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Death of Stoma Guard Cells in Leaf Epidermis under Disturbance of Energy Provision

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Abstract—Cyanide is an apoptosis inducer in stoma guard cells from pea leaf epidermis. Unlike CN^-, the uncoupler of oxidative and photosynthetic phosphorylation carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the combination of CCCP, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU), benzylhydroxamate (BH), myxothiazol, antimycin A, and a glycolysis inhibitor 2-deoxyglucose (DG) did not induce destruction of guard cell nuclei for 20 h of incubation of epidermal peels in the light. DCMU prevented the effect of CN^- as a programmed cell death (PCD) inducer. CCCP, the combination of DCMU and CCCP, or the combination of DCMU, CCCP, BH, myxothiazol, antimycin A, and DG supplemented by CN^- caused destruction of cell nuclei; the number of the cells lacking nuclei in this case was higher than with CN^- alone. DG and CCCP caused cell destruction after longer incubation of the isolated epidermis – after 2 days and to a greater degree after 4 days. The effect of DG and CCCP was reduced by illumination. Cell destruction during long-term incubation was prevented by the combination of DG and CCCP. From data of electron microscopy, DCMU and dinitrophenyl ester of iodonitrothymol (DNP-INT) prevented apoptotic changes of the nuclear ultrastructure induced by CN^-. The suppression of the destruction of the guard cell nuclei under combined action of DG and CCCP was apparently caused by switching of cell death from PCD to necrosis. Thus, the type of cell death – via apoptosis or necrosis – is controlled by the level of energy provision.

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Programmed cell death (PCD) is a physiological process of self-destruction of the cell. Different forms of PCD, such as apoptosis, autophagy, autolysis, and their mixed forms can pass into necrosis, a non-programmed cell death. PCD in animals leads to destruction of the nucleus and complete disappearance of the cell due to phagocytosis (heterophagy). PCD in plants also leads to decay of the nucleus, but the cell wall remains untouched or undergoes destruction at later stages of the process. Formation of internucleosomal DNA fragments (each is a multiple of 180-200 bp), which give a ladder on electrophoresis, is an essential feature of apoptosis [1, 2]. In necrosis, the plasma membrane is disrupted, whereas the nucleus – at early stages of the process – maintains its integrity or undergoes insignificant morphological changes [3, 4]. The nucleus also remains unchanged for a long time in both autophagy [5] and a special PCD form resembling autophagy called paraptosis [6].

In human and other animal cells, their energy state determines the type of cell death. A cell depleted of ATP dies via necrosis. Apoptosis depends on ATP [3, 4]. Similarly, autophagy also depends on ATP: even a small decrease in ATP level leads to significant increase in both AMP level and activity of AMP-dependent protein kinase regulating activity of the serine/threonine kinase mTOR (mammalian target of rapamycin) and inhibiting autophagy [7].

The number of cells dying via necrosis depends on duration of their stay in de-energized state: in ATP deficiency, the number of necrotic cells (cultured rat myocar-

Abbreviations: BH) benzylhydroxamate; CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; DCF) 2′,7′-dichlorofluorescein; DCFH) 2′,7′-dichlorofluorescin; DCMU) 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DG) 2-deoxyglucose; DNP-INT) dinitrophenyl ester of iodonitrothymol; PCD) programmed cell death; ROS) reactive oxygen species.

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diocytes) was insignificant after 2 h and reached 100% after 4 h [8]. Necrotic cell death is evoked by decrease in mitochondrial transmembrane potential (depolarization of the inner mitochondrial membrane) and, like apoptosis, depends on reactive oxygen species (ROS) [8]. Depolarization of mitochondria in hepatocytes also leads to apoptosis [9]. The energized state of the cell can be maintained by the mitochondrial respiratory system, as well as by glycolysis [3, 4]. In particular, inhibitors of respiration, protonophore uncouplers, and oligomycin, which inhibit both synthesis and hydrolysis of ATP in mitochondria, did not evoke apoptosis of HeLa cells, whereas additional blockage of glycolysis by 2-deoxyglucose (DG) induced apoptosis or – after more prolonged and severe decrease in ATP level in cells – necrotic death [10]. The role of mitochondria in apoptosis in animals is not limited by energy provision. Mitochondria are also providers of ROS and some other apoptogenic factors, such as cytochrome *c*, the flavoprotein AIF (apoptosis inducing factor), caspase activator Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with low p*I*), serine protease Omi/HtrA2, and endonuclease G [11-13].

