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Programmed Cell Death in Plants: Effect of Protein Synthesis Inhibitors and Structural Changes in Pea Guard Cells

E. V. Dzyubinskaya[1], D. B. Kiselevsky[1], L. E. Bakeeva[2], and V. D. Samuilov[1]*

[1]*Department of Physiology of Microorganisms, Biological Faculty, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3807; E-mail: vdsamuilov@mail.ru*
[2]*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181*

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Abstract—Pea leaf epidermis incubated with cyanide displayed ultrastructural changes in guard cells that are typical of apoptosis. Cycloheximide, an inhibitor of cytoplasmic protein synthesis, and lincomycin, an inhibitor of protein synthesis in chloroplasts and mitochondria, produced different effects on the dynamics of programmed death of guard cells. According to light microscopy data, cycloheximide reinforced and lincomycin suppressed the CN^--induced destruction of cell nuclei. Lincomycin lowered the effect of cycloheximide in the light and prevented it in the dark. According to electron microscopy data, the most pronounced effects of cycloheximide in the presence of cyanide were autophagy and a lack of apoptotic condensation of nuclear chromatin, the prevention of chloroplast envelope rupturing and its invagination inside the stroma, and the appearance of particular compartments with granular inclusions in mitochondria. Lincomycin inhibited the CN^--induced ultrastructural changes in guard cell nuclei. The data show that programmed death of guard cells may have a combined scenario involving both apoptosis and autophagy and may depend on the action of both cytoplasm synthesized and chloroplast and mitochondrion synthesized proteins.

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Key words: programmed cell death, apoptosis, autophagy, cycloheximide, lincomycin, guard cells, nucleus, chloroplasts, mitochondria, light microscopy, electron microscopy, *Pisum sativum* L.

In eukaryotes cell death can be either programmed (apoptosis and autophagy) or non-programmed (necrosis) [1]. Mitochondria as suppliers of some apoptogenic factors including reactive oxygen species (ROS) play an important role in programmed cell death (PCD) [2-6].

We have demonstrated that chloroplasts are involved in PCD in plants [7-10]. We used in our experiments pea leaf epidermis, a monolayer composed of stoma guard cells (guard cells) containing mitochondria and chloroplasts and basic epidermal cells (epidermal cells) containing mitochondria but not chloroplasts. Light microscopy observations have shown that cyanide, an inducer of PCD, caused fragmentation of nuclei with following disappearance of nuclear fragments in guard and epidermal cells. Illumination significantly accelerated the death of guard, but not epidermal cells. In both cell types, the process was prevented by anaerobiosis and antioxidants. Photoactivation of CN^--induced destruction of guard cell nuclei was inhibited by artificial electron acceptors, inhibitors of the photosynthetic redox chain of chloroplasts, and inhibitors of protein kinases and serine and cysteine proteases. Experiments with pea mutants, deficient in Photosystem II and/or Photosystem I, have shown that photoactivation of PCD is associated with Photosystem II. The process depends on both ROS and functionally active plastoquinone in center *o* of cytochrome b_6f complex of chloroplasts.

The data of electron microscopy [11] suggests that already after 1 h of incubation of isolated epidermis with CN^-, the main features of apoptosis become observable: chromatin condensation and margination (chromatin folds up to form mooned aggregate along the nuclear

Abbreviations: PCD) programmed cell death; ROS) reactive oxygen species.

* To whom correspondence should be addressed.

membrane), vacuolar volume increase and cytoplasmic volume decrease. More prolonged incubation of epidermal peels with CN^- led to profound changes in ultrastructure of guard cells. These changes had no sign of necrosis. The plasma membrane maintained its continuity, but the nuclear membrane lost integrity. Nonetheless, the nuclear contents did not interfuse with cytoplasm and kept morphology of individual structure. Such state is characteristic of apoptosis in mammals [12, 13]. Mitochondria and chloroplasts of guard cells were disposed in the nuclear area in direct contact with chromatin. Similarly to the cell nucleus, chloroplasts lost continuity of their membranes but did not swell, and they maintained stroma and integrity of the thylakoid system. The antioxidant 2,6-di-*tert*-butyl-4-hydroxytoluene prevented the development of CN^--induced ultrastructural changes in guard cells [11].

One of distinctive features of apoptosis that allows its differentiation from necrosis [2] is a sensitivity of apoptosis to protein synthesis inhibitors. Apoptosis of animal [14], plant [15-17], and yeast [18, 19] cells induced by various factors is either precluded or decelerated by cycloheximide, an inhibitor of protein synthesis in the cytoplasm of eukaryotes. Cycloheximide itself can evoke apoptosis, as shown in rat hepatocytes [20] and maize root meristem [21].

The involvement of mitochondria and chloroplasts in PCD allows a hypothesis that selective inhibition of protein synthesis in these organelles might influence apoptosis. Chloramphenicol is a well-known inhibitor of protein synthesis in bacterial cells, mitochondria, and chloroplasts. However, its effect cannot be univocally interpreted because chloramphenicol has side effects associated with ROS production. In suspensions of illuminated thylakoids, chloramphenicol acts similarly to methyl viologen: it is reduced by Photosystem I with subsequent autooxidation by O_2 to produce superoxide anion-radical [22]. In the same manner, chloramphenicol seems to interact with components of the mitochondrial respiratory chain possessing low E_o' values [22]. So, other inhibitors, such as lincomycin, should be used instead of chloramphenicol in studies on protein synthesis in mitochondria and chloroplasts [23, 24].

In the present work, we studied effects of protein synthesis inhibitors on CN^--induced apoptosis of guard cells. We have tested cycloheximide inhibiting the protein synthesis in cytoplasm of eukaryotic cells and lincomycin inhibiting the protein synthesis in mitochondria and chloroplasts. Effects of these inhibitors were estimated by changes observed using light and electron microscopy.

