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Involvement of cAMP and calmodulin in endocytic yolk uptake during *Xenopus laevis* oogenesis

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Summary

The aim of the present study was to show the participation and physiological role of calmodulin (CaM) and cAMP during vitellogenin endocytic uptake in the amphibian *Xenopus laevis*. The results showed a differential distribution of CaM in the ovary follicles during oogenesis. The CaM intracellular localization was not affected by gap junction's downregulation and CaM inhibition did not completely abolished the endocytic activity of oocytes. We showed that cAMP was able to completely rescue the endocytic competence in follicles in which gap junctional communication had been disrupted by octanol. Moreover cAMP was capable of restoring oocyte endocytic capability in the presence of octanol and stelazine, a CaM inhibitor. We propose that, in Vtg uptake regulation, cAMP is upstream of CaM during the endocytic signalling pathway.

Keywords: Calmodulin, cAMP, Signalling pathways, Vitellogenesis, *Xenopus laevis*

Introduction

The process of vitellogenesis, common to all oviparous species, is characterized by hepatic synthesis and secretion of vitellogenin (Vtg) into the blood stream. Vtg is then taken up by growing oocytes and provides the main nutritional reserves necessary for embryo development (Wallace, 1985). In *Xenopus* the synthesis and transport of Vtg have been well elucidated (Wallace *et al.*, 1983; Wall & Patel, 1987; Marilley *et al.*, 1998), although the onset and continuation of the Vtg uptake process by the oocyte remain unclear.

Oogenesis in the amphibian *Xenopus laevis* can be divided into six stages (I–VI) according to the morphology of the developing oocyte. Stages I and II are both previtellogenic. Vitellogenesis begins during stage III and continues through stage V. It is known that the ovarian development of *X. laevis* is asynchronous and follicles at all stages can be found in the same ovary at any time (Dumont, 1972). Thus, oocytes at different stages of development are exposed to the same hormonal environment, although only vitellogenic follicles are actively involved in Vtg endocytosis. In addition, vitellogenic oocytes (stages III–V) cultured *in vitro* without follicle cells grow indefinitely at a rate that is strictly dependent on the external concentration of hepatic Vtg added to the medium (Wallace *et al.*, 1980; Wallace *et al.*, 1981). Thus, for species in which both previtellogenic and vitellogenic follicles are simultaneously present and active, factors other than hormonal control would be present. This fact supports the idea that follicle cells may be involved in the normal vitellogenic process.

Gap junction intercellular communication is ubiquitous in the majority of cells and is indispensable for the proper development and function of most tissues (Rossello & Kohn, 2010). Our previous studies have demonstrated that direct gap junctional communication is a requirement for the acquisition

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of endocytic competence by *Xenopus laevis* oocytes. Thus, oocytes with uncoupled or downregulated gap junctions are unable to begin Vtg uptake (Monaco *et al.*, 2007). In addition, Anderson & Woodruff (2001) showed that a gap junctionally transmitted epithelial cell signal regulates endocytic yolk uptake in the insect *Oncopeltus fasciatus*, while Brooks & Woodruff (2004) reported that the 17–19 kDa protein calmodulin (CaM) is involved in the process. A possibility to be considered is that this mechanism for regulating the vitellogenic process through CaM is conserved in some oviparous vertebrates.

Gap junctions provide direct communication channels for the diffusion of ions and second messenger molecules such as adenosine 3,5-cyclic monophosphate (cAMP), inositol 1,4,5-triphosphate (IP3) and calcium ions (Saez *et al.*, 1989; Giaume, *et al.*; 1997; Kam *et al.*, 1998).

Conversely, Lawrence *et al.* (1978), using cocultures of rat ovarian granulosa cells and mouse myocardial cells showed the first evidence of the passage of cAMP molecules through gap junction channels. cAMP is a ubiquitous second messenger that affects cellular functions by interacting with effector molecules such as cAMP-dependent protein kinases, hyperpolarization activated channels and cyclic nucleotide-gated ion channels (Bedner *et al.*, 2006). Several investigators have proposed cAMP to be one of the molecules involved in meiotic arrest (Eppig & Downs, 1984; Tsafriri *et al.*, 1996; Chen *et al.*, 2009). In the anuran *Bufo arenarum* oocytes we demonstrated that cAMP exerts an inhibitory effect on the maturation induced by the uncoupling of gap junctions when oocytes are pretreated with octanol (Villecco *et al.*, 2000). However, there is no detailed information concerning cAMP participation during *X. laevis* vitellogenesis.

