Cathepsin B-mediated yolk protein degradation during killifish oocyte maturation is blocked by an \( H^{+} \)-ATPase inhibitor: effects on the hydration mechanism

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Raldúa, Demetrio, Mercedes Fabra, María G. Bozzo, Ekkehard Weber, and Joan Cerdà. Cathepsin B-mediated yolk protein degradation during killifish oocyte maturation is blocked by an \( H^{+} \)-ATPase inhibitor: effects on the hydration mechanism. Am J Physiol Regul Integr Comp Physiol 290: R456–R466, 2006. First published September 1, 2005; doi:10.1152/ajpregu.00528.2005.—In teleost oocytes, yolk proteins (YPs) derived from the yolk precursors vitellogenins are partially cleaved into free amino acids and small peptides during meiotic maturation before ovulation. This process increases the osmotic pressure of the oocyte that drives its hydration, which is essential for the production of buoyant eggs by marine teleosts (pelagophil species). However, this mechanism also occurs in marine species that produce benthic eggs (benthophil), such as the killifish (Fundulus heteroclitus), in which oocyte hydration is driven by \( K^{+} \). Both in pelagophil and benthophil teleosts, the enzymatic machinery underlying the maturation-associated proteolysis of YPs is poorly understood. In this study, lysosomal cysteine proteinases potentially involved in YP processing, cathepsins L, B, and F (CatL, CatB, and CatF, respectively), were immunolocalized in acidic yolk globules of vitellogenic oocytes from the killifish. During oocyte maturation in vitro induced with the maturation-inducing steroid (MIS), CatF disappeared from yolk organelles and CatL became inactivated, whereas CatB proenzyme was processed into active enzyme. Consequently, CatB enzyme activity and hydrolysis of major YPs were enhanced. Follicle-enclosed oocytes incubated with the MIS in the presence of bafilomycin A₁, a specific inhibitor of \( \mathrm{H}^{+} \)-ATPase, underwent maturation in vitro, but acidification of yolk globules, activation of CatB, and proteolysis of YPs were prevented. In addition, MIS plus bafilomycin A₁-treated oocytes accumulated less \( K^{+} \) than those stimulated with MIS alone; hence, oocyte hydration was reduced. These results suggest that CatB is the major protease involved in yolk processing during the maturation of killifish oocytes, whose activation requires acidic conditions maintained by a vacuolar-type \( H^{+} \)-ATPase. Also, the data indicate a link between ion translocation and YP proteolysis, suggesting that both events may be equally important physiological mechanisms for oocyte hydration in benthophil teleosts.

Fundulus heteroclitus; oocyte maturation; hydration; vacuolar-type \( H^{+} \)-ATPase; cathepsin

In oviparous vertebrates, very-low-density lipoproteins and vitellogenins (Vgs) are produced in the liver under estrogen regulation, secreted into the bloodstream, and delivered to growing oocytes by means of receptor-mediated endocytosis. During the incorporation into the oocyte, these precursors are cleaved into smaller molecular weight polypeptides, the yolk proteins (YPs) lipovitellin (Lv), phosvitin, and \( \beta \)-component, apparently by the action of the lysosomal aspartic protease cathepsin D (4, 5, 23, 44, 51, 63). The YP are then packed together into yolk spheres, which are stored in developing oocytes as an energy source for embryonic development.

In most marine teleosts, an additional processing of YPs derived from Vg1 and Vg2 takes place during hormone-induced meiosis reinitiation or oocyte maturation (3, 7, 20, 33–35, 43, 54, 56). In species that produce highly hydrated, pelagic (floating) eggs, named pelagophils, the second maturation-associated proteolysis of Lvs, phosvitins, and \( \beta \)-components is related to the production of cleavage peptides and free amino acids (FAAs) that contribute to the colligative osmotic pressure required for oocyte hydration (10, 16, 34–35, 43, 51, 53, 54). In other marine teleosts that spawn less hydrated, nonfloating eggs, named benthophils, such as the killifish (Fundulus heteroclitus), we have recently shown that a different pattern of YP processing with respect to that of pelagophil species occurs during oocyte maturation, resulting in a limited proteolysis of Vg1-derived Lv (29). In these species, only a small increase in FAAs is observed, and thus the main osmotic effectors for oocyte hydration appear to be the accumulation of inorganic cations, such as \( K^{+} \) and \( Na^{+} \) (8, 21, 57).

