromolecule content, and that not all of them undergo acidification during embryogenesis (Postlethwait and Giorgi, 1985; Wallace, 1985; Fagotto, 1995; Chestkov et al., 1998; McNeil et al., 2000; Fausto et al., 2001).

Our findings that $[Ca^{2+}]$ elevation *in vivo* is coincident with the appearance of more LYGs, combined with the fact that LYG formation and membrane label transfer could be achieved *in vitro* after Ca^{2+} treatment provide evidence for the suggestion that *R. prolixus* YGs undergo a process of membrane fusion. LYG formation observed at day 3 could also be the result of SYGs fusing together, and participation of medium sized YGs in this process cannot be disregarded. However, scanning electron microscopy observations of day 3 YGs commonly evidenced SYGs in association with LYGs (data not shown), suggesting that this type of fusion event might occur more frequently. The observation of higher H^+-PPase activity in SYGs at the beginning of embryogenesis and the elevation of this activity in LYGs during the third day of development, when the massive YG fusion events are taking place, in combination with an increase of AP activity, suggest the presence of a functional mechanism of macromolecule transference from SYGs to LYGs during the third day of development. This is consistent with this being the time when yolk mobilization begins in *R. prolixus* embryogenesis (Fialho et al., 2005).

However, it is important to emphasize that H^+-ATPase and CD levels were unaltered between days 0 and 3, being very

similar within the different YG fractions, indicating that these enzymes are not affected by calcium-induced events. Fialho et al. (Fialho et al., 2005) showed that *Rhodnius* CD and AP are involved in VT degradation in a process in which CD is only able to induce yolk degradation after previous VT dephosphorylation, mediated by AP. This model of modulation of yolk degradation is consistent with our findings that only AP seemed to be transferred by fusion events and could be used for modulation of yolk degradation. Analysis on VT location showed that it seems to be present in all YGs and this pattern does not alter between days 0 and 3, which suggests that VT location is independent of calcium-induced events.

A basic feature of eukaryotic cells is that compartmental organization works through regulated mechanisms of macromolecule sorting to their appropriate compartment. In this context, membrane fusion is a fundamental cellular process, being indispensable for compartmental organization (Alberts et al., 2001).

Altogether, the results indicate that calcium-induced events could have a role in the transfer of components of yolk machinery, regulating the access of hydrolases and/or proton pumps to the yolk proteins and modulating their degradation. This hypothesis is further supported by the finding that VT proteolysis is stimulated by calcium-induced events. Incubation of day 0 YGs in the absence of Ca^{2+} resulted in no VT proteolysis, even when acidified in the presence of ATP and PPi. During day 3, where the membrane fusion events take place, VT proteolysis (higher than on day 0) could also be stimulated by calcium. As Ca^{2+} did not induce any significant increase in CD activity, VT degradation seems to be achieved after YG fusion events, suggesting that it brings all components together, allowing the assembly of the yolk degradation machinery.

Our findings, therefore, provide evidence that calcium-regulated YG membrane fusion is a potential mechanism for macromolecule transfer between different compartments inside the egg. This process probably works on modulation of yolk degradation during embryogenesis, which is essential for VT degradation. Understanding exactly how this process occurs could clarify the dynamics of yolk degradation during insect embryo development. Further studies on this mechanism, such as the presence of calcium binding proteins (sensors and buffers) and calcium stores inside the egg, isolation and location of enzymes involved with yolk degradation and characterization of proteins involved with membrane fusion are currently under intense investigation by our group.

List of abbreviations

AP	acid phosphatase
CD	cathepsin D
DIC	differential interference contrast
LYG	large YG
NaPPi	sodium pyrophosphate
SYG	small YG
VT	vitellin
YG	yolk granule

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