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A cytochemical study on the role of ATPases during pollen germination in *Agapanthus umbelatus* L'Her.

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Summary. Cytochemical detection of ATPase activity in the pollen grain (PG) and pollen tube (PT) of *Agapanthus umbelatus* showed that the enzymes concerned presented specific patterns of membrane distribution according to their ionic dependencies and to the time-course of germination and tube growth. In the pollen tubes Ca^{2+}-ATPases were mainly localized in mitochondria and ER membranes, while Mg^{2+}-ATPases were found especially in the tonoplast and in the membrane of the P-particles. K^+-ATPases showed a high activity at the plasma membrane. In the pollen grain similar patterns of ATPase activity were observed. The highest activity of all three types was observed at the plasma membrane of the grain and at the intine and inner exine layers of the cell wall. The activity observed in the pollen grain cell wall decreased with germination time. In vivo germination studies in the presence of specific inhibitors of the ATPases showed patterns of inhibition that could be correlated with the corresponding ATPase putative role.

The results are discussed in terms of the ultrastructural organization of the PG and PT, especially those correlated with (1) formation and maintenance of ionic gradients throughout the PT, (2) polarized growth and (3) hydrodynamics of PT elongation.

Key words: Pollen germination – ATPases – Calcium – Magnesium – Potassium – *Agapanthus umbelatus*

Introduction

The oriented growth of PTs has been the subject of many diversified studies, in particular those concerning ultrastructure (Cresti et al. 1985) and the tip-to-base ionic gradient formation (Reiss et al. 1983). Recently, the capacity of electric fields to change the oriented growth pattern of PTs has been correlated with the existence of external ionic currents in the culture medium (Malhó et al. 1991). These ionic currents, simultaneously with the electric field action, probably act at the plasma membrane level to determine a polarity phenomenon which, in turn, could originate intracellular asymmetrical distributions. A few studies have tried to establish the distribution of membrane proteins in pollen tubes, namely those of calcium channels (Bednarska 1989) and acid phosphatases (Lin et al. 1977). In both cases asymmetrical distributions were observed.

Asymmetrical protein distribution should be of great importance, especially where it concerns ATPases, since these enzymes are generally involved in the control of membrane polarization, in the regulation of solute transport into and out of the cytoplasm (Poole 1978) and in numerous other physiological processes (see Sze 1985; Schroeder and Hedrich 1989). More emphasis has been placed on the importance of ATPases in pollen since the acidification of the medium, which occurs during germination, was correlated with the action of H^+-ATPases (Rodríguez-Rosales et al. 1989).

Studies on ATPases activity are, however, made more difficult by the fact that they can use several ions as co-substrates (namely Ca^{2+}, K^+ and Mg^{2+}) and have different subcellular localizations (Sze 1985; Jiao et al. 1988; Fichmann et al. 1989, and references therein). These difficulties are usually overcome by the use of specific subcellular inhibitors (Sze 1985).

In the work reported here the role of ATPases on pollen germination and tube elongation was analyzed by means of cytochemical detection in PTs and PGs of *Agapanthus umbelatus* using Ca^{2+}, K^+ and Mg^{2+} as co-substrates. This detection was complemented with studies using several specific ATPase inhibitors (to tonoplast, plasma membrane and ER/mitochondria), with the action of these drugs being monitored on in vivo pollen germination and tube growth.

Abbreviations: PT, Pollen tube; PG, pollen grain; PTW, pollen-tube wall; PGW, pollen-grain wall; ER, endoplasmic reticulum; NEM, *N*-ethylmaleimide

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Material and methods

Pollen from *Agapanthus umbelatus* L'Her. was collected from dehiscent anthers of flowers from plants collected at the Lisboa Botanical Garden. All reagents were analytical grade.

Fixation and cytochemistry

Pollen was sown in the culture medium described by Brewbaker and Kwack (1963) containing 1% sucrose. Fixation of PG's was performed at 20, 30, 40 and 60 min after sowing.

ATPase activity was detected using a protocol modified from Hulstaert et al. (1983). Germinated pollen was pre-fixed in 1.5% glutaric dialdehyde in 0.05 *M* cacodylate buffer and 1% sucrose, pH 6.9, for 30 min at 4° C, followed by pre-incubation in 1 m*M* $CeCl_3$, 0.05 *M* TRIS-maleate and 1% sucrose, pH 6.9, for 3 h at 4° C. The incubation was carried out in 1 m*M* $CeCl_3$, 3.4 m*M* ATP (sodium salt, Merck, Darmstadt, FRG), 5 m*M* ($CaCl_2$/KCl/$MgCl_2$), 0.05 *M* TRIS-maleate and 1% sucrose, pH 6.9, for 2 h at 37° C with constant shaking.

