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Programmed Cell Death in Plants: Ultrastructural Changes in Pea Guard Cells

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Abstract—Treatment with cyanide of epidermal peels isolated from pea leaves resulted in destruction of nuclei in the guard cells of stomata, which is visible with a light microscope. The process was accelerated by illumination. Electron microscopy revealed significant CN^- induced changes in the ultrastructure of guard cells, which increased with time. Margination of chromatin, which is one of the first signs of apoptosis, was observed in the guard cells even after 1 h incubation of the isolated epidermis with CN^-. Subsequent chromatin condensation, swelling of the endoplasmic reticulum with formation of large tanks covered with ribosomes, changes in the structure of dictyosomes, and a slight swelling of mitochondria were observed after 3 h of the epidermis incubation with CN^-. After 6 h of incubation with CN^-, the bulk volume of the guard cells was filled with vacuoles, the cytoplasm occupied the thin marginal layer, the nucleus was in the center similarly to the control experiment, but it was polylobal, extended in narrow cytoplasmic bands, and, despite the loss of the nuclear envelope integrity, appeared to be a self-dependent structure. In the envelope-free open regions of the nucleus, mitochondria and chloroplasts directly contacted with chromatin. Much like the cell nucleus, chloroplasts lost the integrity of the membrane, but did not swell and retained the stroma and integrity of the thylakoid system. An antioxidant di-*tert*-butyl-4-hydroxytoluene prevented ultrastructural changes in the cells observed after 6 h of incubation with CN^-. Thus, the CN^- induced death of the guard cells of stomata occurs through the mechanism of apoptosis.

Key words: programmed cell death, apoptosis, guard cells, ultrastructure, nucleus, mitochondria, chloroplasts, antioxidants, *Pisum sativum* L.

Experimental data of recent years have shown the generality of mechanisms of programmed cell death (PCD) in animals, plants, and fungi, and the involvement of bioenergetic structures in PCD. Mitochondria are important in apoptosis as suppliers of apoptogenic factors, such as cytochrome *c*, flavoprotein AIF (apoptosis inducing factor), procaspases, regulatory proteins of PCD, and also reactive oxygen species [1-7]. Indirect data suggest that chloroplasts are involved in PCD. The content of DS9 protein is decreased in tobacco leaves infected with tobacco mosaic virus (TMV). This chloroplast protein is a homolog of the bacterial metalloprotease FtsH. The decrease in the DS9 protein level in TMV-infected leaves correlated with the accelerated death via a hypersensitive response [8], which is the plant defensive reaction to infection with a pathogen. Electron microscopy showed that swelling of chloroplasts succeeded by changes in their ultrastructure preceded the death of mesophyll cells in maize leaves caused by mutation of the *lls1* gene [9].

Cyanide can also induce apoptosis in plants [10, 11]. We have studied the effect of CN^- in experiments with isolated epidermal peels from pea leaves, which is a suitable model of apoptosis in plants [12]. Epidermis is a monolayer consisting of the guard cells of stomata and the basic epidermal cells, and these cells are different in both structure and function. Thus, the guard cells contain chloroplasts and mitochondria, whereas the basic epidermal cells contain only mitochondria. Chloroplasts of the guard cells contain the usual pigments; the electron transport, evolution of O_2, and photophosphorylation are provided by photosystems I and II [13].

We have shown by light microscopy that CN^- causes fragmentation and disintegration of nuclei in the cells of epidermis isolated from pea leaves. Illumination significantly increased the CN^- induced fragmentation of nuclei in guard cells, but not in the basic epidermal cells

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[12]. These phenomena could be prevented in both cell types by anaerobiosis and antioxidants. Compounds used as electron acceptors in the Hill reaction prevented the effect of CN^- in the guard cells [14, 15]. The photostimulation of the CN^- induced apoptosis of the guard cells was abolished with 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, which inhibits the electron transfer between plastoquinones Q_A and Q_B in photosystem II. Quinone analogs, such as dinitrophenylether of iodonitrothymol and stigmatellin, competitively inhibiting the plastoquinol oxidation at the *o*-site of the chloroplast cytochrome b_6f complex displayed the same effect [14, 15]. Thus, it was suggested that chloroplasts should be involved in regulation and realization of the fragmentation and separation of the nucleus fragments observed by us in the guard cells under the influence of cyanide. This process depends on reactive oxygen species and seemed to be regulated by the redox state of plastoquinone at the *o*-site of the b_6f complex of the photosynthetic chain of electron transfer [14, 15].

