The effect of intracellular calcium level regulators on the synthesis of pollen tube callose in *Oenothera biennis* L.

ELŻBIETA BEDNARSKA

Department of Plant Cytology and Genetics, Institute of Biology, N. Copernicus University,
Gagarina 9, 87-100 Toruń, Poland

(Received: September 12, 1988. Accepted: November 17, 1988)

Abstract

It is shown that callose synthesis in the *Oenothera biennis* pollen tube is regulated by the endogenous Ca^{2+} level. Calcium antagonists reduced the amount of callose in the wall above the tip of the pollen tube (Verapamil — calcium channels blocker) and at the tube tip after stopping tube growth (La^{3+} — a Ca^{2+} substitute). Ruthenium red and ionophore A 23187, which raise the Ca^{2+} level in the cytoplasm, induced callose synthesis at the tip of pollen tube.

Key words: pollen tube, callose synthesis, calcium channel blocker, ionophore A 23187, ruthenium red, *Oenothera biennis*

INTRODUCTION

Callose synthesis plays a key role in pollen tube growth. Both *in vitro* (Górska-Bryllass 1965) and *in vivo* (see rev. Heslop-Harrison 1987) callose in growing pollen tubes is deposited above the tip in the form of lamellae on the inner side of the pecto-cellulose wall and in the form of so-called callose plugs. Vesicles, which become detached from the endoplasmic reticulum, take part in the synthesis of callose (Cresti and Van Went 1976). Under *in vitro* conditions the depositions of callose at the tip of pollen tube

occurs as a result of the pollen tube's injury, several minutes after its growth has stopped (Górska-Bryl 1965). *In vivo*, the synthesis of this polysaccharide at the tip of the pollen tube is connected with the physiological self-incompatibility process and constitutes the so-called callose response after rejection of a self-pollen tube (rev. Knox 1984, Gaude and Dumas 1987).

Biochemical studies carried out by Kauss et al. (see rev. Kauss 1987) in suspension-cultured soybean cells have revealed that callose synthesis depends on local increase of Ca^{2+} ions concentration in the cytoplasm which is a necessary condition for the activation of 1,3- β -glucan synthase in the plasma membrane.

Increased Ca^{2+} concentration in the callose synthesis zone has been found by Tretyn et al. (1987) in the dyads and tetrads of *Larix decidua*. In pollen tubes, which are characterized by a tip-to-base Ca^{2+} gradient (Reiss and Herth 1978), a significant drop in calcium concentration takes place outside the zone of callose wall synthesis (Bednarska et al. 1988). The calcium gradient in the pollen tube of *Oenothera biennis* is formed with the participation of active influx of Ca^{2+} ions into the cytoplasm through calcium channels. Studies with $^{45}\text{Ca}^{2+}$ have demonstrated that the amount of Ca^{2+} uptake into pollen tubes increased after ruthenium red treatment, while La^{3+} and the calcium channel blocker, Verapamil, reduced Ca^{2+} uptake from the medium (Bednarska 1989). The effects of overoptimal and underoptimal Ca^{2+} concentrations in the cytoplasm on the synthesis of pollen tube callose in *Oenothera biennis* have been investigated in this study.

MATERIALS AND METHODS

Pollen grains for germination were taken from freshly opened anthers of *Oenothera biennis* L. *Oenothera biennis* pollen grains are interconnected with strands of viscin, which made it possible to form them into clusters of similar size. The clusters were placed in Petri dishes ($\varnothing$ 20 cm) with 10 cm³ of water containing 0.003% CaCl_2 , 0.001% boric acid and 10% sucrose (standard medium). After 2 hours, clusters of germinating pollen were delicately transferred to media with: 1) 10^{-5} M or 10^{-4} M Verapamil (calcium channel blocker), 2) 10^{-4} M La^{3+} ions (substitute for Ca^{2+} ions), 3) 50 μM ruthenium red (RR) or 4) 10 μM ionophore A 23187. The time of incubation in La^{3+} and ruthenium red was 5 to 15 min., since after that time *Oenothera* pollen tubes lost their viability. Incubation in Verapamil was for 0.5, 1 and 2 h., in ionophore A 23187 — from 15 min. to 8 hours. Part of the clusters before being transferred into the experimental media were placed for 5 min. in 1 mM ethylene glycol-bis-(β -aminoethyl ether)-N,N,N',N' tetraacetic acid (EGTA).