After the material was rinsed in the same buffer (pH 6.0), it was fixed in 2.5% glutaric dialdehyde in 0.05 *M* cacodylate buffer and 1% sucrose, pH 6.9, for 2 h at 4° C and post-fixed in 1% buffered osmium tetroxide for 2 h at room temperature. The material was then dehydrated by the acetone series and embedded in Epon-Araldite according to Mollenhauer (1964). Unstained ultrathin sections were observed with a JEOL 200CX at 80 KV.

The controls were performed by substrate (ATP) omission or by the addition of specific inhibitors (Sze 1985) to the incubation medium: sodium vanadate (1 m*M*) (inhibitor of plasma membrane ATPases); sodium azide (5 m*M*) (inhibitor of mitochondrial ATPases); *N*-ethylmaleimide (NEM) (0.2 mM) (inhibitor of ATPases from the tonoplast, mitochondria and probably ER). All of the inhibitors were from Sigma, St. Louis, USA.

Inhibitors

To test the ATPases inhibitors (vanadate, azide and NEM) we added them to the culture medium to a final concentration of 0.1 and 1.0 m*M* and made observations with a Leitz-Wetzlar Dialux optical microscope 60 min after sowing. Percentage inhibition was determined in samples of at least 10,000 pollen grains. PT's longer than the PG long axis were considered to be full grown (aproximately 50 μm). All the results shown are significantly different from their controls without inhibitor ($P<0.001$, ANOVA).