Although various approaches of molecular biology and biochemistry are used, the identification of apoptosis based on changes in ultrastructure still remains the most impressive. Therefore, the purpose of the present work was to study ultrastructural changes in the guard cells of stomata under the influence of cyanide.

MATERIALS AND METHODS

In experiments, we used peels of the isolated lower epidermis of leaves of 8-14-day-old pea (*Pisum sativum*, Alpha cultivar) seedlings, grown under constant illumination at 20-24°C [12]. The epidermis was separated as peels with tweezers and placed into distilled water. To accelerate the delivery of reagents into the cells, the epidermal peels were infiltrated by incubation in vacuum for 1-2 min. Specimens were placed in polystyrene plates and incubated in 0.1 M Na^+,K^+-phosphate buffer (pH 7.4) at room temperature, with addition of 2.5 mM KCN, under illumination with a luminescent lamp at the light intensity of ~1000 lx [12].

For investigation by electron microscopy, the specimens were fixed with 3% glutaric dialdehyde in 0.1 M Na^+,K^+-phosphate buffer (pH 7.4) for 2 h at 4°C, then fixed further with 1% osmium tetroxide in the same buffer for 1.5 h, and dehydrated in ethanol solutions of increasing concentration. The 70% ethanol was saturated with uranyl acetate (1.5%). The material was embed with Epon-812 (Fluka, Germany) epoxide resin. The work was performed using serial ultrathin sections prepared with an LKB-III (LKB, Sweden) ultramicrotome. The sections were mounted on blends covered with a formvar film and stained with lead as described in [16]. The resulting preparations were examined and photographed with a HU-11B electron microscope (Hitachi, Japan).

RESULTS

Figure 1a presents a stomate of isolated pea epidermis after 6 h of incubation in 0.1 M phosphate buffer (pH 7.4) without additions, and this was the maximum incubation time in our experiments. The guard cells of stomata of pea leaves had normal structure corresponding to the literature data on the ultrastructure of stomata in the majority of plant taxons. Each guard cell had in the center a large round-shaped, slightly lens-shaped nucleus, which is characteristic for dicotyledons (in monocotyledons nuclei are extended along the guard cells). Chromatin structures of the nucleus had a spatial organization specific for interphase nuclei. The cytoplasm of the guard cells contained multiple spherical or elongated mitochondria with variously oriented numerous cristae and distinct matrix. Chloroplasts were lens-shaped. In the chloroplast stroma one could see plastoglobules, i.e., structures containing galactolipids, carotenoids, plastoquinone, and more than 12 different polypeptides [17]. The membrane system of chloroplasts of the guard cells was poorly developed—chloroplasts contained a small number of grana. The guard cells had separate vacuoles of various size.

The ultrastructure of stomata from the epidermal peels fixed immediately after removal from the leaf (Fig. 1b, control, the "null" time) was virtually unchanged after 6 h of incubation (Fig. 1a), only a slight swelling of mitochondria and chloroplasts was observed.

The ultrastructure of the guard cells of stomata was noticeably changed even after 1 h of incubation of the epidermal peels in the presence of cyanide (Fig. 2a). Chromatin was displaced to the nucleus margin, and margination of chromatin is a specific sign of apoptosis in animals and plants. The chromatin margination occurred in the nuclei of both cells of the stomate (Fig. 2a). Moreover, the volume of vacuoles was significantly increased alongside with decrease in the cytoplasm volume. There were no pronounced changes in the ultrastructures of chloroplasts and mitochondria, but unusual contacts of dictyosomes with cytoplasmic lipid globules were observed. Figure 2b shows a lipid globule encircled from one side with a pile of dictyosomal cisterns and with a chain of secretory vesicles from another side.