In both pelagophil and benthophil teleosts, however, the proteases involved in the processing of YPs during oocyte maturation are not well known. Recent studies suggest the role of the lysosomal cysteine proteinase cathepsin L (CatL) in the processing of Lv during oocyte maturation in the pelagophil gilthead sea bream (Sparus aurata) (5). Similarly, the activities of acid phosphatases and also of CatL have been implicated in YP degradation in some fish embryos and larvae (26, 38, 51) and during atresia-associated yolk proteolysis in ovarian follicles (58). However, in other fish species, including the killifish, as well as in some amphibians, insects, and sea urchin, cathepsin B (CatB)-like cysteine proteinases and serine proteases have been found or suggested as the enzymes involved in the processing of yolk materials during oocyte maturation and early embryogenesis (6, 9, 23, 27, 32, 36). The causes for these divergent enzymatic mechanisms among teleosts, specially for YP processing during oocyte maturation, remain intriguing.
Cumulative evidence indicates that pH may be the key regulator of YP degradation during embryogenesis in both invertebrates and lower vertebrates. In insects, the major acid proteases are stored in the yolk bodies as a latent, acid-activatable proenzymes (9, 13, 39, 60). These yolk bodies are initially neutral, but they become acidic during development, causing maturation and/or activation of CatL (in the tick) or CatB (in mosquito and silk moth) proenzymes and yolk degradation. Acidification of yolk platelets and its relationship with the onset of yolk proteolysis have also been reported in Xenopus laevis (14, 15) and sea urchin (32, 62), where in the latter the activity of a CatB-like enzyme is regulated by changes in pH. The transient decline in the pH of yolk bodies is established by a vacuolar-type H⁺-ATPase (V-ATPase), which appears to be developmentally regulated, thereby controlling the timing of yolk processing (14, 15, 32).

In teleosts, information on the role of acidification and its mechanism of action for YP proteolysis during oocyte maturation is very scarce. Bafilomycins and concanamycins are two groups of macrolide antibiotics that prevent the acidification of vacuolar compartments such as lysosomes through the inhibition of the V-ATPase at nanomolar levels; hence, lysosomal proteolysis would be suppressed (2, 11, 55). By using bafilomycin A₁ (BA₁), Selman et al. (50) recently reported in the pelagophil black sea bass (Centropristes striata) that V-ATPase inhibitors were responsible for the activation of yolk proteolysis, generation of FFAs, and concomitant oocyte hydration. However, the specific mechanism and potential proteases involved have not been yet identified. In the present work, we have investigated the role of yolk acidification in the regulation of cysteine proteases potentially involved in YP degradation during killifish oocyte maturation. CatL, CatB, and cathepsin F (CatF) were immunolocalized for the first time in fish oocytes, and their changes during the transition from vitellogenesis into maturation were documented. Furthermore, by using the V-ATPase inhibitors BA₁ and concanamycin A (ConA) on in vitro experiments, we investigated the role of V-ATPase-mediated acidification on the activation of CatL and CatB proenzymes and subsequent enzyme activity.

**MATERIALS AND METHODS**

**Animals and chemicals.** F. heteroclitus males and females were collected from the salt marshes of the Bay of Cádiz (South Spain) and maintained in the laboratory as described (12). All chemical reagents, culture medium, and hormones were purchased from Sigma, unless indicated otherwise. The procedures for the sampling of fish and death employed were approved by the Ethical Committee from IRTA (Spain).

**Culture of ovarian follicles in vitro.** Collection of fully grown ovarian follicles and induction of oocyte maturation using the naturally occurring maturation-inducing steroid (MIS) 17α,20β-dihydroxy-4-pregnen-3-one (17,20-P) were carried out as described (29). The effect of V-ATPase inhibitors, BA₁ and ConA, both from 1 to 100 nM, were tested by preincubation of follicles (n = 20–25) with each of the drugs for 1 h before addition of the steroid. Incubations were carried out at 25°C for up to 48 h in a temperature-controlled incubator. The occurrence of oocyte maturation in vitro was scored by the incidence of germinal vesicle breakdown (GVBD). The effect of BA₁ on oocyte hydration was determined on follicles cultured individually in 96-well plates by measuring changes in oocyte volume calculated from the oocyte diameter measured with an ocular stereomicroscope to the nearest 0.01 mm until full hydration was observed. Water content of groups (n = 25–35) of fully grown and mature follicle-enclosed oocytes in vitro, in the presence or absence of 100 nM BA₁, was measured gravimetrically to constant weight at 60°C.

**Enzyme activity of CatL and CatB.** The enzyme activities of CatL and CatB were determined in ovarian follicles undergoing oocyte maturation in vitro in the presence or absence of 100 nM BA₁, using Z-Arg-Arg-7-aminomethylcoumarin (AMC) and Z-Phe-Arg-AMC as substrates for CatB and CatL, respectively, as described (29).

