Yolk Platelets in Xenopus Oocytes Maintain an Acidic Internal pH Which May Be Essential for Sodium Accumulation

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Abstract. Yolk platelets constitute an embryonic endocytic compartment that stores maternally synthesized nutrients. The pH of Xenopus yolk platelets, measured by photometry on whole oocytes which had endocytosed FITC-vitellogenin, was found to be acidic (around pH 5.6). Experiments on digitonin-permeabilized oocytes showed that acidification was due to the activity of an NEM- and bafilomycin A_1-sensitive vacuolar proton-ATPase. Proton pumping required chloride, but was not influenced by potassium or sodium. Passive proton leakage was slow, probably due to the buffer capacity of the yolk, and was dependent on the presence of cytoplasmic monovalent cations. In particular, sodium could drive proton efflux through an amiloride-sensitive Na^+/H^+ exchanger. 8-Bromo-cyclicAMP was found to increase acidification, suggesting that pH can be regulated by intracellular second messengers. The moderately acidic pH does not promote degradation of the yolk platelets, which in oocytes are stable for weeks, but it is likely to be required to maintain the integrity of these organelles. Furthermore, the pH gradient created by the proton pump, when coupled with the Na^+/H^+ exchanger, is probably responsible for the accumulation and storage of sodium into the yolk platelets during oogenesis.

MANY organelles of the secretory and endocytic pathways (vacuolar compartments) have an acidic lumen. The pH of these organelles varies from barely lower than neutrality (*trans*-Golgi elements) to 5.0 and below (lysosomes, plant vacuoles) and appears to be crucial for the various functions each compartment must fulfill, such as prohormone processing in secretory granules, receptor–ligand interaction, and sorting along the endocytic pathway, or degradation in lysosomes (Al-Awqati, 1986; Mellman et al., 1986; Maxfield and Yamashiro, 1991). All these organelles are acidified by a vacuolar proton-ATPase, but little is known about the regulation of the process, which must be both precise and rapid, given the highly dynamic nature of these compartments.

The endosomes have been the subject of most of the investigations in the past few years, since they are readily accessible to exogenous pH probes (Maxfield, 1989). The intralumenal pH decreases along the endosomal pathway to lysosomes, and this is evidence that a negative regulation moderates acidification in early compartments, but not in late endosomes and lysosomes (Mellman et al., 1986). Since the H^+-ATPase is electrogenic, its activity requires simultaneous charge compensation, either by anion influx or cation efflux; if ionic conductance is low, the H^+-ATPase activity establishes a membrane potential that in turn blocks proton translocation (Harvey, 1992). In all organelles studied so far, acidification was found to depend on a parallel, but separate, chloride conductance (lysosomes, Schneider, 1981; multivesicular bodies, Van Dyke, 1985; Golgi apparatus, Glickman et al., 1983; chromaffin granules, Moriyama and Nelson, 1987; osteoclast plasma membrane, Blair et al., 1991; coated vesicles, Mulberg et al., 1991; phagosomes, Lukacs et al., 1991; endosomes, Hilden et al., 1988; Fuchs et al., 1989*a*; Reenstra et al., 1992; Zen et al., 1992). This conductance is high, thus not limiting, in late endosomes and lysosomes, but much lower in early endosomes. Modulation of the chloride conductance has been proposed as a major mechanism for pH regulation (Al-Awqati et al., 1992).

Other mechanisms have also been proposed: Na^+,K^+-ATPase, which cycles between the plasma membrane and the early endosomal compartments, is electrogenic and can therefore inhibit acidification in early endosomes by establishing an inside-positive membrane potential (Fuchs et al., 1989*b*, Cain et al., 1989). In endosomes from rabbit renal cortex, Na^+/H^+ exchange increased the endosomal pH in vitro (Hilden et al., 1990). Also, different isoforms of the vacuolar H^+-ATPase are present in mammalian tissues, which might display functional differences (Hemken et al., 1992; Chatterjee et al., 1992; Puopolo et al., 1992). A peptide inhibitor and an activator have been reported (Zhang et al., 1992*a*,*b*), and phosphorylation might regulate the proton pump activity (Nanda et al., 1992). These regulatory

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mechanisms were found in early stages of endocytosis, but not in late endosomes and lysosomes. The way these late compartments regulate their pH is unknown.

Yolk platelets (YPs,[1] or yolk granules) are huge embryonic organelles formed by multiple fusions of vesicles originating from the endocytic pathway (Wallace, 1985). Their content, the yolk proteins, are derived from vitellogenin (Vg), a phospho-lipo-glycoprotein present in large amounts in the blood of females. Vg is taken up by the growing (vitellogenic) oocyte through receptor-mediated endocytosis and then proteolytically processed to large fragments before being accumulated into the final compartment, the YPs. In Xenopus, the cleavage products are lipovitellins 1 and 2 and phosvitin, an unusual phosphoprotein with a very long repetitive sequence of phosphorylated serine residues. The yolk is a major component of the egg. In Xenopus, it accounts for 50% of the volume, and 80% of the total protein content. In constitutes the main storage compartment that will supply the developing embryo with amino acids, sugars, various lipids, phosphate, and ions.

YPs are an intriguing exception among endosomes: acid hydrolases have been demonstrated to be present within the yolk of Xenopus (Wall and Meleka, 1985) and in many other species (Pasteels, 1973; Vernier and Sire, 1977; Armant et al., 1988; Medina and Vallejo, 1989; Fagotto, 1990*a*), yet no degradation (at most limited processing in some cases) occurs during vitellogenesis, which can last many weeks, or during early embryonic development. Recent work on invertebrates has presented evidence that yolk degradation is triggered by a developmentally regulated acidification of the YP pH (Fagotto, 1990*b*, 1991; Nordin et al., 1991).

The actual pH of the Xenopus YPs has not been studied, despite the potential implications of yolk degradation for the physiology of the developing embryo. We considered that the Xenopus oocyte would be the most suitable model to examine the pH regulation in YPs since vitellogenesis is well characterized in this species and many aspects of ion regulation have been studied (Dascal, 1987).

In this paper we show that Xenopus YPs are moderately acidic (pH 5.6), that a vacuolar H^+-ATPase is responsible for the YP acidification, and that an amiloride-sensitive Na^+/H^+ exchanger is present. This antiporter may mediate the accumulation of sodium into the YPs. Also, we show that the pH of the YPs is regulated by 8-bromo-cAMP.