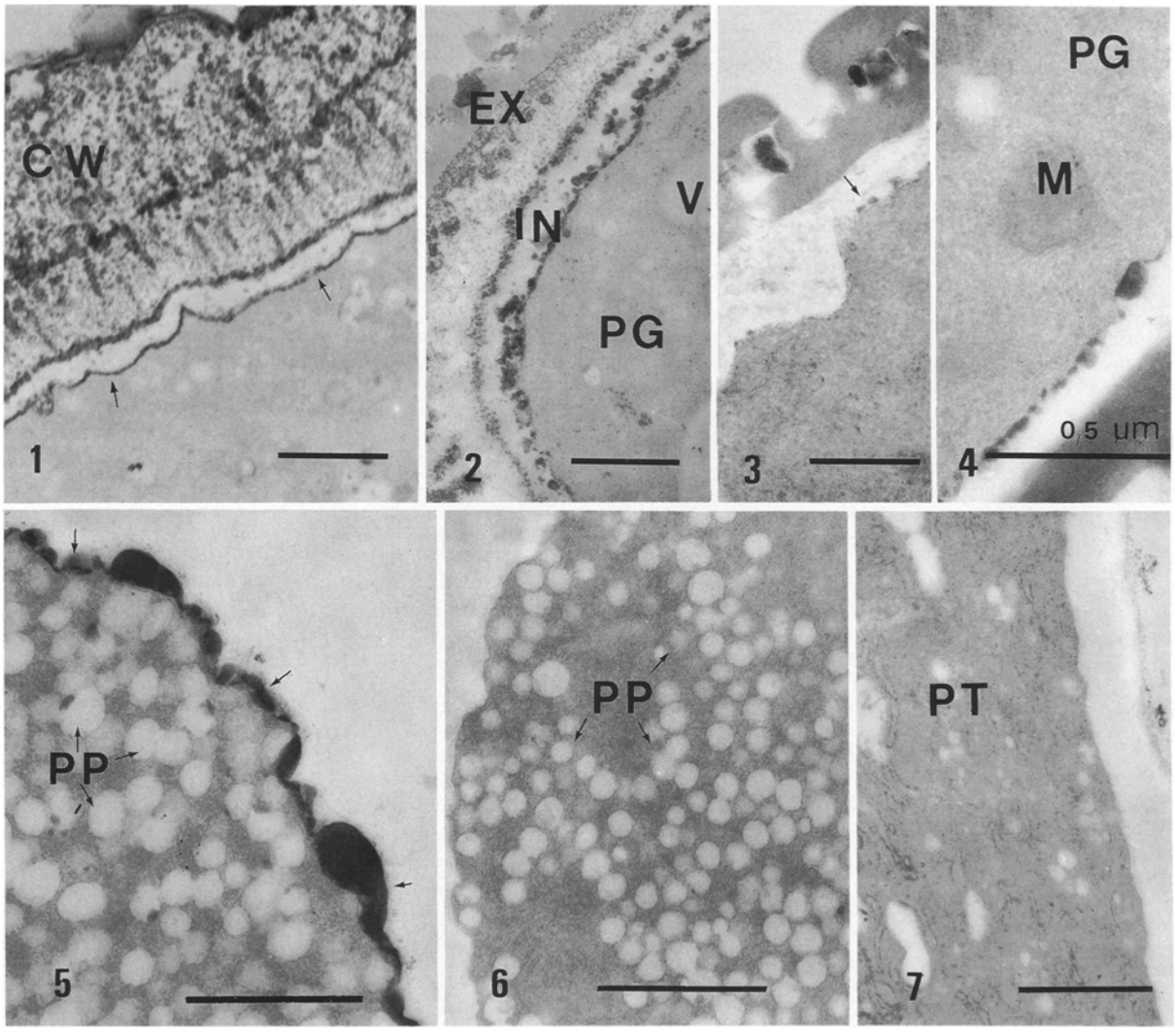
Results

A summary of the cytochemical results is presented in Table 1. In addition to obtaining a reaction on the pollen-grain plasma membrane and cell wall that was qualitatively similar in all tests, different localizations were obtained when the substrate was changed.

Table 1. Results of the cytochemical tests of Ca^{2+}-, Mg^{2+}- and K^+-ATPases (TEST) and their controls with *N*-Ethylmaleimide (NEM), Vanadate (VAN), Azide (AZID) and without substrate (W/SUB). Reaction intensity is described in arbitrary units from absent (−) to very intense (+++)

	Ca^{2+}-ATPases		MG^{2+}-ATPases		K^+-ATPases		
	PG	PT	PG	PT	PG	PT	
Wall	+++	−	++	−	++	−	T
Plasmalemma	+++	−	+++	+	+++	+++	E
Tonoplast	−	−	+++	++	+	+	S
Mitochondria	+++	+++	−	−	−	−	T
ER	+++	+++	+	+	−	−	
GA	+	+	+	+	−	−	
Wall	−	−	−	−	−	−	N
Plasmalemma	+	−	+	−	+	−	E
Tonoplast	−	−	−	−	−	−	M
Mitochondria	−	−	−	−	−	−	
ER	+	+	+	+	−	−	
GA	−	−	−	−	−	−	
Wall	−	−	−	−	−	−	V
Plasmalemma	+	−	+	−	−	−	A
Tonoplast	−	−	+++	++	+	+	N
Mitochondria	+++	+++	−	−	−	−	
ER	+++	+++	+	+	−	−	
GA	+	+	+	+	−	−	
Wall	−	−	−	−	−	−	A
Plasmalemma	++	−	++	+	++	++	Z
Tonoplast	−	−	+++	++	+	+	I
Mitochondria	−	−	−	−	−	−	D
ER	+	+	+	+	−	−	
GA	+	+	+	+	−	−	
Wall	−	−	−	−	−	−	W/
Plasmalemma	+	−	+	−	+	−	S
Tonoplast	−	−	−	−	−	−	U
Mitochondria	−	−	−	−	−	−	B
ER	−	−	−	−	−	−	
GA	−	−	−	−	−	−	

PG, Pollen grain; PT, pollen tube; ER, endoplasmic reticulum; GA, Golgi



Figs. 1–7. Cytochemical detection of K^+-ATPases. *Bar*: 1 µm, unless indicated otherwise. **Figs. 1–4.** Pollen grain (*PG*). **Fig. 1.** Portion of pollen grain near the pore, 20 min after sowing. The grain cell wall (*CW*) and the plasma membrane (*arrows*) give a strong reaction. No reaction product is seen in the cytoplasm. ×15,000. **Fig. 2.** Pollen grain 60 min after sowing. The staining pattern is similar to that in Fig. 1 but the intensity of the reaction is lower. *EX* Exine, *IN* intine, *V*, vacuole. ×15,000. **Fig. 3.** Control with vanadate at the pollen grain, as in Fig. 2. No reaction product is observed in the wall and in the plasma membrane it is very reduced (*arrows*). ×15,000. **Fig. 4.** Control by substrate omission in pollen grain. A reaction much less intense than that found in the test is observed in the plasma membrane (compare with Figs. 1 and 2). *M* Mitochondria. ×50,000. **Figs. 5–7.** Pollen tube (*PT*). **Fig. 5.** Tip region of the pollen tube. P-particles (*PP*) are conspicuous, and an intense reaction at the plasma membrane is visible (*arrows*). ×24,000; **Fig. 6.** Control with vanadate at the pollen-tube tip, as in **Fig. 5.** No reaction product is observed. ×23,000; **Fig. 7.** Control by substrate omission in pollen tube. There is no reaction at the pollen-tube plasma membrane. ×18,000