In this context, the aim of the present study was to show the participation and physiological role of CaM and cAMP during *Xenopus laevis* vitellogenesis. In addition, we investigated the hierarchical relationship between these two signal molecules in the acquisition of endocytic activity by oocytes.

Materials and methods

Biological material

Adult female *Xenopus laevis* specimens were kept in dechlorinated fresh water tanks at 18–20 °C on a 12 h light/dark cycle and fed three times weekly with chopped heart meat.

Frogs were anaesthetized on ice and ovarian lobes were removed via minilaparotomy. After the incision was sutured, the animal was allowed to recover at room temperature.

The vitellogenic follicles (stages III–V; Dumont, 1972) were dissected manually with the aid of a dissecting microscope and sharpened watchmaker's forceps and incubated in an O-R2 sterile solution.

Preparation of vitellogenin-containing serum (Vtg)

For the study of receptor-mediated oocyte endocytic activity it is essential that the receptor ligands (Vtg) be present in the incubation medium. Injecting *X. laevis* females with a dose of estrogen (4 mg 17β-estradiol dissolved in 0.4 ml propylene glycol per 100 g body weight) initiated Vtg accumulation in the blood. After 2–3 weeks, estrogen-treated animals were anaesthetized and bled exhaustively. The serum obtained was dark green and contained approximately 100–150 mg Vtg/ml (Wallace *et al.*, 1980).

Dye transfer experiments

Oocytes were microinjected with 20–40 nl of 1% aqueous solution of the fluorescent dye Lucifer yellow CH MW: 452.2 (Emax λ 535nm-Molecular Probes). To evaluate dye transfer to follicle cells, oocytes were manually defolliculated 2 h after microinjection with watchmaker's forceps and observed with a Zeiss fluorescence microscope.

Downregulation of gap junction

To block the passage of molecules ≥440 Da, follicles were incubated in octanol (Sigma Chemical Co.) dissolved in ethanol and diluted to 1 mM with sterile O-R2 (final concentration of ethanol <1%). It should be noted that 1 mM octanol has been often used to disrupt dye coupling between oocytes and their surrounding epithelial cells (Cerdá *et al.*, 1993; Patiño & Purkiss, 1993; Adler & Woodruff, 2000).

Endocytic tracer

To detect the pathway of Vtg endocytosis, a biotinylated bovine serum albumin (b-BSA) was used as a tracer. BSA (Sigma Chemical Co., A 7906) was tagged with biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (BNHS, Sigma Chemical Co.) by addition of 10 µl of a 20 mg/ml solution (in dimethylformamide) for each 1 mg of albumin present in the dialysis bag. After 1 h at room temperature, the reaction mixture was dialyzed at 4 °C overnight against an OR-2 solution. The biotinylated protein was stored at –20 °C until used.

Tracer incorporation by oocytes

Experimental groups are described in Table 1.

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Table 1 Experimental groups

Reagents	Experimental groups							
	I	II	III	IV	V	VI	VII	VIII
Gentamicin (1 μg/ml)[a]	+	+	+	+	+	+	+	+
Octanol (1 mM)[a]	−	+	−	+	−	−	+	+
Serum Vtg (40 μl)[b]	+	+	+	+	+	+	+	+
Biotinylated BSA (0.2 mg/ml)[b]	+	+	−	−	−	+	+	+
dbcAMP (1 mM)[b]	−	−	−	−	−	−	+	+
Stelazine (200 μM)[a]	−	−	−	−	+	+	−	+

[a]Added at the beginning of the culture period.
[b]Added 3 h after the onset of the culture period.
Different groups of oocytes were cultured in OR-2 medium for 24 h. Final concentrations are shown in brackets.

Histological procedures and immunohistochemistry

Biotinylated BSA (b-BSA) and calmodulin were determined by immunohistochemistry.

After incubation with b-BSA, follicles were fixed in 4% formol, dehydrated and embedded in paraffin-celloidin, as specified by Manes & Nieto (1983). Sections 5–7 μm thick were serially obtained from blocks and mounted on poly-L-lysine-coated glass slides. Then they were deparaffined, rinsed with phosphate-buffered saline (PBS, pH 7.4) and incubated with 3% BSA, 0.02% Tween 20 in PBS (BSA/Tween 20/PBS) for 1 h at room temperature to prevent non-specific background staining (blocking solution).