Materials and Methods

Reagents

Liebovitz's L-15 medium was purchased from GIBCO BRL (Gaithersburg, MD), FITC from Molecular Probes Inc. (Eugene, OR), creatine kinase and phosphocreatine from Boehringer Mannheim Biochemicals (Indianapolis, IN). Bafilomycin A1 was obtained from Dr. K. Altendorf (University of Osnabrück, Osnabrück, Germany). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Preparation of FITC-Vitellogenin

Xenopus females (Xenopus I, Ann Harbor, MI) were injected twice (days 1 and 7) with 2 mg β-estradiol to increase Vg synthesis, then bled by cardiac puncture at day 10 (Opresko et al., 1980). Vg-rich plasma was labeled without further purification, since: (*a*) purified Vg is easily denatured and thus less efficiently internalized by the oocytes; and (*b*) endocytosis by vitellogenic oocytes is highly selective for Vg versus other serum proteins (Danilchik and Gerhart, 1987). 1 ml of plasma containing 15–25 mg Vg was dialyzed against 100 ml 10 mM Na_2HPO_4, 100 mM NaCl, pH 9.0, containing 3.5 mg FITC, for 5 h at room temperature or overnight at 4°C. Labeling was followed by extensive dialysis at 4°C against 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5. Vg concentration was determined by measuring A_{280} after selective precipitation, according to Wallace et al. (1980). However, we found that Vg precipitation upon addition of EDTA and Mg^{2+} was not due to a selective "cross-link of divalent salts of Vg" (Wiley et al., 1979), but merely to the low pH (4.5) of the final solution.

YPs Labeling

Stage IV oocytes (Dumont, 1972) obtained surgically from unstimulated Xenopus females were manually defolliculated after a brief collagenase digestion (20 mg/ml in OR3 medium for 20 min at 18°C; Akabas et al., 1992). OR3 medium was made of Liebovitz's L-15 medium diluted 1:1, supplemented with 1 mM L-glutamine, 15 mM Hepes, 50 mg/ml gentamycin, and adjusted to pH 7.6 with NaOH. Defolliculated oocytes were stored in OR3 medium at 18°C and used within 3 d. 0.8–1.0-mm diam oocytes were selected because they best combined low vegetal pigmentation and high Vg uptake.

Oocytes were incubated under slow rotation at room temperature, for 5–8 h, with 2.5 mg/ml FITC-Vg in OR3, supplemented with 5 μg/ml insulin (Opresko and Wiley, 1987). They were then briefly rinsed in OR3, and incubated overnight at 18°C in OR3 containing 2.5 mg/ml unlabeled Vg and 5 μg/ml insulin. They were finally rinsed in OR3, and anomalous as well as poorly labeled oocytes were discarded.

pH Measurements

Labeled oocytes were placed individually in plastic wells (24-well cell culture plates; Falcon Plastics, Oxnard, CA) in 1 ml medium. They were observed using an inverted epifluorescence Diavert microscope (E. Leitz Inc., Rockleigh, NJ) equipped with a 100 W mercury lamp, interchangeable blue (450 nm) and green (490 nm) narrow band pass filters, a 10× objective, and a photomultiplier tube for quantitative measurements (Maxfield, 1989). The blue and the green fluorescence of two to four different fields (~0.5-mm diam) of the vegetal, low pigmented pole was measured.

Calibration curves were generated by incubating oocytes at various pHs in the presence of high concentrations of ionophores for several hours before measuring the 490/450 ratio. The FITC fluorescence of the yolk was stable for more than 3 d, which allowed us to measure in some calibrations the same oocyte consecutively at two to three different pHs. The media used for calibration were 100 mM NaCl buffered with 33 mM sodium phosphate (pH 6.5–7), 16.5 mM sodium phosphate and 16.5 mM Tris-maleate (pH 5.5–6) or 33 mM Tris-maleate (pH 4.5–5), containing 20–40 μM nigericin and 50–100 μM monensin. In some experiments, the oocytes were fixed 30 min with 2% paraformaldehyde in saline phosphate buffer, pH 7.4, before equilibration at the various pHs as described above. Calibration curves from unfixed and fixed oocytes were similar (see Fig. 2 *A*). Calibration curves for the soluble FITC-labeled vitellogenin were obtained by measuring the 490/450 ratio of a drop (20 μl) of labeled Vg-rich serum diluted in the various calibration buffers (2.5 mg Vg/ml), by photometry under the microscope.

Permeabilization Experiments

The plasma membrane of the oocytes was permeabilized following a protocol slightly modified from Schmalzing et al. (1989). Up to 30 oocytes were incubated in 10 ml 10 μM digitonin, 100 mM KCl, 10 mM NaCl, 20 mM Hepes-KOH, 2 mM $MgCl_2$, 1 mM Na_2EGTA, pH 7.4, for 30 min at 4°C under slow rotation. They were then rinsed in various digitonin-free media (see below) containing 0.1% BSA, for 40–60 min at 4°C. The oocytes were then transferred in 1 ml medium at room temperature in 24-well plates for the first measurement (time = 0). Unless specifically mentioned, the oocytes were rinsed and incubated for the subsequent experiment in the same medium. Inhibitors and activators were added 5 min before time = 0, and ATP within 5 min after $t = 0$. About 30 oocytes could be measured in 30 min. Routinely, the second and third time points were 50–60 and 100–120 min.

The standard medium used was: 100 mM potassium gluconate, 10 mM NaCl, 20 mM Hepes-KOH, 2 mM $MgSO_4$, 0.1% BSA, pH 7.4. Monova-

1. *Abbreviations used in this paper*: NMDG, *N*-methyl-D-glucosamine; Vg, vitellogenin; YP, yolk platelet.

lent ion concentrations in this medium were close to the physiological values (Palmer et al., 1978; Hasegawa et al., 1992). To test the effect of anions and cations, K^+, Na^+, and Cl^- total concentrations were varied as follows: K^+, 0/20/110-120 mM; Na^+, 0/10/100-120 mM; Cl^-, 0/10/25/110 mM. K^+ and Na^+ were replaced by *N*-methyl-glucosamine (NMDG), Cl^- by gluconate. For example, 20 mM K^+, Na^+-free, 10 mM Cl^- medium contained 10 mM KCl, 10 mM potassium gluconate, 90 mM gluconic acid, 20 mM Hepes, 2 mM $MgSO_4$, 0.1% BSA, titrated to pH 7.4 with NMDG. 100 mM ATP stock solutions (magnesium salt) were titrated to ~pH 7.4 with KOH or NaOH depending on the cation composition of the medium. ATP regenerating systems contained 1 mM MgATP and 10 U/ml creatine phosphokinase + 10 mM creatine phosphate (from 40× concentrated frozen aliquots). Disodium creatine phosphate was used in standard media, but was replaced by the potassium salt when low Na^+- or Na^+-free conditions were studied.

