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 ion 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 Kroner (1990) have suggested that an increase in intracellular calcium might be a prerequisite for the recruitment and insertion into the plasma membrane of the intracellular Na\(^+\),K\(^+\) pump. It was therefore important to examine the hypothesis of 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\(_d\), medium for electrophysiological measurements; OR\(_p\), potassium-depleted OR\(_d\); HP, holding potential; nNPP, para-NitroPhenylPhosphate.
Fig. 1. Membrane depolarization of *Pleurodeles walti* 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 walti***

Resumption of meiosis in *Pleurodeles walti* 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 (n=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 (OR2-Ko⁺ 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 walti*. We can observe a reversible depolarization of the membrane potential by 75 mM DHO in OR2 medium.](image-url)
Regulation of Na⁺,K⁺ ATPase activity by calcium

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).

Regulation of Na⁺,K⁺ ATPase activity

Activation of oocytes by calcium ionophores or by fertilization results in a rise in cytosolic Ca²⁺ (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 Ca²⁺ 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.

To test this hypothesis, we studied the effect of an increase in internal free calcium on mature 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 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.

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 medium can be observed (reversibility has been tested on other oocytes).
These observations suggest that during maturation the ATPase was removed from the plasma membrane of the mature oocytes by internalization. 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 walti 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 Kroner (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 regulates 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) Lonomycin-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 blastocoe formation (Slack et al., 1973). More recently, this contention was confirmed in a mamalian 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; Brecken-
bridge 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 (Brenkbridge 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 start 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, CaCl2 1, MgCl2 1, NaHPO4 0.25, Heps 1, pH 7.2.

Stage VI oocytes (Dumont, 1972) were dissected manually with watchmaker's forceps and left for equilibration overnight at 16°C. When necessary, oocytes were defollicled by previous incubation of 2 h at 0.6 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, Lataire 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 into 2 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 pH

Post-fixation was performed for 1.15 h at room temperature in a 4% solution of OsSO4 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.

Acknowledgements

We are grateful to Pr. Julian Smith and Dr. Philippe Cochard for correction of the English version, and to Dr. P. Guerrier for helpful discussions. This work was supported by CNRS (Centre National de la Recherche Scientifique), MESR (Ministère de l'Enseignement et de la Recherche), AFM (Association Française contre les Myopathies, n° 4350432) and by CNES (Centre National d'Études Spatiales). A grant to C.S. was provided by MHT (Ministère de la Recherche et de la Technologie, grant n°226UPS91).

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Accepted for publication: January 1995