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*Original Article*

# Regulation of $Na^+$, $K^+$ ATPase activity during meiotic maturation of *Pleurodeles waltl* oocytes. Role of calcium

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**ABSTRACT** **Changes in activity of the $Na^+$,$K^+$ ATPase of maturing *Pleurodeles waltl* were followed by measuring the resting potential in presence or absence of the specific inhibitor dihydroouabain. Corresponding currents were measured in voltage clamp conditions to eliminate the differences in resting potential at the origin and at the end of the meiotic maturation process. Our data confirm previous results obtained on *Xenopus*, indicating that the $Na^+$,$K^+$ pump activity disappears from the plasma membrane during progesterone-induced maturation and can be reactivated by an increase in internal $Ca^{2+}$ triggered by ionomycin. Moreover we show by ultrastructural histochemistry that these modulations are likely to depend on the internalization and reinsertion of the transporter into the plasma membrane.**

KEY WORDS: *$Na^+$,$K^+$ ATPase, calcium, meiosis, amphibian, Pleurodeles waltl*

## Introduction

Regulation of ion fluxes across the plasma membrane is an integral part of the programs implicated in developmental or physiological processes (Nuccitelli, 1988). Modulation of ion channels or transporters by mitogens, hormones, growth factors or determination inducers leads to changes in intracellular ion concentrations, which might be a signal leading ultimately to cell growth and differentiation (Geering, 1986).

$Na^+$,$K^+$ ATPase is one of the transport systems subject to developmental regulation in a variety of models. Interesting examples of developmental modulation of $Na^+$ transport mediated by the $Na^+$,$K^+$ ATPase are found in *Xenopus laevis* oocytes.

Full-grown amphibian oocytes remain arrested in the first prophase stage of meiosis. Stimulation by progesterone initiates the re-entry into the cell cycle until a new arrest occurs in second meiotic metaphase ($M_2$). By this time, the properties of the plasma membrane have changed dramatically (for review see Moreau *et al.*, 1984). In particular, the plasma membrane of amphibian oocytes depolarizes during maturation, a phenomenon that reflects variations in intracellular ionic concentrations and permeabilities. In urodelan oocytes, changes in intracellular $Na^+$ and $K^+$ activity have been reported (*Pleurodeles*: Rodeau and Vilain, 1987; *Ambystoma*: Barish and Baud, 1984; Baud and Barish, 1984).

In anurans such as *Rana* or *Xenopus*, it has been found that depolarization is accompanied by a decrease in $Na^+$,$K^+$ ATPase (or $Na^+$,$K^+$ pump) activity (*Xenopus*: Richter *et al.*, 1984; *Rana:* Weinstein *et al.*, 1982). Furthermore, Vitto and Wallace (1976) have reported that the inhibition of the $Na^+$,$K^+$ ATPase in *Rana* oocytes by ouabain facilitates the progesterone-induced maturation, suggesting a role in this process.

When maturation is completed in the second meiotic metaphase, all $Na^+$,$K^+$ ATPases reside in the cytoplasm (Schmalzing *et al.*, 1990), suggesting that the decrease of $Na^+$,$K^+$ ATPase activity is mediated by endocytosis of the functional pump.

To test this hypothesis, we have followed the evolution of the activity in oocytes from immature to progesterone-matured stage and we have localized the functional protein by cytochemical staining at the electron-microscope level.

$Na^+$,$K^+$ ATPase has been involved during certain steps of *Xenopus* embryogenesis. Han *et al.* (1991) have proposed that the up-regulation of the $Na^+$,$K^+$ ATPase during early development could be carried out by post-translational regulation.

In the *Xenopus* oocyte, Schmalzing and Kröner (1990) have suggested that an increase in intracellular calcium might be a prerequisite for the recruitment and insertion in the plasma membrane of the intracellular $Na^+$,$K^+$ pump. It was therefore important to examine the hypothesis of the regulation of the $Na^+$,$K^+$ ATPase activity by intracellular calcium.

Using electrophysiological techniques we demonstrate here the disappearance of the $Na^+$,$K^+$ ATPase activity from the plasma membrane of the *Pleurodeles* oocyte during progesterone-induced meiosis. The results of histochemical staining suggest an

*Abbreviations used in this paper:* DHO, di-hydro-ouabain; DMSO, dimethyl sulfoxide; GV, germinal vesicle; GVBD, germinal vesicle breakdown; $OR_2$, medium for electrophysiological measurements; $OR_2$-$K_0$, potassium-depleted $OR_2$; HP, holding potential; pNPP, para-NitroPhenylPhosphate.