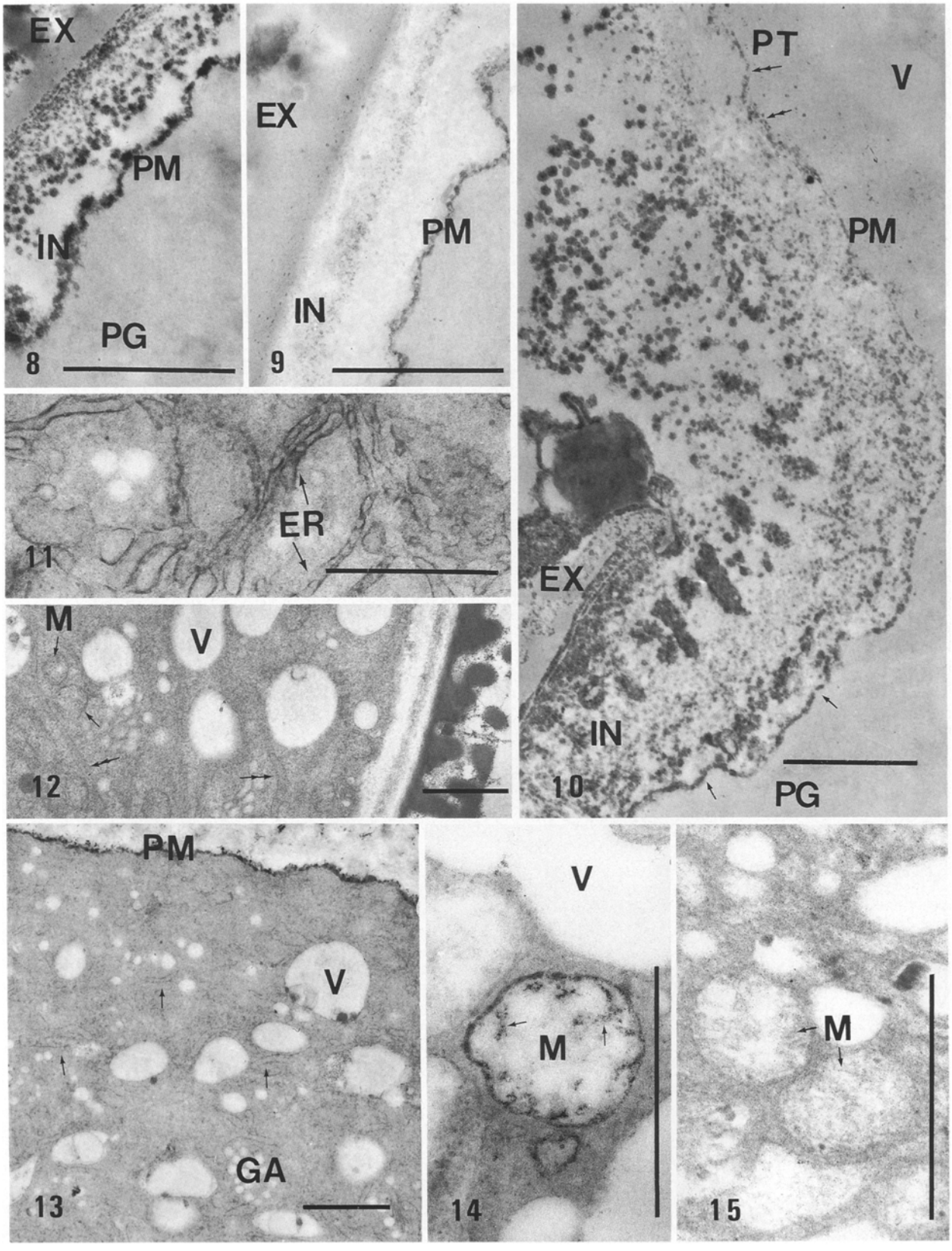
K^+-ATPases

This test revealed an intense reaction on the plasma membrane of both PGs and PTs (Figs. 1, 2 and 5) and a particularly intense one in the tube tip (Fig. 5). In some cases a slight reaction was observed at the tonoplast, but no reaction could be detected in the other organelles (Table 1). In the first 20 min after sowing, the pollen-grain wall (PGW) also showed very dense deposits especially at the intine and inner exine layers (Fig. 1). This reaction decreased in intensity with germination time (Fig. 2, 60 min)

