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Polar localization of plasma membrane Ca^{2+}/Mg^{2+} ATPase correlates with the pattern of steady ionic currents in eggs of *Lymnaea stagnalis* and *Bithynia tentaculata* (Mollusca)

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Summary. During extrusion of the first polar body in eggs of *Lymnaea stagnalis* and *Bithynia tentaculata* a localized Ca^{2+}/Mg^{2+} ATPase activity was detected, using Ando's enzyme-cytochemical method for electron microscopy [Ando et al. (1981) Acta Histochem Cytochem 14:705–726]. The enzyme activity was distributed in a polar fashion, along the cytoplasmic face of the plasma membrane. In the eggs of *Lymnaea* it was found only in the vegetal hemisphere, whereas in *Bithynia* eggs it was localized both in the vegetal hemisphere and at the animal pole. This pattern of enzyme activity corresponds to the polar pattern of transcellular ionic currents measured with the vibrating probe, which we showed to be partially carried or regulated by calcium [Zivkovic and Dohmen (1989) Biol Bull (Woods Hole) 176 (Suppl):103–109]. The characteristics of the ATPase were studied using a variety of approaches such as ion and substrate depletions and substitutions, addition of specific inhibitors of ATPase activity, treatment with EDTA/EGTA and electron energy-loss spectrometry. The results indicate that, in *Lymnaea*, there are at least two enzymatic entities. The first one is a Ca^{2+}/Mg^{2+} ATPase localized along the membrane and in the cortex of the vegetal hemisphere. The second one is a Ca^{2+}-stimulated ATPase (calcium pump of the plasma membrane) localized in a small region of the membrane at the vegetal pole. We speculate that in the eggs of *Lymnaea* and *Bithynia* a functional relationship exists between the plasma-membrane-associated ATPase activity and the transcellular ionic currents measured in the same region.

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Key words: Ca^{2+}/Mg^{2+} ATPase – Molluscan eggs – Ionic currents – Polarity – Calcium

Introduction

In the development of molluscs, as in that of many other animals, the animal-vegetal polarity of the egg determines to a large extent the basic organization of the body plan. The animal pole region of the molluscan egg develops into the head structures, whereas the vegetal pole region will give rise to the blastopore. The factors that govern these regional differentiations have not yet been identified at the molecular level, but there is a large amount of evidence that specific substances localized at the poles act as the determinants of early axial development (see Davidson 1986).

A number of studies have demonstrated that the polar organization of the cytoplasm in molluscan eggs correlates with specific structural properties of the egg cortex, the plasma membrane, or the surface architecture (see Dohmen 1983; Dohmen and van der Mey 1977; Speksnijder et al. 1985a, b). Moreover, centrifugation experiments have shown that some cytoplasmic structures may be tightly attached to the polar cortex (van Dam et al. 1982). These data suggest that the plasma membrane and its adjacent structures may be responsible for establishing or maintaining the polar organization of the cytoplasm. Ionic currents may play a role in this process, as suggested by Jaffe (1982).

Steady ion fluxes can be detected with the vibrating probe technique (Jaffe and Nuccitelli 1974). These ionic

currents have been observed in nearly every cell and organism investigated thus far, and they often correlate with an axis of polarity such as the apical-basal axis in epithelial cells or the animal-vegetal axis in egg cells (see Nuccitelli 1988). Ions enter or leave a cell through ion channels or ion pumps that are localized in the plasma membrane. When ion channels and/or pumps are distributed or activated in a non-random fashion, a net transcellular ionic current occurs. As a consequence, an extracellular electric field is generated, that can be detected by measuring the voltage gradient non-invasively with the vibrating probe.

The role of ionic currents in establishing cellular polarity has been studied most extensively in eggs of the brown algae *Pelvetia* and *Fucus* (see Nuccitelli 1988). The site of inward current predicts the position of subsequent rhizoid outgrowth, which is the first morphological manifestation of polarity in these algal eggs (Niccitelli 1978). This inward current is partly carried by calcium ions, the influx of which may generate an intracellular gradient of calcium that polarizes the egg. As yet there is no direct evidence for the presence of a cytoplasmic calcium gradient during the polarization process that precedes rhizoid outgrowth (Brownlee 1989), and calcium influx is not required for this initial axis formation since it occurs in the absence of external calcium and in the presence of calcium channel blockers (Kropf 1989). For the subsequent process of rhizoid outgrowth an intracellular gradient of calcium is required as shown by Speksnijder et al. (1989), who injected BAPTA [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] buffers that shuttle calcium from regions of high concentration to regions of low concentration. As a result the intracellular calcium gradient will be disturbed and consequently tip growth will be blocked.

In the eggs of *Lymnaea* and *Bithynia*, a transcellular ionic current has been detected that correlates with the animal-vegetal axis (Dohmen et al. 1986; Zivkovic and Dohmen 1989; Zivkovic 1990). In the egg of *Lymnaea*, the current is at least partly carried by calcium ions as inferred from its decrease in the presence of calcium-channel blockers. Direct evidence for a concentration of calcium channels or calcium-pump sites in the plasma membrane that correlates with the site of influx or efflux of a polar current would be desirable but is lacking in any system as far as we know. We chose, therefore, to study the distribution of Ca^{2+}-stimulated plasma membrane ATPase, which serves to pump calcium out of the cell (Carafoli 1987, 1988). In addition to Ca^{2+}-stimulated ATPase (the classical calcium pump of the plasma membrane), there is yet another calcium-activated, membrane-bound enzyme, a high-affinity Ca^{2+}/Mg^{2+} ATPase, which has also been suggested to serve as a calcium pump in some cells (see Dhalla and Zhao 1988). Ca^{2+}-stimulated ATPase appears to be present in the plasma membrane of most if not all animal cells, and it provides the fine regulation of the cytoplasmic concentration of calcium in the submicromolar range. In contrast, the Ca^{2+}/Mg^{2+} ATPase is activated by higher concentrations (micromolar to millimolar) of either calcium or magnesium. The two enzymes also differ in their sensitivity to inhibition by vanadate. Ca^{2+}-stimulated ATPase, like other membrane-bound ion-motive ATPases such as the Na^+/K^+ pump, is inhibited by vanadate, whereas Ca^{2+}/Mg^{2+} ATPase is not affected. The substrate required by Ca^{2+}-stimulated ATPase is MgATP, whereas Ca^{2+}/Mg^{2+} ATPase utilizes ATP (see Carafoli 1988; Dhalla and Zhao 1988). Experimentally the two enzymes may thus be distinguished from each other by using vanadate or by removing magnesium from the medium, since Ca^{2+}/Mg^{2+} ATPase does not require this cation for its activity, whereas Ca^{2+}-stimulated ATPase requires both calcium and magnesium for its activity.

Ando et al. (1981) devised a one-step cytochemical method for the electron-microscopical localization of calcium-activated ATPase activity. Using this method, we provide evidence that there is a localized Ca^{2+}/Mg^{2+} ATPase activity in the eggs of *Lymnaea* and *Bithynia* that coincides with the polar transcellular current pattern previously detected with the vibrating probe.

Materials and methods

Lymnaea stagnalis and *Bithynia tentaculata* are freshwater snails. The eggs are fertilized internally and we used spontaneously laid egg masses. The eggs were allowed to develop in their capsules until they had reached the desired stage of development. Then they were decapsulated and rinsed several times in filtered copper-free tap water.

