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Original Article

Polar ionic currents around embryos of *Lymnaea stagnalis* during gastrulation and organogenesis

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ABSTRACT Embryos of *Lymnaea stagnalis* generate ionic currents which can be measured with the vibrating probe. Here we investigated the presence and origin of the currents during late embryonic development. During gastrulation the current pattern correlates with the animal-vegetal polarity and during organogenesis it is correlated to the newly formed antero-posterior axis. The origin of the ionic currents was studied by inhibition of the Na^+/K^+-pump with ouabain and by enzyme-cytochemical detection of the Ca^{2+}-pump. Ouabain treatment resulted in a reduced current density around the embryo, indicating that the Na^+/K^+-pump contributes significantly to the net current. The Ca^{2+}-pump was found to be localized in the vegetal blastomeres during gastrulation and in the larval kidney during organogenesis. It seems likely that this Ca^{2+}-pump renders only a minor contribution to the net current in late embryonic development. Ionic currents have now been described in *Lymnaea* from the uncleaved egg up to the juvenile snail. During this period the overall current pattern changes only twice, demonstrating that the voltage gradient generated by the embryo remains stable during prolonged periods in development.

KEY WORDS: *molluscan development, gastrulation, voltage gradients, ionic current, calcium pump*

Introduction

Ionic currents are known to be generated by a large variety of developing and regenerating organisms (Nuccitelli, 1990). In most instances, the function of these currents remains obscure, but there are a few cases in which ionic currents are clearly associated with processes that contribute to the establishment of polarity in biological systems. For example in the brown alga, Fucus, an inward current predicts the axis of polarity of the egg (Nuccitelli, 1978). This current is partially carried by Ca^{2+}. It contributes to the formation of an intracellular concentration gradient of Ca^{2+} (Brownlee and Wood, 1986) that appears to be required for the polarization of the egg (Robinson and Cone, 1980; Speksnijder *et al.*, 1989). Besides generating intracellular concentration gradients, ionic currents also generate intracellular and intraembryonic voltage gradients. These voltage gradients may have various effects that are relevant for morphogenesis. For example, in insect follicles it may act as the driving force behind electrophoresis of charged molecules from nurse cells to the oocyte (Woodruff and Telfer, 1980). Voltage gradients may also provide positional information to migrating cells or outgrowing axons, which are known to be highly sensitive to weak voltage gradients, as can be inferred from their galvanotropic behavior in imposed electric fields (see Robinson, 1985). Recently, an intriguing experiment has been reported, involving the manipulation of the endogenous voltage gradient in chick embryos by implantation of conductive tubes (Hotary and Robinson, 1992). The embryos with a conductive tube displayed a defective tail development, suggesting that the voltage gradient plays a role in morphogenesis of the chick embryo.

In the molluscs, ionic currents are prominently present from the meiotic divisions of the egg onwards (Zivkovic and Dohmen, 1989, 1991). They conform to the general pattern of currents as found in other organisms in that they correlate with the polarity of the embryo. During early cleavage stages of the embryo an oscillation of the current has been observed that correlates with the cell cycles (Zivkovic *et al.*, 1991a). At the 24-cell stage, when an important rearrangement of cells takes place that results in the induction of the mesodermal stem cell, the direction of the current pattern is reversed, suggesting that ionic currents may be involved in this induction process (Zivkovic *et al.*, 1991b). In this paper, we show that the currents are associated with the animal-vegetal polarity during gastrulation and with the antero-posterior axis during organogenesis. The role of ion-pumps in generating these currents was investigated by inhibition of the Na^+/K^+-pump and by enzyme-cytochemical detection of the Ca^{2+}-pump.

Abbreviations used in this paper: CFTW, copper free tap water; ATP, adenosine 5'-triphosphate