**SDS-PAGE of YPs.** The effect on H⁺-ATPase inhibitors on steroid-induced yolk proteolysis was determined on samples from 10–15 ovarian follicles incubated with 17,20-P in the presence of BA₁ or ConA. Follicles were placed in 1.5-ml Eppendorf tubes containing 100 μl of 1× Laemmli sample buffer (28) and immediately homogenized and heated for 5–10 min at 97°C. The tubes were allowed to cool, and 10 U of benzonase were added to the homogenates to digest DNA for ~20 min at room temperature (RT). The homogenates were briefly centrifuged at 12,000 g for 3 min, and the supernatant was stored at −20°C until electrophoresis. SDS-PAGE was carried out using 10% or 15% acrylamide mini-gels (7 × 10 cm). Molecular weight standards and follicle homogenates (0.05 follicles/lane) were placed in wells and electrophoresed at constant voltage (180 V). Protein bands were visualized by fixing gels in 12% TCA for 1 h, overnight staining in 0.2% Coomassie blue R-350 (Amersham-Pharmacia Biotech) in 30% methanol plus 10% acetic acid, and final destaining in 25% methanol and 7% acetic acid.

**Measurement of K⁺.** Atomic absorption spectroscopy was used to determine concentrations of K⁺ in follicle-enclosed oocytes undergoing oocyte maturation in the presence or absence of increasing doses of BA₁. Single follicles from each treatment (n = 12–15) were digested with HNO₃ and H₂O₂ (Baker Instra) for 12 h at 90°C. Samples were diluted with 6 ml of redistilled water and analyzed using a Unicam PU 9200X atomic absorption spectrophotometer. Control (blank) tubes were treated as described above, and all analyses were done in duplicate.

**Production of antibodies against killifish CatL, CatB, and CatF.** The CatL, CatB, and CatF antiseras were produced against synthetic peptides corresponding to the deduced amino acid sequences of the corresponding CDNAs (12). Amino acids 317–335 (YMAKDRKNHCGIATAASYP) and 318–330 (CGIESSEVAGIPK) and 235–249 (ETDYSYKHKQT) from the corresponding CatL, CatB, and CatF proenzymes, respectively, were selected for peptide synthesis. The peptides were conjugated to keyhole limpet hemocyanine and injected into rabbits, and the specificity of the antisera obtained were tested by ELISA. The CatL and CatB antisera were affinity purified on thiopropyl sepharose 6B coupled to the synthetic peptide. A rabbit antisera raised against a preparation of pure salmon CatL (52), which showed cross-reactivity with killifish CatL, was also used.

**Immunoprecipitation and immunoblotting.** Biochemical determination of CatL and CatB proenzyme activation in follicles treated with ethanol or 17,20-P in vitro, with or without 100 nM BA₁, was carried out by immunoprecipitation followed by immunoblotting to reduce yolk contamination. For immunoprecipitation, total proteins were extracted from 30 follicles by homogenizing the samples in Triton X-100-containing lysis buffer [1% Triton X-100, 1 mM CaCl₂, 150 mM NaCl, 10 mM Tris, pH 7.4, 0.5 mg/ml PMSF, and a cocktail of protease inhibitors (mini-EDTA-free; Roche)], followed by a centrifugation at 12,000 g for 10 min at 4°C. The whole lysates were precleared by incubation with approximately the same amounts of free protein-A Sepharose beads (Amersham) for 30–60 min; they were subsequently incubated overnight at 4°C with 15 μg of anti-salmon CatL or 10 μg of anti-killifish CatB antisera. Freshly prepared beads were then absorbed to the lysates for 1 h at 4°C, and bead-coupled antibodies were separated by a short centrifugation at 12,000 g and washed with cold homogenization buffer followed by PBS. Bound proteins were eluted with SDS-PAGE sample buffer at 95°C and processed for immunoblotting.
Follicle total proteins separated by SDS-PAGE (15%) were electroblotted on nitrocellulose or PVDF membranes (Bio-Rad) using glycine transfer buffer (190 mM glycine, Tris 25 mM, pH 8.6, 20% methanol). After blocking incubation with Tris-buffered saline with 0.1% Tween 20 and 5% milk powder for 1 h, the membranes were incubated with killifish CatL (1:100) and CatB (1:500) antiseras overnight at 4°C. Bound antibodies were detected with horseradish peroxidase-coupled rabbit secondary antibodies (1:8,000) using the enhanced chemiluminescence method (Amersham).

**Immunocytochemistry and electron microscopy.** Acidic compartments within killifish oocytes were visualized by a modified indirect immunocytochemical protocol (24, 32). Follicle-enclosed oocytes undergoing maturation in vitro, in the presence or absence of 100 nM BA1, were incubated in L-15 medium with 50 μM of the cell-permeable acidic pH probe N-3-(2,4-dinitrophenyl)-amino[propyl-N-(3-aminopropyl)-methylamine, dihydrochloride (DAMP-HCl; Molecular Probes). Follicles at different stages during maturation were removed and washed with fresh medium without DAMP, fixed with PBS containing 2% glutaraldehyde, 2% paraformaldehyde (PFA), and 0.5% DMSO for 4 h at RT, and embedded in paraplast. The sections (10 μm) were either stained with eosin or permeabilized in PBS containing 1% SDS for 10 min and blocked with 5% rabbit serum and 0.1% BSA in 0.01% PBST (1% BSA and 0.01% Tween 20 in PBS). These sections were subsequently incubated with rabbit anti-dinitrophenyl fluorescein-conjugated antibody (Molecular Probes) in PBS at 1:100 dilution overnight at 4°C. After sections were washed with PBS, the specimens were mounted on glass slides and viewed under a Leica DMLB microscope equipped with fluorescence optics.