Enzyme cytochemistry for electron microscopy. Eggs were fixed for 45 min in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, at 4° C. After rinsing overnight in cacodylate buffer containing 0.25 M sucrose, the eggs were incubated for 30 min at 37° C in Ando's medium (Ando et al. 1981). This medium is called standard medium (SM) and consists of 2 mM lead citrate in 250 mM glycine/50 mM KOH buffer, 10 mM $CaCl_2$, 8 mM levamisole (Sigma), and 3 mM ATP (disodium salt, Pharmacia), at a final pH of 9.0 After six rinses of 5 min each in cacodylate buffer, the eggs were postfixed for 1 h in 1% OsO_4 in the same buffer. The eggs were then oriented in 2% agar in distilled water (van der Wal and Dohmen 1978), dehydrated in a graded series of ethanol concentrations and embedded in Epon via propylene oxide. Ultrathin sections were cut parallel to the animal-vegetal axis. Unstained sections were examined in a Zeiss EM-10 microscope. Each type of incubation was repeated at least three times.

To determine the type of ATPase involved and to estimate the specificity of the reaction several controls were carried out in *Lymnaea* eggs: (a) inactivation of the enzyme by preheating the eggs for 30 min at 70° C before incubation in SM, after the fixation step; (b) omission of the substrate (ATP); (c) omission of the activating calcium ion; (d) replacement of calcium by magnesium; (e) addition of the inhibitors vanadyl sulphate (vanadate) or ouabain. Both inhibitors were dissolved as 10 mM stock in distilled water immediately prior to use. In order to exclude a contribution of endogenous calcium to the activation of the ATPase, the eggs were depleted of calcium, after fixation, by treating them for about 45 min at 4° C with 10 mM EGTA in cacodylate buffer pH 8.0 containing 0.25 M sucrose. These eggs were subsequently rinsed for 1 h in cacodylate buffer prior to incubation in one of the following media: (a) SM, (b) SM without calcium, (c) SM without ATP, (d) SM without calcium with 5 mM EGTA added. Eggs pretreated in the same manner with EDTA instead of EGTA were incubated in the following media: (a) SM, (b) SM without calcium, (c) SM without ATP, (d) SM containing 10 mM vanadyl sulphate.

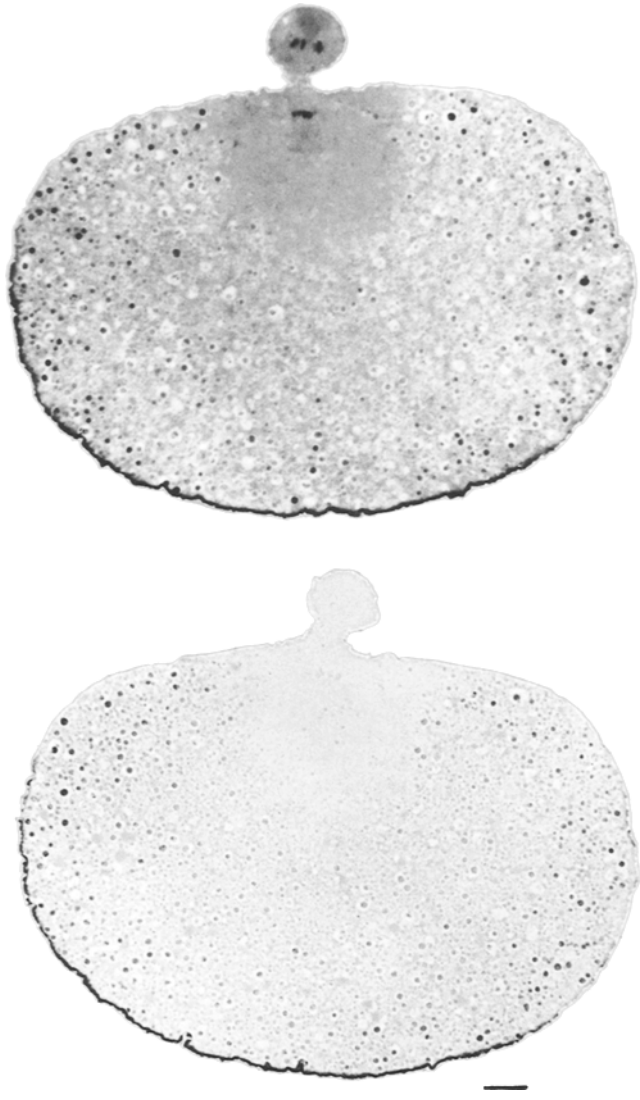


Fig. 1. Ca^{2+}/Mg^{2+} ATPase activity at the vegetal pole of a *Lymnaea stagnalis* egg after incubation in standard Ando's medium and treatment with ammonium sulfide. The *upper section* was stained with methylene blue, whereas the *lower section* was not stained. *Bar* represents 10 μm

In order to test the specificity of the enzyme for different substrates, we first removed possible endogenous substrate reserves by treating fixed eggs for 20 min with the detergent Nonidet P40 (0.2% in cacodylate buffer) and rinsing them three times for 10 min in cacodylate buffer. The eggs were then either rinsed overnight at 4° C in the same buffer before incubation in SM without ATP, or treated overnight at 20° C with 0.0015 unit/ml exogenous ATPase isolated from rabbit kidney (Sigma) in 10 mM Na-HEPES +0.9% NaCl pH 7.2. The eggs were then rinsed in cacodylate buffer for 1 h (six changes). Subsequently, the ATPase-treated eggs were incubated in one of the following media: (a) SM without substrate, (b) SM containing 3 mM ATP, (c) SM containing 3 mM GTP instead of ATP, (d) SM containing 3 mM UTP instead of ATP, (e) SM containing 3 mM GTP and 3 mM UTP in addition to 3 mM ATP. The same incubations were performed in another series of experiments, in which SM was supplemented with 3 mM $MgCl_2$. Prior to the cytochemical ATPase reaction (1 h at 37° C) the eggs were treated for 2 h with cacodylate buffer containing the corresponding substrate(s). This was done to allow sufficient time for diffusion of the substrate from the extracellular medium into the substrate-depleted egg.