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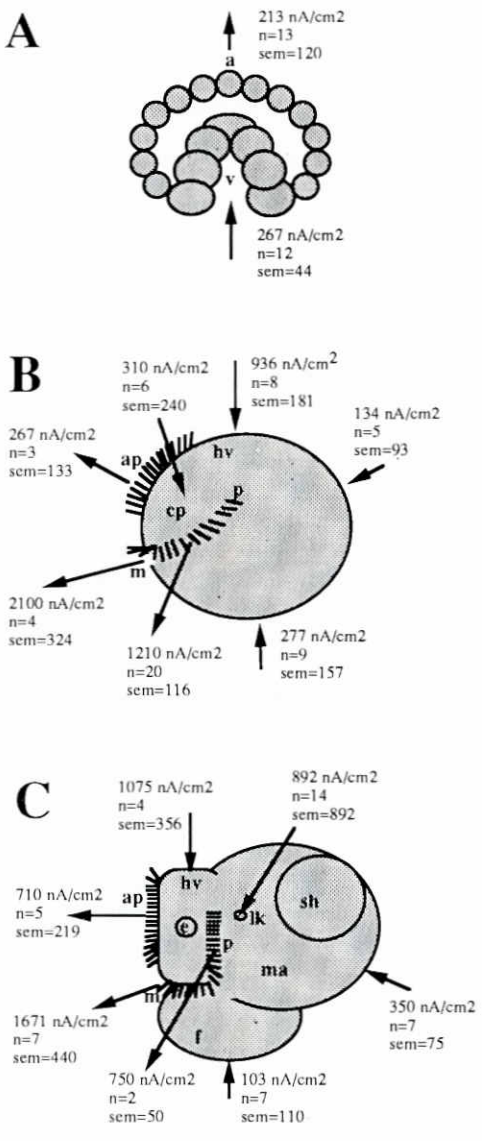

Fig. 1. Polar ionic currents generated by *Lymnaea* embryos in CFTW+$CaCl_2$. (A) *Current pattern around the gastrula.* **(B)** *Current pattern around the trochophore larva.* **(C)** *Current pattern around the veliger. a, animal pole; v, vegetal pole; ap, apical plate; hv, head vesicle; p, prototroch; cp, cephalic plate; m, mouth; ma, mantle; f, foot; e, eye; sh, shell; lk, larval kidney; n, number of embryos; sem, standard error of the mean.*

Results

Ionic currents during late embryonic development

Ionic currents were measured during gastrulation and organogenesis. Current densities ranged from approximately 1 μA/cm^2 inward to 2 μA/cm^2 outward. A polar current was measured around the mid-gastrula stage (28-32 h after first cleavage). The current was outward at the animal pole and inward at the vegetal pole (Fig. 1A). The current density decreases gradually when approaching the equator of the embryo. When measuring all around the equator of one embryo, the current density varied strongly and could even change its direction. Possibly, these local differences in current pattern reflect the dorso-ventral polarity of the embryo during gastrulation. However, evidence supporting this idea is difficult to obtain, because the dorso-ventral axis during the gastrula stage can not be observed *in vivo*. During gastrulation, the vegetal region invaginates towards the interior of the embryo and thereby forms the endomesoderm. When gastrulation is completed, the embryo develops into a trochophore larva (for embryonic stages see Verdonk, 1965; Meshcheryakov, 1990). The trochophore contains ciliated cleavage-arrested cells (apical plate and prototroch), unciliated cleavage-arrested cells (head vesicle) and regions with a high mitotic activity (mantle and cephalic plates). The current pattern around trochophores was measured between 48 and 55 h after first cleavage. The mouth and the ciliated regions showed an outward current. An inward current was measured over the remaining surface of the embryo, with the maximum current density at the cleavage-arrested cells of the head vesicle (Fig. 1B). The trochophore larva develops into a veliger by the formation of several differentiated structures such as a beating heart, a shell, a foot, a larval kidney and two eyes. Veligers, 4 days after first cleavage, generated a current pattern quite similar to that of the trochophore larva. An outward current was measured at the mouth and the ciliated cells (prototroch and apical plate) and an inward current was measured over the remaining surface of the embryo. The maximum inward current was found at the cleavage-arrested head vesicle cells and the larval kidney (Fig. 1C). In both the trochophores and the veligers the outward current was found only in head structures (apical plate, mouth and prototroch), demonstrating a correlation of the current pattern with the antero-posterior axis. After describing the current pattern during late embryonic development, we studied the origin of the ionic currents. This was done by measurement of the transepithelial potential, by inhibition of the Na^+/K^+-pump and by studying the distribution pattern of the Ca^{2+}-stimulated ATPase.