The control were clusters of germinating pollen transferred to the standard medium for 2, 4, and 8 hours. All the experiments are shown in Table 1.

Calcium ions were localized by means of chlorotetracycline (CTC) after Reiss and Herth (1978) on unfixed material in a K. Zeiss fluorescence microscope (Fluoval) fitted with a BG 12 excitation filter. For monitoring CTC- Ca^{2+} emission, a narrow-pass interference barrier filter with a peak at 532 nm was used. The localization of callose was carried out by aniline blue fluorescence (Eschrich and Currier 1964) in pollen tubes fixed in 2.5% glutaraldehyde in cacodyl buffer at pH 7.2 containing 10% sucrose. One hundred tubes were studied in each experiment.

Table 1

Intracellular calcium level regulators used in experiments with pollen tubes of *Oenothera biennis* L.

Control	Verapamil		La^{3+} , 10^{-4}M	RR, 50 μM	Ionophor A 23187, 10 μM
	10^{-5}M	10^{-4}M			
2, 4, 8 h.	0.5, 1, 2 h. EGTA 5 min. + Verapamil 0.5, 1, 2 h.	0.5, 1, 2 h. EGTA 5 min. + Verapamil 0.5, 1, 2 h.	5-15 min. EGTA 5 min. + La^{3+} 5-15 min.	5-15 min. EGTA 5 min. + RR 5-15 min.	15 min.-8 h. EGTA 5 min. + Ionophore 15 min.-8 h.

RESULTS

After 4-10 hours of germination of pollen grains in standard medium, callose was localized in the wall above the growing tip in 80-90% of pollen tubes (Fig. 1). Only few pollen tubes showed the presence of callose over their entire length, which testified to growth inhibition or was the result of the tubes being injured during the experiment. CTC fluorescence showed that a typical tip-to-base Ca^{2+} gradient occurs in growing pollen tubes (Fig. 2). The five-minute incubation of pollen tubes in EGTA resulted in a complete lack of CTC- Ca^{2+} fluorescence.

Ruthenium red and ionophore A 23187 caused callose synthesis over the entire length of the pollen tube. However, the effects of those substances on the

rate of callose synthesis were different. After 10-15 min. in ruthenium red strong callose fluorescence appeared at the pollen tube tips (Fig. 3) in the form of a continuous wall or large callose plugs. They were present in about 96% of pollen tubes. After 5 min. preincubation in EGTA followed by incubation in RR (5-10 min.), callose plugs were formed generally just above the tube tip (Fig. 4). Sometimes there were also small callose plugs at the pollen tube tip (Fig. 5). A short 2-3 min. incubation in RR did not disturb the tip-to-base CTC-Ca²⁺ gradient (Fig. 6). After a longer, 10-15 min. incubation, the pollen tubes showed hardly any CTC-Ca²⁺ fluorescence.

Ionophore A 23187 did not affect the synthesis of pollen tube callose so drastically. After 15-30 min. of incubation only a few pollen tubes had a thin callose wall in the tip. The number of such tips increased as incubation in the ionophore continued. After 4-6 hours, 50-60% of pollen tubes exhibited anomalies in the structure of the tip (distensions, lack of agranular zone), and about 90% of the pollen tubes were surrounded with a strongly fluorescent callose wall (Figs. 7a, b). Fluorescence of callose was definitely stronger than in control tubes. Preincubation in EGTA reduced the number of pollen tubes which, after incubation in ionophore A 23187, were surrounded with a strongly fluorescent callose wall. Generally large callose plugs appeared over the whole length of the pollen tube (Fig. 8).