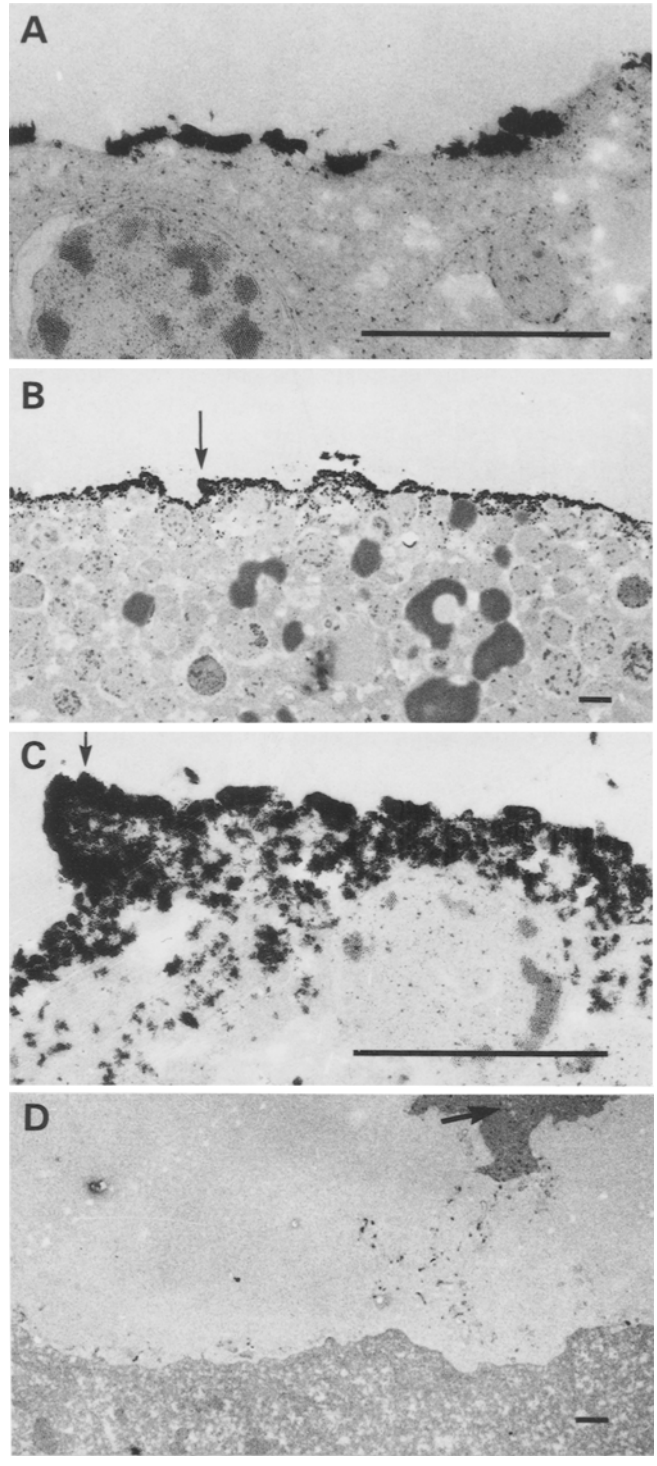


Fig. 2A–C. Ca^{2+}/Mg^{2+} ATPase activity along the membrane of the vegetal pole of *Lymnaea stagnalis* eggs after incubation in the standard Ando's medium. The reaction product in eggs at prometaphase of the first meiotic cell cycle is localized mainly at the outer membrane surface **A**, whereas it is localized at the cytoplasmic face of the membrane during first polar body extrusion (meiotic telophase) **B**, **C**. In **C** a detail from **B** is shown (*arrow*). The animal pole was free of reaction product during polar body formation (**D**, *arrow points to the polar body*). All *bars* represent 1 μm

The permeability of fixed eggs as a result of Nonidet treatment was tested by observing the diffusion of 70-kDa fluorescein-isothiocyanate(FITC)-dextran (Sigma) into the interior to the egg.

Enzyme cytochemistry for light microscopy. The eggs were treated as for electron microscopy until after the incubation in SM. Then the eggs were rinsed four times in distilled water, incubated for 2 min in 1% ammonium sulfide in distilled water, and rinsed again four times in distilled water. The eggs were further processed as for electron microscopy, except that there was a reduction of the post-fixation time in OsO_4 from 1 h to 15 min. Sections of about 1 μm were cut with glass knives and photographed in a Zeiss microscope.

Oxalate/pyroantimonate (OPA) technique. In order to precipitate loosely bound calcium, and to localize the precipitate in the electron microscope, a combined oxalate/pyroantimonate technique was used (Borgers et al. 1977). Briefly, *Lymnaea* eggs were fixed at the time of first polar body extrusion for 2 h at 4° C in 3% glutaraldehyde in 0.09 M potassium oxalate in distilled water, supplemented with 1.4% sucrose at a final pH 7.4. Then the eggs were rinsed for 15 min in 0.09 M potassium oxalate in distilled water supplemented with 7.5% sucrose. After a 2-h postfixation at 4° C in 1% OsO_4 in distilled water containing 2% potassium pyroantimonate and a rinse in distilled water adjusted to pH 10 with 0.1 M KOH, the eggs were oriented in 2% agar in distilled water, dehydrated in a graded series of ethanol concentrations, passed through propylene oxide, and embedded in Epon.

Controls. The ability of EGTA to dissolve calcium containing deposits was tested by incubating sections in 10 mM EGTA, pH 7.8, at 60° C, for 30 min. Ultrathin sections, which were incubated in distilled water, and pH-adjusted to 7.8 with KOH, served as controls.

Electron energy-loss spectrometry (EELS). Unstained 40 to 50-nm thick sections on 400-mesh copper grids were analysed with a Zeiss EM 902 electron microscope equipped with an electron energy-loss spectrometer. Prior to measurement, the spectrometer was calibrated using biostandards containing 6.8% w/w calcium (BioRad-Polaron). EELS measurements were performed on sections from two separate OPA experiments.

Results

Ca^{2+}/Mg^{2+} ATPase cytochemistry

Good preservation of cellular detail and a dense, well-localized reaction product were obtained after fixation in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde. Fixation times exceeding 45 min, or the use of fixatives of higher concentrations resulted in a decrease or disappearance of the reaction product.

In eggs of *Lymnaea* fixed at the time of first polar body formation (telophase of the first meiotic cell cycle) a dense, well-localized reaction product was found along the cytoplasmic face of the plasma membrane and in the cortical area of the vegetal hemisphere (Figs. 1, 2B, C and Table 1, expt. 0). However, in eggs fixed at prometaphase of the first meiotic cell cycle, the reaction product was localized mainly along the extracellular surface of the plasma membrane at the vegetal pole (Fig. 2A and Table 3, expt. 25). The extent of the ATPase-positive region was variable, ranging from a small area strictly localized at the vegetal pole, to a large area occupying the whole vegetal hemisphere including the equator. The plasma membrane of the animal hemisphere was devoid of reaction product (Fig. 2D). At both prometaphase and telophase, precipitate was absent from the cytoplasm.

These data show that, in eggs of *Lymnaea*, there is a polar localization of membrane-bound Ca^{2+}/Mg^{2+} ATPase during the first meiotic cell cycle, which correlates with the site of outward transcellular current measured with the vibrating probe in these eggs (Zivkovic and Dohmen 1989).

Similar experiments were done with eggs of the molluscs *Bithynia tentaculata* and *Nassarius reticulatus* (data not shown) and with eggs of the ascidian *Phallusia mamillata* (data not shown). However, only in the eggs of *Bithynia* did we observe Ca^{2+}/Mg^{2+} ATPase activity.

Table 1. Effect of ion and substrate depletion and ion substitutions on ATPase activity in intact and EGTA/EDTA pretreated eggs of *Lymnaea* during first polar body formation

Experiment no.	Pretreatment	Cytochemical reaction medium[a]	Localization and density of reaction product	Number of eggs
0	–	SM		12
1	–	SM-ATP		8
2	–	SM-Ca^{2+}		8
3	–	SM-Ca^{2+}; +10 mM Mg^{2+}		4
4	–	SM+10 mM vanadyl sulphate		6
5	–	SM+2 mM ouabain		5
6	70° C (30 min)	SM		4
7	10 mM EGTA (45 min)	SM		4
8	10 mM EGTA (45 min)	SM-Ca^{2+}		3
9	10 mM EGTA (45 min)	SM-ATP		4
10	10 mM EGTA (45 min)	SM-Ca^{2+} +5 mM EGTA		4
11	10 mM EDTA (45 min)	SM		3
12	10 mM EDTA (45 min)	SM-Ca^{2+}		4
13	10 mM EDTA (45 min)	SM-ATP		3
14	10 mM EDTA (45 min)	SM+10 mM vanadyl sulphate		4

[a] SM, standard medium (complete Ando's medium)

In this species, a polar lobe is constricted off at the vegetal pole during first and second cleavage (Dohmen and Verdonk 1974). A similar constriction, though far less conspicuous, occurs concomitantly with the extrusion of the first and second polar bodies.