The transepithelial potential

The electrical gradient between the external medium and the lumen inside the embryo was measured with micro electrodes (Fig. 2). For these experiments the embryos were cultured in an artificial medium (medium C), since the embryos cultured in CFTW$^+CaCl_2$ poorly survive small epithelial wounds caused by impalement with a micro electrode. The current pattern in this medium was re-examined with the vibrating probe (Fig. 2A) and was shown to be quite similar to the current pattern in CFTW$^+CaCl_2$ (Fig. 1). Measurements with the micro electrodes revealed that the inside of the embryo is positively charged as compared to the surrounding medium (Fig. 2B). This positive transepithelial potential combined with low concentrations of salts in the culture medium suggests that active pumping mechanisms are involved in the uptake of positive ions by the embryo. The contribution of the Na^+/K^+-pump to the current pattern and the distribution of the Ca^{2+}-pump were further investigated.

The Na^+/K^+-pump

Ionic currents generated by 4-day-old embryos were examined after inhibition of the Na^+/K^+-pump with ouabain. In the ouabain-containing medium, the current density decreased until 30 min after addition of ouabain (Fig. 3). Previous experiments with this concentration (0.1 mM) of ouabain showed that ouabain-treated *Lymnaea* embryos fail to form cleavage cavities, suggesting that the Na^+/K^+-pump plays an important role in osmoregulation of the embryo

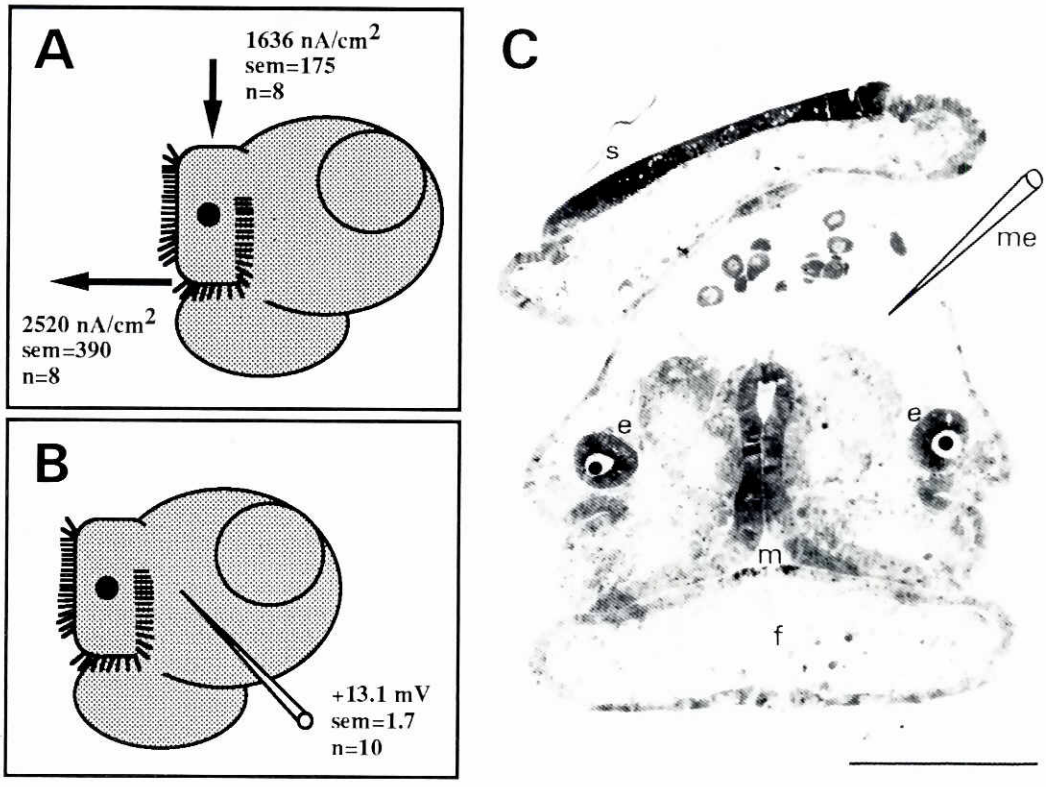

Fig. 2. Measurement of the transepithelial potential in 4-day-old veligers in medium C. (A) *Current pattern in medium C.* **(B)** *The transepithelial potential.* **(C)** *Frontal section through a veliger indicating the position of the micro electrode after impalement through the epithelial layer. n, number of embryos; sem, standard error of the mean; s, shell; e, eye; m, mouth; f, foot; me, microelectrode. Scale bar, 100 μm.*

(Zivkovic, 1990). The current density 30 min after addition of ouabain, was compared to the current density just before the treatment (Fig. 3). The current pattern was not altered by the ouabain treatment and remained outward at the mouth and inward at the head vesicle. However, the current densities at the mouth and the head vesicle were considerably reduced by the ouabain treatment (41%, 67% resp.), suggesting that the Na^+/K^+-pump contributes, either directly or indirectly, to the polar currents around the embryo.