Calculations

In each experiment, four to seven oocytes were measured for each condition, which was tested in at least three experiments. The relative pH variations were expressed as the difference between the 490/450 ratio at a given time point and the initial ratio at time = 0. These differences were calculated for each individual oocyte, with no background subtraction, and then averaged for each experiment.

For pH calibrations each oocyte was measured twice: in intact embryos, and after equilibration at one given pH in the presence of ionophores. Variations of the 490/450 ratios were large because the oocytes were heterogeneous with regard to autofluorescence, pigmentation and labeling intensity. For this reason, a more accurate pH estimation was obtained by plotting the difference between the initial 490/450 ratio in intact oocytes and the ratio at a given pH, calculated for each individual oocyte (null point method).

Results

pH of Xenopus Yolk Platelets

Internalized FITC-labeled macromolecules have been widely used to measure the internal pH of various endocytic compartments since fluorescein has a pH-sensitive excitation spectrum (Okhuma and Poole, 1978; Maxfield, 1989; Dunn and Maxfield, 1990). pH values can be determined from the ratio of FITC fluorescence with 490 and 450 nm excitation. We used this methodology to measure the YP pH.

In Xenopus oocytes, YPs are the terminal destination for endocytosed Vg. To enter this last compartment, Vg must be converted into lipovitellin and phosvitin by a proteolytic process taking place in the primordial yolk platelets (Opresko et al., 1980). Inhibition of this cleavage, e.g., by denaturation, blocks delivery to the mature YPs (Opresko et al., 1980). Under our experimental conditions, however, FITC-Vg reached the correct destination, since FITC fluorescence was almost exclusively found in mature YPs (Fig. 1), and few smaller organelles resembling the primordial yolk platelets (~0.3 μm; Busson et al., 1989) were labeled. YPs are highly heterogenous in size, ranging from 1 to 10 μm. The core of the full grown oocytes is filled with large, old YPs which result from a large number of fusions of primordial YPs, initiated early during vitellogenesis. New YPs continue to be added at the periphery during the late stages used in the present experiments. They are small, being produced by a lower number of fusions (Wallace, 1985). The YPs labeled in our experiments represented only a very minor fraction of the total YPs. They were close to the surface, and most were small (average ~2 μm), newly formed YPs (Fig. 1, *filled arrows*), though older, larger YPs also had some fluorescence (*outlined arrows*).

An attempt was made to isolate the YPs, but they turned out to be extremely fragile. A large fraction of them were disrupted during cell fractionation, even when very gentle conditions were used (data not shown). Therefore, measurements were performed on whole, intact or permeabilized oocytes, by photometry using an epifluorescence microscope. Under these conditions, YPs were very stable, and the pH could be measured for many hours.

Fig. 2 *A* shows a pH calibration curve obtained by averaging the 490/450 ratio from unfixed (*filled circles*) and paraformaldehyde fixed (*empty circles*) oocytes with FITC-Vg labeled YPs equilibrated at the indicated pHs in the presence of the ionophores monensin and nigericin. The measurements were corrected for the background fluorescence, estimated from unlabeled oocytes. The arrow indicates the

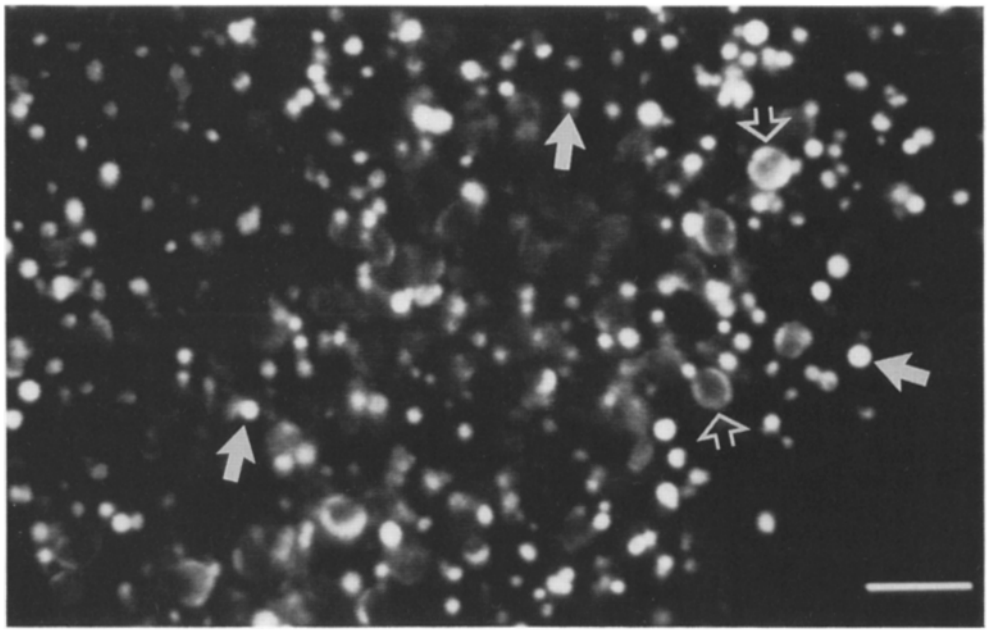

Figure 1. Confocal scanning microscopy of a FITC-Vg-labeled stage IV oocyte. Oocytes were incubated with 2.5 mg/ml FITC-Vg for 5 h as described in Materials and Methods. Living cells were imaged with a Bio-Rad MRC-600 confocal system on a Zeiss Axiovert microscope using a Zeiss 63X, 1.4 NA objective. The figure shows a single optical section obtained near the bottom of an oocyte oriented with its pigmented side up. (*Filled arrows*) FITC-positive, small, newly formed YPs. (*Outlined arrows*) Fluorescence in larger, older YPs. Bar, 10 μm.