La³⁺ and Verapamil decreased the amount of callose synthesized in pollen tubes. After 5-15 min. incubation with La³⁺, pollen tube growth was completely inhibited. In spite of this, however, in about 90% of the tubes no callose was observed at the tip (Fig. 9). Preincubation of pollen tubes in EGTA did not in principle change the localization of callose. Sometimes, distinct callose plugs appeared above the tip (Fig. 10). Several minutes of incubation of pollen tubes in La³⁺ resulted in dilution and extinction of CTC-Ca²⁺ fluorescence (Fig. 11).

Verapamil 10⁻⁵ and 10⁻⁴ M significantly reduced the amount of synthesized callose. Since in Verapamil pollen tubes grow, though more slowly, for 1-2 hours (Bednarska 1989) it was possible to observe the gradual reduction of callose deposited in the pollen tube wall. After 2 hours incubation in Verapamil, the callose wall was in the form of small agglomerations, whose number decreased towards the pollen tube tip (Figs. 12, 13, cf. Fig. 1 of a control pollen tube). Only 30% of pollen tubes whose growth had been stopped by Verapamil displayed agglomerations of callose at the tip. In the pollen tubes preincubated in EGTA and then treated with Verapamil, callose synthesis was inhibited nearly completely, only few small callose plugs were observed along a pollen tube (Fig. 14).

Pollen tubes growing one hour in 10⁻⁵ M Verapamil were characterized by a very strong reduction of CTC-Ca²⁺ fluorescence (Fig. 15).

PLATE I

Figs. 1-2. Pollen tubes growing at optimal Ca^{2+} concentration. $\times 400$. Fig. 1 — No callose at pollen tube tip. Fig. 2 — Fluorescence of the CTC- Ca^{2+} complex at pollen tube tip

PLATE II

Figs. 3-6. Pollen tubes growing in 50 μ M ruthenium red. $\times 400$. Fig. 3 — Callose appearing at pollen tube tip after 10 min. Figs. 4-5 — Pollen tubes preincubated in EGTA. Fig. 4 — Large callose plugs above tip devoid of callose. Fig. 5 — Small callose plugs in the region of pollen tube tip. Fig. 6 — Fluorescence of the CTC- Ca^{2+} complex at the pollen tube tip after 3 min. in RR

PLATE III

Figs. 7-8. Pollen tubes incubated in 10 μ M ionophore A 23187. $\times 400$. Figs. 7a-b — Strongly fluorescent callose wall in pollen tubes incubated for 4 hrs. (Fig. 7a), 6 hrs. (Fig. 7b).
Fig. 8 — Numerous callose plugs along the entire pollen tube preincubated in EGTA

Figs. 9-11. Pollen tubes incubated in 10^{-4} M La^{3+} . $\times 400$. Fig. 9 — No callose in the inhibited, distended pollen tube tip. Fig. 10 — No callose in tip of pollen tube preincubated in EGTA. Fig. 11 — Weak (cf. Fig. 2) fluorescence of CTC- Ca^{2+} complex after 5 min. in La^{3+}

PLATE V

Figs. 12-15. Pollen tubes growing in 10^{-4} M and 10^{-5} M Verapamil. $\times 400$. Fig. 12 — No continuous callose wall after 2 hrs. in 10^{-5} M Verapamil. Fig. 13 — Small callose grains in the wall after 2 hrs. in 10^{-5} M Verapamil. Fig. 14 — No callose in the long distance above the tip of pollen tube preincubated in EGTA. Fig. 15 — Weak fluorescence (cf. Fig. 2) of CTC- Ca^{2+} complex in pollen tube growing for 1 hr. in 10^{-5} M Verapamil

DISCUSSION

The present studies have revealed that callose synthesis in *Oenothera biennis* pollen tubes growing *in vitro* is influenced by the intracellular Ca^{2+} level. This is in agreement with the results of Kauss et al. (see rev. Kauss 1987), who, studying suspension-cultured soybean cells, found that activation of both the enzyme 1,3- β -glucan synthase and callose synthesis are regulated by the concentration of calcium ions.