The cytochemical experiments on *Bithynia* eggs demonstrate a bipolar localization of the reaction product, which is in contrast to the unipolar localization in *Lymnaea* described above. At the vegetal pole, extending as far as the constriction of the polar lobe (Fig. 3C, D) we observed a reaction product (Fig. 3D, E) similar in appearance and subcellular localization to that observed at the vegetal pole of *Lymnaea* eggs (Fig. 2B, C). At the animal pole, we found a reaction product localized along the plasma membrane of the extruding polar body (Fig. 3A, B). This precipitate was distinct from that on the vegetal pole and from that observed in *Lymnaea* by its sharp localization and regular distribution along the membrane. It did not extend into the cortical layer (Fig. 3B). The difference between *Bithynia* and *Lymnaea* in the localization of ATPase activity can be correlated with the difference in ionic current pattern (for current patterns in *Bithynia* eggs see Zivkovic 1990). During the first polar body formation, current enters the vegetal pole in *Bithynia*, whereas it leaves the vegetal pole in *Lymnaea*. During the same period, the current at the animal pole is first outward and then inward in *Bithynia*, whereas it is continuously inward in *Lymnaea*. In both eggs, maximum currents were measured concurrently with anaphase and telophase of the meiotic division. The ATPase activity at the extruding polar body of *Bithynia* may be responsible for the opposite direction of the current in *Bithynia* as compared with *Lymnaea*, in which no ATPase activity is found at the animal pole.

In *Lymnaea*, a number of control experiments have been carried out. To check the enzymatic nature of the reaction, eggs were preheated at 70° C (Table 1, expt. 6), resulting in the complete absence of reaction product (Fig. 4A). This result excludes the possibility of non-enzymatic hydrolysis of ATP by lead, one of the potential causes for formation of non-specific precipitates (Moses and Rosenthal 1968; Jacobsen and Jorgensen 1969). Using the inhibitor vanadate (Bond and Hudgin 1980) (Table 1, expt. 4) resulted in a significant decrease of membrane-bound reaction product, whereas the cortical precipitate was nearly normal (Fig. 4C). This indicates that the reaction at the plasma membrane may be due to the presence of Ca^{2+}-stimulated ATPase (Ca pump), whereas the cortical reaction may be due to the

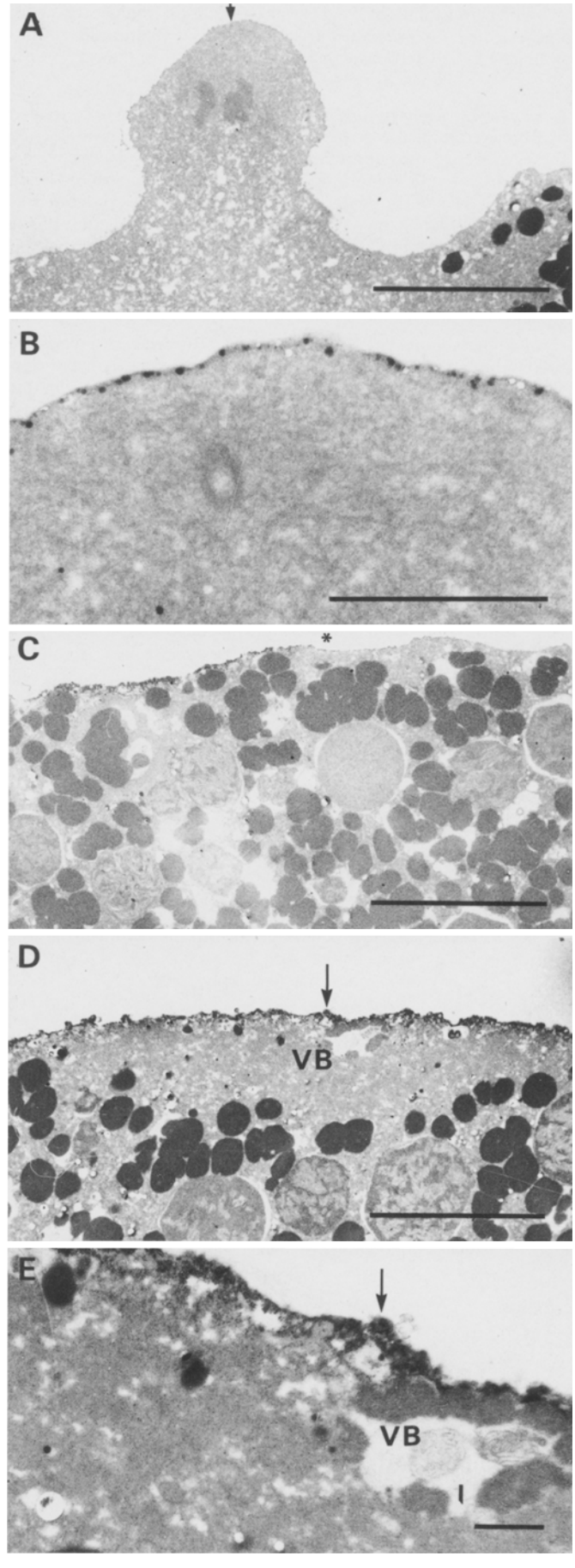
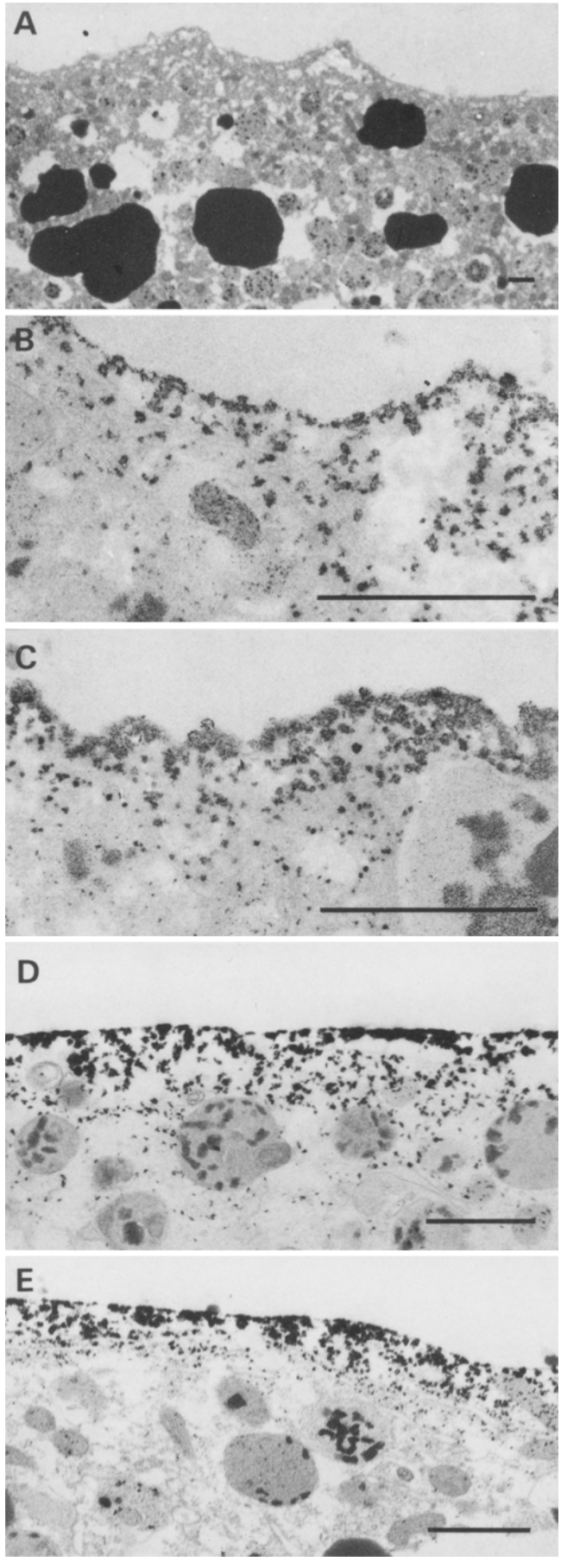