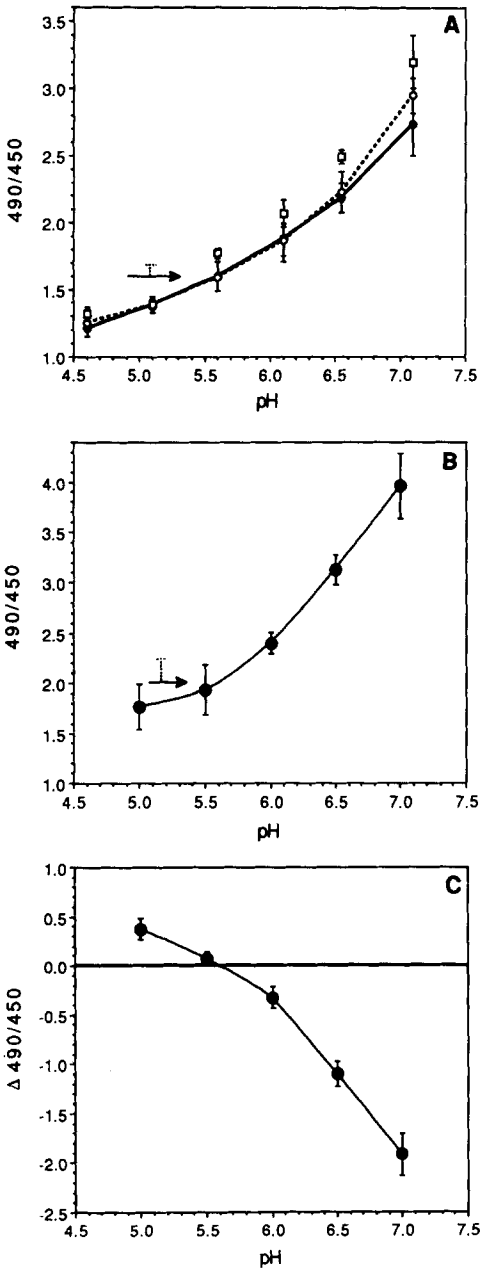

Figure 2. pH calibration of FITC-Vg-labeled yolk platelets. (*A*) pH calibrations were generated by incubating FITC-Vg-labeled, unfixed (●), or paraformaldehyde fixed (○) oocytes at various pHs in the presence of ionophores as described in Materials and Methods. The 490/450 excitation ratios were calculated after subtraction of an average background fluorescence measured on unlabeled oocytes. Empty squares represent the pH dependence of the 490/450 excitation ratio of soluble FITC-labeled vitellogenin measured under the microscope. Each point is the average of the 490/450 excitation ratios of four oocytes ±SD. The arrow indicates the average value measured on 40 intact oocytes, and the half error bar shows the corresponding SD. (*B*) Calibration curve using unfixed FITC-Vg-labeled oocytes incubated in the presence of ionophores. No background was subtracted. Each point is the average of the 490/450 ratios of four oocytes. The arrow indicates the average value for 20 intact oocytes ±SD. (*C*) Null point determination using the same measurements presented in *B*: each point is the average of the difference between the ratio in the intact oocyte and the ratio at equilibrium at the indicated pH, calculated for each individual oocyte. The intersection of the curve with the abscissa (Δ490/450 = 0) gives the initial pH value (~5.6). Values are average ±SD.

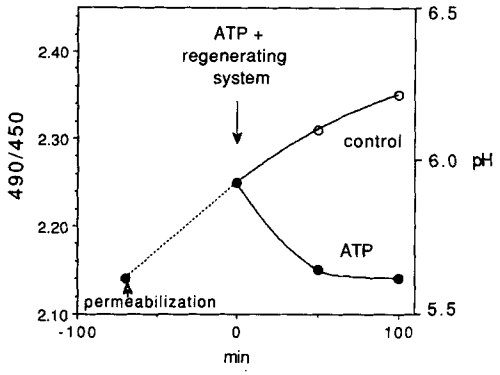

Figure 3. Representative experiment of photometry measurement of pH variations in FITC-Vg-labeled, digitonin-permeabilized oocytes. After permeabilization (*arrow*), 10 oocytes were rinsed at 4°C for 40 min in a 10 mM Na^+, 110 mM K^+, 10 mM Cl^- medium (physiological conditions), and then incubated at time 0 at room temperature in the same medium, with (---●---) or without (---○---) 1 mM MgATP and an ATP regenerating system (5 oocytes per condition) as described in Materials and Methods. At the end of the experiment the oocytes were incubated at various pHs in the presence of monensin and nigericin to establish a pH calibration (*right*).

average 490/450 value found in intact oocytes, which corresponds to pH 5.6. Fig. 2 *A* also shows the 490/450 ratio of a solution of fluorescent vitellogenin adjusted to various pH values, measured under the microscope by photometry (*empty squares*). The similarity of the calibration curves indicated that the internal YP pH could be effectively clamped by the addition of ionophores to intact oocytes. Note that the ratios shown in Fig. 2 *A* cannot be compared with the ratios of the other experiments presented below, because a different set of filters was used.

A disadvantage of using whole oocytes was that the strong autofluorescence of the yolk, the uneven pigmentation of the vegetal pole, and the variable FITC-Vg uptake affected the fluorescence ratios. These parameters could not be estimated to correct our measurements for background fluorescence with sufficient precision to detect small pH variations. The fact that the fluorescence ratios were quite different from oocyte to oocyte is reflected in the standard deviations shown in Fig. 2 *A*. In all experiments presented below, the 490/450 ratio was directly calculated without background subtraction. The problem of variability in oocyte fluorescence was overcome by calculating the variations in the 490/450 fluorescence ratio for each oocyte, and averaging data on the variations from several oocytes. An example of calibration curve calculated from uncorrected fluorescence measurements on FITC-labeled, unfixed oocytes is shown in Fig. 2 *B*. The comparison of six determinations on different days indicated an average pH of 5.6 and a standard error of ~0.1 pH units. Fig. 2 *C* shows a null point pH determination using the data from the same experiment shown in Fig. 2 *B*. The negative or positive deflection of the initial ratio compared to the ratio after equilibration at a given pH was calculated for each oocyte. The intersection of the plot with the abscissa gave the YP pH in intact oocytes. This method was more precise because it was less influenced by the variability of the background fluorescence. It confirmed that the YP pH was 5.6, and showed that individual oocytes all displayed a homogenous pH. The 490/450 ratio from intact oocytes did not

Table I. Sensitivity of ATP-dependent YP Reacidification to H^+-ATPase Inhibitors

Inhibitor	Percent relative reacidification
None	100
200 μM NEM	20 ± 10
20 nM bafilomycin	−15 ± 30
100 nM bafilomycin	−10 ± 25
1 mM sodium orthovanadate	85 ± 20
3 mM sodium azide	100 ± 20

FITC-Vg-labeled, permeabilized oocytes were rinsed in a 10 mM Na^+, 110 mM K^+, 10 mM Cl^- medium and then incubated in the same medium with or without MgATP and an ATP-regenerating system, in the presence of various inhibitors. The variation in fluorescence ratio (Δ490/450) during the first 50 min of incubation was measured by photometry. The relative reacidification was calculated as the difference Δ490/450 (+ATP) − Δ490/450 (−ATP) and expressed as percent of the control. Values are average of three experiments ± SEM.

change for at least 2 d, indicating that the YPs maintained a stable pH.