During pollen tube growth callose is continuously synthesized and deposited in the wall above the tip. Very rapid callose synthesis also takes place at the tip of the tube after injury *in vitro* (Górska-Bryl 1965), or in inhibited self-pollen tubes *in vivo* (see rev. Knox 1984, Gaude and Dumas 1987). The present studies have demonstrated that both the synthesis of a continuous callose wall above the tip and callose deposition at the tip depend on calcium. This is particularly clearly demonstrated by the results obtained after treatment with Verapamil and La^{3+} . After treatment with this Ca^{2+} blockers callose synthesis in pollen tubes was reduced. Verapamil, which blocks slowly calcium channels in mammalian cells (Fleckenstein 1985) and which binds to plant membranes (Marmé 1985), significantly reduces the uptake of $^{45}\text{Ca}^{2+}$ into *Oenothera biennis* pollen tubes from the medium (Bednarska 1989). The decrease in the Ca^{2+} concentration in pollen tubes was reflected in a reduction of CTC- Ca^{2+} fluorescence. In pollen tubes growing in Verapamil, the amount of callose deposited in the above tip clearly dropped. There were only a few callose grains.

La^{3+} which is a substitute for Ca^{2+} ions, very drastically reduces $^{45}\text{Ca}^{2+}$ uptake into the pollen tubes of *Oenothera biennis* and inhibits their growth within a few minutes (Bednarska 1989). However, most of the tubes did not produce callose at the tip. This shows that La^{3+} ions, which inhibit 1,3- β -glucan synthase *in vitro* (Kauss and Jeblick 1985), also inhibit callose synthesis in pollen tubes. La^{3+} also has a destructive influence on the ultrastructure of the pollen tube tip of *Tradescantia virginiana* (Picton and Steer 1985). Calcium channel blockers (Nifedipine and Verapamil) and La^{3+} ions affect inhibition of callose synthesis in suspension-cultured cells of *Glycine max* and *Catharanthus roseus*, although the specificity of the effect of Verapamil and La^{3+} is questioned (Waldmann et al. 1988).

Treatment with ruthenium red and ionophore A 23187 induced callose synthesis in the pollen tubes of *Oenothera biennis*. After ruthenium red the uptake of $^{45}\text{Ca}^{2+}$ from the medium strongly increased (Bednarska 1989). A response to the increased Ca^{2+} level in the pollen tube cytoplasm is synthesis of callose usually in the form of large deposits, which surround the tube tip within a few minutes. This experiment is in agreement with the biochemical demonstration that ruthenium red activated 1,3- β -glucan synthase (Kauss

and Jeblick 1985). Ruthenium red also causes disturbances in the ultrastructure and thickening of the pollen tube tip wall (Picton and Steer 1985).

Ionophore A 23187 induced synthesis much more slowly than ruthenium red. The fluorescence of callose in the wall and at the tip of *Oenothera biennis* pollen tubes increased and after 4-8 hours was definitely stronger than in the control. The slow synthesis of callose after ionophore A 23187 may result from the process of removing excess Ca^{2+} with the participation of calcium pumps in the plasmalemma. Ionophore A 23187 also induces callose synthesis in suspension-cultured cells of *Glycine max* and *Catharanthus roseus* (Waldmann et al. 1988).

Preincubation of *Oenothera biennis* pollen tubes in EGTA reduced or inhibited callose synthesis. After the pollen tubes had been transferred to conditions decreasing Ca^{2+} influx into the cell (La^{3+} and Verapamil), callose synthesis was practically blocked. Transferring the pollen tubes to conditions increasing calcium influx caused unblocking of this process. However, in the pollen tubes transferred to ruthenium red or to ionophore A 23187 the amount of synthesised callose was definitely smaller than in tubes which had not been preincubated in EGTA. Preincubation in EGTA also inhibited callose synthesis in soybean cells (see rev. Kauss 1987).

The result pointing the participation of Ca^{2+} in pollen tube callose synthesis under *in vitro* conditions may suggest that these ions play an essential role in callose response during rejection of self-pollen tubes in the pollen-pistil interaction.

Acknowledgment

I thank Dr. Andrzej Tretyn and Doc. Alicja Górską-Brylass for helpful discussion, Prof. Bohdan Rodkiewicz for correction of manuscript and Mgr. Ewa Sikorska for technical assistance.