MATERIALS AND METHODS

The experiments were carried out on peels of lower leaf epidermis of pea (*Pisum sativum* L. Alpha) seedlings grown for 8-14 days under continuous illumination at 20-24°C [7]. Epidermal peels were separated with forceps and placed into distilled water. The infiltration method involving incubation of the epidermis in vacuum for 1-2 min was used for rapid influx of added reagents into the cells. The samples were placed into polystyrene plates and incubated either in distilled water or in 0.1 M sodium-potassium phosphate buffer, pH 7.4, with additives (the composition is given in legends for figures) at room temperature either in dark or under illumination with a luminescent lamp at the light intensity of ~1000 lx.

After the incubation, the samples were maintained in distilled water for 5 min and then treated for 5 min with Battaglia fixative (the mixture of chloroform, 96% ethanol, glacial acetic acid, and 40% formaldehyde, 5 : 5 : 1 : 1). Then the samples were washed with ethanol for 10 min for removal of the fixative, incubated for 5 min in water, and stained with the nuclear dye Carazzi's hematoxylin for 20 min. The stained epidermal peels were washed with tap water and inspected using a light microscope. Experiments were repeated no less than twice (commonly three times). The fraction of cells with destroyed nuclei and lack of nuclei was determined from 300-500 inspected cells [7].

For electron microscopy, the material was preincubated for 6 h in the light followed by fixation with 3% glutaraldehyde solution in 0.1 M sodium-potassium phosphate buffer, pH 7.4, for 2 h at 4°C, then with 1% osmium tetroxide solution in the same buffer for 1.5 h, and dehydrated through ethanol. Ethanol (70%) was saturated with uranyl acetate (1.5%). The material was embedded in the epoxy resin Epon 812. Serial ultrathin sections were prepared using an LKB-III (LKB, Sweden) ultramicrotome. The sections were placed on blends covered with a formvar film and stained with Reynolds' lead citrate [25]. The grids were observed and photographed using a HU-11B electron microscope (Hitachi, Japan). Figures demonstrate the data of typical experiments.

RESULTS

Light microscopy. Judging from the light microscopy observations (Fig. 1), cyanide caused destruction and disappearance of guard cell nuclei in pea leaf epidermis peels. The effect of cyanide increased under illumination or upon treatment of the peels with cycloheximide inhibiting protein synthesis in the cytoplasm. The effect of cycloheximide was exhibited both in the dark and in the light. Lincomycin, an inhibitor of protein synthesis in mitochondria and chloroplasts, suppressed the CN^--induced nucleus destruction; its effect was more expressed in the dark than in the light. A comparison of effects of lincomycin and cycloheximide + lincomycin on the CN^--induced destruction of nuclei showed that lin-

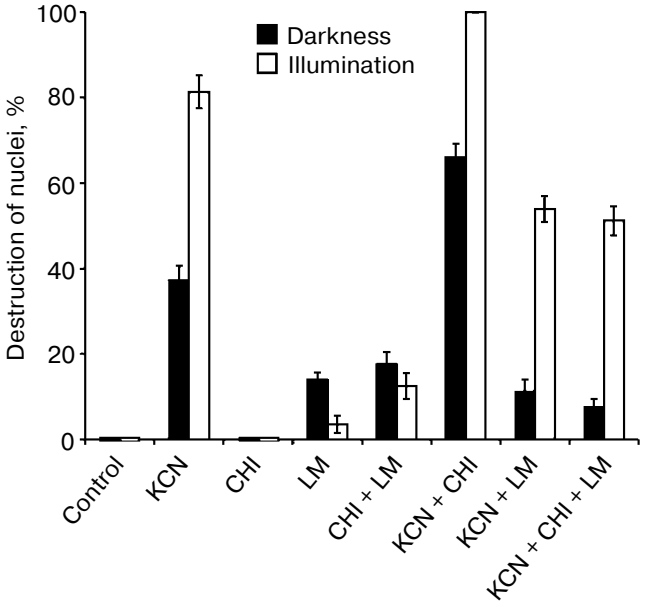

Fig. 1. Effects of cycloheximide (CHI) and lincomycin (LM) on CN^--induced destruction of guard cell nuclei in pea leaf epidermis peels in the dark and in the light. Additives: 2.5 mM KCN, 0.2 mM CHI, and 2 mM LM. The epidermal peels were submerged into distilled water following infiltration with CHI and/or LM and incubated for 30 min in the dark; then the peels were treated with KCN followed by infiltration and incubated for 20 h either in the dark or in the light. Destruction of nuclei was estimated using light microscopy.

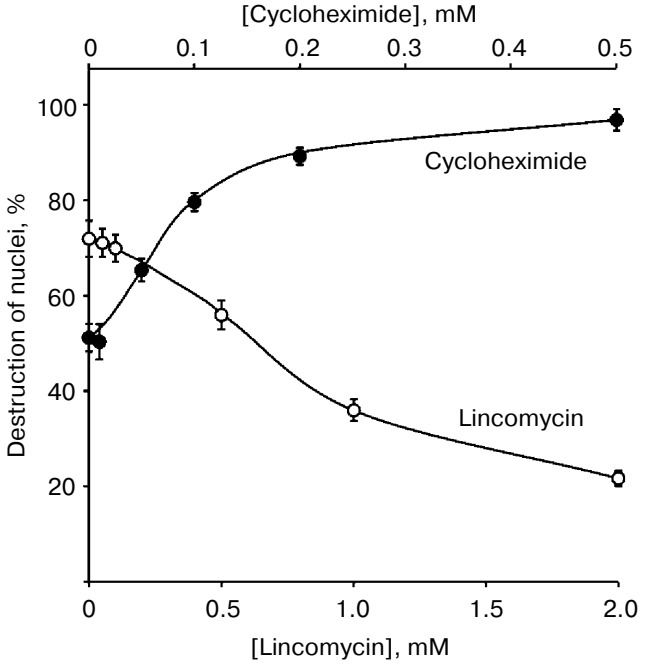

Fig. 2. CN^--induced destruction of guard cell nuclei in pea leaf epidermis peels at various concentrations of cycloheximide and lincomycin in the light. Incubation time was 16 h with cycloheximide and 21 h with lincomycin. Other conditions were the same as in the Fig. 1. Destruction of nuclei was not observed in controls without KCN or with 0.2 mM cycloheximide and was 3.5 ± 2% in presence of 2 mM lincomycin. Destruction of nuclei was estimated using light microscopy.

comycin reduced the effect of cycloheximide in the light and completely prevented it in the dark (Fig. 1).