The pH of the YPs was stable, and it equilibrated with the external pH very slowly even in the presence of large amounts of ionophores because of the buffering capacity of the yolk (see below). Generation of standard curves with multiple points required up to 3 d. Therefore only relative fluorescence variations, but not absolute pH values were determined in many of the permeabilization experiments described below. Despite these technical difficulties, our method was sensitive enough to detect variations of less than 0.1 pH units.

Characterization of the Proton-ATPase

To analyze the mechanisms involved in regulating the YP pH, FITC-labeled oocytes were permeabilized with digitonin, then incubated under various conditions. Digitonin treatment as described in Materials and Methods, for limited times at 4°C, acted specifically on the plasma membrane, while leaving YPs intact and able to maintain an acidic pH. Harsher permeabilization conditions, such as increased digitonin concentration or higher temperature led to YP permeabilization, as monitored by the rapid collapse of the pH gradient.

In the presence of physiological intracellular ion concentrations in the external (cytoplasmic) medium, but in the absence of an energy source, the pH gradient dissipated slowly (Fig. 3). Leakage could be enhanced by adding the ionophores nigericin and monensin, which exchange protons with other cations, indicating that membrane permeability was a limiting factor. However, even in the presence of ionophores, proton leakage was still slow when compared with organelles such as endosomes and lysosomes from mammalian cells (Maxfield, 1982).

When MgATP was added, the YPs reacidified and eventually recovered their initial pH (Fig. 3). Strikingly, the YPs never reached pHs lower than the initial value, which indicated that the regulatory mechanisms were still effective in permeabilized oocytes. As shown in Table I, reacidification was due to a proton pump having the characteristics of a typical vacuolar H^+-ATPase (Harvey, 1992): it was NEM sensitive and completely blocked by low concentrations of bafilomycin A1, a specific inhibitor of the vacuolar H^+-ATPase (Bowman et al., 1988). Azide and vanadate, respectively, inhibitors of the mitochondrial H^+-ATPase and of the P-ATPases such as the Na^+,K^+-ATPase, had no effect.

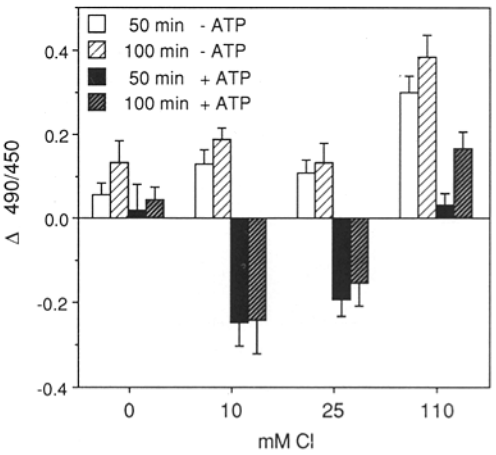

Figure 4. Chloride dependence of passive proton leakage and ATP-dependent reacidification. Permeabilized oocytes were rinsed in 10 mM Na^+, 110 mM K^+ media containing various Cl^- concentrations ranging from 0 to 110 mM, and then incubated in the same media with or without MgATP and an ATP regenerating system. The change in 490/450 ratio was measured by photometry. Values are mean ± SEM.

When proton gradient dissipation was allowed to proceed too far, addition of ATP could not restore the initial pH, but it merely slowed the leakage. One interpretation is that YP integrity requires an acidic internal pH (see below), and YPs were damaged above a certain critical pH. This critical value was probably between pH 6 and 6.5, but could not be estimated more precisely, because, at the scale of our measurements (thousands of YPs per field), the loss of the capacity to reacidify appeared progressive.

Effect of Chloride

We next investigated the role of cytoplasmic chloride on both passive leakage and reacidification. Keeping K^+ and Na^+ concentrations at physiological levels (110 mM/10 mM) and varying Cl^- from 0 to 25 mM had no effect on proton leakage (Fig. 4). On the other hand, reacidification was maximal at 10 and 25 mM Cl^-, but was completely blocked in a Cl^--free medium, which indicates that Cl^- was required for acidification, as found for most vacuolar organelles studied previously (Al-Awqati, 1986). High Cl^- (110 mM) gave complex results, since leakage was apparently increased, while reacidification was poor (ATP merely slowed alkalinization).

Effect of Potassium and Sodium, Evidence for a Na^+/H^+ Antiporter

The effects of variations in the sodium and potassium concentrations were determined in the presence of 10 mM Cl^-. Similar results were obtained when chloride was increased to 25 mM (not shown). Passive proton leakage as measured by the change in 490/450 ratio over a 1-h period was found to be strongly dependent on the presence of permeant monovalent cations in the medium (Fig. 5 *A*). Replacing both sodium and potassium by NMDG, a non-permeant compound, reduced leakage close to 0. Proton leakage could be