After 3 h of incubation of the epidermal peels with CN^-, the volume of vacuoles continued to increase and the ultrastructure of the nucleus was changed: chromatin was significantly condensed (Fig. 3a). At this stage of incubation of the epidermal peels other cell organelles were also affected. Mitochondria were slightly swollen, rounded, outlines of cristae were changed, and the matrix was enlightened. Unusual structures were found in the cytoplasm, which in separated sections were produced by concentric layers of closed double membranes (Fig. 3b). These structures were supposed to be significantly changed dictyosomes. This hypothesis is supported by the

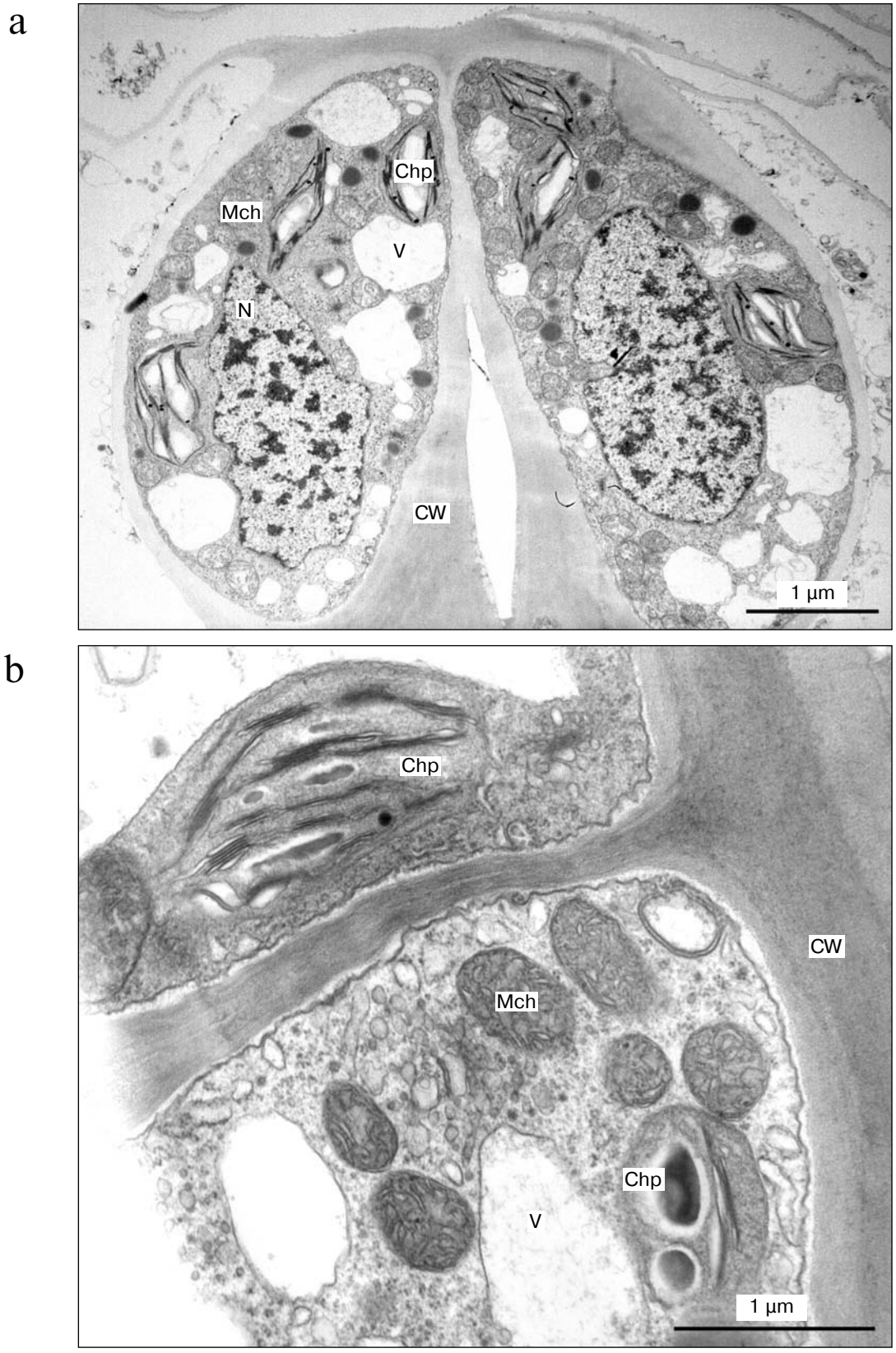

Fig. 1. Guard cells of stomata in the pea epidermis: a) in peels preincubated for 6 h in 0.1 M Na^+,K^+-phosphate buffer (pH 7.4) without additions; b) in peels fixed immediately after removal from the leaf. Here and in Figs. 2-4: V) vacuole; CW) cell wall; Mch) mitochondrion; Chp) chloroplast; N) nucleus.