In order to detect b-BSA, the slides, after blocking, were treated with a 1:3000 dilution of streptavidin-FITC conjugate (Sigma Chemical Co.) and incubated at 4 °C in a moist chamber overnight. After extensive washing with PBS, an anti-FITC antibody conjugated with alkaline phosphatase was applied in a 1:3000 dilution for 2 h at room temperature. The slides were then washed and alkaline phosphatase activity was detected by incubation with NBT/BCIP (Roche Biochemicals) as a substrate. The reaction was stopped by rinsing the sections with absolute methanol. Then the slices were hydrated, mounted and observed with a Zeiss microscope.

In order to detect CaM, the sections were deparaffined, rinsed with PBS (pH 7.4) and incubated with 0.3% H_2O_2 in methanol for 30 min to inactivate the endogenous peroxidase. Slides were incubated with blocking solution. After blocking, the slides were treated with a 1:100 dilution of a mouse anti-calmodulin monoclonal antibody (Sigma Chemical Co.) and incubated at 4 °C in a moist chamber overnight. After extensive washing with PBS, the slides were treated with a 1:50 dilution of an ExtrAvidin–peroxidase (Sigma Chemical Co.,) conjugate for 1 h at room temperature. Peroxidase activity was detected by incubation with 3,3′-diaminobenzidine (Sigma Chemical Co.) in H_2O_2 at 37 °C for 10 min. The reaction was stopped by rinsing the samples with distilled water and slices were mounted and observed with a Zeiss microscope.

Controls included parallel and adjacent sections incubated without the first antibody. All images were obtained under the same conditions.

Results

Calmodulin immunolocalization during *Xenopus laevis* oogenesis

Ovarian sections of *Xenopus laevis* were immunostained for CaM in order to analyze its presence and distribution throughout oocyte development. Figure 1 shows that intracellular CaM localization depends on the oocyte stage.

During the initial oocyte stage (corresponding to previtellogenic oocytes), the occurrence of CaM was noted in the cytoplasm in a weak and uniform way, probably due to endogenous synthesis of CaM (Fig. 1*A*,*B*). It is important to note that a strong signal in the follicular cells was observed, suggesting a substantial presence of CaM (Fig. 1*A*,*B*).

During the onset of vitellogenesis (stage III) CaM localization was mainly found in the cortical cytoplasm. The periphery of the oocytes was strongly stained and coincided with the nascent forms of small yolk platelets (Fig. 1*A*,*C*). Oocyte at stages IV and V showed a uniform cytoplasmic label of CaM between yolk platelets concomitant with the vitellogenic process (Fig. 1*A*,*D*). When ovarian sections were incubated without the first antibody (Fig. 1*E*), no signal was detected.

These results showed that during *Xenopus laevis* oogenesis there is a differential distribution of CaM and this fact could indicate that CaM would be synthesized both in the follicular cells and in the oocytes.

Effect of gap junction uncoupling on the uptake of Vtg and CaM distribution during vitellogenic process

To establish follicle cell–vitellogenic oocyte coupling through gap junctions, transfer fluorescent dye experiments were performed. The fluorescent dye Lucifer yellow CH is widely used to determine gap junction functionality, since it can pass freely through gap junctions but does not readily permeate membranes as long as the cell is healthy. Incubation with 1 mM octanol is an efficient method for downregulating/uncoupling gap junctional communication in ovarian follicles. Lucifer yellow CH was microinjected through the follicular layer into 40–50 vitellogenic oocytes both with and without octanol.

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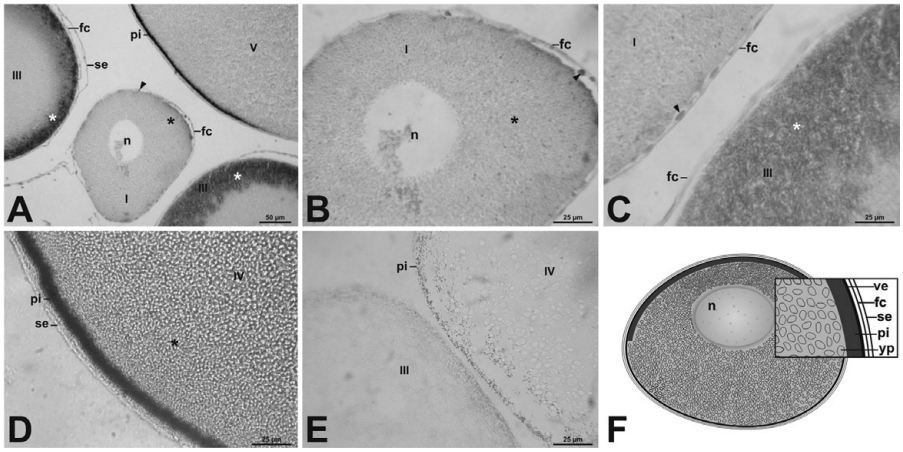