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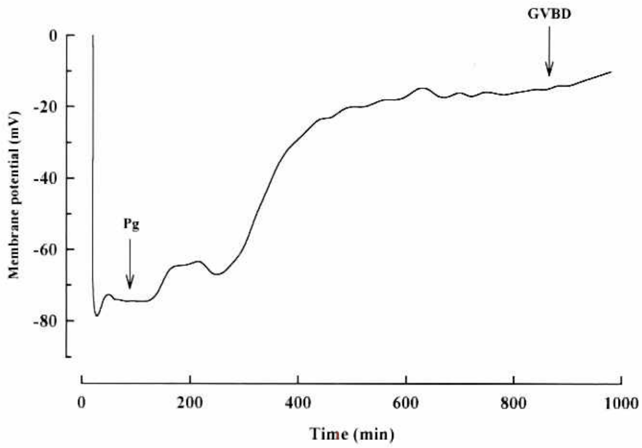


**Fig. 1. Membrane depolarization of *Pleurodeles waltl* oocytes triggered by progesterone-induced meiosis (typical experiment).** *The arrows correspond to progesterone addition (Pg) and germinal vesicle breakdown (GVBD) characterized by a white spot at the animal pole.*

internalization of the $Na^+,K^+$ pump during this process. Reinsertion of this transporter in the plasma membrane can be provoked by increasing internal calcium concentration by ionomycin.

## Results

### *Resumption of meiosis in Pleurodeles waltl*

Resumption of meiosis in *Pleurodeles waltl* can be triggered by mating or by hormonal stimulation using progesterone. In the annual sexual cycle of this amphibian, there exists a period of sexual-arrest of at least three months (June to September) during which, addition of progesterone cannot trigger resumption of meiosis *in vitro*. *In vivo*, naturally, no fertilization can occur. During the remainder of the sexual-cycle, the morphological effect of hormonal stimulation can be easily followed during resumption of meiosis by direct observation of oocytes through a dissecting microscope. In *Pleurodeles*, when 1 mg/ml progesterone was added to the external medium, the first cytological signs of maturation appeared only 5-6 h after treatment, by which the germinal vesicle (GV) had reached the animal pole. Germinal vesicle breakdown (GVBD) occurred only later, some 12 to 14 h following hormone addition at 18°C.

### *Electrophysiological studies*

The effect of hormonal stimulation was followed by electrophysiological measurements. Recorded values for the membrane resting potential of full-grown *Pleurodeles* oocytes enclosed in their follicles ranged from -60 to -85 mV. The mean value was -75.5±7.1 mV (n= 8). These values were not significantly different when oocytes were defolliculated.

When 1 mg/ml progesterone was added to immature oocytes, the membrane depolarized gradually after 15 min to reach a steady state potential ranging between -20 and -4 mV (m= -14.65±5.3, n= 5) before GVBD (Fig. 1). This depolarization may also reflect variations in the activity of the $Na^+,K^+$ pump. Indeed, this enzyme which regulates the intracellular concentrations of $Na^+$ and $K^+$, is an electrogenic carrier. We used this property to follow its activity before and after maturation.

We first examined the effect of the specific inhibitor dihydroouabain on the membrane potential of ovarian oocytes. Specific inhibitors of the $Na^+,K^+$ ATPase are the cardiotonic steroids. In our experiments we used the ouabain derivative dihydroouabain (DHO) or *k*-strophantidin that block $Na^+,K^+$ ATPase in oocytes of *Xenopus laevis* reversibly, in contrast to ouabain (Lafaire and Schwarz, 1986). Under voltage clamp, we verified that DHO had no effect on other membrane channels. Addition of 75 μM DHO to the external medium resulted in a depolarization of 25 mV from the initial -75 mV membrane potential (mean= 25.6±16 mV, n= 5). This depolarization led to a steady state potential within 10 to 20 min, which was near -45 mV in most of our experiments. Complete reversal of the phenomenon could be obtained in 45 min when the inhibitor was washed out (Fig. 2). A similar depolarization was observed when defolliculated oocytes were incubated in potassium-depleted medium ($OR_2$-$K_0$, data not shown). Addition of DHO to defolliculated oocytes was followed by a slightly lower depolarization (mean= 16 mV±5, n =5).