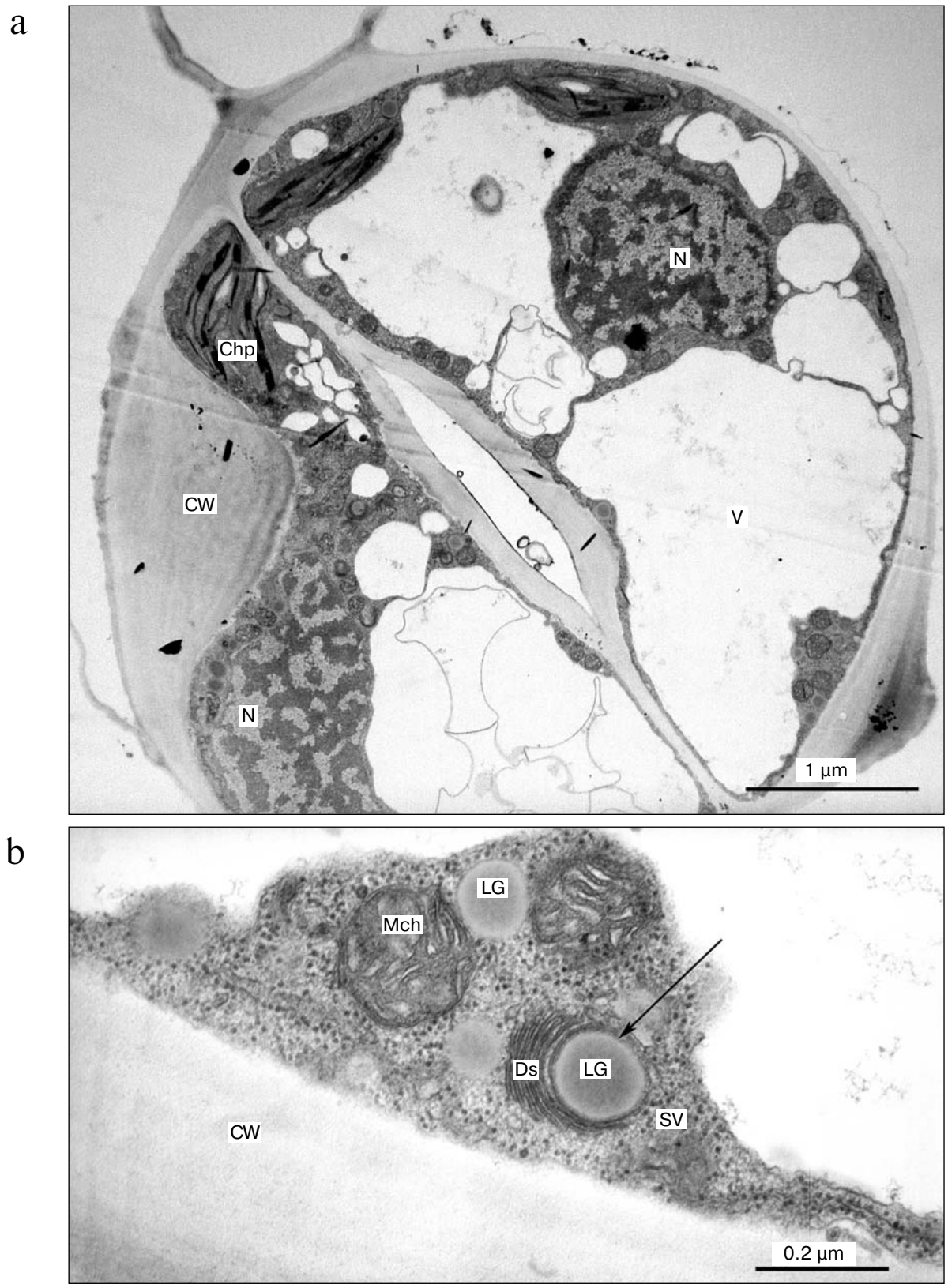

Fig. 2. Guard cells of stomata in the pea epidermal peels preincubated for 1 h in 0.1 M Na^+,K^+-phosphate buffer (pH 7.4) in the presence of 2.5 mM KCN: a) general appearance of a stomate; b) the cell region, the arrow points to the lipid globule encircled from one side with a pile of dictyosomal cisterns and with a chain of secretory vesicles from another side. Notations: Ds) dictyosome; LG) lipid globule; SV) secretory vesicles.