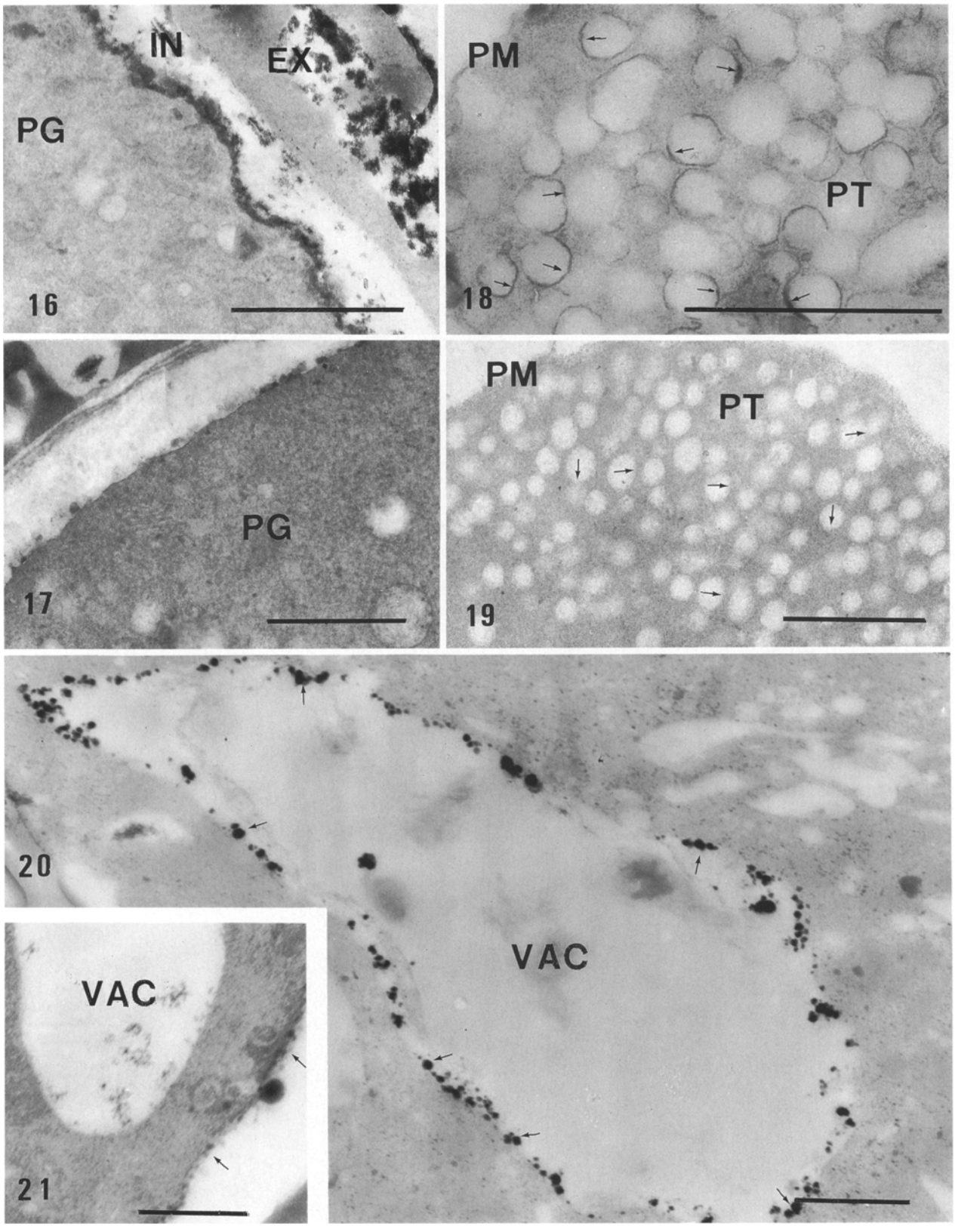
Vanadate completely inhibited the reaction at the plasma membrane of the tube (Fig. 6), but at the plasma membrane of the grain a very weak reaction was still observed (Fig. 3). The control without substrate revealed a similar pattern (Figs. 4 and 7, compare with Figs. 1 and 2). The inhibitory effect of NEM was observed mainly at the level of the PGW (Table 1).