Figure 2 shows that destruction of guard cell nuclei in illuminated epidermal peels increases with increase in cycloheximide concentration (10-500 μM) and decreases with increase in lincomycin concentration (0.05-2 mM). It was reported that cycloheximide taken at concentration of 50 μM precluded, whereas at 100 μM enhanced the apoptosis of palisade parenchyma cells in the leaves of transgenic tobacco plants with reduced activity of catalase [26]. We did not observe such effect of cycloheximide on pea guard cells (Fig. 2).

Unlike cycloheximide, lincomycin—alone or in combination with cycloheximide—caused appreciable (up to 10-15%) destruction of guard cell nuclei during incubation of epidermal peels for 20 h in absence of CN^- (Fig. 1). Figure 3 shows that further incubation of epidermal peels does not result in significant destruction of nuclei in the presence of lincomycin or lincomycin + cycloheximide, whereas almost 100% destruction of nuclei is observed in the presence of cycloheximide after 48-h incubation in the light or 72-h incubation in the dark.

The effect of cycloheximide on CN^--induced destruction of nuclei is time-dependent. After 6 and 18 h of incubation, the destruction of nuclei in the presence of CN^- + cycloheximide was lower than that in presence of

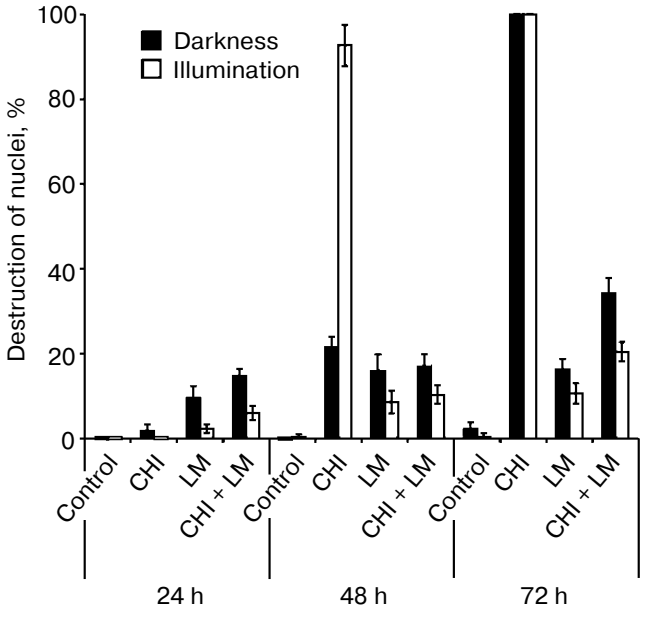

Fig. 3. Destruction of guard cell nuclei depending on time of incubation of pea leaf epidermis peels with cycloheximide (CHI), lincomycin (LM), and CHI + LM in the dark and in the light (in absence of CN^-). Additives: 0.2 mM CHI and 2 mM LM. Destruction of nuclei was estimated using light microscopy.

Effect of cycloheximide on CN^--induced destruction of guard cell nuclei from pea leaf epidermis in the light (from the data of light microscopy)

Incubation time, h	CN^-	CN^- + cycloheximide
6	5.6 ± 1.3	2.0 ± 1.0
18	51.3 ± 3.3	37 ± 2.5
19.5	63.9 ± 2.8	63.5 ± 3.5
24	89.1 ± 3.0	99.3 ± 1.3

Note: Experimental conditions: age of seedlings, 12 days; infiltration with cycloheximide, 1 min; preincubation, 30 min; addition of KCN and infiltration, 1 min; incubation time is specified in the table. Additives: 2.5 mM KCN and 0.2 mM cycloheximide.

CN^- only (table). However, cycloheximide enhances the CN^--induced degradation of nuclei after 24 h of incubation: it becomes higher than in the presence of CN^- alone.

Electron microscopy. Judging from electron microscopy, CN^- caused ultrastructural changes in nuclei (Fig. 4, a and b). A significant number of nuclei lacked membranes. Nuclear membrane fragments were freely disposed in the cytoplasm, but nonetheless the nuclear contents did not interfuse the cytoplasm: the nucleus in the form of an individual structure was stretched in thin cytoplasmic bands in the middle of the cell. Chromatin was in condensed state characteristic of apoptosis. Moreover, vacuolization of cytoplasm and destructive changes of chloroplasts (breaks in their membranes) appeared. At the same time, the plasma membrane remained uninterrupted.

When epidermis was incubated with cyanide and cycloheximide the guard cell nucleus also contained membraneless areas; however, chromatin was decondensed, and it was hard to draw the line between the nuclear contents and surrounding cytoplasm in open areas (Fig. 5, a and b). Despite partial obliteration of the nuclear membrane, there were no destructive changes in cytoplasm and cellular organelles in guard cells. However, characteristic features of apoptosis were obvious. Vacuoles appeared and tended to fill up the guard cell volume (Fig. 5, a and b), whereas the volume of cytoplasm appropriately decreased. In epidermal peels incubated with CN^- and cycloheximide (Fig. 5) compared with the peels incubated with CN^- only (Fig. 4b), the guard cell vacuoles contained a significant amount of membrane-circled cytoplasmic particles as a result of constriction of cytoplasmic fragments.