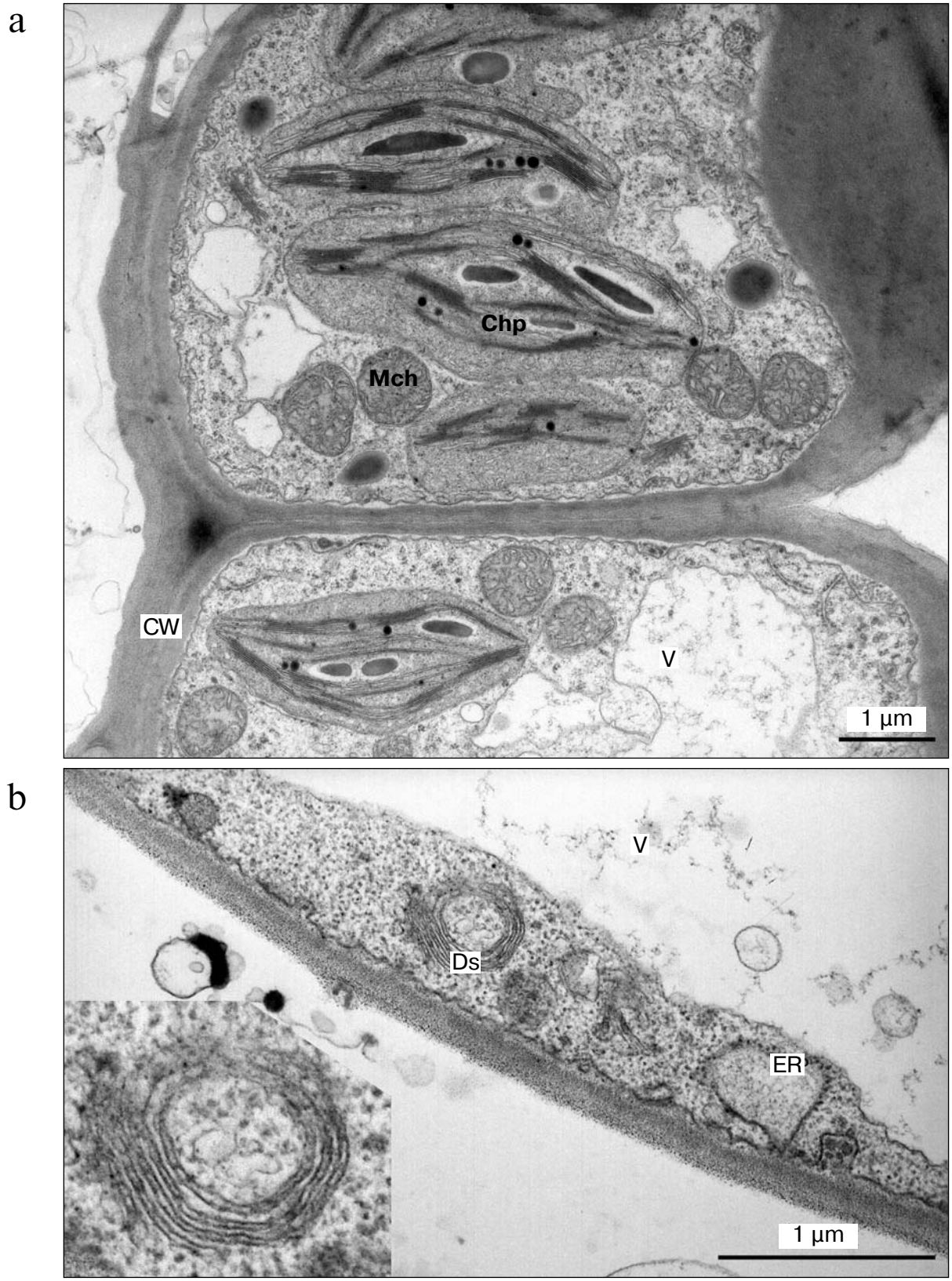

Fig. 3. Guard cells of stomata in the pea epidermal peels preincubated for 3 h in 0.1 M Na^+,K^+-phosphate buffer (pH 7.4) in the presence of 2.5 mM KCN: a) general appearance of a stomate; b) the cell region which contains a swollen endoplasmic reticulum and a dictyosome with changed ultrastructure; separately a dictyosome is shown under greater magnification. ER, endoplasmic reticulum.

presence near these structures of membrane vesicles, which are components of the Golgi apparatus together with dictyosomes. The endoplasmic reticulum was markedly swollen and formed large cisterns with ribosomes on their surface (Fig. 3b). However, the ultrastructure of chloroplasts (Fig. 3a) was the same as that of chloroplasts in the control guard cells (Fig. 1a).