For immunocytochemistry, ovarian pieces or isolated ovarian follicles undergoing oocyte maturation in vitro were fixed in 4% PFA in PBS for 4–6 h at RT and subsequently dehydrated and embedded in paraplast. Sections of ~6 μm were blocked with 5% goat serum in PBST and incubated overnight at 4°C with anti-salmon CatL (1:300) and anti-killifish CatB (1:100) antisera or for 1 h at RT with anti-killifish CatF (1:300) antisera and 5% goat serum in PBST. After four washes of 5 min each with PBS, the sections were incubated with FITC anti-rabbit secondary antibodies (1:300 in PBS) for 1 h, washed three times with PBS, and mounted with Vectashield (Vector Labs). In some sections, CatF and CatL were also immunolocalized with the avidin-biotin-peroxidase complex method using a commercial kit according to the manufacturer’s instructions (Vectastain avidin-biotin-peroxidase complex kit, Vector Labs). Using the preimmune sera or preincubation of the antisera with the synthetic peptide for 37°C previous to its application onto the sections did not reveal any staining (not shown), which demonstrated the specificity of the signals. Immunofluorescence and immunoperoxidase staining were observed and documented with a Leica DMLB microscope equipped with a Leica DMLB microscope equipped.
with fluorescence optics or with a Nikon TE 300 inverted microscope equipped with Nomarski optics, respectively.

Transmission electron microscopy of ovarian follicles was carried out on samples fixed in 0.1 M cacodylate buffer, 2% PFA, and 2.5% glutaraldehyde for 48–72 h at 4°C. The samples were processed for standard electron microscopy as described (19) and observed and photographed using a JEOL JEM 1010 electron microscope.

Statistical analysis. Data are presented as means ± SE. Data were statistically analyzed by either the Student’s t-test or one-factor ANOVA, after arcsine transformation of the data when needed, followed by the Tukey’s multiple-range test. Differences were considered significant at $P \leq 0.05$.

RESULTS

Immunolocalization of CatL, CatB, and CatF in the killifish ovary. In a previous study, our group (12) reported that CatL, CatB, and CatF mRNAs were expressed in killifish ovarian follicles throughout oocyte growth (vitellogenesis) and maturation. To elucidate the subcellular localization of these proteases in ovarian follicles, immunofluorescence and immunoperoxidase experiments on ovarian sections were carried out. Immunocytochemical analysis showed clearly distinguishable signals for CatL, CatB, and CatF in late vitellogenic oocytes, although their pattern of subcellular localization was slightly different (Fig. 1). Immunoreactivity for CatL, CatB, and CatF appeared surrounding yolk globules in vitellogenic oocytes as well as at the edges of the central mass of liquid yolk, where fusion of yolk globules occurs (Fig. 1, A, B, D, and E). However, CatF (Fig. 1E) apparently showed a higher level of immunoreactivity at the yolk mass when compared with CatL and CatB (Fig. 1, A, B, and D). Interestingly, CatF immunoreaction, but not CatL or CatB, was also found within nascent cortical alveoli in cortical alveoli-stage oocytes (Fig. 1F, arrowheads), which are previtellogenic and do not contain yolk (48), thus suggesting that this protease may be involved in the processing of the alveoli content in addition to yolk processing.

To observe possible changes in the subcellular localization of CatL, CatB, and CatF during oocyte maturation, immunofluorescence was performed on manually isolated follicle-enclosed oocytes undergoing maturation in vitro in response to the naturally occurring MIS, 17,20-P (Fig. 2). When matura-

![Fig. 2. Immunolocalization of CatL (A, D), CatB (B, E), and CatF (C, F) during oocyte maturation in vitro. Isolated fully grown follicle-enclosed oocytes were incubated with 0.1 µg/ml 17α,20β-dihydroxy-4-pregnen-3-one (+17,20β-P; D-F) or ethanol vehicle (−17,20β-P, A-C). After −12 h from steroid stimulation, follicles were fixed and processed for immunofluorescence (see MATERIALS AND METHODS). Panels show phase-contrast (top) and corresponding immunofluorescence (bottom) images. Positive signals for both CatL (A, D) and CatB (B and inset, E and inset) appear more intense at the edges of the yolk mass (asterisks) during maturation (arrows), whereas CatF immunoreaction at the same sites seems to disappear (arrows in C and F). B, inset: cytoplasmatic, CatB-positive yolk globules (arrows) in fully grown oocytes. E, inset: detail of the surrounding area of the central yolk mass in oocytes undergoing maturation where small yolk globules (arrows) are merging. Scale bars, 100 µm (A and D), 50 µm (B, E and F), and 25 µm (C).]
tion was induced, immunoreactions for both CatL and CatB in yolk globules were greatly reduced, whereas a strong signal, specially for CatB, was found at the edges of the central yolk mass (Fig. 2, A, B, D, and E). By contrast, CatF staining surrounding yolk inclusions and yolk mass (Fig. 2C) completely disappeared (Fig. 2F).