The membrane current was also measured under voltage-clamp conditions. After addition of DHO, we observed an inward reversible current of 20 nA (Fig. 3). This inward current thus represented the current elicited by the ATPase (mean= 22.5±8.1 nA, n =4, holding potential = -70 mV).

Interestingly, no variation of potential could be triggered by DHO in immature oocytes during the sexual-arrest period, although the membrane potential was still near -70 mV. These data demonstrate the presence of an active $Na^+,K^+$ ATPase in the plasma membrane of immature oocytes.

The effect of DHO on the membrane potential was next investigated for mature oocytes. Figure 4 shows a typical effect of DHO on the membrane potential of a mature oocyte 15 h after progesterone treatment. The potential of this oocyte was -10 mV. 75 μM DHO did not induce any significant depolarization. This result suggests that the $Na^+,K^+$ ATPase was no longer active. However, the $Na^+,K^+$ ATPase may in fact function at a level below our detection threshold, due to variations of permeabilities during maturation and modifications in electrochemical gradients (Rodeau and Vilain, 1987). The electrochemical gradients, particularly of

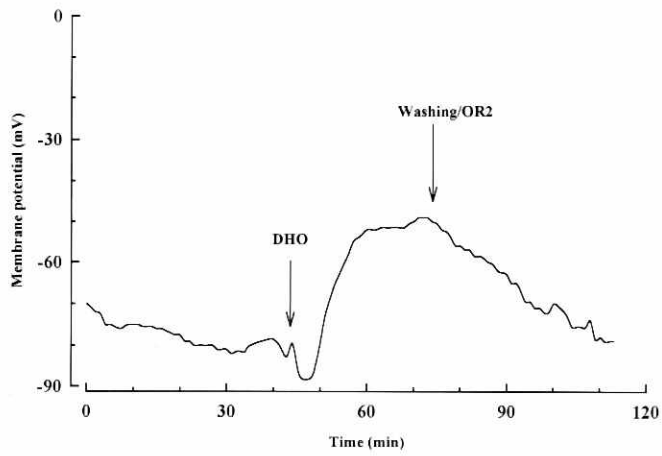


**Fig. 2. Evidence for the presence of $Na^+,K^+$ ATPase activity in the plasma membrane of an immature oocyte of *Pleurodeles waltl*.** *We can observe a reversible depolarization of the membrane potential by 75 mM DHO in $OR_2$ medium.*

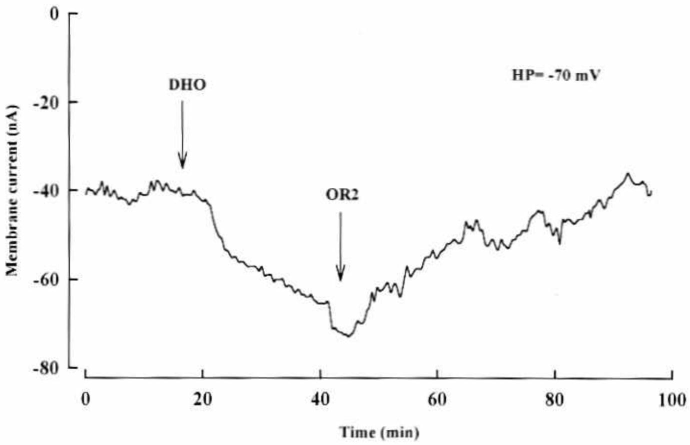


**Fig. 3. Inhibition of $Na^+$,$K^+$ ATPase triggers an inward current.** *Current pump recorded in voltage clamp after inhibition of $Na^+$,$K^+$ pump activity by DHO. Such inward currents were reversed when DHO was washed out (HP= -70 mV).*

$Na^+$, are less important at depolarized potentials than at more negative potentials.