Like in animals, decrease in mitochondrial transmembrane potential in plants is an early PCD sign [14-17] preceding cytochrome *c* release from the intermembrane space of mitochondria [18]. However, cytochrome *c* release from mitochondria does not always correlate with PCD in plants. When protoporphyrin IX was taken as PCD inducer, the release of cytochrome *c* from *Arabidopsis thaliana* mitochondria occurred only after the death of the cells [18]. Alternatively, $\Delta\psi$ decrease in mitochondria preceded cytochrome *c* release into cytoplasm, when ceramide was taken as PCD inducer [18]. Cytochrome *c* release in plants might be not a harbinger of PCD but rather a consequence of mitochondrial destruction in PCD, so further molecular-biological and genetic studies are required to elucidate this [19].

During apoptosis of cells from suspended cultures isolated from the callus on soybean stems, the levels of ATP and glucose-6-phosphate remained high, whereas necrosis was accompanied by significant decrease in both levels [17]. The treatment of *A. thaliana* cells from suspended cultures with H_2O_2 generated in the glucose oxidase reaction also led to decrease in ATP level, cytochrome *c* release from mitochondria, and cell death [20].

One can suppose *a priori* that chloroplasts play a role in energy supply during PCD. There are some data on involvement of chloroplasts in PCD. Experiments with pea leaf epidermis have demonstrated that cyanide, an inducer of PCD, causes condensation and margination of chromatin and fragmentation of nuclei followed by disappearance of nuclear fragments from stoma guard cells [21, 22]. Nuclei also disappeared in basic epidermal cells. Illumination significantly accelerated death of guard cells (containing both chloroplasts and mitochondria) but not epidermal cells (containing mitochondria only) of pea leaves [21]. Substances used as electron acceptors in the Hill reaction prevented the effect of CN^- on guard cells [23, 24]. 3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of electron transfer between plastoquinones Q_A and Q_B in the Photosystem II (PS II), canceled the light stimulation of CN^--induced destruction of guard cell nuclei. The same effect was incident to quinone analogs, such as dinitrophenyl ester of iodonitrothymol (DNP-INT) and stigmatellin, the competitive inhibitors of plastoquinol oxidation on site *o* of chloroplast cytochrome b_6f complex [23, 24]. Earlier we have tested pea mutants defective in either Photosystem I (PS I) or PS II or both systems [25]. The experiments demonstrated that light stimulation of CN^--induced destruction of guard cell nuclei and its withdrawal by DCMU are associated with functioning of PS II [25]. Thus, chloroplasts are involved in PCD. The process depends on ROS and seems to be regulated by redox state of plastoquinone on the site *o* of chloroplast cytochrome b_6f complex of the photosynthetic electron-transfer chain [23, 24].

Nevertheless, defects or even absence of chloroplasts are not a cause of plant cell death during ontogenesis [26]. Etiolated plantlets, as well as plantlets of mutants *albina* and *xantha* with defective chloroplasts, can normally grow till depletion of reserve substances. Leaves with viral mosaic (foliage variegation) develop until the size of photosynthesizing tissue becomes insufficient for maintenance of life of non-photosynthesizing tissue. The loss of green color caused by degradation of chlorophyll is one of first signs of aged leaves. Chlorosis also develops under plant starvation. Both isolated cells and callus cultures of plants possess restricted photosynthetic activity and so are only able to maintain chemoorganotrophic metabolism.

What is the effect of energy metabolism disturbance on the state of plant cell nuclei? This study was intended to elucidate this question. It has been shown that the type of cell death is determined by the level of cell de-energization. Cells died via apoptosis when carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of oxidative and photosynthetic phosphorylation, was added. Glycolysis inhibition by 2-deoxyglucose (DG) also induced apoptosis. The combination of CCCP and DG led to the breakage of cell membranes and necrotic cell death without changes in the nuclei.

MATERIALS AND METHODS

The experiments were carried out on lower epidermis isolated from leaves of pea (*Pisum sativum* L. cv Alpha) seedlings grown for 7-15 days under continuous illumination at 20-24°C [21]. Epidermal peels were separated with

forceps and placed into distilled water. The infiltration method of incubation of the epidermis under vacuum for 1-2 min was used for rapid influx of added reagents into the cells. The samples were placed in polystyrene plates and incubated in distilled water with additives (the composition is given in legends for figures) at room temperature either in the dark or under illumination with a luminescent lamp at the light intensity of ~1000 lx.