Fig. 3A–E. Ca^{2+}/Mg^{2+} ATPase activity in eggs of *Bithynia tentaculata* during extrusion of the first polar body (meiotic telophase) after incubation in standard Ando's medium. At the animal pole the reaction product is localized in the membrane surrounding the polar body (*arrow* in **A**, and detail in **B**). At the vegetal pole it is localized along the cytoplasmic face of the plasma membrane surrounding the polar lobe (**D**, and detail in **E**; *arrow* points to the precipitate near the vegetal body, *VB*). The enzyme activity extends from the vegetal pole up to the constriction of the polar lobe (*, **C**). *Bars* represent 1 μm in **B** and **E**, and 10 μm in **A**, **C**, and **D**



activity of Ca^{2+}/Mg^{2+} ATPase, since the former enzyme is reported to be sensitive to vanadate, whereas the latter enzyme is not (e.g. Zhao and Dhalla 1988).

Ouabain, a specific inhibitor of the Mg^{2+}-dependent Na^+/K^+ ATPase (Na^+/K^+ pump of the plasma membrane), did not affect the observed ATPase activity, which shows that the reaction product is not due to Na^+/Ka^+ ATPase (Table 1, expt. 5). This is supported by the fact that Na^+/K^+ ATPase is extremely sensitive to inhibition by lead, which we used as a capture ion for phosphate from hydrolysed ATP (Ernst and Hootman 1981). Since we did not use a standard medium to detect the ouabain-sensitive K^+-dependent *p*-nitrophenylphosphatase, which uses *p*-nitrophenylphosphate as substrate instead of ATP, and dimethylsulphoxide as an activator (which shifts the pH optimum of the enzyme to alkaline values), the inhibiting action of lead ions was not compensated by the activating action of dimethylsulphoxide (Mayahara et al. 1980). Taken together with the lack of inhibition by ouabain, the chance that the detected ATPase activity in *Lymnaea* eggs is due to the Na^+/K^+ pump seems negligible. However, we cannot completely exclude the involvement of a proton-translocating ATPase (Araki et al. 1990) or H^+/K^+ ATPase (Ogawa et al. 1987), since we did not use specific inhibitors of the two enzymes. The reaction media for the detection of both these enzymes are similar to the medium used for Na^+/K^+ pump cytochemistry, and they detect the *p*-nitrophenylphosphatase activity in the presence of dimethylsulphoxide. Ando's medium in itself would perhaps allow the detection of these enzymes, but the inhibiting action of lead and the absence of the activating effect of dimethylsulphoxide render this possibility unlikely in the present experiments.

In order to check whether enzyme activity is dependent on the presence of calcium or magnesium, $CaCl_2$ was omitted from the SM (Table 1, expt. 2). As a result, the intensity of the reaction diminished significantly, but the pattern remained unchanged (Fig. 4B) as compared to that in the complete medium (Fig. 2B, C). The formation of reaction product in calcium-free medium suggests the existence of an alternative source of calcium, such as endogenous calcium stores or micromolar calcium present in the chemicals used to compose SM.

Substitution of $CaCl_2$ by equimolar $MgCl_2$ (Table 1, expt. 3) resulted in the presence of a reaction product along the plasma membrane and the cortical area of

Fig. 4A–E. Ca^{2+}/Mg^{2+} ATPase activity at the vegetal pole of *Lymnaea* eggs incubated in the standard medium. **A** After preheating at 70° C, resulting in the absence of the reaction product. **B** Without calcium, showing decrease in the activity as compared to Fig. 2B, C. **C** Supplemented with 10 mM vanadyl sulphate, resulting in partial inhibition mainly of membrane-bound activity. **D** Reaction product was found in fixed eggs pretreated with 0.2% Nonidet P40 in cacodylate buffer, followed by incubation in exogenous ATPase and thereafter incubated in standard medium containing 3 mM ATP, 3 mM GTP, and 3 mM UTP. **E** Same pretreatment as **D**, but final incubation in standard medium containing 3 mM ATP and 3 mM $MgCl_2$. All *bars* represent 1 μm

the whole egg. The precipitate was less dense and less coarse at the animal pole. This result indicates that the ATPase activity in the animal hemisphere depends on millimolar magnesium and may be due to a separate enzyme, a Mg^{2+} ATPase, with a more extended distribution than the Ca^{2+} ATPase. In the literature there is no consensus on the question whether Ca^{2+} ATPase and Mg^{2+} ATPase are separate enzymes or constitute one Ca^{2+}/Mg^{2+} ATPase complex (see Dhalla and Zhao 1988). Since the reaction product, which is formed in the animal hemisphere when calcium is substituted by magnesium, is resistant to treatment with 2 mM ouabain (data not shown), it is unlikely that it is due to the activity of the plasma membrane Na^+/K^+ pump.

The contribution of endogenous calcium and magnesium to the ATPase activity was studied by pretreating the eggs with EGTA or EDTA followed by incubation in modified SM. When pretreatment with EGTA was followed by incubation in calcium-free SM to which EGTA was added, the reaction product was entirely absent (Table 1, expt. 10), showing that rigorous elimination of calcium abolishes the enzyme activity completely. When pretreatment with EDTA was followed by incubation in calcium-free SM without EDTA or EGTA (Table 1, expt. 12) a narrow zone of reaction product was found exclusively localized at the vegetal pole (Fig. 5B, C). So under less stringent conditions there is still some enzyme activity at the vegetal pole, suggesting that the enzyme can be activated by very low concentrations of calcium, which is a characteristic of Ca^{2+}-stimulated ATPase. This enzyme has a high affinity for calcium and consequently micromolar concentrations of calcium suffice for its activation. Pretreatment with EGTA, followed by incubation in calcium-free SM (Table 1, expt. 8) resulted in a reduction of the cortical reaction product similar to that observed after EDTA pretreatment (Table 1, expt. 12). However, after EGTA pretreatment the most dense reaction product was localized laterally from the vegetal pole. At present we cannot explain the latter result. The discrepancy in localization of reaction product may be caused by the difference in chelating properties between EDTA and EGTA, the latter being more specific for calcium ions (see Campbell 1983). Thus, experiments 8 and 12 show that even after pretreatment with EDTA or EGTA, the remaining calcium is sufficient to activate the enzyme(s), although in a very restricted area.