To investigate the role of membrane potential on the activity of the pump, we documented in immature oocytes the properties of the current pump elicited by the $Na^+$,$K^+$ ATPase by voltage-clamp experiments, from the hyperpolarized potentials of immature oocytes to the depolarized potentials of mature oocytes. Under voltage-clamp conditions, the variation of current triggered by application of DHO represents a direct measure of pump activity. We observed that the pump current decreased when the potential was changed to depolarized values: when the immature oocyte was clamped to -70 mV, addition of 75 μM DHO induced an inward current of 20 nA; a steady state was reached in 10 to 20 min (Fig. 3). In contrast, when the holding potential was clamped to -20 mV, the inward current induced by addition of DHO was 2 nA. This indicates that the $Na^+$,$K^+$ ATPase activity depends on the membrane potential in immature oocytes.

In order to avoid this limiting effect of potential during investigation of $Na^+$,$K^+$ ATPase activity in mature oocytes, these cells were then polarized to near the potential of immature ones under current-clamp conditions. When mature oocytes were polarized to near -70 mV, the addition of 75 μM DHO induced no significant depolarization (Fig. 4). Nor did we observe any inward current. These results clearly demonstrate the absence of activity of the $Na^+$,$K^+$ ATPase and raise the question as to whether the $Na^+$,$K^+$ pump is absent from the membrane of mature oocytes or is inactivated.

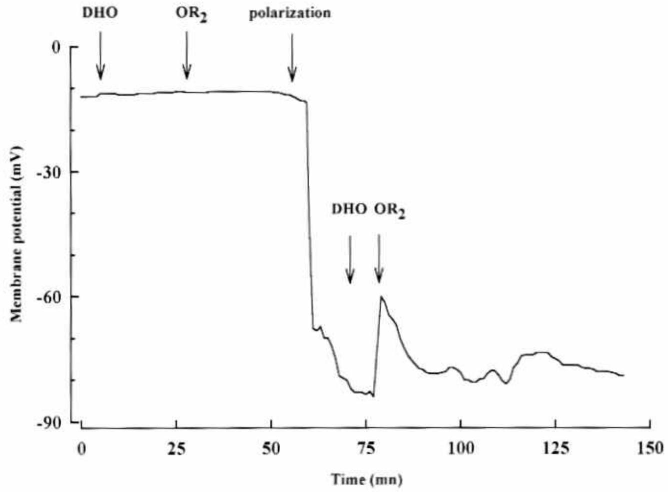


**Fig. 4. Absence of $Na^+$,$K^+$ ATPase activity in the plasma membrane of a mature oocyte.** *75 mM DHO did not depolarize the membrane of the matured oocyte. The arrow indicates the polarization (-80 mV) obtained by injection of a constant current. Under these conditions, no $Na^+$,$K^+$ ATPase activity was detected.*

### *Regulation of $Na^+$,$K^+$ ATPase activity*

Activation of oocytes by calcium ionophores or by fertilization results in a rise in cytosolic $Ca^{2+}$ (for review see Shen, 1992) followed by changes in the plasma membrane, such as expansion of microvilli. In anuran oocytes, it also triggers fusion of cortical granules (Busa and Nuccitelli, 1985). Since $Ca^{2+}$ has been shown to be involved in vesicle fusion (for review see Schuel, 1985), it can be suggested that internal calcium may control the insertion of the $Na^+$,$K^+$ pump proteins into the plasma membrane.

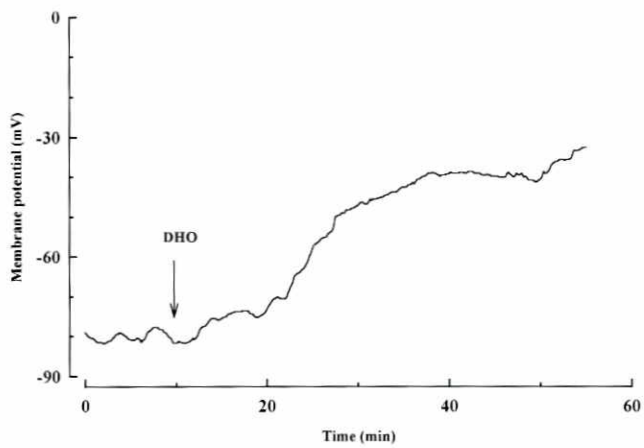


**Fig. 5. Effect on mature oocytes of 10 min treatment with 5 mM ionomycin.** *Depolarization of the membrane potential of these oocytes polarized to -80 mV by 75 mM DHO in $OR_2$ medium can be observed (reversibility has been tested on other oocytes).*

To test this hypothesis, we studied the effect of an increase in internal free calcium on matured oocytes. Oocytes, after 15 h of progesterone stimulation, were incubated for 10 to 30 min in 5 μM ionomycin. Ionomycin triggered a depolarization of the plasma membrane from -12 mV towards -10 to -2 mV. In some cases, the membrane potential became positive. The presence of the $Na^+$,$K^+$ ATPase activity in these oocytes was then examined.