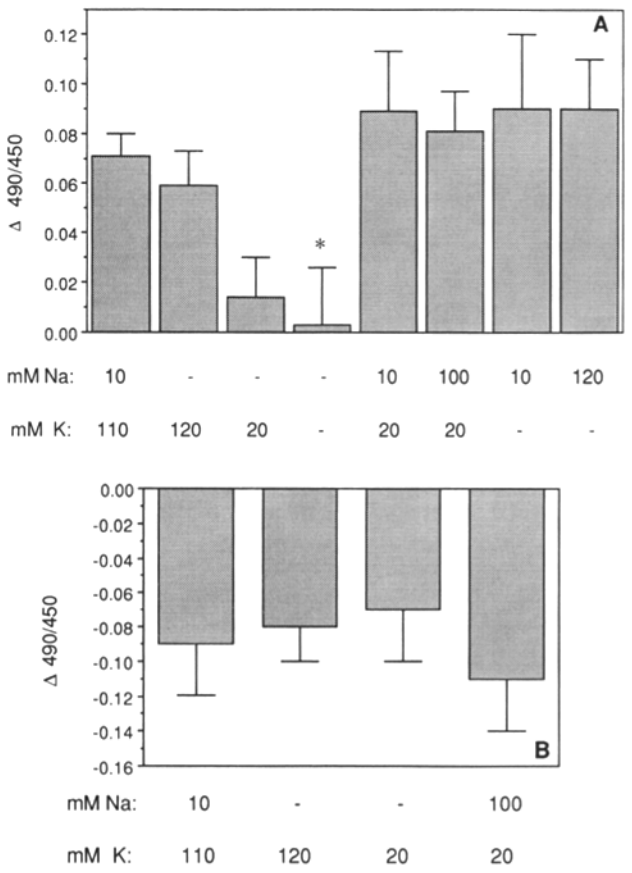

Figure 5. K^+ and Na^+ dependence of passive proton leakage (*A*) and ATP-dependent reacidification (*B*). (*A*) Permeabilized oocytes were rinsed and then incubated for 50 min in media containing 10 mM Cl^- and various concentrations of Na^+ and K^+ in the absence of ATP. (*) This condition was tested in a separate set of experiments. (*B*) Permeabilized oocytes were rinsed for 20 min in Na^+-free, 120 mM K^+, 10 mM Cl^- medium to allow proton leakage, for 20 min in Na^+-free, 20 mM K^+, 10 mM Cl^- medium, and then incubated for 50 min with various combinations of Na^+ and K^+, in the presence of 10 mM Cl^-, MgATP and an ATP regenerating system. Values are mean ± SEM.

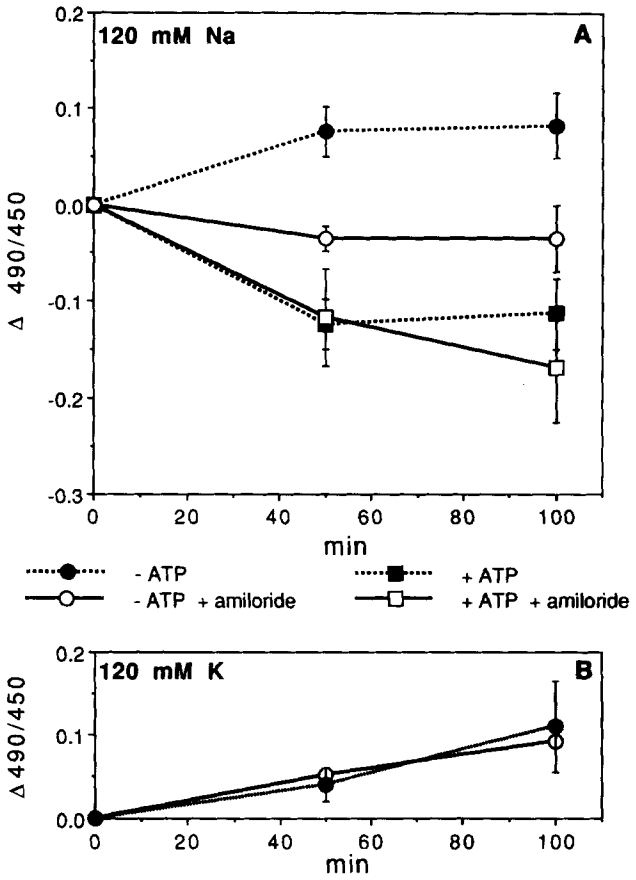

Figure 6. Effect of amiloride on proton leakage and reacidification. (*A*) Permeabilized oocytes were rinsed in a 120 mM Na^+, K^+-free, 10 mM Cl^- medium, and 1 mM amiloride was added at time 0, with or without MgATP and an ATP regenerating system. (*B*) Same experiment using a 120 mM K^+, Na^+-free, 10 mM Cl^- medium, without ATP. Values are mean ± SEM.

sustained by either sodium or potassium. However the dependence for these two ions was strikingly different: in the absence of Na^+, leakage was significant only with high K^+ (110–120 mM), while it was already high at 10 mM Na^+ with or without K^+. Furthermore 1 mM amiloride, an inhibitor of Na^+ channels and Na^+/H^+ antiporters, inhibited Na^+-driven, but not K^+-dependent, proton leakage (Fig. 6). No inhibition was observed with 10 μM amiloride (not shown). Such a low concentration should completely block Na^+ channels, which have a K_i for amiloride <1 μM, but not Na^+/H^+ antiporters, which are much less sensitive to this inhibitor (Garty and Benos, 1988; Moolenaar, 1986). These data indicate that an Na^+/H^+ antiporter is present in YP membranes.

Unlike passive leakage, reacidification was insensitive to cytoplasmic monovalent cations (Fig. 5 *B*) (Na^+/K^+-free conditions were not tested in these experiments, because no Na^+/K^+-free ATP regenerating system was available). Also, amiloride had no significant effect on acidification in the presence of high sodium (Fig. 6 *A*).

Na^+,K^+-ATPase has been proposed to limit acidification in some early endosomes by creating an internal positive potential (Fuchs et al., 1989*b*; Cain et al., 1989). Orthovanate had no effect on acidification of the YPs (Table I), even if valinomycin was added to increase potassium permeability (not shown), which should stimulate Na^+,K^+-ATPase activity (Fuchs et al., 1989*b*). We conclude that Na^+,K^+-ATPase is probably not important for the regulation of the YP pH. In fact, Na^+,K^+-ATPase was not found in the YPs by immunofluorescence, while it was clearly detected at the plasma membrane and in cortical vesicles (Pralong-Zamofing et al., 1992; Fagotto, F., unpublished results).

Effect of 8-Bromo-Cyclic AMP

Cyclic AMP modulates acidification of early endosomes (Gurich and DuBose, 1980; Mulberg et al., 1991; Reenstra et al., 1991; Zen et al., 1992). This is considered, at least in some cases, to be mediated by modifying the chloride conductance (Mulberg et al., 1991; Reenstra et al., 1991), but other channels are known to be cAMP regulated (Kim, 1991). We tested the effect of 8-bromo-cyclic AMP, a membrane permeant, weakly hydrolyzable cAMP analog, on YP acidification. In this series of experiments permeabilized oo-