After the incubation, the samples were treated for 5 min with Battaglia fixative (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 Carazzi's hematoxylin for 20 min. The stained peels were washed with tap water and inspected under a light microscope. The portion of cells with destroyed nuclei and lack of nuclei was determined from 300-500 inspected cells (for each epidermal peel) [21].

For electron microscopy, the samples were incubated in 0.1 M Na^+,K^+-phosphate buffer, pH 7.4, followed by fixation with 3% glutaraldehyde solution in the same buffer for 2 h at 4°C, then with 1% osmium tetroxide solution in the same buffer for 1.5 h, and dehydrated using ethanol. A 70% ethanol solution 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 ultramicrotome (LKB, Sweden). The sections were placed on Formvar coated grids and stained with Reynolds' lead citrate [27]. The grids were observed and photographed using an HU-11B electron microscope (Hitachi, Japan).

DNA was isolated from lower epidermis of 14-day-old pea seedlings. For DNA isolation, about 25-35 mg of epidermis was used. Following incubation with reagents the samples were powdered by grinding in a mortar with liquid nitrogen, suspended, and dissolved in lytic buffer solution (0.05 M Tris-HCl, pH 7.4, 0.025 M EDTA, and 1% sodium dodecyl sulfate) at room temperature. Homogenates were treated with 5 M NaCl to discharge DNA-associated proteins and then twice deproteinized by agitation with phenol. Nucleic acids from aqueous phase separated by centrifugation were precipitated with isopropanol. Precipitate was dissolved in TE-buffer (0.05 M Tris-HCl, pH 7.5, 0.005 M EDTA) and treated with RNase A solution (Sigma, USA) added to the concentration of 100 μg/ml for 20 min at 37°C. Following repeated deproteinization with phenol, DNA was precipitated with isopropanol and dissolved in TE-buffer. Electrophoresis of DNA specimens was conducted in 1% agarose gel in 0.04 M Tris-acetate buffer, pH 8.0, containing 0.5 μg/ml of ethidium bromide for 1.5 h at 4-5 V/cm.

Fluorescence of 2′,7′-dichlorofluorescein (DCF) was measured on a VersaFluor fluorimeter (Bio-Rad, USA). Epidermis was fixed on a polystyrene plate by its intact surface, submerged in 50-μM 2′,7′-dichlorofluorescin (DCFH) diacetate solution, incubated in the dark for 10 min, washed with distilled water, and placed into a cell with 25-mM HEPES-NaOH, pH 7.2. DCF fluorescence was excited by light with λ 485-495 nm and recorded at 515-525 nm.

Experiments were repeated 3-4 times. Typical data are given.

RESULTS

Cyanide induced destruction and disappearance of stoma guard cells in epidermal peels isolated from pea leaves (Fig. 1). The nuclei decomposed in more than 60% of stoma guard cells after 20 h of incubation in the light. Cyanide has multiple effects on cells: it inhibits mitochondrial cytochrome *c* oxidase, inactivates ribuloso-1,5-bisphosphate carboxylase/oxygenase in chloroplasts [28], and inhibits activity of Cu,Zn-superoxide dismutase, catalase, and peroxidases including chloroplast ascorbate peroxidase [29]. Unlike CN^-, DCMU inhibiting electron transport from primary plastoquinone to secondary plastoquinone in PS II and proton uncoupler CCCP had no