Cytoplasmic acidification and yolk proteolysis during oocyte maturation. The macrolide antibiotics BA1 and ConA were employed to study the physiological role of V-ATPase during acidification and yolk proteolysis in killifish oocytes. However, before testing the effect of these compounds on yolk processing, we first evaluated their effect on oocyte maturation in vitro. Fully grown follicle-enclosed oocytes were incubated with 0.1 μg/ml 17,20βP in the presence or absence of BA1 and ConA (1–100 nM) (Fig. 3). Oocytes in prematuration follicles revealed by DAMP immunolocalization was already detected in prematuration oocytes and in oocytes undergoing maturation in vitro in response to 17,20βP (Fig. 4, A and B). However, no positive signals for DAMP were detected in fully mature oocytes (Fig. 4C), suggesting a further increase in pH of the cytoplasm and yolk structures after meiosis resumption before ovulation. Notably, acidic compartments in vitellogenic and maturing oocytes were detected in the cytoplasm, but also surrounding the yolk globules and at the edges of the central yolk mass (Fig. 4, A and inset), thus at the same regions where CatL, CatB, and CatF were immunolocalized. However, treatment with BA1 during steroid-induced maturation strongly reduced the acidification of the oocyte cytoplasm and yolk globules, in both prematuration and maturing oocytes (Fig. 4, D and E), indicating the role of a V-ATPase in the acidification mechanism of killifish oocytes.

Electron microscopy was employed to document potential ultrastructural alterations in yolk globules during maturation in the presence of BA1 (Fig. 5). Prematuration oocytes showed the typical dissociation and fusion of noncrystalline, electron dense yolk globules into a central mass of fluid yolk (Fig. 5, A–C). These processes appeared to be accelerated in response to the MIS, concomitant with an enhanced proteolysis of YPs (29), and finally, a single central mass of yolk filled with liquid and transparent material was formed in fully mature, not ovulated oocytes, which filled most of the oocyte cytoplasm (Fig. 4, D and E). However, the ultrastructural changes of yolk globules during steroid-induced maturation appeared disturbed by the presence of BA1, since in these oocytes the yolk globules partially disassembled and were not completely fused into the central yolk mass (Fig. 5, F–H).

To correlate the BA1-induced structural alterations of yolk globules during oocyte maturation with yolk proteolysis, the changes in major YPs were characterized by SDS-PAGE and staining with Coomassie blue (Fig. 6). In the absence of BA1, the 122-kDa and 45-kDa bands visible in immature follicles, which correspond to Lv heavy chain (Lv H122) and Lv light chain (Lv L45) of killifish Vg1, respectively (29), were processed into smaller YPs (Lv H103 and Lv L42, respectively) during MIS-induced oocyte maturation (Fig. 6, lanes 2 and 3). However, the presence of BA1 inhibited in a dose-response manner the typical processing of Lv H122 and Lv L45 during maturation, maximum inhibition being at 100 nM BA1 (Fig. 6, lanes 4–6). Thus oocyte maturation in the presence of BA1 appears to have occurred without yolk globule acidification and YP proteolysis, both of which normally accompany maturation both in vitro and in vivo.
CatL and CatB proenzyme activation and enzyme activity.

To ascertain whether the inhibitory effect of BA1 on yolk proteolysis was mediated by an alteration of the functional properties of CatL and/or CatB, the enzyme activities of these proteases, as well as the activation of the corresponding proenzymes, were determined at ~24 h after MIS-induced oocyte maturation in vitro in the presence or absence of 100 nM BA1 (Fig. 7). As reported earlier (29), CatL enzyme activity in MIS-treated oocytes decreased markedly (P < 0.05) as maturation progressed, whereas in control, ethanol-treated follicles its activity remained approximately constant during the same culture period (Fig. 7A). The presence of BA1 did not have a significant effect on CatL activity, although in control follicles exposed to BA1 the activity was slightly lower than in those not treated with BA1. In contrast, the enzyme activity of CatB in both control and MIS-treated follicles increased after 24 h, although this increase was significantly higher (P < 0.05) in follicles undergoing GVBD (Fig. 7B). In both cases, treatment with BA1 significantly (P < 0.01) inhibited CatB activity.