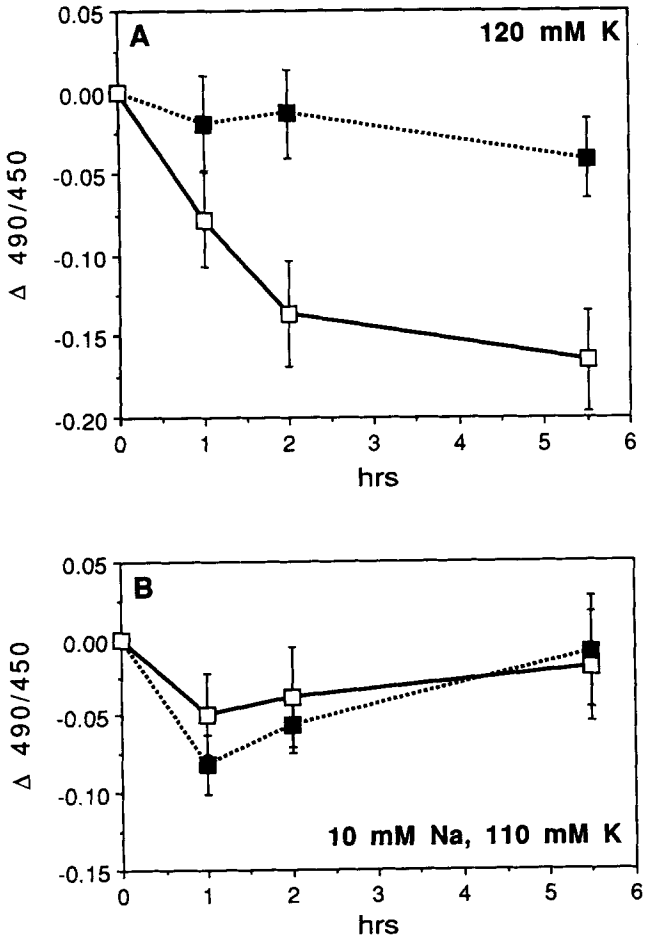

Figure 7. Effect of 8-br-cAMP on acidification. After permeabilization, the oocytes were rinsed in a Na^+,K^+-free medium (minimal proton leakage), and then incubated in a Na^+-free, 120 mM K^+, 25 mM Cl^- medium (*A*), or a 10 mM Na^+, 110 mM K^+, 25 mM Cl^- medium (*B*). 1 mM KMgATP and an ATP regenerating system were added at time 0, with (-□-) or without (--■--) 0.5 mM 8-Br-cAMP. Values are mean ± SEM.

cytes were rinsed with a Na^+/K^+-free medium to minimize proton leakage and thus keep the pH as close as possible to the initial value before the first measurement. As shown in Fig. 7 *A*, when oocytes were incubated with MgATP in a high K^+, Na^+-free medium, the YP pH did not change much, which is consistent with regulation limiting acidification to the in vivo pH. However, the addition of 8-bromo-cAMP significantly activated acidification. 8-bromo-cAMP had no significant effect on acidification in the presence of Na^+ (Fig. 7 *B*).

Buffering Capacity of the Yolk and pH Dependence of YPs Integrity

YPs were isolated in unbuffered 100 mM KCl, broken in distilled water, and the yolk crystals were titrated in 100 mM KCl. The titration curve showed that the yolk is a buffer over a broad pH range, with a pKa around 6.6 (not shown). This effect is likely to be due to the presence of the highly phosphorylated phosvitin.

We tested whether failure of the YPs to reacidify once equilibrated at neutral pH was due to YPs disruption; a crude oocyte homogenate was incubated for 1–2 h at pH 7.4 in the presence of potassium and nigericin to alkalinize the YPs, and was then examined under a microscope after addition of 0.01% Azur A. This dye is excluded from intact organelles, but stains broken YPs dark blue. Extensive breakage occurred as compared to control YPs kept at low pH.

Discussion

The present work has shown that Xenopus YPs are acidic organelles, maintaining an internal pH around 5.6. Endocytosed macromolecules must pass through several compartments before reaching the YPs (pinocytic vesicles, early tubular endosomes, multivesicular bodies). Tracing experiments as well as enzymatic studies indicate that YPs correspond to late endosomes or modified lysosomes (Wall and Patel, 1987; Wall and Meleka, 1985). The pH of 5.6 found in the YPs is in good agreement with values found for late endocytic compartments (pH 5–6), but is clearly lower than the pH of early endosomes (>6) (Maxfield and Yamashiro, 1991).

The YP pH has also been determined using a pH-sensitive dye, NERF, suitable for dual excitation measurements by confocal microscopy. These experiments (Fagotto, F., and F. R. Maxfield, manuscript submitted for publication) confirmed that the YP pH in Xenopus oocytes is 5.6. Furthermore, since the fluorescence of individual YPs was measured, we could analyze the pH as function of the size of the organelles. A similar value was found for small and large yolk platelets, as well as for the primordial YPs. This indicates that though the YPs are highly heterogeneous in size, their pH is uniform.

The YP pH remains stable during oogenesis, but reaches a lower value during embryonic development (Fagotto, F., and F. R. Maxfield, manuscript submitted for publication), which probably triggers yolk degradation as in other species (Fagotto, 1990*b*, 1991; Nordin et al., 1991; Mallya et al., 1992). The moderately acidic pH found in oocytes is thus not an absolute limit for acidification, but must be maintained by a regulatory mechanism. Using permeabilized oocytes, acidification, and the permeability properties of the YP membrane could be studied. YPs are acidified by a typical vacuolar proton-ATPase, as shown by the ATP dependence and sensitivity to the specific inhibitor Bafilomycin A_1. Interestingly, permeabilized oocytes kept the essential properties for the YP pH regulation since reacidification under physiological ionic conditions invariably led to pHs close to the in vivo value.

Acidification required physiological concentrations of chloride (cytoplasmic Cl^- in oocytes is ~23 mM) (Hasegawa et al., 1992) and was blocked under Cl^--free conditions, in agreement with reports on other vacuolar organelles (Al-Awqati, 1986). Modulation of chloride conductance has been proposed to regulate acidification in early (but not late) endosomes (Hilden et al., 1988; Mulberg et al., 1991; Reenstra et al., 1991; Zen et al., 1992). In oocytes, however, since YP acidification was already maximal at low Cl^- concentrations, chloride was not a limiting factor in the establishment of the pH gradient under our experimental conditions. High Cl^- increased passive proton leakage and prevented ATP-dependent reacidification. The significance of this response to a nonphysiological concentration is unclear.

The YP pH gradient is largely maintained in the absence of proton pump activity, provided the external medium has a low ionic strength. This is probably due to the immobile negative charges of the yolk (Donnan equilibrium), as in other protein-rich, acidic vacuoles, such as lysosomes (Reijngoud et al., 1976; Moriyama et al., 1992) and secretory granules (Grinstein and Furuyu, 1983; Arvan et al., 1984). The Donnan effect is less significant at physiological ionic strength. In the presence of cytoplasmic cations, the pH gradient dissipates slowly because the high phosphate content of the yolk must be titrated. The buffering capacity of the yolk would stabilize the internal pH even if large fluctuations of the proton pump activity and/or of proton leakage occur.