Ca^{2+}-ATPases

In the pollen tube the reaction products were observed to be localized essentially in the endomembrane compartment (ER and some Golgi vesicles) and at the mito-



Figs. 8–15. Cytochemical detection of Ca^{2+}-ATPases. *Bar*: 1 μm. **Figs. 8–12.** Pollen grain (*PG*). **Fig. 8.** Pollen grain 20 min after sowing. Strong reactions are observed at the plasma membrane (*PM*) and intine (*IN*). *Ex* Exine. ×30,000. **Fig. 9.** Pollen grain 60 min after sowing. The reaction at the intine and plasma membrane is much less intense (compare with **Fig. 8**). ×30,000. **Fig. 10.** Pollen grain and pollen tube 30 min after sowing. Note the decrease in the reaction intensity at the plasma membrane of the tube (*double arrow*) when compared with the grain plasma membrane (*arrow*). ×24,000. **Fig. 11.** Pollen grain cytoplasm 40 min after sowing. Reaction is seen at the ER membranes. ×30,000. **Fig. 12.** Control with substrate omission. No reaction product is observed on plasma membrane, mitochondria (*arrows*) or ER (*double arrows*). ×15,000. **Fig. 13.** Control with NEM. Tonoplast and Golgi profiles (*GA*) show no reaction product. ER (*arrows*) and plasma membrane show some reactivity but less intensely than in the test (compare with **Figs. 8 and 11**). ×15,000. **Figs. 14 and 15.** Pollen tube (*PT*). **Fig. 14.** Pollen-tube cytoplasm. Mitochondria show reaction in both cristae (*arrows*) and membranes. ×43,500. **Fig. 15.** Control with sodium azide. No reaction product is observed in the mitochondrial membranes. ×42,500



Figs. 16–21. Cytochemical detection of Mg^{2+}-ATPases. *Bar*: 1 μm. **Figs. 16 and 17.** Pollen grain (*PG*). **Fig. 16.** Pollen grain 30 min after sowing. Intense reaction is seen at the plasma membrane and exine but not at the intine. ×30,000. **Fig. 17.** Control with vanadate as in **Fig. 16.** No reaction product is observed on plasma membrane when compared with the test. ×25,000. **Figs. 18–21.** Pollen tube (*PT*). **Fig. 18.** Tip region of the pollen tube 60 min after sowing. Reaction products are clearly visible at the inner face of the P-particles membrane (*arrows*) (compare to **Fig. 4**). There is no reaction at the plasma membrane. ×45,000. **Fig. 19.** Control with substrate omission in the tip. No reaction product is observed on the "P-particles" (*arrows*). ×25,000. **Fig. 20.** Basal portion of the pollen tube 60 min after sowing. There is a strong reaction (*arrows*) at the tonoplast of a big vacuole (*VAC*). ×20,000. **Fig. 21.** Control with NEM (*N*-ethylmaleimide). Pollen-grain plasma membrane still presents a weak reaction (*arrows*) (similar to **Fig. 4**), but at the tonoplast there is a total absence of reaction. ×20,000

chondrial membranes (Fig. 14). The tonoplast showed no reactivity either in the PG or the PT (Table 1 and Figs. 11 and 14) while at the plasma membrane and cell wall the reaction was observed only in the PG and not in the PT (Fig. 10). At the PGW the reaction was similar to that observed for the K^+-ATPases, a strong reaction that decreased with germination time (Figs. 8 and 9). This reaction was absent in all of the controls performed (Table 1, Figs. 12 and 13).

The control with azide showed total inhibition of the reactivity at the mitochondrial level (Fig. 15), and partial inhibition at the ER and plasma membrane (Table 1). NEM inhibits the reaction at the mitochondria while the ER show a partial inhibition when compared with the test; at the plasma membrane of the pollen grain the results are similar to those obtained for the control of K^+-ATPases (Table 1 and Fig. 13).

The control with substrate omission was negative at the level of the organelle membranes and PGW (Fig. 12, Table 1).

Mg^{2+}-ATPases

The cytochemical detection of Mg^{2+}-ATPases revealed the presence of electron-dense deposits mainly on the tonoplast (Fig. 20), PGW and plasma membrane (Fig. 16). A slight reaction was observed in the endoplasmic reticulum (ER) and dictysomes (Table 1). Membranes of P-particles were also reactive at their inner face (Fig. 18). The reaction at the PGW level was similar to that of the K^+ test, but less intense.

Controls with vanadate (Fig. 17) and NEM (Fig. 21) showed some deposits on the plasma membrane similar to those found for the K^+-ATPases (Table 1). Controls with substrate omission (Fig. 19) and NEM (Fig. 21) both showed a complete absence of reaction in the membrane of the P-particles and in the tonoplast. Azide mainly inhibited activity at the PGW level while leaving activity at the tonoplast, ER and dictyosomes unaffected (Table 1).