On ionomycin-treated oocytes, 75 μM DHO did not induce a significant depolarization (mean= 1.16±0.68 mV, n= 6). This indicates that the $Na^+$,$K^+$ ATPase was not activated or was activated to a level that was not sufficient to be detected. When ionomycin-treated oocytes were hyperpolarized in current-clamp to -80 mV

TABLE 1

**EFFECT OF DHO ON MEMBRANE POTENTIAL AND MEMBRANE CURRENT UNDER VARIOUS CONDITIONS**

| stages | depolarization after DHO | variation of membrane current after DHO (HP= -70 mV) |
|---|---|---|
| GV | 25 mV | 22 nA |
| $M_2$ | 0 | 0 |
| $M_2$ after ionomycin | 31 mV | 25 nA |

GV, germinal vesicle stage; $M_2$, metaphase 2 stage.

(Fig. 5), addition of 75 µM DHO induced a depolarization of 42 mV. These results are similar to those obtained with the same dose of DHO on immature oocytes (mean= 31.54±4.64, n= 8).

Recording of the current pump under voltage-clamp conditions showed, after addition of DHO, an inward current of 25 nA, which was similar to that recorded in immature oocytes. These data indicate that increasing the internal calcium concentration reactivates the $Na^+$,$K^+$ ATPase. Our electrophysiological measurements are summarized in Table 1.

To precise our electrophysiological measurements, we performed experiments to localize the functional $Na^+$,$K^+$ ATPase by histochemical staining at the electron-microscope level.

### *Histochemical localization*

In immature oocytes, an extensive labeling of the plasma membrane was observed (Fig. 6A). However, the distribution of the lead precipitate was not homogeneous: it was observed in clusters (0.57 to 0.35 µm). This observation thus complemented our electrophysiological experiments, and further demonstrated the presence of the functional $Na^+$,$K^+$ pump protein in the plasma membrane of immature oocytes.

In mature oocytes, 15 h after hormone stimulation, when GVBD had occurred, no staining could be observed (Fig. 6B). This demonstrated that the functional form of the $Na^+$,$K^+$ ATPase was absent from the plasma membrane of the mature oocyte.

Schmalzing *et al.* (1990) have shown that all the ATPase proteins are located in the cytoplasm when maturation is completed. As many changes occur in the plasma membrane during maturation (Kado *et al.*, 1981), we examined the hypothesis that ATPase could be internalized during resumption of meiosis by a process of endocytosis of membrane patches. To allow the penetration of reagents, mature oocytes were permeabilized 30 min by Triton 100X. Figure 6C shows part of the cortex of a mature oocyte permeabilized before histochemical reaction. Cytochemical labeling is observed at the internal aspect of small vesicles located in the cortex of the oocyte. These vesicles have a loose, irregular shape and they could not be mistaken for vitelline vesicles, which are darker. These observations suggest that during maturation the ATPase was removed from the plasma membrane of the mature oocytes by internalization.

An interesting explanation to our experiments with ionomycin, demonstrating a reactivation of the $Na^+$,$K^+$ pump activity could be the hypothesis that the $Na^+$,$K^+$ pump becomes reinserted into the plasma membrane by a vesicle-fusion mechanism. To test this hypothesis, we localized the $Na^+$,$K^+$ ATPase activity by cytochemical staining. Mature oocytes 15 h after progesterone treatment, were treated for 30 min with 5 µM ionomycin before cytochemical staining. Figure 6D shows part of such treated oocyte. Lead precipitate was essentially located in the plasma membrane, as in the case of immature oocytes (Fig. 6A). Some internal stores of $Na^+$,$K^+$ ATPase were also visualized and figures of vesicle fusion were observed. The labeling was heterogeneous, showing clusters (0.72 to 0.48 µm) of active sites. These observations provide evidence that a rise of intracellular calcium results in the insertion of $Na^+$,$K^+$ ATPases in the plasma membrane by vesicle fusion.