The ultrastructure of chloroplasts and mitochondria in specimens treated with CN^- and cycloheximide (Fig. 5, c and d) is in a sharp contrast with that of control (Fig. 4a). Chloroplasts had increased electron density of stroma, thylakoid grana were dislodged to one side of the organelle, and the membrane envelope formed multiple invaginations, so that the chloroplast stroma looked perforated (Fig. 5c). Mitochondria had ultracondensed matrix, a characteristic feature of apoptosis (Fig. 5d). Moreover, an enclosed space surrounded by membrane and filled with granules appeared in the central part of mitochondria.

Lincomycin prevented the CN^--induced changes in ultrastructure of guard cells (Fig. 6a). The nucleus maintained its integrity and did not differ in ultrastructure from the control nucleus (Fig. 4a). Vacuoles did not differ in size from vacuoles in control cells; however, both chloroplasts and mitochondria demonstrated significant

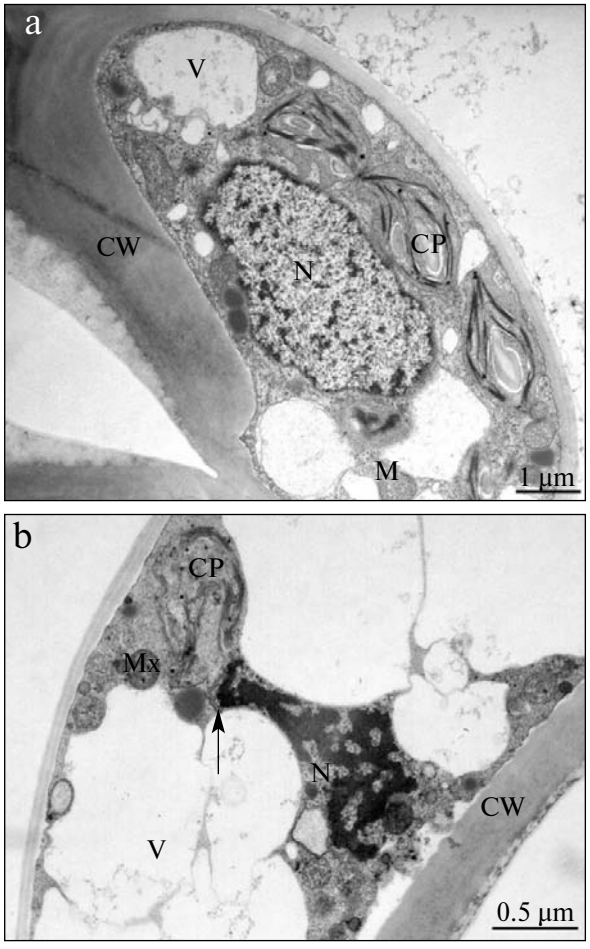

Fig. 4. Electron micrographs of stoma guard cells of pea epidermis: a) in peels incubated in 0.1 M sodium-potassium phosphate buffer, pH 7.4, without additives; b) in peels incubated in 0.1 M sodium-potassium phosphate buffer, pH 7.4, containing 2.5 mM KCN. The arrow points to nuclear membrane fragments freely disposed in the cytoplasm. Here and further: CP, chloroplast; CW, cell wall; M, mitochondria; N, nucleus; V, vacuole.