Figure 1 Immunolocalization of the calcium-binding protein calmodulin in tissue sections of *Xenopus laevis* ovary. (*A–E*) Micrographs of ovary sections treated with anti-calmodulin antibody and visualized using Extravidin–biotin system. (*A*) Note the weak and uniform signal in the previtellogenic oocyte cytoplasm (black asterisk). Follicular cells show a strong signal signifying a noteworthy presence of calmodulin (CaM) (arrowhead). At later stages, the signal become cortical and stronger (white asterisk). (*B–D*) Higher magnifications of micrograph (*A*). (*E*) Control performed without the first antibody. (*F*) Schematic diagram of stage IV oocyte describing its main characteristics. Roman numerals indicate oocyte stages. fc, follicular cells; n, nucleus; pi, pigment; se, surface epithelium; ve, vitelline envelope; yp, yolk platelets.

Figure 2 shows that follicle cells from vitellogenic follicles incubated without 1 mM octanol fluoresce after dye injection (Fig. 2*A*,*B*). In contrast, follicle cells from vitellogenic follicles treated with 1 mM octanol did not show fluorescence (Fig. 2*C*,*D*). These results demonstrate that during the vitellogenic process there are functional gap junctions at the oocyte/follicle cells interface that might lead to a metabolic coupling between the oocyte and the surrounding follicle cells.

In order to assess the role of gap junctions in Vtg uptake, the formation of labelled yolk platelets was monitored through non-specific fluid-phase labelling with biotinylated albumin. Results showed that untreated control follicles developed newly labeled yolk platelets in the oocyte cortex (Fig. 2*E*, Group I), while follicles treated with octanol showed no sign of nascent yolk sphere formation (Fig. 2*F*, Group II). Follicles in which gap junction communication between epithelial cells and oocytes was disrupted by octanol ceased endocytic uptake of the yolk precursor. In consequence, these results show that both gap junction coupling and Vtg uptake were temporally coincident events during *Xenopus laevis* vitellogenesis and that without the appropriate signal from surrounding epithelial cells oocytes were unable to endocytose vitellogenin.

On the other hand, the results showed that downregulation of gap junctions treated with 1 mM octanol did not change the intracellular localization of CaM in vitellogenic oocytes (Fig. 2*G*, Group III and Fig. 2*H*, Group IV), suggesting that the presence of this protein in the oocytes could be independent of functional/coupled gap junctions.

Effect of CaM inhibition on the Vtg uptake during *Xenopus laevis* vitellogenesis

In order to gain an insight into the role of CaM during the endocytic uptake of Vtg, a specific inhibitor of CaM, stelazine, was used. In the presence of calcium, stelazine binds to CaM rendering it incapable of activating the calcium dependent protein or any other enzyme in the system. A previous link between CaM and receptor-mediated endocytosis of Vtg has been found in insects (Brooks & Woodruff, 2004).

Figure 3 shows experiments monitoring the pathway of labelled yolk platelets through incorporation of b-BSA as an endocytic tracer in the absence or presence of 200 μM stelazine (Fig. 3*A*,*B*, Group I; Fig. 3*D*,*E*, Group VI).

Vitellogenic oocytes displayed a labelled yolk pathway in untreated control follicles, while in stelazine treated oocytes a diffuse and less intense way of yolk incorporation could be observed. Additionally, we showed that, in stelazine incubated oocytes, CaM maintained a uniform labelling in the cytoplasm, indicating that the inhibition of its activity did not change CaM immunolocalization (Fig. 3*C*, Group III and Fig. 3*F*, Group V).