The Ca^{2+}-pump

Ca^{2+}/Mg^{2+}-ATPase activity can be detected using Ando's enzyme cytochemical method for electron microscopy (Ando *et al.*, 1981). In early embryonic stages of *Lymnaea*, localized ATPase activity was detected and characterized by 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 (Zivkovic *et al.*, 1990). Two types of ATPases were detected. The first is a Ca^{2+}/Mg^{2+}-ATPase and the second is a Ca^{2+}-stimulated ATPase, which represents the Ca^{2+}-pump of the plasma membrane. In this study, we used a low-calcium medium in which only the Ca^{2+}-pump is active due to its high affinity calcium sites. In gastrulas, the pump was found along the intracellular face of the plasma membranes facing the archenteron (Fig. 4A,B). In the trochophore larva no reaction product was detected. In veligers, the Ca^{2+}-pump was present in the larval kidney (Fig. 4C,D). Some dispersed reaction product was found in the larval liver cells. The localization of this reaction product was not associated with the plasma-membrane.

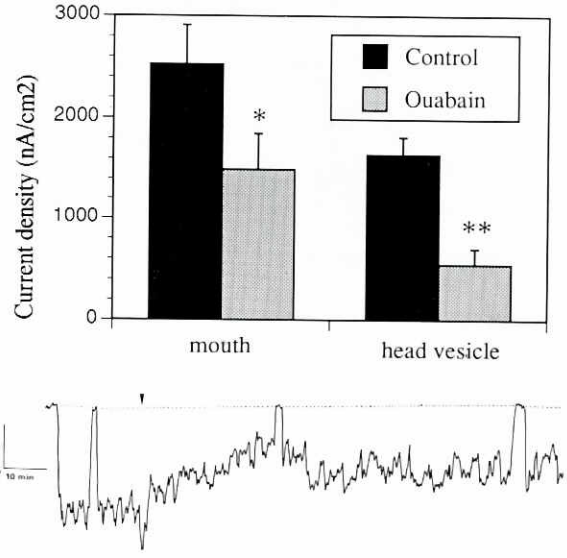

Fig. 3. Inhibition of the Na^+/K^+-pump. *Ionic currents were measured around 4-day-old embryos in medium C, 30 min after addition of ouabain to a final concentration of 0.1 mM. Differences in current densities between the control and treated embryos were indicated by an asterisk when being significant at a 90% confidence limit and by a double asterisk when being significant at a 95% confidence limit (Student's t test). An individual measurement shows how the current through the mouth decreases in time, after addition of ouabain (arrow head). The stippled line represents the reference position of the vibrating probe, in which the currents are considered to be zero.*