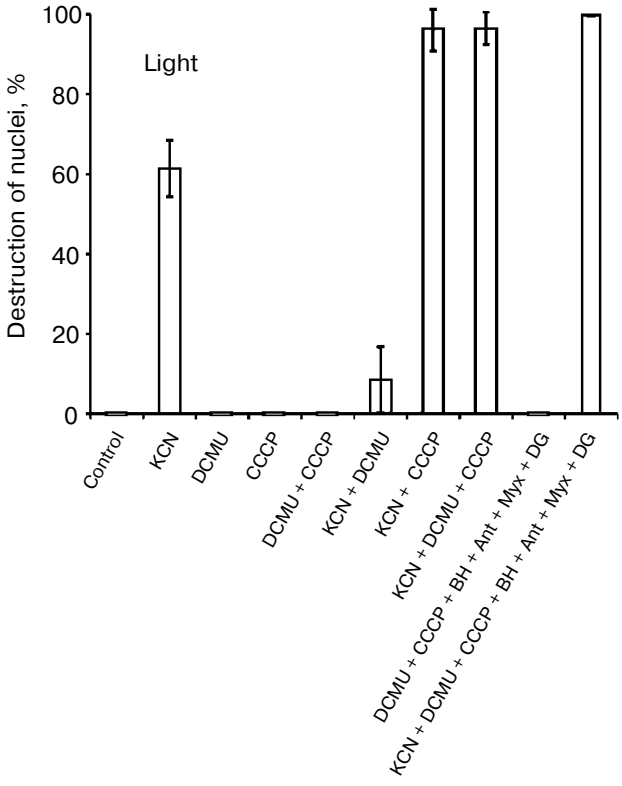

Fig. 1. Light microscopy data on the effect of energy metabolism inhibitors on the nuclei of guard cells of pea leaf epidermis in the light. Epidermal peels after infiltration with 10 μM DCMU, 5 μM CCCP, 10 mM BH, 5 μM myxothiazol (Myx), 5 μM antimycin A (Ant), and 10 mM DG were incubated in the dark for 30 min and thereafter, when indicated, they were infiltrated with 2.5 mM KCN and incubated for 20 h in the light.

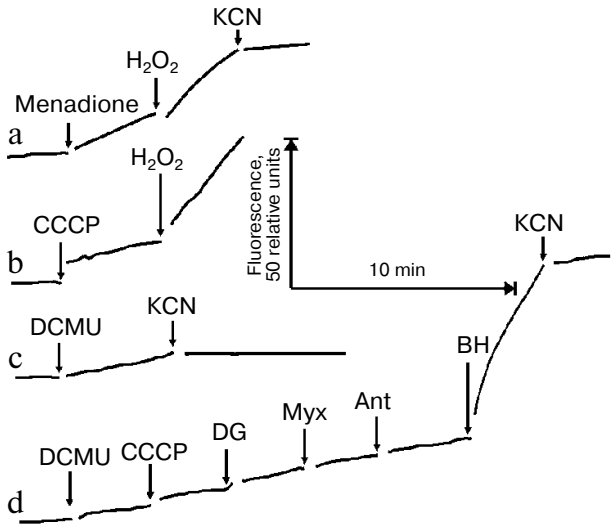

Fig. 2. DCF formation from DCFH diacetate added to epidermal peels from pea leaves. Additives: menadione, 100 μM; H_2O_2, 1 mM; CCCP, 10 μM; KCN, 2.5 mM; DCMU, 10 μM; DG, 10 mM; myxothiazol (Myx), 5 μM; antimycin A (Ant), 5 μM; BH, 5 mM.

influence on nuclei of guard cells. The combination of CCCP with DCMU is ineffective as well. According to data published earlier [23, 24], DCMU decreased the CN^--inducing effect on PCD. In contrast, destruction of nuclei with CN^- and CCCP was higher than with CN^- alone. Moreover, CCCP abolished the inhibiting effect of DCMU on degradation of nuclei induced by CN^-. Cell de-energization by the combination of DCMU, CCCP, BH (an inhibitor of mitochondrial alternative oxidase), myxothiazol (an inhibitor of cytochrome bc_1 mitochondrial complex), antimycin A (an inhibitor of ferredoxin:plastoquinone reductase, a component of cyclic electron transport in chloroplasts [30, 31]), and DG (a glycolysis inhibitor) induced destruction of cell nuclei after 20 h of incubation only when this mixture of inhibitors of energy metabolism was supplemented with cyanide (Fig. 1).

ROS (without which the apoptotic decomposition of guard cell nuclei does not occur) are effectively removed in the absence of CN^-, particularly under anaerobic conditions or addition of antioxidants [23, 24]. Formation of fluorescent dichlorofluorescein (DCF) from non-fluorescent dichlorofluorescin (DCFH) is a sensitive indicator for ROS. H_2O_2-dependent oxidation of DCFH to DCF is accelerated on addition of Fe^{2+} and catalyzed by peroxidase [32]. Figure 2a shows the increase in DCF fluorescence in response to menadione addition. Menadione, being reduced by components of the photosynthetic and respiratory chains, was spontaneously oxidized by O_2 to form of O_2^- and H_2O_2 (Fig. 2a). CCCP and DCMU induced a slight increase in DCF fluorescence (Fig. 2, b and c). Subsequent addition of DG, myxothiazol, and antimycin A had no influence, and only BH increased the fluorescence (Fig. 2d). In all variants, the process was inhibited by CN^-.