It is important to note that in stelazine-incubated oocytes the endocytic activity was not completely abolished, suggesting that another signal molecule

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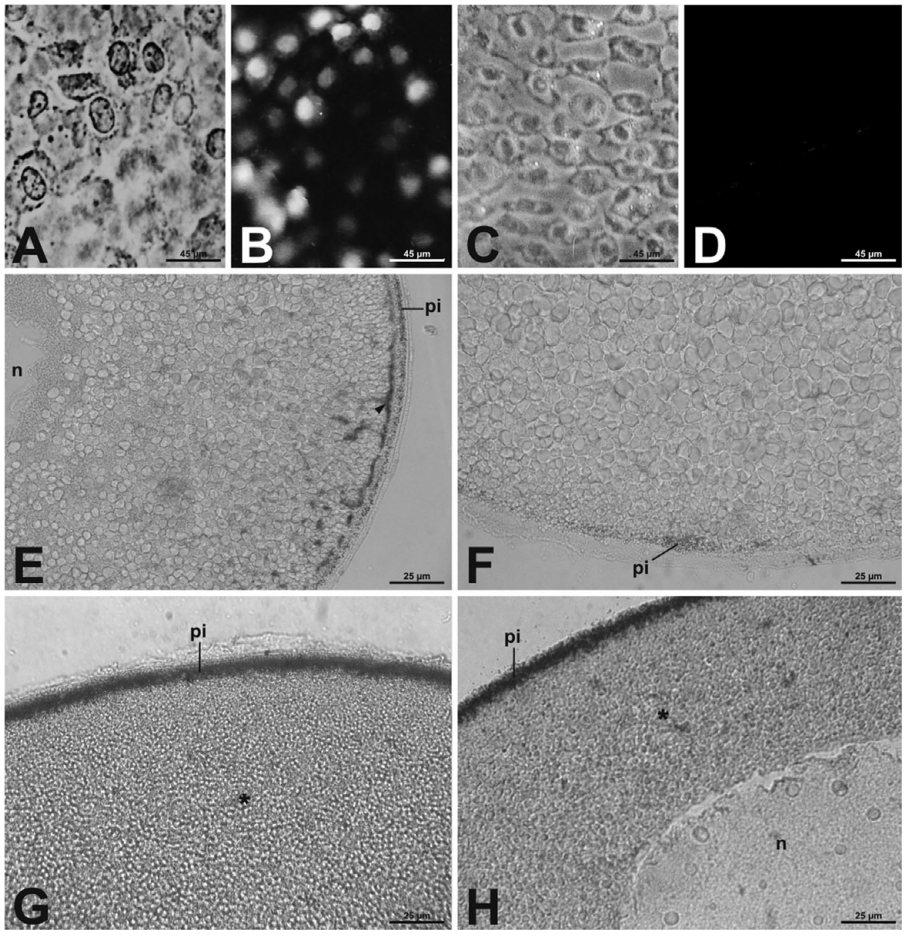

Figure 2 Gap junction uncoupling study. (*A*,*B*,*E*,*G*) Stage IV oocyte cultured without octanol treatment. (*C*,*D*,*F*,*H*) Treated with 1 mM octanol. (*A*,*C*) Follicle cells epithelia examined for phase contrast. (*B*,*D*) Fluorescence micrographs of (*A*,*C*). (*E*–*H*) Micrographs of oocyte sections revealed with alkaline phosphatase system for b-BSA detection (*E*,*F*) and treated with anti-calmodulin antibody (*G*,*H*). (*B*) Notice the fluorescence resulting from the dye transference from vitellogenic oocyte to follicle cells in untreated follicles. (*D*) No fluorescence is observed. (*E*) Cortical mark (arrowhead) indicated Vtg incorporation corresponding to oocytes with coupled gap junctions. (*F*) No mark is observed in oocytes with uncoupled gap junctions. (*G*,*H*) No difference is observed in calmodulin (CaM) distribution (black asterisk), in both octanol untreated and treated oocytes. n, nucleus; pi, pigment.

different from CaM could be involved in regulating Vtg uptake during *Xenopus laevis* oogenesis.

Rescue of Vtg endocytic uptake in follicles with downregulated gap junctions

To establish the participation of cAMP as a possible molecular signal for the acquisition of endocytic competence by vitellogenic oocytes, rescue experiments were performed using the stable cell-permeable cAMP analogue (dbcAMP (N^6,2′-*O*-dibutyryladenosine 3′-5′-cyclic monophosphate) in the incubation medium.

Vitellogenic oocytes were treated with 1 mM octanol and incubated in the presence of 1 mM dbcAMP. The formation of labelled yolk platelets was monitored through non-specific fluid-phase labelling with biotinylated albumin. Results showed that there was an intense new yolk platelet pathway when dbcAMP was incorporated into a medium in which gap junctions had been downregulated with octanol, rescuing the follicle endocytic activity (Fig. 4*A*,*B*, Group VII).