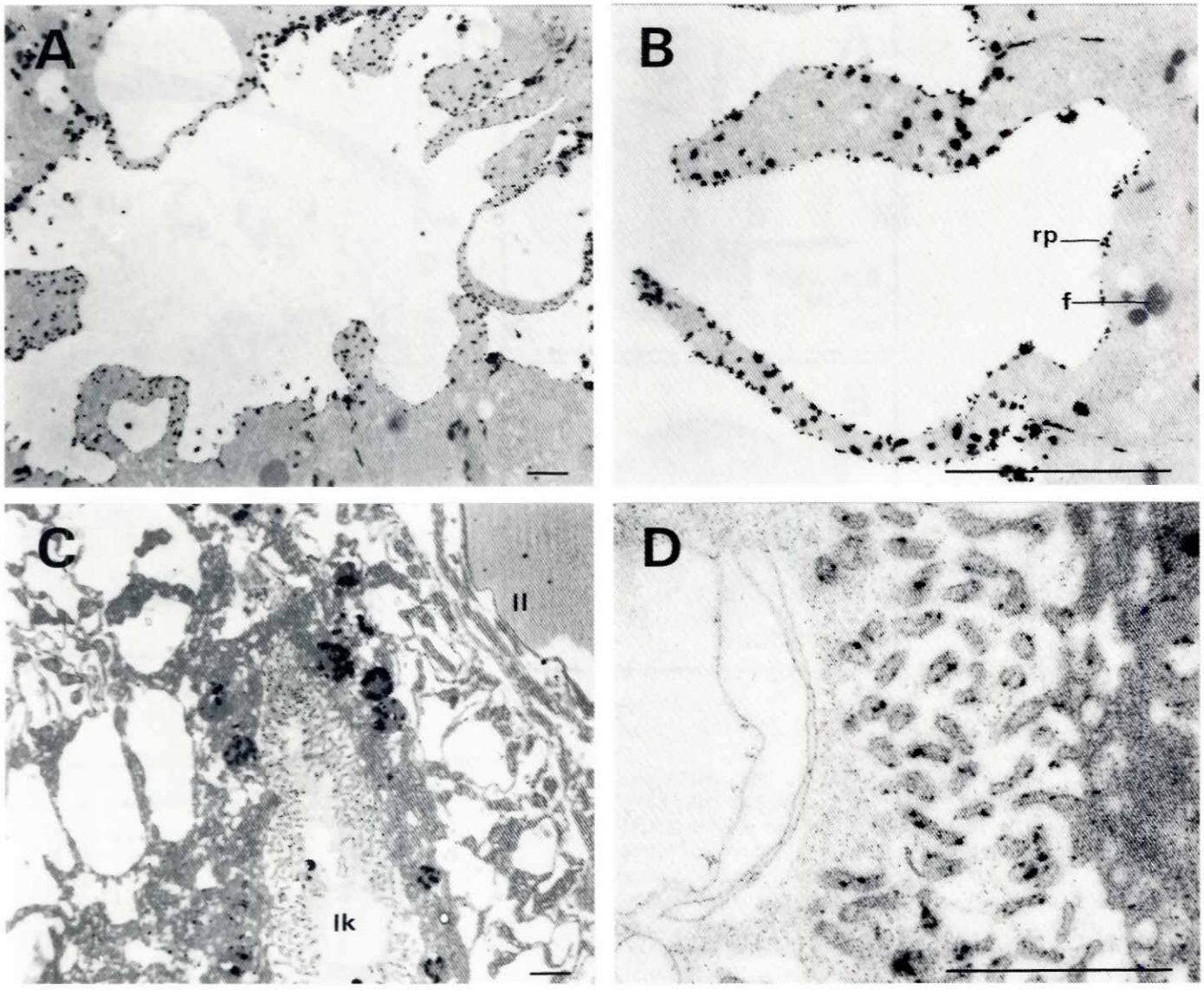

Fig. 4. Localization of the Ca^{2+}-stimulated ATPase. (A) *In gastrulas the reaction product is present along the cell membranes facing the newly formed archenteron.* **(B)** *At higher magnification the reaction product (rp) can be distinguished from ferritin (f) by the presence of a crystal pattern in the latter.* **(C)** *In veligers, the reaction product is present in the larval kidney (lk) and some dispersed reaction product is present in the larval liver (ll).* **(D)** *The reaction product is localized along the plasma membrane of ciliated cells facing the lumen of the larval kidney. Scale bar, 10 μm.*

Discussion

The present results complete a description of the current pattern and the localization of the Ca^{2+}-pump from the uncleaved egg up to the juvenile *Lymnaea* snail (Fig. 5). In early development, the current pattern is correlated with the animal-vegetal polarity (Fig. 5A). It fluctuates during cell cycles (Fig. 5B) and reverses direction prior to mesoderm induction (Fig. 5C). In the present paper we show that an inward current is associated with the involution of the vegetal region during gastrulation (Fig. 5D). Cellular movements during gastrulation result in the formation of an antero-posterior axis. The blastopore through which the vegetal cells involute moves towards the apical plate. The blastopore develops into the mouth and the apical plate becomes the most anterior region of the embryo. With the formation of the mouth from the blastopore the current direction reverses from inward (blastopore) to outward (mouth). This current reversal is associated with the termination of cellular movements. The correlation of ionic currents with involuting cells during gastrulation has been described previously in chick, mouse and *Xenopus* (reviewed by Nuccitelli, 1990). In contrast to *Lymnaea*, each of these vertebrates showed an outward current associated with the involuting cells. This difference in current direction may be due to the cellular configuration during gastrulation. In the vertebrates mentioned above, the migrating cells constitute a thin layer between the blastocoel and the culture medium. This is not the case in *Lymnaea*; the large vegetal cells that invaginate during gastrulation constitute