After 6 h of incubation of the epidermal peels with CN^-, the ultrastructure of the guard cells was sharply different from the control (Fig. 4a). The cell volume was filled with vacuoles. The cytoplasm was represented by a thin marginal layer. The nucleus was located in the center of the cell in narrow cytoplasmic cords that, on observation with a light microscope, seems to be a picture of fragmentation and destruction of nuclei. A significant part of the nucleus was open, because the envelope was lost. Fragments of the nuclear envelope were found in the cytoplasm (Fig. 4c). However, the internal ultrastructure of the nucleus retained its characteristic morphology. The nuclear content was not mixed with the cytosol but was a self-dependent structure despite the loss of integrity of the nuclear envelope. On the open regions of the nucleus, chromatin directly contacted with mitochondria and chloroplasts (Fig. 4, b and c). In Fig. 4b, the mitochondrion was located in the nucleus region and closely contacted with chromatin. For the incubation time, mitochondria manifested significant swelling (Fig. 4, b, c, d).

At this stage of incubation with CN^- (6 h), chloroplasts, similarly to the nucleus, lost integrity of the membrane envelope and seemed to be open (Fig. 4c). The membrane envelope was only partially retained, but chloroplasts were not swollen, their stroma and integrity of the membrane thylakoid system were retained. Figure 4c shows that the open regions of chloroplasts are, as a rule, adjacent to the open regions of the nucleus.

After 6 h of incubation with CN^-, in the cytoplasm of the guard cells structures were detected which corresponded in organization to autophagosomes of animal cells (Fig. 4d). The mitochondria located in the direct vicinity with these structures were markedly degenerated in structure.

An antioxidant ionol (di-*tert*-butyl-4-hydroxytoluene) prevented the CN^- induced changes in the ultrastructure of guard cells (Fig. 5). After 6 h of incubation in the presence of 2.5 mM KCN and 100 μM ionol, the ultrastructure of the guard cells was similar to that of the control cells preincubated for 6 h without additions.

DISCUSSION

Current concepts about apoptosis are based on biochemical studies and electron microscopy data. Initially, apoptosis as a specific kind of cell death was discovered based on data on the cell ultrastructure [18]. At present, apoptosis in animals is described by a definite sequence of morphological, and first of all, ultrastructural changes in the cell, and these changes are used as characteristics of this process [1]. But data on ultrastructural changes in plant cells during apoptosis are fragmentary. The main characteristics of apoptosis in plants are mainly described by analogy with the known data for animals.

We have recorded margination of chromatin in the guard cells of stomata even after 1 h of incubation of the isolated epidermis with CN^-, and such chromatin margination is one of the main signs of apoptosis; changes were also detected in other cell organelles. The volume of vacuoles sharply increased, and the cytoplasm volume decreased. Plant cells have no lysosomes. Enzymes responsible for degradation of the cell components are located in vacuoles. Therefore, a significant increase in the volume of vacuoles and the presence in them of membrane structures suggested the initiation of apoptosis at this stage of our experiment.

The further incubation of epidermal peels with cyanide dramatically changed the ultrastructure of the guard cells of stomata. These changes were not destructive, as distinguished from necrosis. The nucleus lost the integrity of the envelope, and fragments of the latter were found in the cytoplasm. However, the nuclear content was not mixed with the cytoplasm and retained the morphology of a self-dependent structure. Mitochondria and chloroplasts were located directly in the region of the nucleus, in close contact with chromatin. Such an ultrastructure of the nucleus characterizes apoptosis in animals [19, 20].