In addition, when uncoupled gap junction oocytes were incubated with stelazine, the labelled yolk platelets were also observed by dbcAMP treatment (Fig. 4*C*,*D*, Group VIII).

These results demonstrate that cAMP is the signal that triggers vitellogenesis and that CaM is involved downstream in the vitellogenic pathway of *X. laevis.*

Discussion

X. laevis oogenesis is a continuous, asynchronous process and oocytes at all stages of development

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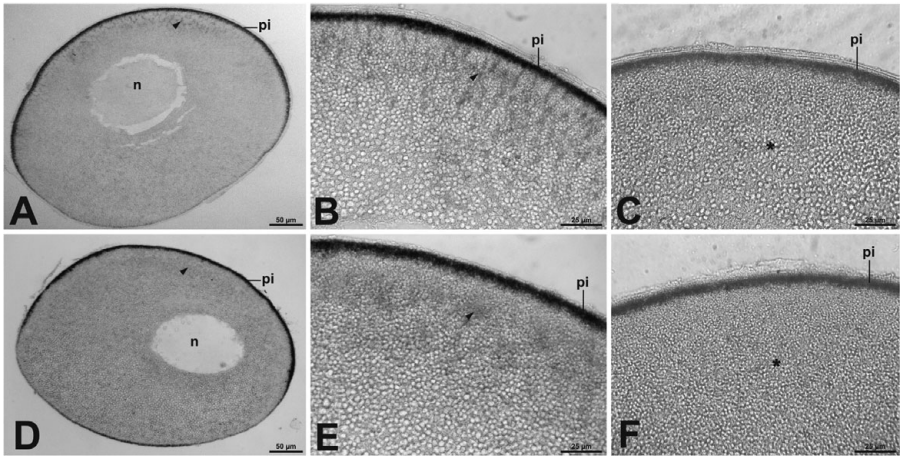

Figure 3 Calmodulin immunolocalization and b-BSA detection in tissue sections of oocytes treated and untreated with stelazine. (*A*–*C*) Stage IV oocytes without stelazine treatment. (*D*–*F*) Oocytes treated with stelazine. (*A*,*B*,*D*,*E*) Oocyte sections revealed by b-BSA detection. Vtg uptake was observed with and without stelazine treatment (arrowhead). Note the lower and diffused signal in oocytes treated with stelazine. (*C*,*F*) Oocytes sections treated with anti-calmodulin (anti-CaM) antibody. No difference is noted in CaM distribution (black asterisk) in both stelazine untreated and treated oocytes. n, nucleus; pi, pigment.