The presence of endogenous substrate reserves was checked by incubating eggs in standard medium without ATP. Eggs fixed at the time of first polar body extrusion

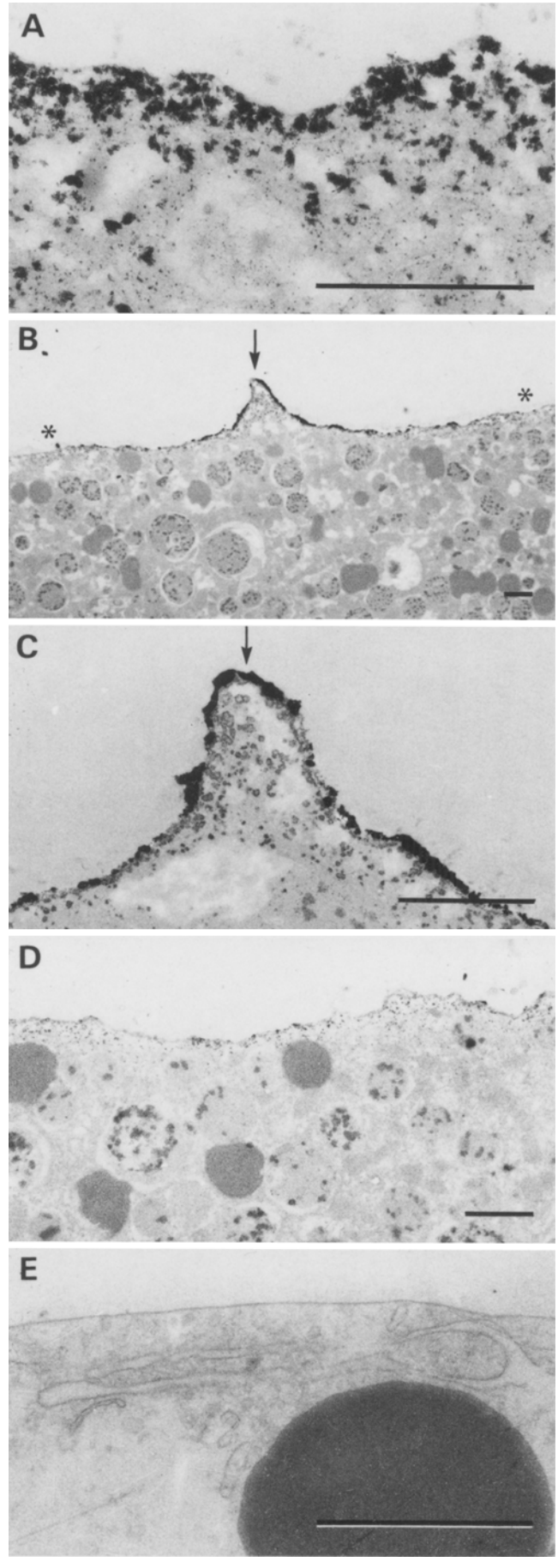


Fig. 5. Ca^{2+}/Mg^{2+} ATPase activity at the vegetal pole of *Lymnaea* eggs after pretreatment in 10 mM EDTA **B**, **C**, **E** or EGTA **A**, **D** in cacodylate buffer followed by: incubation in **A** standard medium (*SM*); **B**, **C** SM without calcium, showing localized activity area at the vegetal pole (between **). Detail in **C** (*arrow*); **D** SM without ATP, showing diminished activity as compared to **A**; **E** SM to which 10 mM vanadyl sulphate was added, showing complete inhibition of the activity remaining after EDTA pretreatment. All *bars* represent 1 μm

showed a normal staining pattern (Table 1, expt. 1). This result shows that there is a cytoplasmic pool of ATP, which is not washed out during fixation and rinsing of the eggs. However, eggs fixed at prometaphase of the first meiotic cell cycle lacked the reaction product normally present at the extracellular face of the plasma membrane at the vegetal pole up to this stage and absent afterwards (Table 3, expt. 26). This precipitate on the outer egg surface may be due to a Ca^{2+}/Mg^{2+} ecto-ATPase as reported for mammalian cells (Knowles 1988). Such an ecto-ATPase hydrolyses ATP from an extracellular pool and in *Lymnaea* eggs it may be a remnant of ecto-ATPase activity at the basal membrane of the oocyte in the ovarium before oviposition.

To assess the substrate specificity of the ATPase under investigation, the eggs first had to be depleted of endogeneous nucleotides, which apparently were not washed out following fixation and rinsing (Table 1, expt. 1). We approached this by treating the fixed eggs with the detergent Nonidet P40 for 20 min and subsequently rinsing them overnight in cacodylate buffer at 4° C (Table 2, expt. 15). Since subsequent incubation in standard medium without ATP resulted in the presence of reaction product at the vegetal hemisphere (Table 2, expt. 15) we concluded that treatment with Nonidet and subsequent rinsing does not deplete eggs of their internal substrate stores. As a control of the effect of Nonidet, the diffusion of FITC-dextran into the eggs was monitored. This large (70-kDa) molecule penetrated into the eggs within a few minutes. The much smaller ATP molecules would be expected to have diffused out of the fixed egg if diffusion were not hampered by binding or compartmentalization of the ATP.

The presence of substrate in the eggs after Nonidet treatment was shown by an additional treatment with exogenous ATPase (Table 2, expts. 16–24). This double treatment, first Nonidet for 20 min and then exogenous ATPase overnight, resulted in the complete absence of reaction product when the eggs were incubated in medium without ATP (Table 2, expt. 16). This proves two

Table 2. Substrate specificity of the ATPase[a]

Experiment no.	Incubation (1)	Incubation (2)	Incubation (3)	Cyto-chemical reaction medium	Localization and density of reaction product	Number of eggs
15	0.2% Nonidet P40 20 min	CB, 4° C overnight	–	SM-ATP		5
16	0.2% Nonidet P40 20 min	Exogenous ATPase 0.0015 unit/ml, 20° C, overnight	–	SM-ATP		3
17	0.2% Nonidet P40 20 min	Exogenous ATPase 0.0015 unit/ml, 20° C, overnight	3 mM ATP (2 h)	SM (ATP)		4
18	0.2 Nonidet P40 20 min	Exogenous ATPase 0.015 unit/ml, 20° C, overnight	3 mM GTP (2 h)	SM-ATP; +GTP		3
19	0.2% Nonidet P40 20 min	Exogenous ATPase 0.0015 unit/ml, 20° C, overnight	3 mM UTP (2 h)	SM-ATP; +UTP		3
20	0.2% Nonidet P40 20 min	Exogenous ATPase 0.0015 unit/ml, 20° C, overnight	cocktail (2 h)	SM-ATP; +cocktail		2/2
21	0.2% Nonidet P40 20 min	Exogenous ATPase 0.0015 unit/ml, 20° C, overnight	3 mM ATP (2 h)	SM +3 mM Mg^{2+}		2
22	0.2% Nonidet P40 20 min	Exogenous ATPase 0.0015 unit/ml, 20° C, overnight	3 mM GTP (2 h)	SM-ATP; +GTP +3 mM Mg^{2+}		3
23	0.2% Nonidet P40 20 min	Exogenous ATPase 0.0015 unit/ml, 20° C, overnight	3 mM UTP (2 h)	SM-ATP; +UTP +3 mM Mg^{2+}		2
24	20° C Nonidet P40 20 min	Exogenous ATPase 0.0015 unit/ml, 20° C, overnight	Cocktail	SM-ATP; +cocktail +3 mM Mg^{2+}		2