REFERENCES

- Bednarska E., 1989. The effect of exogenous Ca^{2+} ions on pollen grain germination and pollen tube growth — investigations with the use of $^{45}\text{Ca}^{2+}$, and Verapamil, La^{3+} and ruthenium red. Sex. Plant Reprod. 2: 53-58.
- Bednarska E., Tretyn A., Górską-Brylass A., 1988. Localization of Ca^{2+} and peroxidase activity in the zone of callose synthesis in *Haemanthus albiflos* L. pollen tube. Acta Physiol. Plant. 10: 237-241.
- Cresti M., Van Went J. L., 1976. Callose deposition and plug formation in *Petunia* pollen tubes *in situ*. Planta 133: 35-40.
- Eschrich W., Currier H. B., 1964. Identification of callose by its diachrome and fluorochrome reactions. Stain Technol. 39: 303-307.

- Fleckenstein A., 1985. Calcium antagonists and calcium agonists: Fundamental criteria and classification. In: Bayer Symposium IX. Cardiovascular effects of dihydropyridine-type-calcium antagonists and agonists. Springer Verlag, Berlin-Heidelberg, pp. 3-31.
- Gaude T., Dumas C., 1987. Molecular and cellular events of self-incompatibility. *Int. Rev. Cytol.* 107: 333-366.
- Górska-Bryllass A., 1965. Cytomorphological studies of callose in pollen tubes. *Acta Soc. Bot. Pol.* 34: 757-762.
- Heslop-Harrison J., 1987. Pollen germination and pollen-tube growth. *Int. Rev. Cytol.* 107: 1-78.
- Kauss H., 1987. Some aspects of calcium-dependent regulation in plant metabolism. *Ann. Rev. Plant Physiol.* 38: 47-72.
- Kauss H., Jeblick W., 1985. Activation by polyamines, polycations, and ruthenium red of the Ca^{2+} -dependent glucan synthase from soybean cells. *FEBS* 2613, 185: 226-230.
- Knox R. B., 1984. Pollen-pistil interaction. In: Cellular interactions. H. F. Linskens and J. Heslop-Harrison (eds.), Springer Verlag, Berlin-Heidelberg-New York-Tokyo. pp. 508-608.
- Marmé D., 1985. The role of calcium in the cellular regulation of plant metabolism. *Physiol. Vég.* 23: 945-953.
- Picton J., Steer M. W., 1985. The effects of ruthenium red, lanthanum, fluorescein isothiocyanate and trifluoroperazine on vesicle transport, vesicle fusion and tip extension in pollen tubes. *Planta* 163: 20-26.
- Reiss H. D., Herth W., 1978. Visualisation of Ca^{2+} -gradient in growing pollen tubes of *Lilium longiflorum* with chlorotetracycline fluorescence. *Protoplasma* 97: 373-377.
- Tretyn A., Bednarska E., Górska-Bryllass A., 1987. Localization of Ca^{2+} ions and peroxidase activity in the zone of callose wall synthesis in dyads and tetrads of *Larix decidua* Mill. *Cell Biol. Intern. Rep.* 11: 157-164.
- Waldmann T., Jeblick W., Kauss H., 1988. Induced net Ca^{2+} uptake and callose biosynthesis in suspension-cultured plant cells. *Planta* 173: 88-95.

Wpływ regulatorów wewnątrzkomórkowego poziomu wapnia na syntezę kalozy łagiewkowej Oenothera biennis L.

Streszczenie

Stwierdzono, że synteza kalozy w łagiewkach pyłkowych *Oenothera biennis* jest regulowana endogennym poziomem Ca^{2+} . Antagoniści wapnia redukują ilość kalozy w ścianie powyżej wierzchołka łagiewki pyłkowej (Verapamil — inhibitor kanałów wapniowych), a także na wierzchołku łagiewki pyłkowej po zatrzymaniu jej wzrostu (La^{3+} — substytut Ca^{2+}). Czerwień rutenowa i jonofor A 23187, które podwyższają poziom wapnia w cytoplazmie, indukują też syntezę kalozy na wierzchołku łagiewki pyłkowej.