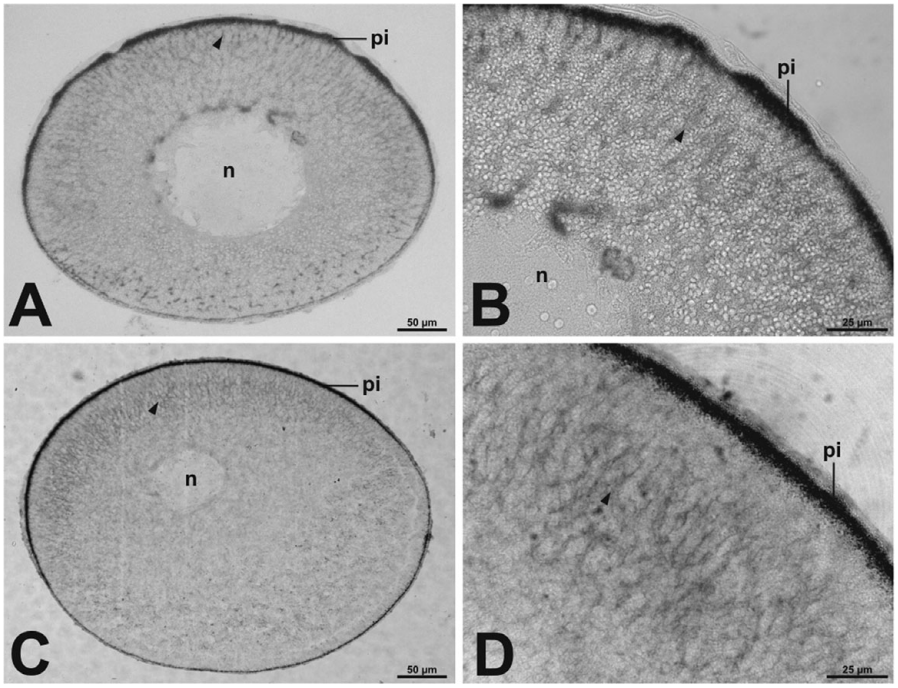

Figure 4 Rescue experiments with dbcAMP. Micrographs of stage IV oocyte sections revealed by b-BSA detection in order to display Vtg incorporation. (*A*,*B*) Oocytes cultured with octanol and dbcAMP. The cell-permeable cAMP analogue restored the vitellogenic process (arrowhead). (*C*,*D*) Oocytes cultured with octanol, stelazine and dbcAMP. Note that vitellogenesis could be completely rescued (arrowhead) even in the presence of the calmodulin (CaM) inhibitor. n, nucleus; pi, pigment.

are present in the ovary at all times during adult life (Dumont, 1972). Moreover, yolk formation or vitellogenesis is the oldest most common feature by which the mother supplies the zygote for embryonic development in most reproductive strategies, with only few exceptions among invertebrates and only *Eutheria* among vertebrates (Polzonetti-Magni *et al.*, 2004). In amphibian species in which previtellogenic and vitellogenic follicles coexist simultaneously, both hormonal regulation and local control mechanisms, whose follicle cells would participate through the gap junctions, should be present.

In the present paper we proposed that cAMP could be the signal transmitted through coupled

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gap junctions, which is necessary for the endocytic vitellogenic pathway in *Xenopus laevis* oocytes. We also suggested that CaM is probably implicated in the signalling cascade for the acquisition of follicle endocytic competence.

In this sense, we have shown that direct gap junctional communication is a requirement for endocytic activity as, without the appropriate signal from surrounding epithelial cells, *Xenopus laevis* oocytes were unable to endocytose the yolk precursor protein (Monaco *et al.*, 2007). Also, in insects, this communication channel has been confirmed to occur via gap junctions and the control molecule transported from follicle cells to oocytes has been identified as the 17 kDa protein calmodulin (Brooks & Woodruff, 2004). Moreover, Curran & Woodruff (2007) showed that CaM in its elongated shape was able to transit the vertebrate gap junctions between oocytes and the epithelial cells that surround them in the amphibian *X. laevis* and in fishes *Danio rerio* and *Oryzias latipes* (Curran & Woodruff, 2007).

One of the hypotheses tested was whether vitellogenesis triggering by CaM could have been maintained during evolution for anuran amphibians, although its participation in the vitellogenic process has not been completely defined. Previous results showed only the relationship between CaM and meiosis resumption in amphibian oocytes (Wasserman & Smith, 1981). Moreover, Chien & Dawid (1984) reported that *X. laevis* contains two functional CaM genes that appear to be expressed coordinately in different tissues and that testis, brains, and ovaries contain the highest concentrations of CaM mRNA (Chien & Dawid, 1984).

The developmental significance of different patterns of CaM synthesis and localization during amphibian oogenesis is not clear. To understand this significance, in this report we describe for first time the ovary CaM immunolocalization in the anuran *Xenopus laevis.* On the basis of cytological analysis, we showed that CaM is preferentially localized in the oocyte cytoplasm throughout vitellogenesis. Thus, this study provides the evidence that the cortical oocyte cytoplasm increased its immunoreactivity to the anti-CaM antibody from stage III parallel to the vitellogenic process and continued throughout stages IV and V. In agreement with this, measurements of CaM synthesis at all developmental stages indicated that it was being synthesized continuously during oogenesis and that the rate of synthesis increased during vitellogenesis (Cicirelli & Smith, 1986). This information is consistent with results of this study where we show a strong CaM label from stage III oocytes.

The evidence provided in this report reveals that the pathway regulating endocytic activity includes transfer from epithelial cells to the oocyte of a diffusible molecular signal. Furthermore, this signal must be transferred via open gap between the follicular cells and the oocytes. Experiments in which gap junctions were octanol down-regulated have shown that Vtg receptor-mediated uptake was inhibited by this treatment. Interestingly, the intracellular location of CaM was unchanged after octanol gap junction disruption.