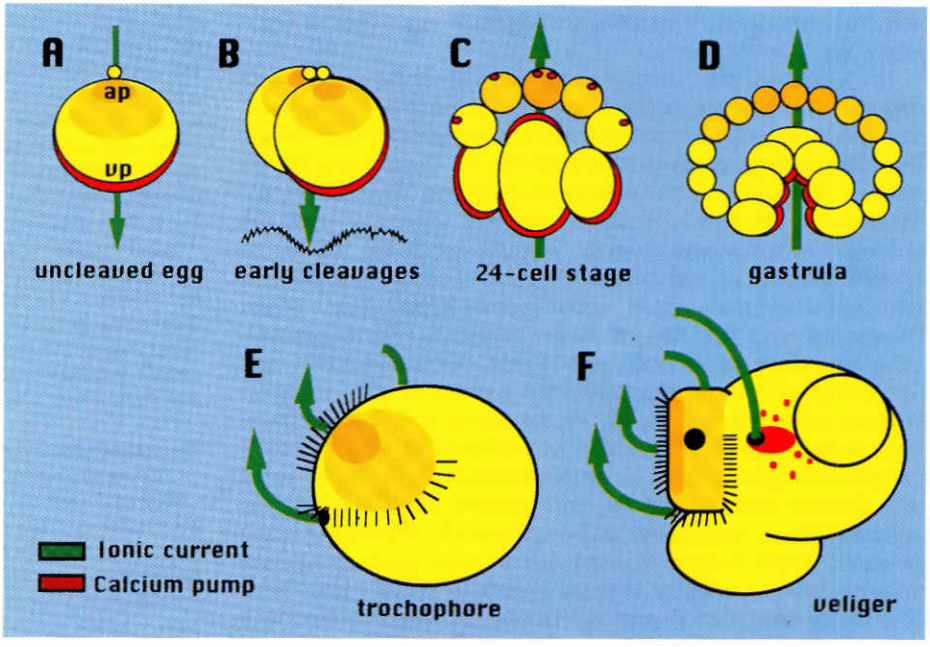

Fig. 5. Summary of currents and localization of the Ca^{2+} pump during development of the *Lymnaea* embryo. (A) *Both the current pattern and the localization of the Ca^{2+}-pump are associated with the animal-vegetal polarity in the uncleaved egg (Zivkovic and Dohmen, 1989; Zivkovic et al., 1990; Créton et al., 1992).* **(B)** *The current oscillates during early cell cycles (Zivkovic et al., 1991a).* **(C)** *During mesoderm induction, the current direction reverses and the Ca^{2+}-pump is active at the tip of the mesentoblast (Zivkovic et al., 1991b).* **(D)** *An inward current and an activated Ca^{2+}-pump remain present at the vegetal pole as the cells invaginate during gastrulation.* **(E)** *The current leaves the embryo through the mouth and ciliated areas in the head and returns through more posterior regions.* **(F)** *Again, the current leaves the head and returns through more posterior regions. The inward current at the larval kidney is associated with a localized activity of the Ca^{2+}-pump. The fate of the animal pole (ap) and the vegetal pole (vp) can be followed throughout development.*

a thick layer between the blastocoel and the culture medium. The current leaves the gastrula at the apical plate cells, which start to form cilia.

In both trochophores and veligers, the outward current remains associated with ciliated cells (Fig. 5E,F). This asymmetrical pattern of currents provides the embryo with a voltage gradient along its antero-posterior axis. The voltage gradient correlates with the direction of cell migration. In *Lymnaea*, the cephalic plate cells $1b^{12121}$ and $1b^{12122}$ move to more lateral-posterior regions. These mid-cephalic plate cells are originally surrounded by structures that generate high density outward currents, such as the mouth, the prototroch and apical plate. The cephalic plate cells move aside and thereby reach cephalic plate regions which generate inward currents. This movement of cells along with the currents in the culture medium is consistent with the direction of cell migration in imposed electrical fields (Robinson, 1985), suggesting that the voltage gradient around the *Lymnaea* trochophore larva might act as the driving force behind the migration of the mid cephalic plate cells. Whether or not the voltage gradients play a role in polarization of cells may be studied in the ciliated cells of *Lymnaea*, which are highly polarized according to the proximal-distal axis of the embryo. It is known that cell-substratum and cell-cell interactions play a decisive role in establishing cell polarity in epithelial layers (Rodriguez-Boulan and Nelson, 1989). The possibility of an additional role for electrical gradients in this process has not been investigated in epithelial cells as far as we know, but it does not seem unlikely that electrical gradients are involved in the process of polarization because epithelial cells are subjected to a considerable electrical gradient. In *Lymnaea* embryos, this electrical gradient is approximately 13 mV over the cell diameter (see Fig. 2), which is much larger than applied electrical gradients used to affect various cell types (Robinson, 1985).