## Discussion

The main objective of this study was to follow the regulation of $Na^+$,$K^+$ ATPase activity during meiosis resumption in *Pleurodeles waltl* oocytes. We have also studied the role of calcium in the regulation of the activity of this enzyme.

By means of electrophysiological measurements, we observed that the transition of prophase-to-metaphase triggers the arrest in the activity of the $Na^+$,$K^+$ pump. In immature oocytes, the activity could be detected, whereas it had disappeared when maturation was completed. This down-regulation had been previously observed in other amphibian oocytes. In mature *Xenopus laevis* oocytes, Schmalzing *et al.* (1990) reported that $Na^+$,$K^+$ pump proteins were accessible to ouabain when oocytes were permeabilized by digitonin or SDS. They suggested that the down-regulation of the ATPase activity is mediated by the removal of protein from the plasma membrane through internalization of membrane patches.

By cytochemical staining we have shown here that the $Na^+$,$K^+$ ATPases were functional in the cytoplasm of the mature oocytes and were located in small vesicles in the cortex. These vesicles are not vitelline vesicles, which appear darker and display different shapes. They could be endocytotic vesicles, but further histological analysis will be necessary to determine their exact nature. The selective removal of the pump proteins may be accomplished by endocytosis regulated by *Rab*-proteins. These GTP-binding proteins are thought to be involved in membrane trafficking and protein sorting (for review see Novick and Brennwald, 1993) and they might be used as markers.

Our results further strengthen the idea of an internalization of the $Na^+$,$K^+$ ATPase during maturation. However, cytochemical staining allowed us to detect only functional proteins. Therefore we can not definitely rule out the possibility that a fraction of the $Na^+$,$K^+$ pumps remains in the plasma membrane in an inactive state.

Another interesting finding in our study concerns the recovery of $Na^+$,$K^+$ ATPase activity in mature oocytes after an increase in intracellular free calcium triggered by a calcium ionophore. Schmalzing and Kröner (1990) showed that an increase in intracellular calcium led to an increase of the ouabain-binding sites in permeabilized mature oocytes.

By cytochemical staining, we have shown that a rise in intracellular calcium triggers the reinsertion of the $Na^+$,$K^+$ pump proteins in the plasma membrane of mature oocytes. Moreover, our results suggest that this insertion is probably mediated by vesicle fusion. These reinserted proteins undoubtedly arise from the large pool of ATPases present in the cortex of the oocyte. Thus, we tentatively conclude that intracellular calcium controls $Na^+$,$K^+$ ATPase activity by insertion of the protein in the plasma membrane.

The regulation of the activity of the $Na^+$ pump may play an important role in development since its activity is involved in a