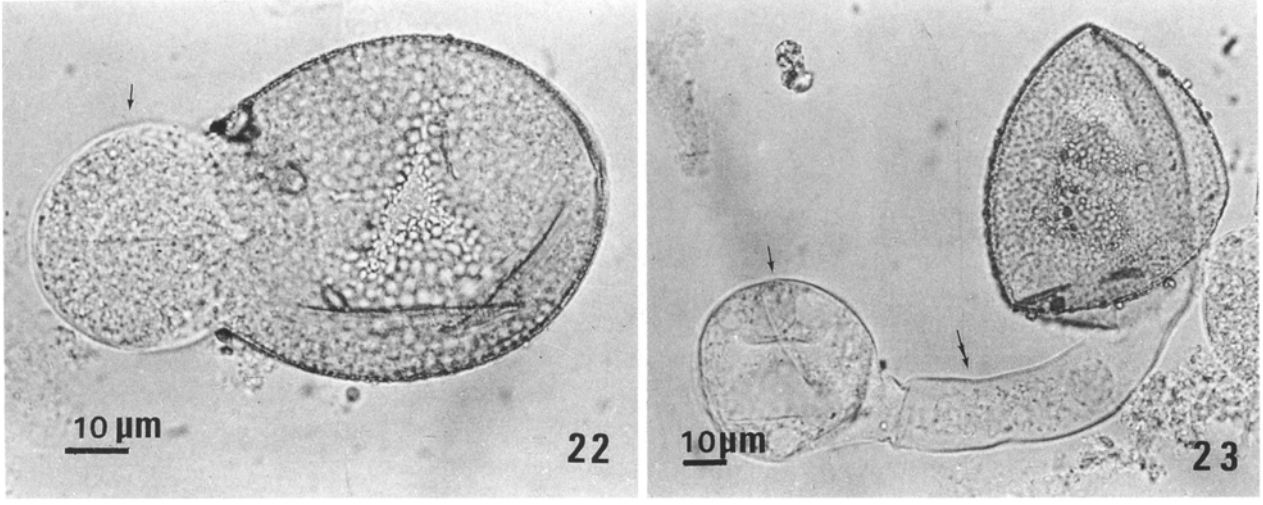
Table 2. Inhibition of pollen germination and pollen-tube growth in the presence of sodium azide (AZ), Sodium vanadate (VAN) and *N*-Ethylmaleimide (NEM). All of the results shown are significantly different from their controls without the drug ($P<0.01$, ANOVA)

Inhibitor (m*M*)		% Inhibition	
		Germination	Tube growth
AZ	0.1	21.53±1.22	100
	1.0	100	100
VAN	0.1	28.20±3.16	85.32±7.51
	1.0	100	100
NEM	0.1	66.71±5.43	95.34±7.69
	1.0	100	100

Inhibitors

Pollen germination and pollen-tube (PT) growth are significantly inhibited by azide (AZ), vanadate (VAN) and NEM (Table 2). PT growth is more inhibited than pollen germination.

While the use of AZ and VAN resulted in growth inhibition, there were no visible changes in PT morphology. NEM induced the formation of a swollen tube tip (Figs. 22 and 23) followed, in most cases, by tip burst. NEM was also the most effective inhibitor of pollen germination. AZ was the most effective inhibitor of pollen-tube elongation.



Figs. 22 and 23. Pollen germination in the presence of NEM. In both cases NEM induced swelling of the tip (*arrows*). The tube is also swollen (*double arrow*). Fig. 22: ×800, Fig. 23: ×600

Discussion

Our cytochemical results clearly show that ATPases have distinct and well-characterized space, time and functional patterns during the germination process of the pollen grains. Not only were strictly polarized distributions detected (e.g. the K^+-ATPases in the tube tip, or Mg^{2+}-ATPases in the tonoplast of the basal part of the tube), but general alterations in activities were also detected during the time-course of pollen germination and tube growth (e.g. the decrease of activity in the pollen-grain membrane and cell wall in all tests).

The inhibition of the plasma membrane, tonoplast, mitochondria and ER ATPases by their specific inhibitors in the different controls confirms the true ATPase nature of the activities detected. Other phosphatase activities could hardly explain the specificity of different ionic dependencies, and, simultaneously, the inhibition of a given organelle ATPase by a specific inhibitor.

According to Katz et al. (1988) the use of lead (acting as a chelating agent) enables the localization of phosphatases and not of ATPases. The use of cerium chloride seems, however, to be much more specific for different phosphatase activities, as shown by Hulstaert et al. (1983). On the other hand, Katz et al. (1988) defined two major conditions for a correct cytochemical detection of ATPases activity: (1) the localization of electron-dense deposits at the inner face of the membrane and (2) inhibition by specific inhibitors. The last was completely verified. As to the first, the only exception was the reaction appearing at the level of the PGW, which could be due to the presence of non-specific phosphatases, enzymes already detected at this level (Mascarenhas 1975). This assumption is, somehow, confirmed by the complete inhibition of PGW reactivity with azide, a known inhibitor of acid phosphatase (Gallagher and Leonard 1982). A slight reaction was also observed at the plasma membrane in all of the other controls. Again this activity may be due to the presence of non-specific phosphatases, as already described by Lin et al. (1977). The difference in reaction between controls and test does, however, account for a major ATPase activity at this level, especially in the case of K^+-ATPases in the pollen-tube tip.