How do the embryos generate their currents? *Lymnaea* embryos exhibit a transepithelial potential, the inside of the embryo being positive. This transepithelial potential, together with the low concentrations of ions in the culture medium, suggests that the embryo utilizes active pumping mechanisms to take up positive ions into its hemolymph, because these ions must be transported against the electrical gradient as well as the concentration gradient (see also Schlichter, 1981; De With *et al.*, 1989). The Na^+/K^+-pump was shown to be involved in generating the current pattern since inhibition of this pump with ouabain resulted in a reduced current density. These results, as well as the presence of Na^+ currents in multicellular stages (Taylor, 1977; Zivkovic, 1990) and the importance of the cleavage cavity in formation of the current pattern (Zivkovic *et al.*, 1991a,b), suggest that Na^+ plays a crucial role in the formation of a polar current pattern in late embryonic development. Most likely, Na^+ is pumped into intercellular spaces resulting in an excess Na^+ as well as a positive charge in the lumen of the embryo. This electrochemical gradient will result in an outward Na^+ current through a path of low resistance.

These mechanisms of current generation differ considerably from those utilized by the uncleaved egg of *Lymnaea*. A role for Ca^{2+} in generating the polar current pattern during meiotic divisions was indicated by the results of ion-substitution experiments using the vibrating probe (Zivkovic, 1990). The location of inward current depends upon the site where cytokinesis occurs (Créton *et al.*, 1992), whereas the outward current at the vegetal pole is associated with the presence of the Ca^{2+}-pump (Zivkovic *et al.*, 1990). Thus, the current pattern of the uncleaved egg seems to be generated by a combined activation of ion channels and ion pumps, in which Ca^{2+} plays an important role, either as the ion which carries the current or as an activator of ionic currents. In the present study, it was shown that the location of the inward current at the vegetal pole during gastrulation and at the site where the larval kidney is present in the veliger, is associated with the presence of Ca^{2+}-pumps. Although it is tempting to attribute to these Ca^{2+}-pumps the function of a battery generating the current pattern around the embryo, some evidence argues against this idea. In gastrulas, the pumps are located in the cell membranes that make contact with the archenteron (Fig. 5D). If Ca^{2+} were pumped through these membranes, it would cause an outward current instead of an inward

one. Possibly, the Ca^{2+} currents are negligible as compared to the larger Na^+ currents.

In conclusion, ionic currents in *Lymnaea* are generated through different mechanisms during embryonic development. Two major changes in current direction are observed during development from the uncleaved egg to the juvenile snail (Fig. 5). The first is a current reversal associated with mesoderm induction (Zivkovic *et al.*, 1991b). The second is a current reversal associated with the formation of the mouth (outward current) out of the blastopore (inward current). As the current changes direction only twice, it remains stable for prolonged periods in development. These stable voltage gradients may act back on the embryo by affecting voltage-sensitive processes. In *Lymnaea* embryos, both cell migration and cell polarization are aligned with the pattern of ionic currents. Whether or not these currents are the driving force behind these processes remains to be shown by manipulation of the current pattern around the embryo. Since the inhibition of the Na^+/K^+-pump with ouabain seems to affect more general processes such as osmoregulation, more subtle approaches will be explored in future research. Some interesting examples of such approaches have been described previously. In frogs, it was shown that implantation of a battery could stimulate regeneration of an amputated limb (Borgens *et al.*, 1977). Furthermore, applied currents are clinically used in the healing of fractured bones. These currents seem to mimic the fracture currents which have a similar current density (Borgens, 1984). A clear example, in which ionic currents are manipulated in embryonic development, is the conductive tube implanted in chick embryos (Hotary and Robinson, 1992). These implants alter the endogenous current pattern and subsequently affect tail development. Whether or not these effects on development and regeneration correspond to the voltage-sensitive processes in cell culture (Robinson, 1985) is largely unknown. In this respect, the molluscan system may provide new insights, because the invariant cleavage pattern and early differentiation allows one to study the effects of voltage gradients on individual cells in the developing embryo.