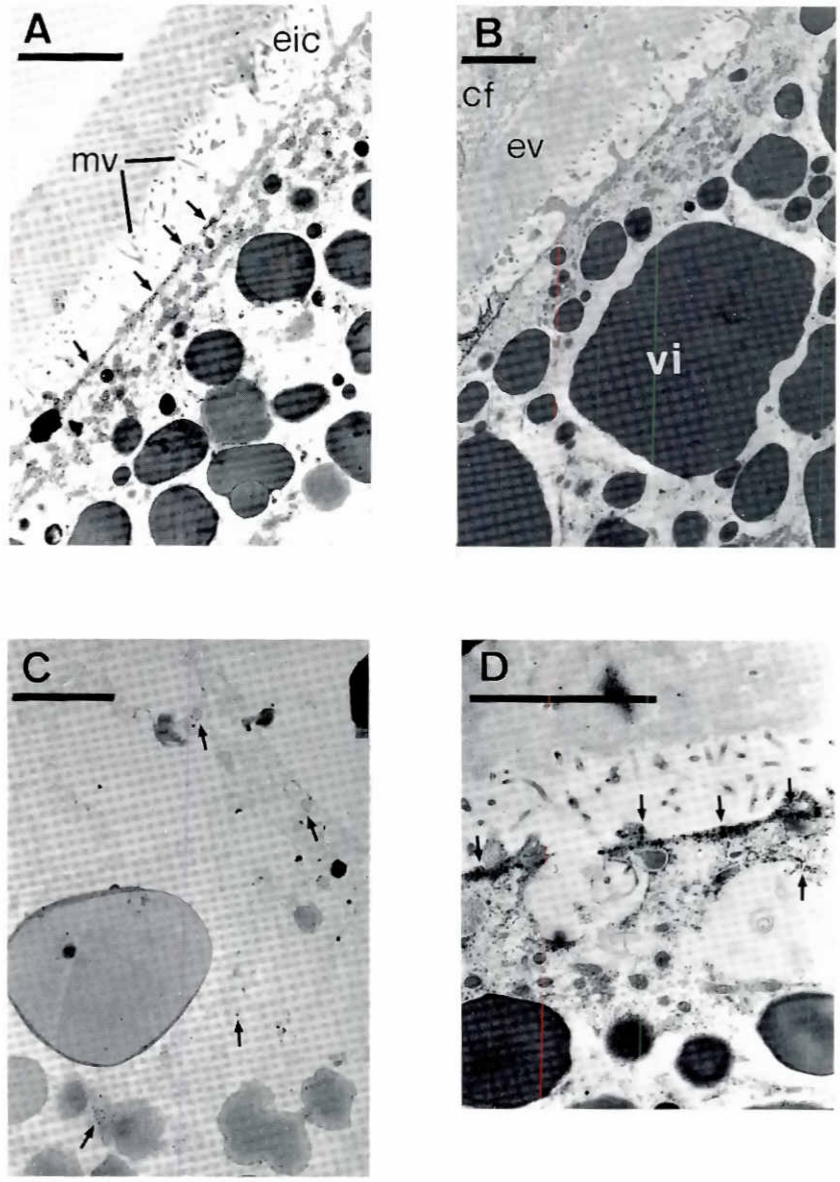


**Fig. 6. Histochemical staining of the active $Na^+,K^+$ pump. (A)** *Immature oocyte. The oocyte is surrounded by follicular cells (cf) which have established contacts with the oocyte by microvilli (mv) through the intercellular space (eic). A heterogeneous staining can be observed in the plasma membrane (arrow).* **(B)** *Mature oocyte. Number of the microvilli is reduced. No staining can be observed in the plasma membrane.* **(C)** *Permeabilized mature oocyte. Some staining (arrow) can be observed in small vesicles located in the cortex of this mature oocyte.* **(D)** *Ionomycin-treated mature oocyte. A heterogeneous staining (arrow) can be observed in the plasma membrane and in vesicles about to fuse. Bar, 2 mm.*

number of developmental processes. Moreover, modulations in the activity of the $Na^+,K^+$ pump are often accompanied by variations of intracellular calcium. The possible role of the down-regulation of $Na^+,K^+$ ATPase activity in maturation has been discussed by Vitto and Wallace (1976). They reported that inhibition of $Na^+,K^+$ pump shortens the timing of progesterone-stimulated maturation of *Xenopus* oocytes. In *Pleurodeles*, our own experiments have never revealed such a phenomenon (Moreau, unpublished results). However, this does not necessarily mean that there is no relationship between the activity of the sodium pump and maturation in *Pleurodeles*. In fact, an interesting observation was made during the arrest season of sexual activity. During this period, GVBD can not be induced by progesterone, and $Na^+,K^+$ ATPase activity can not be inhibited by ouabain when studied by electrophysiological techniques. This could result either from a disappearance of the $Na^+,K^+$ pump or from a functional inhibition. On the other hand, we do not know whether this lack of activity inhibition is a cause or a consequence of the inhibition of meiosis. The modulation of the $Na^+,K^+$ ATPase activity may modify the intracellular ionic concentrations, particularly of sodium. An increase in intracellular sodium activity may be capable of activating a $Na^+/H^+$ antiporter. This activation, accompanied by the disappearance of a $H^+$ current (Baud and Barish, 1984), could be responsible for the alcalinization of the oocyte during maturation. Thus, modulation of $Na^+,K^+$ ATPase activity may be involved in some of the events of progesterone-induced maturation.