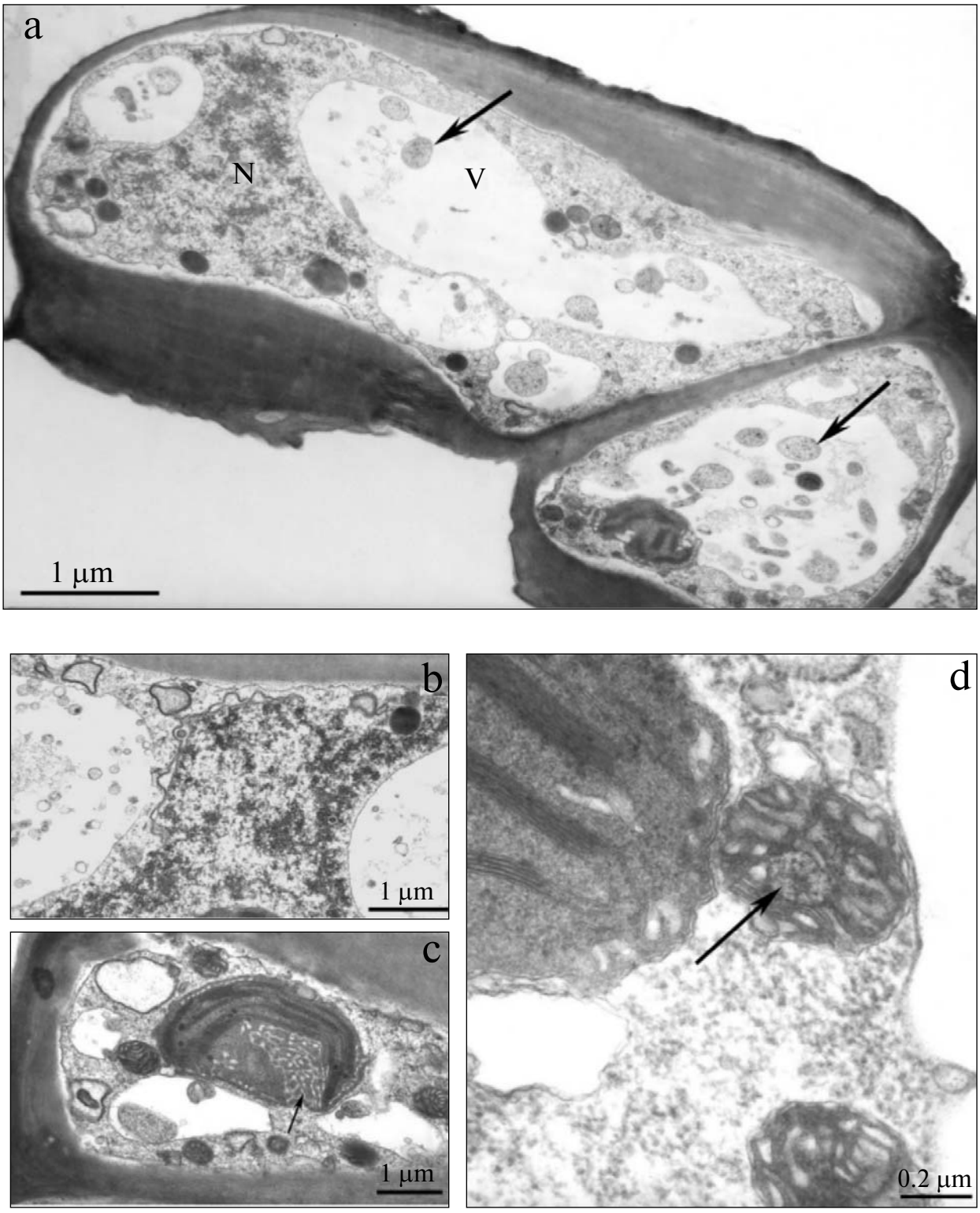

Fig. 5. Electron micrographs of stoma guard cells of pea epidermis incubated with 2.5 mM CN^- and 0.2 mM cycloheximide: a) general view of stoma, arrows point to cytoplasmic particles surrounded by membrane; b) cell part with nucleus and damaged nuclear membrane; c) cell part with chloroplast, arrow points to an invagination of membranous envelope with formation of perforated stroma; d) cell part with mitochondrion, arrow points to a restricted membranous area filled with granular inclusions.

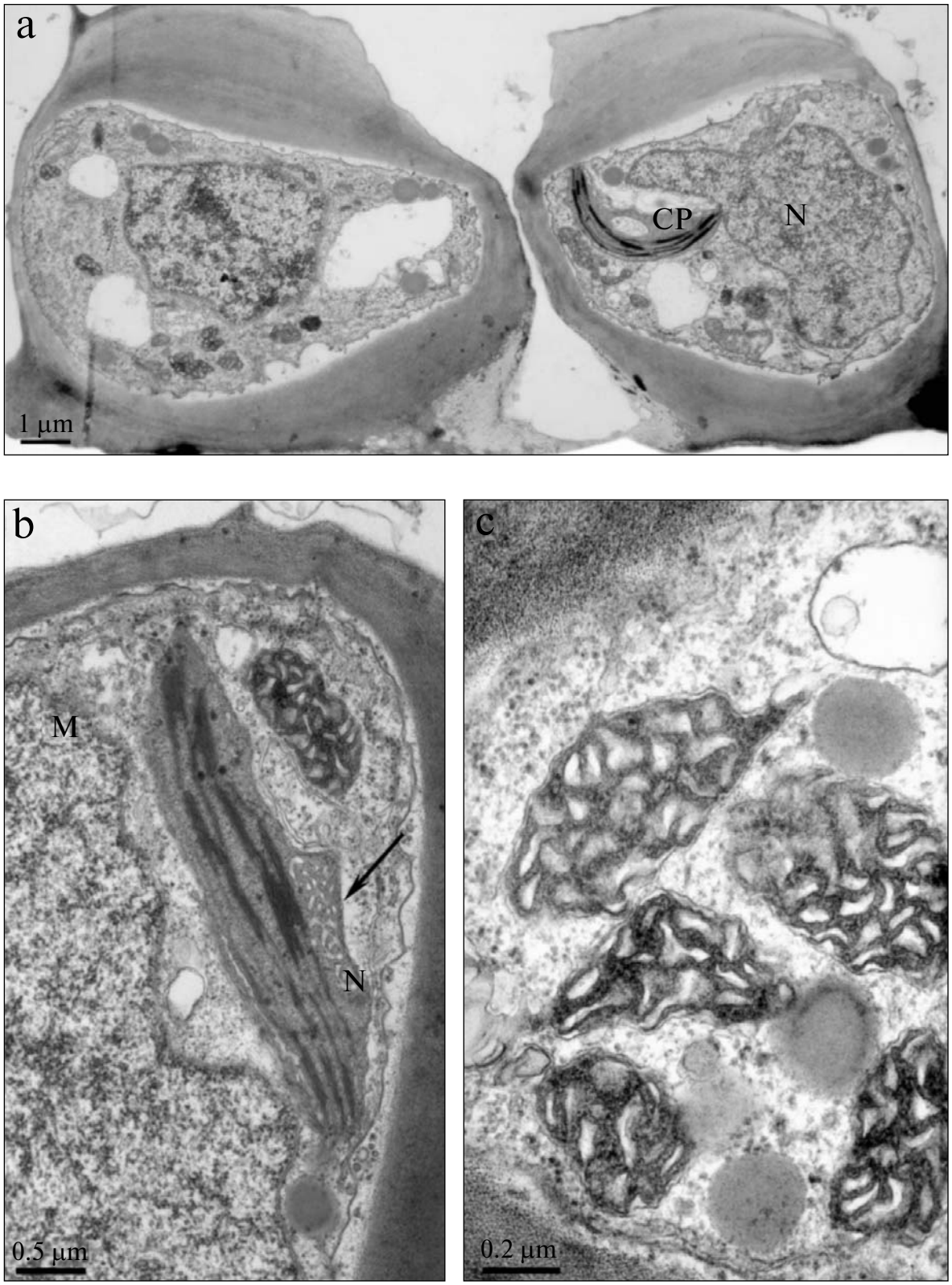

Fig. 6. Electron micrographs of guard cells of pea epidermis incubated with 2.5 mM CN^- and 2 mM lincomycin: a) general view of stoma; b) cell part containing chloroplast, arrow points to a perforated area of stroma; c) cell part containing mitochondria.