[a] Depletion of endogenous substrate stores from fixed *Lymnaea* eggs using Nonidet P40 (0.2%) in cacodylate buffer (CB; 0.1 M, pH 7.4) and exogenous rabbit kidney ATPase (0.0015 unit/ml in 10 mM/NaHEPES/0.9% NaCl, pH 7.2); cocktail consists of 3 mM ATP, 3 mM GTP and 3 mM UTP

points: first, that a large molecule like exogenous ATPase can enter a Nonidet-treated egg by diffusion, and second, that intracellular, apparently non-diffusable ATP is hydrolysed by this exogenous ATPase. Unexpectedly, however, subsequent addition of ATP, GTP, or UTP to the incubation medium did not result in the formation of a precipitate under these conditions (Table 2, expts. 17–19). Only after addition of the three nucleotides together (Table 2, expt. 20) or supplementation of SM, containing any of the substrates, with magnesium (Table 2, expts. 21–23) did the precipitate form. Incubation in the mixture of three nucleotides (Fig. 4D), resulted in a precipitate in two out of four eggs at the vegetal hemisphere and, in the other two eggs, along the whole plasma membrane and egg cortex, with the highest density at the vegetal pole. After incubation in substrates supplemented with magnesium the reaction product was found along the whole plasma membrane and the cortex in all eggs (Fig. 4E). These results suggest that enzyme function may be impaired by these experimental treatments and that only at high substrate concentrations, or after addition of magnesium, does activity become apparent. The cumulative effect of three different nucleotides suggests that the enzyme, after being treated in the described manner, has a broad nucleotide-hydrolysing capacity, but further research is needed to elucidate this point.

Another approach to depleting internal substrate reserves was to dissociate and wash out intracellularly bound ATP. Binding of ATP may be in the form of a membrane-associated protein-ATP-Ca^{2+} complex, as has been proposed for the ATP-release site in myocytes (see Rovetto 1985). Removing Ca^{2+} by treatment with EGTA (Table 1, expt. 9) or EDTA (Table 1, expt. 13) might then release ATP. Subsequent incubation of the eggs in SM without substrate (expts. 9, 13) resulted in a significant decrease of the reaction product (Fig. 5D) as compared to eggs pretreated with EGTA (Table 1, expt. 7) or EDTA (Table 1, expt. 11) and subsequently incubated in standard medium including ATP (Fig. 5A). We infer from these data that the chelating pretreatment improved the washout of ATP from the endogenous stores and that, consequently, calcium or magnesium may play a role in binding of intracellular ATP.

Oxalate/Pyroantimonate (OPA) technique and electron energy-loss spectrometry (EELS)

Using the OPA cytochemical method to demonstrate the presence of precipitable calcium (Borgers et al. 1977), both fine and coarse electron-opaque deposits were found on the outer surface of the plasma membrane within the vitelline layer. The cytoplasm was free of precipitate. The distribution of the precipitate on the surface was not homogeneous: at the vegetal pole it was coarse and densely packed (Fig. 6B), whereas both coarse and fine, but less dense precipitate was present at the animal

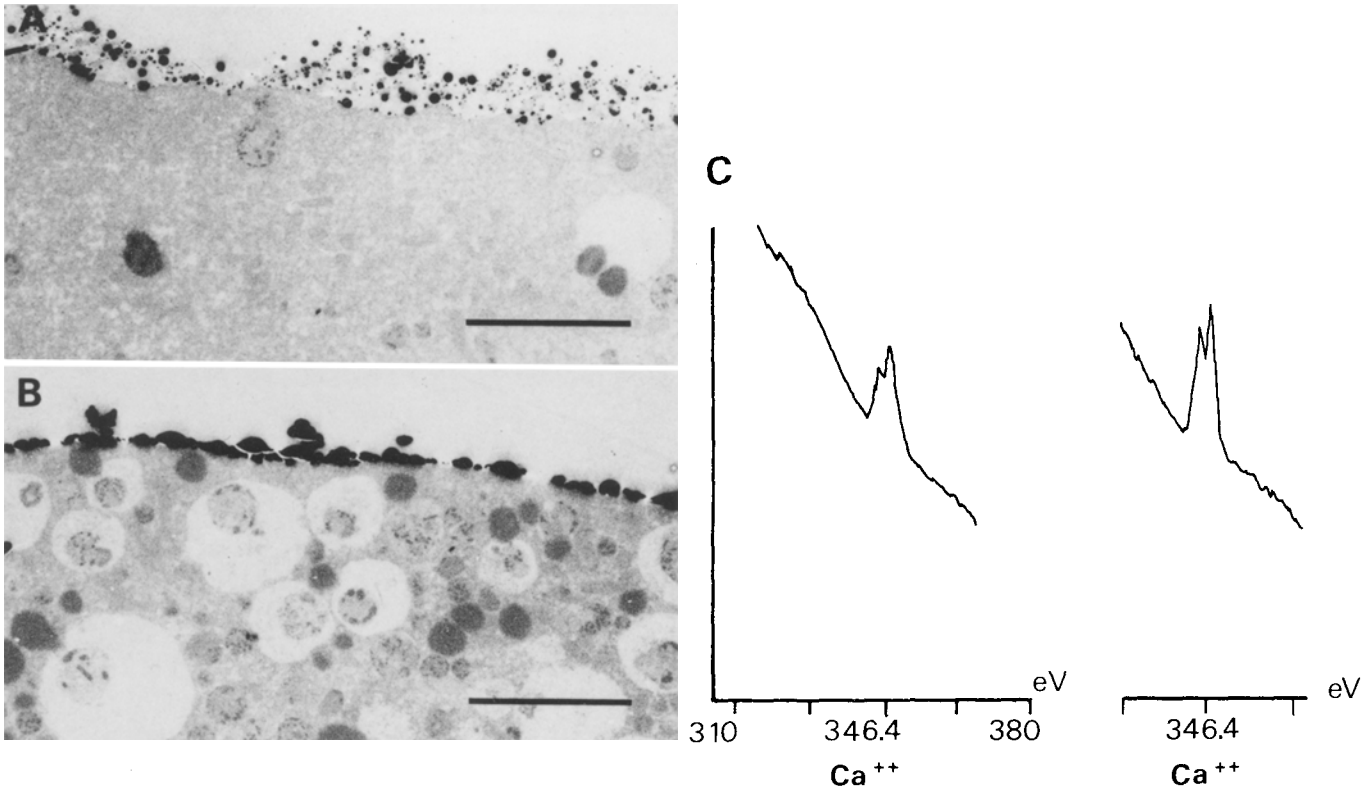


Fig. 6. Calcium-antimony precipitate (using the oxalate/pyroantimonate method) localized at the animal **A**, and vegetal **B** pole of *Lymnaea* eggs. The precipitate is associated with the egg surface. **C** Measurements of precipitate at the animal (*left*) and vegetal (*right*) poles using electron energy-loss spectrometry, showing the presence of calcium. *Bars* represent 1 μm

pole (Fig. 6A). Laterally a fine, less dense deposit was found predominantly.

As a control, sections were treated with EGTA, which dissolved the electron-dense deposits. Incubation in distilled water did not affect the deposits (data not shown). These data support the view that endogenous calcium reserves exist in the eggs of *Lymnaea*.