The effect of BA1 on the activation of CatL and CatB proenzymes was then investigated by immunoprecipitation followed by immunoblotting (Fig. 7, insets). Detergent extracts from follicles undergoing MIS-induced oocyte maturation in vitro, with or without 100 nM BA1, were immunoprecipitated with CatL and CatB antibodies and subsequently analyzed for the presence of CatL and CatB peptides by Western blot. Immunoprecipitated proteins from ovarian follicle extracts revealed two specific polypeptides reacting with CatL antibodies of approximately 35 and 24 kDa molecular mass, corresponding to CatL proenzyme and CatL enzyme, respectively (Fig. 7A, inset). The ratio of CatL proenzyme and enzyme remained similar between control and MIS-treated follicles over culture, regardless of BA1 treatment (Fig. 7A, inset, lanes 1-5), thus indicating that the reduction of CatL enzyme activity in maturing oocytes previously observed was not caused by a reduction of the amount of CatL enzyme.

Follicle proteins immunoprecipitated with anti-CatB antibodies also revealed two CatB-related polypeptides of approximately 36 and 27 kDa molecular mass, corresponding to the proenzyme and enzyme, respectively. In this case, an increase in the relative amount of CatB enzyme with respect to the proenzyme was detected in control follicles after 24 h of culture (Fig. 7B, inset, lanes 1 and 2), which agreed with the elevated activity of the enzyme in these follicles. Such activation of the CatB proenzyme in control follicles was not affected by BA1 (Fig. 7B, inset, lane 3), although the enzyme activity of CatB was significantly reduced (Fig. 7B). Follicles treated with the MIS showed a higher increase of the relative amount of CatB enzyme (Fig. 7B, inset, lane 4), in accordance with the higher activity of the enzyme, and this steroid-induced activation of the proenzyme was apparently impaired by BA1 (Fig. 7B, inset, lane 5).

Relationship between yolk proteolysis and oocyte hydration.

Follicles treated with 17,20βP in the presence of BA1 showed several macroscopic alterations after the completion of the maturation process with respect to those incubated with 17,20βP alone (Fig. 8). In BA1-treated mature follicles, the oocyte cytoplasm appeared slightly more opaque with a higher number and smaller lipid droplets than in 17,20βP-treated follicles without BA1 (Fig. 8A). Most noticeable, however, was that the final volume of oocytes progressively decreased with increasing BA1 concentration in the culture medium (Fig. 8B), suggesting that oocytes undergoing maturation in the presence of BA1 hydrated to a lesser extent than those incubated with...
To confirm that BA1 prevented steroid-induced oocyte hydration, the water content of immature follicle-enclosed oocytes, and of in vitro 17,20βP-matured oocytes with or without 100 nM BA1, was determined gravimetrically. As shown in Table 1, the treatment of oocytes undergoing maturation with BA1 reduced the uptake of water, which indicated that inhibition of yolk proteolysis effectively inhibited the hydration of the oocyte.

To explore the possible mechanisms by which BA1 might affect the hydration process, the oocyte content in K\textsuperscript{+}, the main osmotic effector for the hydration of killifish oocytes (21, 57), was determined in MIS-stimulated ovarian follicles in the presence of increasing doses of BA1 (Fig. 9). These experiments showed that MIS stimulation produced an increase of 3.2-fold in the content of K\textsuperscript{+} of mature follicles with respect to immature follicles (from 48.8 to 156.9 nmol K\textsuperscript{+}/follicle), but the accumulation of K\textsuperscript{+} was partially inhibited in a dose-dependent manner by BA1. Thus the effect of BA1 on oocyte hydration in killifish could involve a diminished ability of the oocyte to accumulate K\textsuperscript{+} in response to the MIS.

DISCUSSION

In the present work, we have provided immunologic evidences for the role of the cysteine proteinases CatL and CatB, and more remarkably of CatF, in the mechanism of yolk processing in the killifish oocyte. The mRNAs encoding CatL, CatB, and CatF are expressed in vitellogenic ovarian follicles (12), and their proenzymes appear associated with yolk globules in the oocyte, as well as with the sites of their fusion into the central mass of fluid yolk. Thus this specific subcellular localization resembles that found for cathepsin D in vitellogenic trout oocytes (51), which may enhance the rapid activation of the proenzyme for yolk proteolysis by changes in pH or by the action of another protease. The early localization of cathepsins in yolk globules of vitellogenic oocytes is consistent...
with the specific pattern of yolk formation and degradation in killifish oocytes (47, 48), in which yolk globule fusion, possibly associated with the processing to some extent of Vg-derived YPs, already takes place at later stages of vitellogenesis before oocyte maturation.