We observed changes in chloroplasts that had not been described earlier. Similarly to the nucleus, they lost the integrity of the external membrane, which was only partially retained. There was no swelling of chloroplasts; their stroma and the integrity of the thylakoid membrane system were retained. Similar changes in the ultrastructure of chloroplasts were shown during the development of apoptosis caused by mutation of the *lls1* gene in the mesophyll of maize leaves [9].

Thus, CN^- induced apoptosis of the guard cell of stomata in the isolated epidermis of pea. Margination of chromatin was one of the first ultrastructural signs of apoptosis, and it was detected even after 1 h of incubation of the isolated epidermis with CN^-. But in chloroplasts ultrastructural changes were recorded only after 6 h of incubation when the nucleus was already disintegrated. However, mitochondria of the guard cells displayed no ultrastructural rearrangements specific for apoptosis in animals: small electron-dense mitochondria moving towards the nucleus were not detected [20]. Mitochondria were slightly swollen, similarly to the control specimen. This was in agreement with data on apoptosis in plants [21]. In animal cells, changes in the ultrastructure of mitochondria are one of the first signs of apoptosis, and they appear earlier than changes in the nucleus.

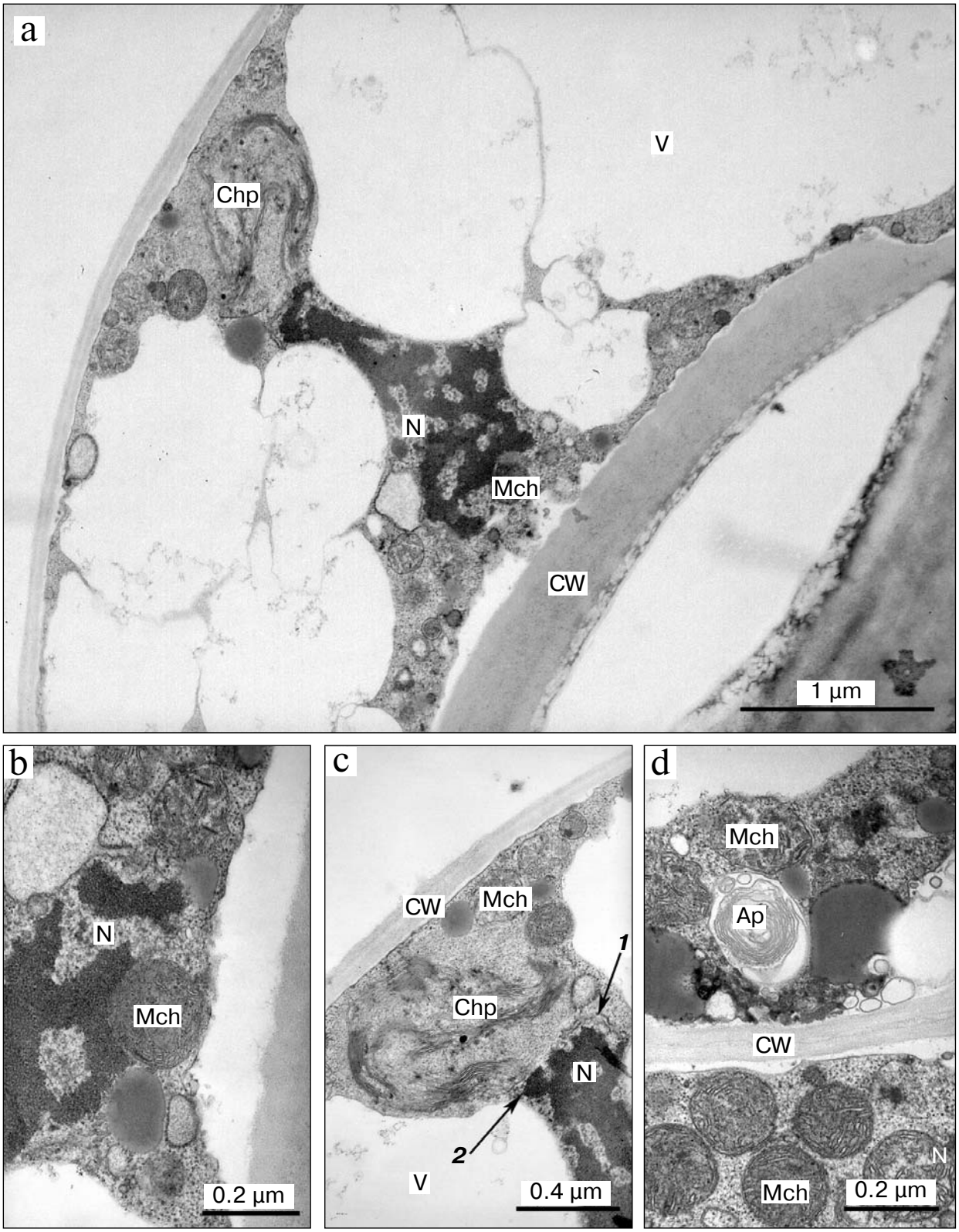

Fig. 4. Guard cells of stomata in pea epidermal peels preincubated for 6 h in 0.1 M Na^+,K^+-phosphate buffer (pH 7.4) in the presence of 2.5 mM KCN: a) general appearance of a stomate; b) the mitochondrion contact with chromatin in an open region of the nucleus; c) the cell region, arrow *1* shows the nuclear envelope, arrow *2* shows the contact of the open region of chloroplast with the open region of the nucleus; d) the cell region with autophagosome and the adjacent mitochondrion with lysed ultrastructure, and also population of mitochondria with normal ultrastructures. Ap, autophagosome.

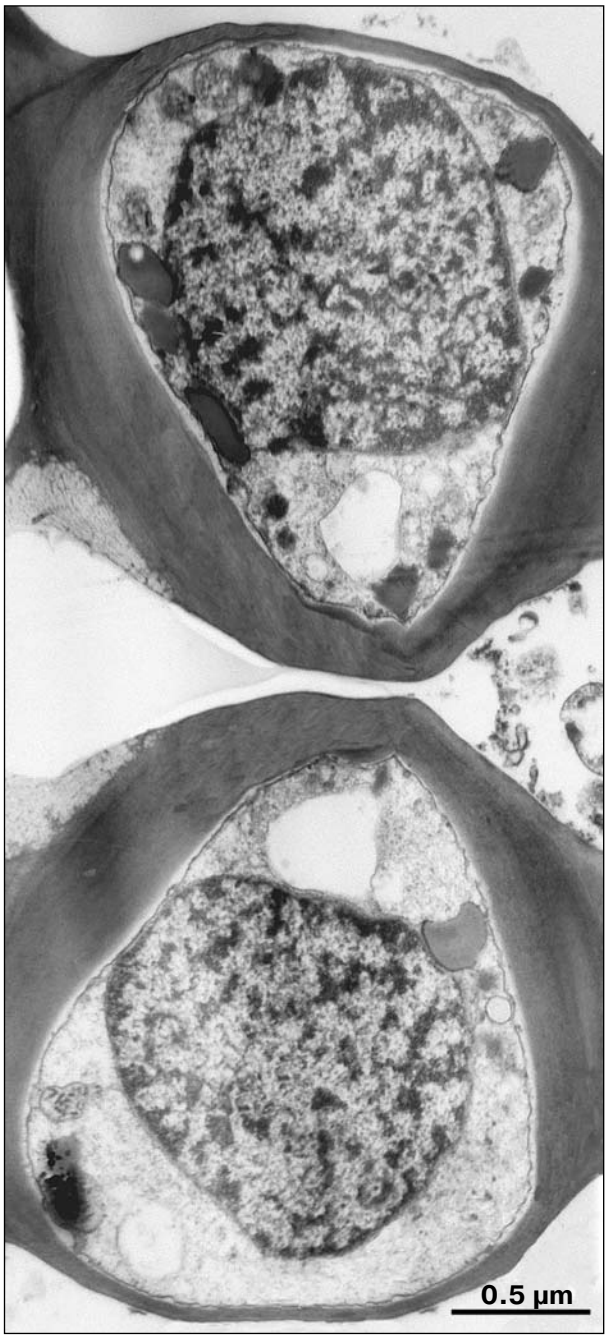

Fig. 5. Guard cells of stomata in pea epidermal peels preincubated for 6 h in 0.1 M Na^+,K^+-phosphate buffer (pH 7.4) in the presence of 2.5 mM KCN and 100 μM ionol.

The antioxidant ionol prevented cyanide-induced ultrastructural changes in the guard cells, and this supported our early data obtained by light microscopy. This suggests the involvement of reactive oxygen species in development of apoptosis in the cells of stomata [12].

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