Considerable insight has been gained into the role of CaM in the regulation of vitellogenic activity by the use of stelazine, a specific CaM inhibitor. In this report we showed that stelazine leads to a decreased uptake of yolk precursors and that oocyte cytoplasm shows a less intense labelling of endocytic tracers. These results are in agreement with Tucciarone & Lanclos (1982), who found that stelazine inhibits Vtg endocytosis by 63% as determined by the decreased uptake of Vtg into yolk platelets.

In this regard, since endocytic activity is only partially blocked by stelazine, we propose that CaM would not be the only molecule involved in the signaling pathway that regulates the vitellogenic process.

Previous studies in invertebrates indicate that the control of yolk protein internalization and vitellogenesis stimulation could be done by modulation of cAMP levels (Medeiros *et al.*, 2004; Zmora *et al.*, 2009). In this report we show that follicles in which gap junctional communication between epithelial cells and oocyte had been disrupted by octanol and vitellogenic uptake had ceased, the dbcAMP (cell-permeable cAMP analogue) was able to completely rescue endocytic competence. Thus, follicles incubated in presence of dbcAMP presented new yolk platelets within cytoplasm oocytes, indicating that they had resumed Vtg uptake. In this sense, previous data from our laboratory show that gap junctions would act as mediators in cAMP transfer between follicle cells and oocytes in *Bufo arenarum* (Villecco *et al.*, 2000).

Conversely, the experiment consisted in incubating uncoupled gap junctions oocytes in presence both dbcAMP plus stelazine allows us to suggest the hierarchical relationship between the cAMP and CaM. This result showed that even in the presence of octanol and CaM inhibitor, cAMP is capable of restoring oocyte endocytic capability. Moreover, this allows us to suggest that CaM would collaborate downstream in the pathway regulating the onset and maintenance of Vtg uptake during *Xenopus laevis* oogenesis.

Furthermore, we propose a probable mechanism of Vtg uptake regulation in which cAMP is located upstream in the signaling pathway with respect to CaM, triggering vitellogenesis (Fig. 5). Nevertheless, additional experiments are necessary to elucidate the whole regulation process.

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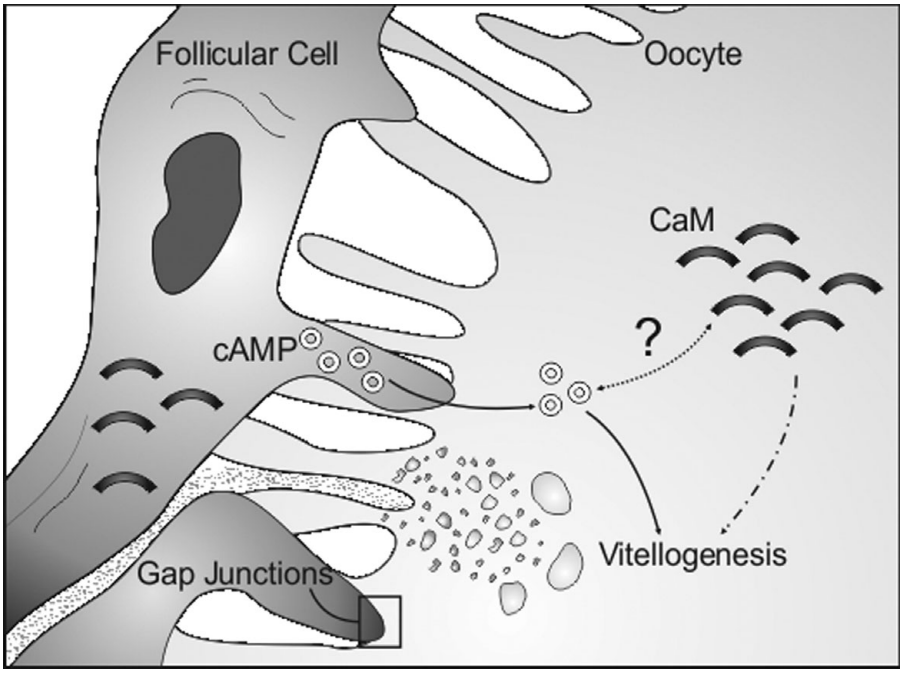

Figure 5 Considering these results, we propose a probable mechanism of vitellogenin uptake regulation in which cAMP is located upstream in the signalling pathway with respect to calmodulin (CaM), triggering vitellogenesis. Nevertheless, additional experiments are necessary in order to elucidate the entire regulation process. Continuous line, established process; dotted line, unproved interaction mechanism; dash-dot line, proved but unknown interaction.

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