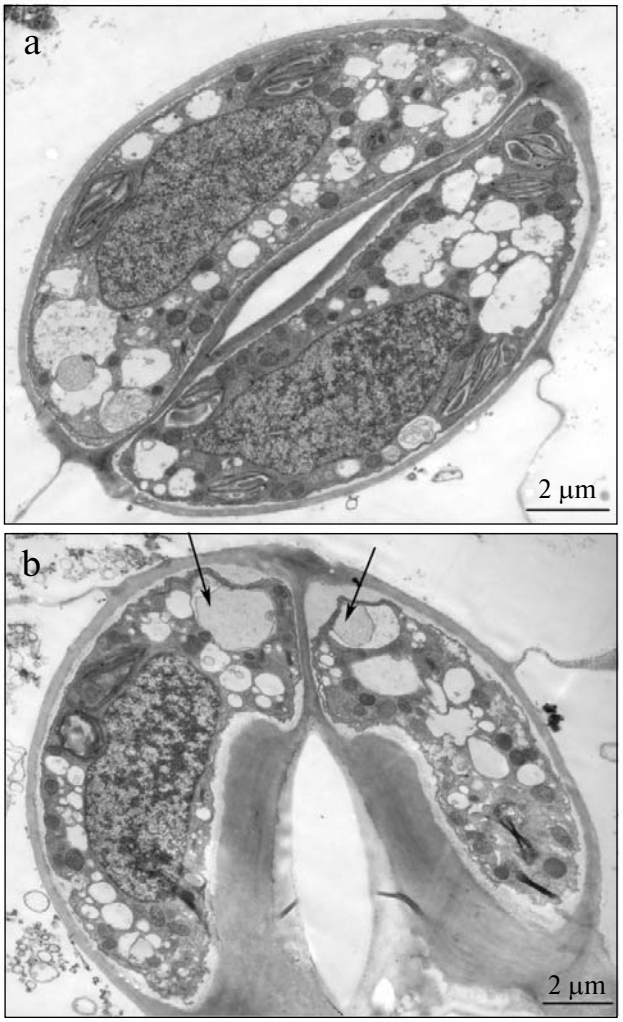

Fig. 7. Electron micrographs of stoma guard cells in pea epidermis incubated with: a) 2.5 mM CN^-, 0.2 mM cycloheximide, and 2 mM lincomycin; b) 0.2 mM cycloheximide and 2 mM lincomycin. Arrows point to autophagosomes in vacuoles.

difference from the control. Chloroplasts had increased electron density of stroma. Like in the presence of cycloheximide, in some chloroplasts thylakoid grana were dislodged to one side of the organelle (Fig. 6a) and some areas of chloroplast stroma had perforated structure as a result of membrane invagination; however, these changes were less expressed (Fig. 6, a and b). Two mitochondrial populations appeared: 1) large, anomalously branched, with electron-transparent matrix and watered intermembrane space, and 2) electron-dense, also exceeding the mitochondria of control cells in size (Fig. 6, a and c).

After 6-h incubation, cycloheximide and lincomycin did not cause characteristic apoptotic changes in the absence of CN^-. Nonetheless, we observed some small change in cell ultrastructure. Although we did not observe significant changes from control in nuclei, chloroplasts, and mitochondria, dark electron-dense globules of possible lipid nature appeared in the cytoplasm (data not presented). Moreover, cycloheximide caused slight enlargement of vacuoles and lincomycin caused perforation of chloroplast stroma areas resembling that observed when the cells were treated with CN^- in the presence of cycloheximide or lincomycin.

Figure 7a displays stoma guard cells of epidermis preincubated with CN^-, cycloheximide, and lincomycin. The cytoplasm fragments stand out in vacuoles lysed by hydrolytic enzymes. As compared to the control (Fig. 4a), the mitochondria are characterized by more electron-dense matrix and watered intermembrane space (Fig. 7a). In epidermis preincubated with cycloheximide and lincomycin (without CN^-), autophagosomes are visible in vacuoles (Fig. 7b). Constriction of cytoplasmic fragments bounded by membrane occurs; these structures resemble those shown in Fig. 5a. Mitochondria with electron-dense matrix are rather larger than those in the control (Fig. 4a).

DISCUSSION

We have studied effects of specific protein synthesis inhibitors on cyanide-induced death of plant cells (pea epidermis). Before proceeding to the discussion of results, we will briefly summarize the observations of cyanide effects on plant cells. The form of cell death is determined by intracellular ATP level: ATP-depleted animal and human cells die via necrosis, whereas apoptosis is ATP-dependent [27-29]. The duration of de-energizing is of significant importance: necrotic cells were in small number under ATP deficit conditions for 2 h and achieved 100% in 4 h [30]. The cell energy status may be maintained by mitochondrial respiration as well as by glycolysis [6, 27-29]. So, respiration inhibitors (rotenone, antimycin A, myxothiazol), protonophore uncouplers, and oligomycin, an inhibitor of ATP synthesis and hydrolysis in mitochondria, did not induce apoptosis in HeLa cells, and induced necrosis in combination with 2-deoxyglucose blocking glycolysis [31].

Cyanide inhibits activities of mitochondrial cytochrome oxidase, heme catalase, peroxidases, and Cu,Zn-superoxide dismutase. CN^- inactivates ribuloso-1,5-bisphosphate carboxylase as well [32]. However, plant mitochondria and chloroplasts do not stop their functioning in the presence of CN^-. The mitochondrial respiratory chain of plants contains alternative oxidase, quinol oxidase, which is resistant to CN^- and catalyzes electron transfer from ubiquinone to O_2 [33]. NADH:ubiquinone oxidoreductase (Complex I) therefore maintains its functional activity. As for chloroplasts, CN^-, inhibiting ribuloso-1,5-bisphosphate carboxylase and thereby depressing $NADP^+$ regeneration via NADPH oxidation in the Calvin cycle, results in inhibition of photosynthetic O_2 release. The chloroplast redox chain can function there-

with in a regimen of cyclic electron transfer involving Photosystem I and cytochrome b_6f complex [34] and pseudo-cyclic electron transfer from H_2O involving Photosystem II, cytochrome b_6f complex, and Photosystem I to O_2 with the formation of H_2O [35]. Thus, in pea epidermis peels treated with CN^- the function of mitochondria and chloroplasts in the cell energy supply ($\Delta\mu H^+$ generation, ATP synthesis from ADP and P_i) is maintained, at least during the time necessary for induction of apoptosis. It has been demonstrated previously [11] that first signs of apoptosis in stoma guard cells are seen already in 1 h after their treatment with cyanide.