Our finding of CatF proenzyme in intracellular sites of yolk fusion in vitellogenic oocytes (Figs. 1 and 2) provides the first evidence in fish for a role of this protease in the mechanisms of yolk processing. This observation agrees with a recent report on the parasitic worm *Paragonimus westermani*, where a CatF-like cysteine proteinase is localized in the vitelline gland, which is responsible for the secretion of vitelline and shell material into the naked ovum (41). Interestingly, CatF was no longer detected in yolk organelles of the oocyte during meiosis resumption (Fig. 2), unlike CatL and CatB that remain at the edges of the central yolk mass at this stage. These data may imply that CatF is involved during protease activation and/or yolk proteolysis specifically during vitellogenesis. The accumulation of CatF mRNA in killifish ovarian follicles that occurs during oocyte maturation both in vivo and in vitro (12) is thus possibly related to the requirement of CatF maternal transcripts during early embryogenesis rather than for the specific processing of YPs during oocyte maturation.

The role of acidification of yolk bodies for the activation of yolk processing at the onset of embryonic development has been documented for both invertebrates and lower vertebrates (e.g., Refs. 15, 32, 39, 62). In pelagophil teleosts, recent findings suggest that a similar pH-regulated mechanism controls the processing of YPs during oocyte maturation. In one of these species, the black sea bass, we reported that BA1 prevented the proteolysis of Lv and further generation of FAAs of gonadotropin-treated ovarian follicles in vitro (50). In the benthophil killifish, direct assessment of cytoplasmic and yolk globule acidification during oocyte maturation by DAMP immunocytochemistry revealed that BA1-sensitive acidic compartments are already detected in postvitellogenic oocytes. Notably, these acidic areas coincided with the sites where CatL, CatB, and CatF were immunolocalized. In addition, as in the black sea bass, BA1 prevented the degradation of LvH 122 and LvL 45 yolk products during oocyte maturation, but it did not affect meiosis resumption. Thus, together, these observations provide further support for the role of acidification of yolk compartments, regulated by V-ATPase, for YP hydrolysis in both pelagophil and benthophil teleosts.

The identity and mechanism of action of lysosomal proteases involved in YP proteolysis during fish oocyte maturation.
differences with respect to follicles incubated with 17,20P alone (*H9252P/H11021 significant (H11011Wet weight, mg 1.74/H11006). Water content of follicle-enclosed oocytes

Table 1. Water content of follicle-enclosed oocytes

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<thead>
<tr>
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<th>Immature (n = 3)</th>
<th>Mature (n = 4)</th>
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<tr>
<td>Wet weight, mg</td>
<td>1.74±0.07*</td>
<td>3.27±0.07‡</td>
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<tr>
<td>Dry weight, mg</td>
<td>0.63±0.03</td>
<td>0.71±0.05</td>
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<td>2.56±0.06§</td>
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<td>%Water</td>
<td>63.8±0.2*</td>
<td>78.5±1.3§</td>
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Values are means ± SE of 3 or 4 experiments (n = 25–35 follicles/treatment). Data indicate the wet and dry weights and % water in immature and matured follicle-enclosed oocytes in vitro with 0.1 μg/ml 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) in the presence or absence of 100 mM baflomycin A1 (BA1). Values within a row with different superscripts are statistically significant (P < 0.001; ANOVA).

are poorly known. Although CatL has been suggested to be the main protease involved in this process in gilthead sea bream (5), CatB seems to be the enzyme responsible for maturation-associated yolk degradation in both barfin flounder (Verasper moseri; Ref. 36) and killifish (29). Consistent with this latter report, we found that partial proteolysis of LvH 122 and LvL 45 during MIS-induced oocyte maturation correlated with an increased CatB enzyme activity (Fig. 7B). In contrast, CatL activity dramatically decreased as maturation proceeded (Fig. 7A) (29), which apparently was not caused by a reduction in the amount of enzyme available (as observed both biochemically and immunologically) but most likely by an inactivation mechanism. Such inhibitory processes may require the action of specific cysteine protease inhibitors (e.g., Refs. 1, 59, 61), since both ovarian CatL and CatB seem to have similar pH for optimum activity (5, 64), and thus inhibition of CatL by MIS-induced changes in pH during maturation seems unlikely. However, conclusive evidences to rule out a pH-mediated mechanism for CatL inactivation during killifish oocyte maturation need further investigation with the use of purified native CatL and CatB enzymes from oocytes.