Figure 3 demonstrates data on the destruction of guard cell nuclei under more prolonged incubation of epidermal peels with inhibitors. Slight destruction of nuclei was observed in control samples (without addi-

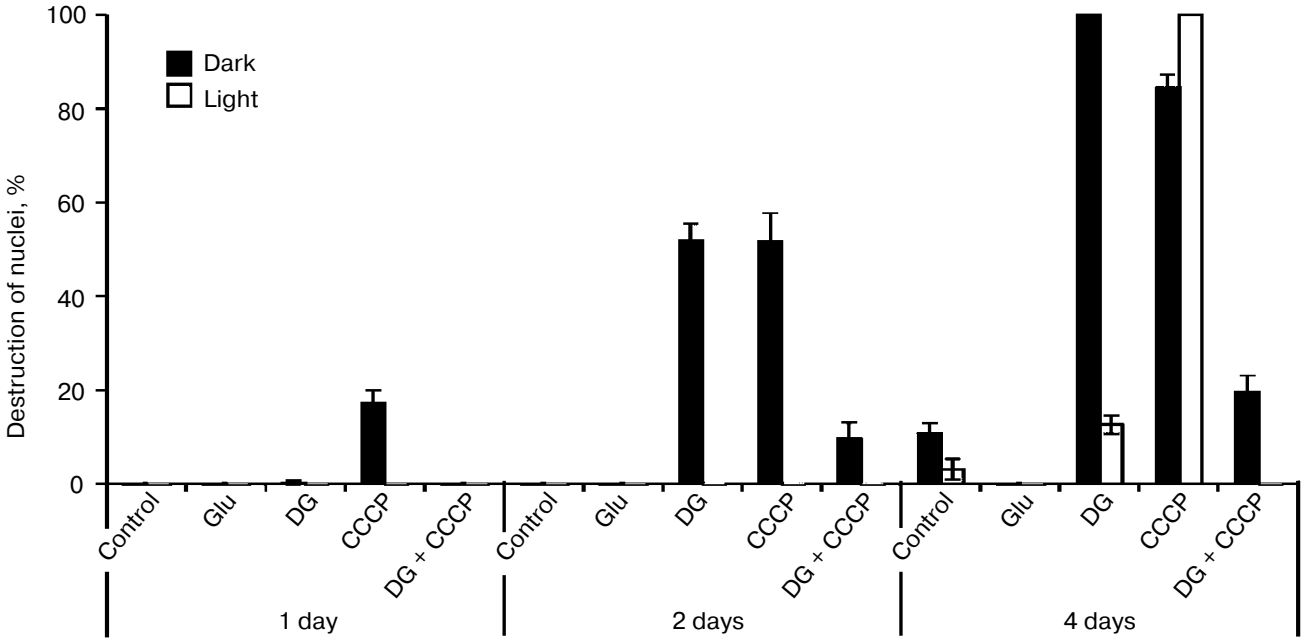

Fig. 3. Light microscopy data on the effect of glucose (Glu), DG, and CCCP on nuclei of guard cells of pea leaf epidermis in the dark and in the light. Epidermal peels after infiltration with 10 mM Glu, 10 mM DG, and 10 μM CCCP were incubated for 1, 2, or 4 days.

tives) only after four days of incubation, which could be prevented by glucose. DG or CCCP induced decomposition of nuclei after two days and, to a greater extent, after four days of incubation. Illumination of samples retarded the effect of DG. The destruction of nuclei with CCCP was also inhibited by light (two days), but under longer incubation (four days) the difference disappeared between the samples incubated either in the dark or in the light. The destruction of guard nuclei was prevented by DG + CCCP (Fig. 3).