The inhibition of catalase and peroxidases must result in accumulation of H_2O_2, which mediates the PCD and participates in many signal transduction/transformation pathways both in animals and plants [36]. H_2O_2 regulates activity of Ca^{2+}-channels and protein phosphorylation and dephosphorylation in plants [37-39]. H_2O_2 significantly influences protein synthesis as well. So, H_2O_2 stimulates synthesis of more than 100 various proteins and represses synthesis of more than 70 proteins in *Saccharomyces cerevisiae* cells [40]. These proteins include proteins with antioxidant function, such as cytochrome *c*-peroxidase, catalase, glutathione reductase, superoxide dismutase, and thioredoxin reductase, heat shock proteins, proteases, translation apparatus proteins, enzymes metabolizing carbohydrates, amino acids, nitrogen bases, and citric acid cycle enzymes. The effect of H_2O_2 may be predictably complex in animal and plant cells. H_2O_2 induces significant alterations in mRNA profile in human cells: it induces and represses genes mainly controlling apoptosis and cell cycle, but not the system of antioxidant defense, which, as concluded by authors [41], in mammals, unlike microorganisms, is constitutive, but not inducible.

Exogenous H_2O_2 is subjected to decomposition by plant cells [42]. High doses of H_2O_2 are necessary to induce PCD in plants. So, aleurone cells treated with abscisic acid were totally insensitive to H_2O_2 added at concentration of 325 mM [43]. Nevertheless, moderate H_2O_2 concentrations (<5 mM) caused induction of glutathione S-transferase and glutathione reductase genes in soybean cells [41]. H_2O_2 accumulation caused by the inhibition of H_2O_2-decomposing enzymes and inhibition of plastoquinone oxidation in site *o* of cytochrome b_6f complex of chloroplasts induced by inactivation of ribulose-1,5-bisphosphate carboxylase—these are two effects of CN^- apparently causing its effect as an inducer of the programmed death of stoma guard cells [8, 10].

Differences in effects of cycloheximide and lincomycin on CN^--induced PCD of guard cells. We have first demonstrated in the present work that cycloheximide and lincomycin differently influence PCD in plants: cycloheximide enhanced, whereas lincomycin inhibited the CN^--induced destruction of nuclei in pea guard cells. Moreover, lincomycin abrogated the effect of cycloheximide in darkness and significantly reduced it in the light. These data suggest the dependence of apoptosis of stoma cells on combined action of proteins synthesized in cytoplasm and in chloroplasts and mitochondria as well. The protein(s) synthesized in cytoplasm preserve(s) the cell nucleus from CN^--induced destruction, whereas proteins synthesized in mitochondria and chloroplasts contrariwise facilitate destruction of the nucleus. It is still difficult to judge the nature of these proteins. For instance, lincomycin inhibits the synthesis of Photosystem II D1 protein in chloroplasts [23] and in *Synechocystis* 6803 cells [24]. The light stimulation of CN^--induced destruction of guard cell nuclei is absent in a pea mutant with a defect in Photosystem II [9]. Lincomycin decreased the cycloheximide effect in the light and totally prevented its action in the dark (Figs. 1 and 3). These facts demonstrate that the contributions of mitochondria and chloroplasts in guard cell apoptosis are different. Data has been obtained earlier that enables a supposition that reduced plastoquinone in chloroplasts plays a role in initiation of apoptotic response of the guard cells [8, 10], whereas the reduced ubiquinone in mitochondria apparently renders antiapoptotic action [10]. The effects of cycloheximide and lincomycin depend on the equilibrium shift between the synthesis of proapoptotic and antiapoptotic proteins, such as caspases or caspase-like proteases, on one hand, and chaperone proteins and antioxidant enzymes on the other hand. In particular, cycloheximide activating various protein kinases and gene transcription factors in animal cells might demonstrate both damaging and protective effects [14]. At low concentrations (100 nM) causing only moderate and/or short-term decrease in protein synthesis, cycloheximide can abrogate cell death via induction of the synthesis of antioxidant enzymes (particularly, Mn-superoxide dismutase) and anti-apoptotic protein Bcl-2 [14]. Another mechanism of antiapoptotic action of cycloheximide is possibly associated with mobilization of cysteine unavailable for protein synthesis and its involvement in the synthesis of glutathione, an effective intracellular antioxidant inhibiting apoptosis [44].

Effect of cycloheximide is expressed autophagy. In accordance with work [11], CN^- induced ultrastructural alterations in guard cells are typical for apoptosis. The most prominent effect of cycloheximide on the background of CN^- and/or lincomycin was autophagy, the appearance of numerous cytoplasm vesicles in vacuoles (compare Figs. 4b, 5a, and 7, a and b).

Autophagy is nonselective decomposition of cytoplasm components with involvement of lysosomal and vacuolar hydrolytic enzymes. This process plays an important role in maintenance of cellular homeostasis under starvation (cell make-up with amino acids and other compounds as a result of hydrolysis of intracellular macromolecules) and in regulation of metabolism (for instance, autophagy of ribosomes and mitochondria in reticulocytes during erythrocyte maturation in mammals)

and in realization of PCD [45-49]. Autophagy has been demonstrated in higher plant cells under carbon and nitrogen starvation [50-53], during the germination of seeds autophagy of starch granules [54], and during aging of leaves [53, 55].