With respect to subcellular localization our results agree with those previously reported on ATPase localization in different plant tissues that were obtained mainly by biochemical methods. K^+-stimulated ATPases were localized mainly in the plasma membrane (Anthon and Spanswick 1986; Briskin 1986). Ca^{2+}-ATPases, in turn, have been localized in the ER (Buckout 1984; Giannini et al. 1987), Golgi vesicles (Hager and Hermsdorf 1981) and secretory vesicles (Schessner and Schnorr 1990), while Mg^{2+}-dependent ATPases have been recognized at the tonoplast (Leigh and Walker 1980; Wagner and Lin 1982; Bush and Sze 1986), Golgi vesicles (Chanson et al. 1984), ER (Bush and Sze 1986) and plasma membrane (Rasi-Caldogno et al. 1987).

If we assume that the ATPase activities detected in PG and PT are probably related to the presence of H^+-ATPases, pollen seems to behave like a typical higher plant cell in having as the primary active transport process, the electrogenic transport of H^+ extruded from the cell across the plasma membrane (Poole 1978) and its pumping from the cytoplasm into the vacuole across the tonoplast (Smith and Raven 1979). As a rule, all these ATPases are associated to a membrane hyperpolarization (Doll and Hauer 1981) resulting in the controlled acidification of the organelles inside due to H^+ uptake (Wagner and Lin 1982) and the formation of an inside positive membrane potential (Doll and Hauer 1981).

The results on the different spatial distribution of ATPases in the PT and PG plasma membrane of *Agapanthus umbelatus* agree with the hypothesis of Weisenseel and Jaffe (1976) according to which the enzymatic composition of the PG and PT could be different. The plasma membrane of the PG presents several types of ATPases (K^+, Ca^{2+}- and Mg^{2+}-dependent) whose activity is particularly intense at the beginning of germination, while in the plasma membrane of the tube only K^+-ATPase activity was detected, and this mainly in the tip region. These enzymes could be involved in an electrochemical gradient generation by H^+ pumping, thus providing the driving force for transport of various solutes (including cations, anions, sugars; Schroeder and Hedrich 1989) and, consequently, in the establishment of the large electrical currents that traverse growing PTs (Weisenseel et al. 1975). With increasing germination time, the PG is progressively isolated from the PTs due to the vacuolization of the basal areas (Cresti et al. 1985). This feature could justify in some way the decrease in the plasma membrane ATPases' activity observed during the germination process. These ATPases could have as a major function the regulation of solute exchanges in the pre-germination period. With the emergence of the PT, the main exchanges that occurred at the level of the grain plasma membrane are transferred to the tube.

The results on the in vivo effect of the inhibitors azide, vanadate and NEM are consistent with this hypothesis. The higher inhibition of germination by vanadate than by azide could indicate that, in this step, the plasma membrane ATPases are more important than the mitochondrial ones. During their ultrastructural studies Elleman and Dickinson (1986) also observed that the plasma membrane is one of the most dynamic parts of the cell during the early germination process.

The localization of Ca^{2+}-ATPases to the membranes of the mitochondria and ER is concordant with the inhibition of pollen germination and PT growth by sodium azide, since these enzymes are involved in the translocation and accumulation of calcium as well as in the production of ATP (Poole 1978). Furthermore, a Ca^{2+}-ATPase has recently been cytochemically localized in secretory vesicles of the pyloric gland cells of cattle, where they presumably participate in calcium homeostasis (Schessner and Schnorr 1990). Therefore, azide could alter the regulation of the entrance and concentration of ionic species in the cytoplasm by the organelles with accumulation capacity. This, in turn, could lead to a modification in the ionic gradients and a reduction of the ATP levels, both processes being essential for PT growth.

The inhibition of PT growth by NEM confirmed the important role of tonoplast ATPase. Inhibition of this enzyme induced the tube apex to swell, probably as a consequence of the drastic reduction in the accumulation of osmotic solutes (ions and sugars) in the vacuole. As ATPases are responsible for an active transport, the resulting uptake of water from the cytoplasm to the vacuole (Hager et al. 1986) is also inhibited, inducing a turgor pressure in the cytoplasm and the swelling in the tip, the only elastic point of the pollen wall.

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