The microstructure of guard cells undergoes drastic CN^--induced alterations in time based on the evidence derived from electron microscopy [22]. Condensation and margination of chromatin in guard cells was observed even after 1 h of incubation of isolated epidermis with CN^-. After 6 h of incubation, the volume of guard cells was filled with vacuoles, the cytoplasm occupied a thin wall layer, the nucleus acquired multi-blade shape, was extended in narrow cytoplasm extensions, and, in spite of loss of nuclear membrane unity, had an appearance of separate structure. Mitochondria and chloroplasts were localized in direct contact with chromatin on exposed areas of the nucleus devoid of membrane. Mitochondria were swollen. Chloroplasts, like the cell nucleus, lost the continuity of their membrane, but were not swollen, and preserved their stroma and integrity of the thylakoid system. The pattern of ultrastructural alterations indicates that the CN^--induced death of guard cells is realized via apoptosis [22]. In fact, electrophoresis of DNA isolated from epidermal peels after 6 h of incubation with CN^- demonstrates the typical "DNA ladder" pattern, the intrinsic feature of apoptotic cell death (Fig. 4).

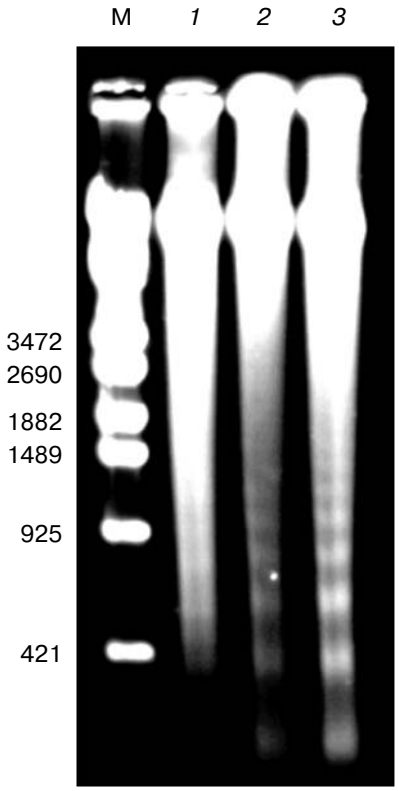

Fig. 4. Electrophoretic separation of oligonucleosomal DNA fragments from epidermal peels of pea leaves preincubated with 2.5 mM KCN in the light. M, DNA markers containing the numbers of nucleotide pairs indicated on the left of panel M; *1*) control, 6 h without additives; *2*) 3 h with KCN; *3*) 6 h with KCN.

DCMU prevented the CN^--induced destruction of guard cell nuclei (Fig. 5a). The ultrastructure of stoma cells was not altered and corresponded to that in control samples after 6 h of incubation of pea leaf epidermis with CN^- and DCMU. DNP-INT prevented the CN^--induced alterations in ultrastructure of nuclei as well. However, sizes of vacuoles were increased, mitochondria were swollen (Fig. 5b), and unusual mitochondrial contacts with nuclei were detected (Fig. 5c).

Unlike DCMU, CCCP induced changes in the spatial organization of chromatin (Fig. 5d). The chromatin was condensed and shifted to nuclear membrane (chromatin margination). However, no significant increase in vacuolar volume or decrease in cytoplasm volume was observed. Chloroplasts were swollen, mitochondria were slightly swollen, became round shaped, the shape of mitochondrial cristae was changed, and matrix was bleached. Similar changes were observed under the influence of DG (data not shown).

When the epidermis was incubated with CCCP + DG, the ultrastructure of stoma cells was substantially changed (Fig. 5e): plasmatic and vacuolar membranes were damaged, chromatin was lumpy, its fine structure was not resolvable, the inner mitochondrial membrane was destroyed, thus resulting in mitochondrial swelling, and chloroplasts were also significantly swollen with their structure degraded.

DISCUSSION

PCD depends on energy provision. Cells in apoptosis have a high level of ATP, whereas necrosis is characterized by significant decrease in ATP level [3, 4, 10, 17]. The duration of de-energization is of great importance. Cell death via apoptosis came on at short period of de-energization, and via necrosis – after longer de-energization of cells [8, 10].

To judge from Fig. 1, incubation for 20 h in the light with the combination of DCMU and antimycin A inhibiting photosynthetic electron transfer, myxothiazol and BH inhibiting mitochondrial respiration, DG inhibiting glycolysis, and CCCP uncoupling both photosynthetic and oxidative phosphorylation does not cause destruction of guard cells. Destruction of nuclei only occurred when this mixture was complemented with cyanide inhibiting heme catalase and peroxidases and thus preventing decomposition of H_2O_2.