Our data show that proton leakage can be driven both by K^+ and Na^+. In the former case, the proton efflux is probably compensated by an influx of K^+ through a K^+ channel, since barium (5 mM), a classical K^+ channel inhibitor, slowed proton leakage in the presence of K^+ alone (not shown). On the other hand, in the presence of Na^+, protons cross the YP membrane through a Na^+/H^+ exchanger, as indicated by the sensitivity to amiloride (Garty and Benos, 1988; Moolenaar, 1986).

Since acidification was found insensitive to vanadate, we conclude that Na^+/K^+-ATPase is probably not involved in regulation of the YP pH. Na^+/K^+-ATPase is known to recycle from the plasma membrane to early endosomes, and, at least in some cell lines, to affect the pH of these organelles (Cain et al., 1989; Fuchs et al., 1989). However, no effect of Na^+/K^+-ATPase on the internal pH has been detected in late endosomes or lysosomes.

That the same pH was reached after ATP-dependent reacidification in the presence of a variety of external ion concentrations suggests that the internal pH value is regulated and does not simply reflect the balance of unregulated ion transport processes. Under certain conditions (i.e., Na^+-free media), YP acidification could be enhanced by the cAMP analog 8-bromo-cAMP. The effect of 8-bromo-cAMP on YP pH is interesting because cAMP levels are known to vary during embryogenesis (Otte et al., 1989), which could explain the stronger YP acidification observed in late embryonic stages (Fagotto, F., and F. R. Maxfield, manuscript submitted for publication). A rise in cAMP, however, is probably not sufficient by itself, since 8-bromo-cAMP did not lower the pH in the presence of Na^+. It is conceivable that proton leakage through the Na^+/H^+ antiporter prevents any acidification to lower pHs (either because the yolk is a less effective buffer, or because some pH "sensor" regulates the ion permeabilities), and that inactivation of the exchanger is required for any further acidification.

In all other examples studied (ticks, Fagotto, 1991; cockroaches, Nordin et al., 1991; sea urchins, Mallya et al., 1992), YPs are initially neutral, and acidify only later during development, in correlation with proteolysis of the yolk proteins. In Xenopus oocytes, however, the function of the mild acidic pH of the YPs is obviously not related to hydrolytic activities, since the YP content is not degraded during oogenesis. So why are YPs kept acidic for weeks?

Low pH may be required to build and maintain the crystal lattice of the yolk. Hen phosvitin examined by Raman spectroscopy displayed an atypical dispersed conformation at neutral pH, due to the high negative charge concentration, but was compacted at low pH (Prescott et al., 1986). Xenopus phosvitin closely resembles its hen homologue and may behave similarly. Its compaction at low pH may be crucial for the yolk crystal structure, which is an optimal way to pack yolk proteins into the smallest possible space. Our finding that YPs are disrupted when their internal pH is neutralized would be consistent with swelling or partial disruption of the crystals.

Significantly, the presence of a Na^+/H^+ antiporter, together with a pH gradient, provides a mechanism for the accumulation of sodium in amphibian YPs. Indeed, yolk platelets contain an unusually high sodium concentration; 35–50% of the total Na^+ in the oocyte was reported to be in the YPs (Dick and Lea, 1967; Morrill, 1965), but since the isolation procedure probably led to extensive disruption of the YPs, the amount of Na^+ concentrated into the YPs was likely to be underestimated. According to the data published on total Na^+ (about 75 mM, Morrill, 1965; Palmer et al., 1978) and free cytoplasmic Na^+ (6–14 mM, Slack et al., 1973; Palmer et al., 1978), intralumenal Na^+ in YPs may be higher than 100 mM. Such high values can certainly be reached under the physiological conditions found in the oocyte, i.e., a large pH gradient across the YPs membrane maintained by a proton pump, a large internal pH buffer, and a cytoplasmic concentration of sodium that is sufficient for maximal transport through the Na^+/H^+ antiporter. The ionic composition of the yolk would result from the balance between the proton pump activity, the potassium-dependent proton permeability and the Na^+/H^+ antiporter, which are all possible candidates for modulation by intracellular messengers.

Many molecules are transported across the plasma membrane using systems coupled to a Na^+ gradient (Scott, 1987). In several intracellular acidic compartments, on the other hand, the proton gradient drives transport of small solutes (efflux of degradation products in lysosomes, see, e.g., Mancini et al., 1989; accumulation of neurotransmitters in synaptic vesicles and other neuronal secretory vesicles, Kanner and Schuldiner, 1987; Moriyama et al., 1990). The plant vacuole takes advantage of the pH gradient to accumulate various small metabolites and ions (Taiz, 1992). We postulate that in Xenopus YPs a pH gradient is used to store sodium. In amphibians, an internal sodium supply is essential for development, since the embryo has to face a low ionic environment (fresh water) (Slack and Warner, 1973). While ion channels and pumps are abundant in the plasma membrane of the oocyte, they are quickly removed during oocyte maturation (Morrill and Ziegler, 1980; Richter et al., 1984; Pralong-Zamofing et al., 1992), and the egg membrane becomes completely impermeant. During cleavage Na^+,K^+-ATPase is inserted in the newly formed basolateral membrane of the blastomeres. There, it transports sodium out of the cells into the extracellular space that swells by osmosis, forming the blastocoelic cavity in the center of the egg (Slack and Warner, 1973; Morrill et al., 1974). The outer, "apical" membrane of the blastomeres being impermeant, sodium cannot be pumped from the environment, as in classical epithelia, and must come from an internal store, most likely the YPs. The Na^+/H^+ antiporter might well operate in the opposite direction at that stage, exchanging inner Na^+ for protons, and in fact the yolk may play the role of an intracellular apical domain.

Though often considered as a simple storage compart-

ment, the yolk most likely has complex interactions with the physiology of the developing organism. Its total membrane surface is much larger than the plasma membrane, and its internal volume is huge compared to the volume of the embryo. The effect of variations in this internal compartment on the embryonic cells may be comparable to the influence of the extracellular environment on adult tissues. It is thus not surprising that the YP membrane contains pumps and channels, as well as regulatory molecules such as G proteins (α_i and α_s, Gallo, C. J., M. Terasaki, T. L. Z. Jones and L. A. Jaffe, University of Connecticut, Storrs, CT, personal communication) similar to the ones classically found at the cell surface.

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