Materials and Methods

Embryos

The fresh-water snail *Lymnaea stagnalis* is a gastropod mollusc from the subclass Pulmonata. Egg-laying was induced by a fresh-water stimulus (Ter Maat *et al.*, 1983). The embryos were reared inside their capsules at 25°C in copper-free tap water (CFTW) until the desired stage of development was reached. The embryos were then decapsulated in CFTW supplemented with 2 mM $CaCl_2$ ($CFTW^+CaCl_2$). The CFTW contains approximately 0.7 mM Na^+, 0.04 mM K^+, 0.8 mM Ca^{2+} and 0.1 mM Mg^{2+}, pH 8. For experimental manipulations we used an artificial medium (medium C; 10 mM NaCl, 0.5 mM KCl, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ in distilled water, pH 6).

Measurement of extracellular currents

Ionic currents were measured using the extracellular vibrating probe (Jaffe and Nuccitelli, 1974) as described previously (Zivkovic and Dohmen, 1989). We used the one-dimensional vibrating probe system equipped with wire electrodes (Scheffey, 1988). The electrodes had platinum black tips of 35 μm and vibrated with an amplitude of 35 μm. The minimum recording time at one position was 15 sec with a 3 sec time constant of the lock-in amplifier. The probes were calibrated by measuring a known current, which was passed through a glass micro electrode filled with 3M KCl, whose tip was considered a source of current point. The measurements were performed either in $CFTW^+CaCl_2$, with a specific resistivity of 1400 Ωcm or in medium C, with a specific resistivity of 500 Ωcm. The current density around multicellular embryos clearly exceeded the detection limit of about 20 nA/cm^2. Embryos were measured in plastic petri dishes (Costar, Cambridge, MA, USA). After the embryos had attached to the bottom of the dish, the probe was positioned with the center of vibration 50 μm away from the surface of the embryo. The plane of vibration was always kept perpendicular to the egg surface by rotating the plastic dishes. Between two measurements the probe was moved to a reference position 300 μm away from the embryo at which the current density was considered to be a zero reference. The current direction depicted by arrows or indicated as inward or outward refers to the flow of positive charge. Ionic currents were measured around embryos during gastrulation and organogenesis. The contribution of the Na^+/K^+-pump was determined by adding ouabain (Sigma) from a 10 mM stock solution to a final concentration of 0.1 mM in medium C. The specific resistance of this ouabain-containing medium is 500 Ωcm. The values of current densities around different embryos were used to calculate the mean and standard error of the mean.

The transepithelial potential

For measurement of the transepithelial potential, 4-day-old embryos were transferred into medium C. Electrodes were made on a Mecanex BB-CH micro-electrode puller using thin-walled capillaries with an internal filament (GC 150TF-10, Clark electromedical instruments). The electrodes, filled with 0.1 mM KCl, had resistances of about 22 MΩ. A micro-electrode connected to a WPI amplifier (Intra 767) was pushed through the epithelial layer into the lumen of the embryo. The transepithelial potentials were displayed on an oscilloscope and chart recorder. Recordings that fluctuated more than 10% within a 3 min period were excluded from calculation of the mean.

Enzyme cytochemistry

Ando's cytochemical method (Ando *et al.*, 1981) was slightly modified to detect the Ca^{2+}-stimulated ATPase at the ultrastructural level as described previously (Zivkovic *et al.*, 1990). Embryos were fixed in a paraformaldehyde (4%)-glutaraldehyde (0.5%) mixture in 0.1 M sodium cacodylate buffer (CB), pH 7.2 at 4°C. After rinsing in CB, they were treated for 45 min with 10 mM EDTA in CB to deplete Ca^{2+} and Mg^{2+} reserves inside the embryo. After extensive rinsing in CB, the embryos were incubated in Ando's cytochemical medium without Ca^{2+}. This medium consists of 2 mM lead citrate in a 250 mM glycine/50 mM KOH buffer, 8 mM levamisole (Sigma) and 3 mM ATP (disodium salt, Pharmacia) at a final pH of 9.0. When a Ca^{2+}-stimulated ATPase is active, the ATP is degraded into ADP and phosphate, which then precipitates the lead. The embryos were post-fixed in 1% OsO_4 in CB, rinsed in distilled water, oriented in 2% agar in distilled water, dehydrated through a graded series of ethanol and embedded in Epon via propylene oxide. Ultra thin sections were cut on a Reichert ultramicrotome. Unstained sections were examined in a Zeiss EM-10 electron microscope.

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