During early development of *Xenopus laevis*, it has been suggested that the $Na^+,K^+$ ATPase could be implicated in blastocoel formation (Slack *et al.*, 1973). More recently, this contention was confirmed in a mammalian developmental system (for review see Wiley *et al.*, 1990). Later in amphibian development, the regulation of $Na^+,K^+$ pump activity has been involved in the maintenance of neural plate potentials (Blackshaw and Warner, 1976) and in neuronal differentiation (Messenger and Warner, 1979; Breckenbridge and Warner, 1982). However, Han *et al.* (1991) have shown that the β subunit of the pump, responsible for insertion of the protein into the plasma membrane, is not synthesized before the end of neurulation, which suggests that modulation of $Na^+,K^+$ ATPase activity during early development can entirely be achieved through post-translational regulation. Thus, this modulation could be elicited by variations in intracellular free calcium. During neurulation, an increase in cytosolic calcium could trigger the activation of the $Na^+,K^+$ pump (Breckenbridge and Warner, 1982). Indeed, differential modulation of ion transporters by differentiation inducers leads to changes in ionic concentrations which may be the first signal leading to cell differentiation (Guerrier *et al.*, 1984; Geering, 1986). It has been shown, for example, that calcium influx at early developmental stages regulates neuronal differentiation of *Xenopus* spinal neurons (Holliday and Spitzer, 1990, 1993). Accordingly, we propose that variations of homeostasis necessary to trigger some differentiation programs may be modulated by the insertion of $Na^+,K^+$ ATPase in the plasma membrane, mediated by a calcium-dependent vesicle-fusion from internal pools. This, in turn, implies that $Na^+,K^+$ pump activity may reflect variations of intracellular free calcium.

## Materials and Methods

### *Oocytes and solutions*

Adult female *Pleurodeles waltlii* were anaesthetized by immersion in 1 g/l MS222 (Aldrich Chemical Co, Milwaukee, Wisconsin, USA). Ovarian lobes were surgically removed and placed in modified OR2 medium (Wallace *et al.*, 1973), which has the following composition in mM: NaCl 82.25, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 1, $NaHPO_4$ 0.25, Hepes 1, pH 7.2.

Stage VI oocytes (Dumont, 1972) were dissected manually with watchmakers forceps and left for equilibration overnight at 16°C. When necessary, oocytes were defolliculated by previous incubation of 2 h in 0.8 mg/ml collagenase solution (Calbiochem) under continuous gentle agitation. Meiotic maturation was induced by addition of progesterone (Sigma, St Louis, Missouri, USA) at a final concentration of 10 µg/ml. Progesterone stock solution was 1 g/ml in absolute ethanol. Ionomycin in stock solution 1 mM in DMSO was used for incubation with matured oocytes at a final concentration of 5 µM. The ouabain is the specific inhibitor of the $Na^+,K^+$ ATPase. In our experiments, we used the labile antagonist Di-Hydro-Ouabain (DHO, Lafaire and Schwartz, 1986) in excess concentrations (75 µM).

### *Electrical recording*

Electrical measurements were performed using 2 conventional electrodes filled with 3 M KCl, with resistance ranging from 1 to 4 MΩ. Measurements were displayed on a pen chart recorder. The bath was perfused continuously by a peristaltic pump except when DHO was added.

### *Cytochemical detection*

ATPase activity was assayed using the paranitrophenylphosphate (pNPP) assay modified from the method described by Ernst and Hootman (1981). Oocytes were fixed in 4% paraformaldehyde 0.1 M cacodylate buffer, pH 7.8 for 24 h at 4°C, cut in two and fixed again for 4 h, then washed overnight at 4°C in cacodylate buffer. The oocytes were incubated with the pNPP solution in Tris-HCl, pH 9, at room temperature for 45 min. They were then washed for 5 min in 2% lead solution, followed by 5 min rinse in sucrose solution (100 mM) and 3 washes with buffers (0.1 M Tris-HCl, then 0.1 M cacodylate buffer) for 5 minutes each. Controls were performed by adding 75 µM DHO, or by removing pNPP.

Post-fixation was performed for $1^{1/2}$ h at room temperature in a 4% solution of $OsSO_4$ in 0.1 M cacodylate buffer (1 vol/1 vol). After washing in buffer, the oocytes were dehydrated and embedded in Epon for observation with a Hitachi 100 transmission electron microscope. Counterstaining by lead citrate was not performed. When necessary, mature oocytes were permeabilized for 30 min with 1% Triton 100X before incubation with pNPP solution.

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