The forms of cell death, via apoptosis or autophagy, are often concomitant to each other [48]. Selective autophagy of depolarized mitochondria (with impaired generation of membrane potential) was observed in hepatocyte apoptosis induced by opening of giant pores in the inner mitochondrial membrane [56] and also in neurons with impaired apoptosis program by the inhibition of caspases [57].

Cycloheximide inhibiting the biosynthesis of proteins encoded by nuclear DNA apparently disturbs the course of CN^--induced apoptosis in guard cells and enhances autophagy, an alternative PCD pathway. This phenomenon was exhibited not only in formation of cytoplasmic vesicles in vacuoles, but also in inhibition of apoptotic condensation of nuclear chromatin (Fig. 5). Cycloheximide, however, did not prevent the appearance of nuclear membrane breakage. The nuclear pore is a complex structure; hence, it can be easily damaged, and the nuclear membrane as a result. To compare, mitochondrial, nuclear pore, and Golgi apparatus proteomes are composed of 615, 174, and 136 polypeptides, respectively [58].

As demonstrated by the light microscopy (table), CN^--induced disappearance of guard cell nuclei was retarded by cycloheximide, but accelerated in the course of time, and eventually the content of enucleated cells with CN^- + cycloheximide became higher than with CN^- alone.

Effect of cycloheximide on ultrastructure of chloroplasts and mitochondria in the presence of CN^-. It has been shown earlier [11] that CN^--induced apoptosis in guard cells is accompanied by disturbance of chloroplast tegument integrity, which does not result in stroma diffluence and disturbance of thylakoid grana. The significant alteration observed with cycloheximide on the background of CN^- is the absence of chloroplast tegument breaking and its invagination into the stroma (Fig. 5, a and c). Cycloheximide apparently inhibits the synthesis of enzymes providing membrane lysis of the chloroplast covering. Chlorosis, the pigment bleaching, is a diagnostic sign of ill-being of plant cells including PCD, aging of leaves, and hypersensitive plant response to microbial attack. Most enzymes participating in chlorophyll degradation are located in the inner membrane of chloroplast tegument [59]. Therefore, the invagination of the envelope, whose appearance looks like perforations, may play a role in spatial approaching of membrane enzymes with thylakoids in which photosynthetic pigments are localized. Chloroplasts *per se* contain a substantial set of proteases [46]; however, vacuolar enzymes apparently participate in degradation of chloroplasts as well [59-61].

Mitochondria of guard cells under CN^--induced apoptosis undergo minor alterations [11]. The treatment of pea leaf epidermis with CN^- and cycloheximide induces increase in the electron density of chloroplast matrix and stroma to a greater extent than the treatment with CN^- alone (Figs. 4b and 5, a and d). Closed formations with granules appeared inside the mitochondria.

Effect of lincomycin on ultrastructure of guard cells in the presence of CN^-. In accordance with the data of light microscopy (Figs. 1 and 2), lincomycin inhibited CN^--induced ultrastructural changes of guard cell nucleus (Fig. 6). This is the evidence for mitochondrial and chloroplastal origin of factors triggering the mechanism of PCD. These factors are of protein nature: their CN^--induced synthesis is inhibited by lincomycin. The preservation of cell nucleus provided by lincomycin on the background of CN^- was accompanied by the perforation of chloroplasts, although less prominently than in the presence of CN^- and cycloheximide, as well as condensation of chloroplast stroma and prominent heterogeneity of mitochondria (their separation by the extent of matrix condensation and the appearance of large organelles).

Effect of cycloheximide and lincomycin in the absence of CN^-. Cycloheximide and lincomycin affected slightly the ultrastructure of guard cells in absence of CN^-. Some enlargement of vacuoles and appearance of electron-dense globules in cytoplasm was observed 6 h after the treatment with cycloheximide, and signs of perforation in chloroplast stroma were observed after the treatment with lincomycin. The globules are apparently reserve substances of lipid nature. The guard cells had prominent autophagosomes in vacuoles in epidermis preincubated with cycloheximide and lincomycin (Fig. 7, a and b).

Under more prolonged observation using light microscopy (Fig. 3), cycloheximide induced decomposition of guard cell nuclei prevented by lincomycin. The process was accelerated under illumination: almost complete decomposition of nuclei with cycloheximide in the light appeared after 48 h, and in dark after 72 h of the observation.

Lincomycin also induced slight destruction of nuclei, more prominently in darkness than in light (Fig. 3). Maximum level of nuclear decomposition with lincomycin was achieved already after 24 h of observation. It did not exceed 15% and was maintained at this level after 48 and 72 h. Two fractions of guard cells apparently exist: one of them is predisposed, and another is resistant to initiation of PCD with lincomycin.

These data support an assumption that the factors initiating the programmed death of guard cells originate from chloroplasts and mitochondria, whereas more extensively from chloroplasts than from mitochondria.

Light and electron microscopy in PCD registration. The data of light microscopy and the data of electron microscopy obtained in the present work are concordant and compliment each other. Electron microscopy reveals

signs of PCD at earlier stages of observation (6 h and shorter [11]) than the light microscopy (15-20 h). Nevertheless, light microscopy enables reliable detection of PCD in plants. This is convenient for express diagnostics of the process, especially with consideration for simplicity and accessibility of the light microscope.

The fragmentation and disappearance of nuclei registered in the light microscope, on one hand, and the condensation and margination of chromatin, the vacuolization and decrease in cytoplasm volume, the breaking of nuclear and chloroplast tegument, the condensation of chloroplast stroma and mitochondrial matrix, appearance of perforations in chloroplasts and similar alterations in mitochondria, and the maintenance of the integrity of cytoplasm membrane, on the other hand, suggest that CN^--induced death of guard cells represents PCD—apoptosis, autophagy, and their combination. All these structural alterations are sensitive to inhibitors of protein synthesis.

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