EELS measurements of precipitates from different regions showed the presence of calcium as a peak at 346 eV at both the animal and vegetal poles (Fig. 6C). This precipitated calcium must have originated from *Lymnaea* eggs, since no other source of calcium in the OPA method is available. Besides calcium, we detected antimony (used in the precipitation technique) at 527 eV. Magnesium could not be detected in the precipitates.

Discussion

We have previously demonstrated the existence of a polar pattern of ionic currents in the egg of *Lymnaea* (Dohmen et al. 1986; Zivkovic and Dohmen 1989). In addition, we have mapped polar extracellular currents around the eggs of *Bithynia* (Zivkovic 1990). Since calcium appears to be an important carrier or regulator of these ionic currents in *Lymnaea* (Zivkovic and Dohmen 1989), we started an investigation on the nature and the distribution of ATPases that might act as calcium pumps. In this paper we demonstrate a polar distribution of membrane-bound, calcium and/or magnesium-activated ATPase in the eggs of *Lymnaea* and *Bithynia*.

We used a cytochemical approach that enabled us to localize the calcium- and/or magnesium-dependent ATPase activity at the ultrastructural level. By varying the experimental conditions, we obtained more detailed information on the factors that stimulate or inhibit enzyme activity and on its substrate specificity, which allowed a more precise characterization of the ATPases. Unfortunately there is much confusion on the terminology of calcium-activated ATPases. In this paper we follow the nomenclature used by Dhalla and Zhao (1988). The classical Ca^{2+} pump of the plasma membrane is called the Ca^{2+}-stimulated ATPase, whereas all other enzyme entities that are activated by calcium and/or magnesium are designated Ca^{2+}/Mg^{2+} ATPase. There is also much uncertainty about the molecular identity of the various functional activities observed in biochemical or cytochemical assays, e.g. whether calcium-dependent and magnesium-dependent enzyme activity should be attributed to two different enzymes or to one single enzyme. Functionally, the vanadate-sensitive high-affinity (K_m for Ca^{2+} <0.5 μM) Ca^{2+}-stimulated ATPase has been characterized as a calcium pump that ejects calcium from the cell even when the intracellular concentration of calcium is very low (Carafoli 1988). The function of the vanadate-insensitive, low-affinity (K_m for Ca^{+} >10 μM) Ca^{2+}/Mg^{2+} ATPase is far less clearly defined. Taking into account the uncertainties mentioned above we propose the existence of two enzymatic entities in the eggs of *Lymnaea*: a Ca^{2+}/Mg^{2+} ATPase and a Ca^{2+}-stimulated ATPase.

Ca^{2+}/Mg^{2+} ATPase

The enzymatic activity present along the cytoplasmic face of the plasma membrane and in the egg cortex in a large part of the vegetal hemisphere conforms to the criteria for a Ca^{2+}/Mg^{2+} ATPase. First, it is activated by micromolar or higher concentrations of either calcium (Table 1, expt. 0) or magnesium (Table 1, expt. 3). Secondly, it has a low affinity for calcium and magnesium, since this activity is no longer observed after EDTA pretreatment followed by incubation in Ca^{2+}- and Mg^{2+}-free medium (Table 1, expt. 12). Thirdly, it is insensitive to vanadate (Table 1, expt. 4).

Ca^{2+}-stimulated ATPase

In a very restricted area of the plasma membrane at the vegetal pole we found an ATPase activity that conforms partly to the criteria for a Ca^{2+}-stimulated ATPase. First, it has a high affinity for calcium since its activity is detected after EDTA pretreatment followed by incubation in Ca^{2+}- and Mg^{2+}-free SM, under which circumstances only micromolar calcium concentration might be expected (Table 1, expt. 12, and Fig. 5B, C). Secondly, it is sensitive to vanadate (Table 1, expt. 14, and Fig. 5E). It differs, however, from the Ca^{2+}-stimulated ATPase because it does not seem to require magnesium for its activity, whereas the Ca^{2+}-stimulated ATPase needs MgATP as a substrate. This enzyme with a high affinity for calcium in *Lymnaea* resembles, with respect to its independence from magnesium, a high-affinity Ca^{2+}/Mg^{2+} ATPase, which has been demonstrated to exist in the plasma membrane of some cell types (see Dhalla and Zhao 1988). However, the latter enzyme appears to be insensitive to vanadate (Kwan and Kostka 1984; Dhalla and Zhao 1988), whereas the high-affinity ATPase in *Lymnaea* is inhibited by vanadate. It is clear that this high-affinity ATPase in *Lymnaea* does not entirely meet the criteria for either Ca^{2+}-stimulated ATPase (Ca^{2+} pump) or Ca^{2+}/Mg^{2+} ATPase. However, it is possible that, in vivo, the enzyme in *Lymnaea* eggs utilizes MgATP as a substrate, and that, as a result of fixation, the enzyme properties such as substrate specificity have been modified in such a way that it can also utilize ATP. This view is supported by the results of substrate-depletion and -substitution experiments (see Table 2), which show that ATP, GTP and UTP can be hydrolysed by the enzyme, provided that magnesium ions are added. Thus it is possible that in vivo the high-affinity ATPase in the *Lymnaea* egg uses MgATP as a substrate, and it would then meet all the criteria for being the Ca^{2+} pump of the plasma membrane.

Significance in early development

The spatial co-occurrence of the calcium-activated ATPases and the ionic currents in the molluscan eggs may indicate that the generation of the ion fluxes is

somehow affected by calcium ions. Movement of calcium ions across the plasma membrane, caused by the pumping activity of the Ca^{2+}-stimulated ATPase, might in itself generate ionic currents. Calcium is also known to influence the plasma membrane's permeability to other ions such as K^+ and perhaps Cl^- (see Hille 1984; Young et al. 1984) and may thus affect transmembrane fluxes of other ions. This view is supported by the fact that calcium-channel inhibitors, when used in low concentrations, only attenuate the ionic current (Zivkovic and Dohmen 1989), which indicates that passive calcium influx constitutes only a small component of the ionic current and that other ion species carry the remaining current.

Unfortunately, we could not inhibit the enzyme activity with vanadate in vivo to see whether this treatment abolishes the ionic currents, since the plasma membrane is impermeable to this compound. This experiment would have provided direct evidence for or against a causal relationship between the ionic currents and the ATPase activity.

The Ca^+/Mg^{2+} ATPase that is detected along the membrane of the whole vegetal hemisphere may, in this region, regulate the activity of calcium channels. It has been suggested that Ca^{2+}/Mg^{2+} ATPase is involved in the gating of calcium channels in heart muscle cells (Dhalla and Zhao 1988). Such regulation might take place by phosphorylation of the membrane proteins that constitute calcium ion channels, in which case the phosphorylation would act as a mechanism required to open the channels (Dhalla and Zhao 1988).

Both the Ca^{2+}-stimulated- and Ca^{2+}/Mg^{2+} ATPases may thus directly or indirectly regulate the calcium flux across the plasma membrane in a localized region of the egg. It is conceivable that such a regulation of calcium fluxes might result in localized changes of free cytosolic calcium concentration and subsequently might affect the organization of the cytoskeleton in that particular region of the egg, thus contributing to its polar differentiation, for example the localized surface differentiations found at the vegetal pole of eggs of several molluscs (Dohmen and van der Mey 1977; Dohmen 1983).

The present report on the spatial restriction and colocalization of ion fluxes and enzymatic activity in molluscan eggs may contribute to our understanding of how a polar ionic current pattern originates in egg cells and what its significance could be in early development.

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