The increase of CatB enzyme activity in both control and MIS-treated killifish ovarian follicles was sensitive to a BA1-induced increase in internal pH, which is consistent with the acidic pH optimum (~5.5) reported for Xenopus laevis embryonic CatB (64). The enhanced enzyme activity of CatB in immature and maturing follicles could be explained by the activation of the proenzyme over culture time, which in MIS-induced follicles was more marked, and most of the proenzyme appeared to be processed into potentially active enzyme (Fig. 7B, inset). The MIS-stimulated processing of CatB proenzyme was pH dependent, since the presence of BA1 partially prevented the maturation of the proenzyme. The nature of this process is still unclear, although autocatalytic cleavage of CatB proenzyme under acidic conditions, as it has been reported for mammalian CatB (31, 46), may be a potential mechanism involved. However, cathepsins are normally delivered to lysosomes as proenzymes (22), and, because BA1 has been found to suppress indirectly the fusion of lysosomes into target vacuoles (42), an inhibition of the delivery of CatB proenzyme to yolk globules during MIS-stimulated oocyte maturation is another potential mechanism that may be involved. In addition, we found that immature oocytes not exposed to the MIS showed partial maturation of CatB proenzyme, which may suggest the existence of an additional mechanism for CatB activation not sensitive to BA1 and thus MIS and pH independent. Therefore, it is apparent that the mechanisms by which CatB becomes activated in killifish oocytes are complex and remain to be elucidated.

The proteolysis of YPs during oocyte maturation in pelagophil teleosts generates the source of FAAs in the oocyte...

Fig. 8. Effect of BA1 on oocyte hydration during 17,20P-induced oocyte maturation in vitro. A: photomicrographs of follicle-enclosed oocytes treated with ethanol vehicle (1), 0.1 μg/ml 17,20βP (2), or 0.1 μg/ml 17,20βP and 100 nM BA1 (3). Scale bar, 500 μm. B: inhibition of oocyte volume increase during 17,20βP (0.1 μg/ml)-induced maturation in vitro in the presence of increasing amounts of BA1. Data are means ± SE of 3 experiments in which follicles were incubated individually in 96-well plates (n = 24 follicles). *Significant differences with respect to follicles incubated with 17,20βP alone (*P < 0.05; **P < 0.001; Student’s t-test).

Fig. 9. Effect of BA1 on K+ accumulation in ovarian follicles during 17,20βP-induced oocyte maturation in vitro. Data are means ± SE of 3 experiments (n = 20–25 follicles/treatment). Values with different superscripts are significantly different (ANOVA, P < 0.05).
necessary for water uptake, which renders the eggs buoyant in sea water (see Introduction). Marine benthophil fish also show varying degrees of Lv degradation during oocyte maturation (17, 20, 25, 29, 49); given that in these fish low or almost no oocyte hydration occurs, the physiological significance of this process is unclear. The degradation of Lv may be related to the generation of FAAs and small peptides for energy production and protein synthesis during embryogenesis (18, 40, 45). However, our present findings suggest that CatB-mediated proteolysis of Lv components during meiosis resumption in killifish may also play an important role during oocyte hydration that was not previously noted (37). In this species, inhibition of Lv proteolysis in vitro by BA1 in K$^+$-containing medium, through the suppression of yolk globule acidification and CatB activity, reduced K$^+$ influx into the oocyte and strongly diminished subsequent hydration (Fig. 9 and Table 1). These surprising observations would however be consistent with Ling’s (30) association-induction hypothesis on the behavior of small ions within cells, which may implicate that chemical modification of YPs in the oocyte could have profound effects on the ion-binding properties of the resulting peptides newly exposed to an aqueous environment. Therefore, it could be speculated that BA1-mediated inhibition of Lv cleavage might reduce the generation of new K$^+$-binding sites within the oocyte, thus preventing the accumulation of K$^+$ through passive diffusion from the capillary beads and hence reducing the increase in the oocyte osmotic pressure. Thus,YP proteolysis-facilitated accumulation of K$^+$ in the oocyte, along an electrochemical gradient, would be the physiological mechanism involved in the hydration of killifish oocytes. Although this model needs to be directly demonstrated, it would suggest that proteolysis of YPs is essentially involved in the process of oocyte hydration in both benthophil and pelagophil teleosts, reinforcing the notion that the hydration of fish oocytes is generated and/or regulated by the oocyte itself rather than by the associated follicle cells.

In summary, we have shown that during maturation of killifish oocytes CatL enzyme activity is reduced, whereas CatB proenzyme is processed into active enzyme, which appears to be the major protease involved in the cleavage of Lv yolk components. As for CatF, the increase in CatL mRNA previously reported during killifish oocyte maturation (12) is likely to be related to specific requirements for this protease during early embryogenesis, which is supported by the finding of CatL maternal transcripts in blastula embryos (A. Tingaud-Sequeira and J. Cerdà, unpublished observations). The activation of CatB during oocyte maturation requires V-ATPase-maintained acidic conditions, although the specific molecular mechanisms involved need to be investigated further. Surprisingly, we also obtained evidences for a role of yolk proteolysis during the hydration of killifish oocytes, which may indicate that ion translocation into the oocyte as well as YP hydrolysis are equally important physiological mechanisms to drive the hydration of the oocyte in benthophil teleosts. However, the molecular events that may link both events are still completely unknown. The uncovering of these mechanisms will undoubtedly contribute to our understanding of the physiological basis of egg formation and early embryo development in teleosts.

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