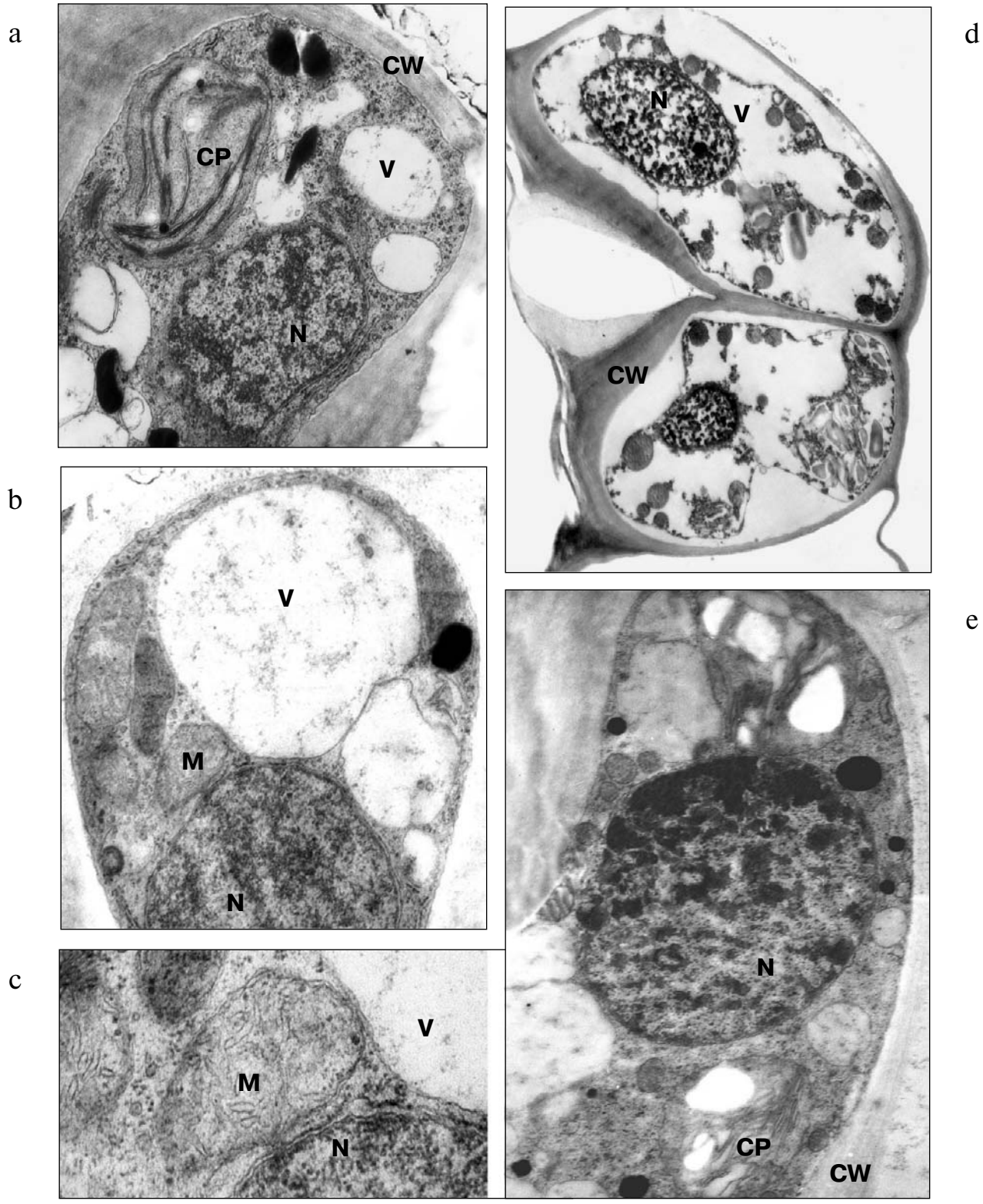

Fig. 5. Electron microscopy data on the effect of KCN in combination with DCMU (a) or DNP-INT (b, c), on the effect of CCCP (d), and DG + CCCP (e) on stoma cell state in pea leaf epidermis in the light. Epidermal peels were incubated for 6 h after their infiltration with 2.5 mM KCN, 10 μM DCMU or 10 μM DNP-INT, 10 μM CCCP and 10 mM DG. Notations: CP, chloroplast; CW, cell wall; M, mitochondrion; N, nucleus; V, vacuole.

H_2O_2-dependent formation of fluorescent DCF from DCFH catalyzed by intracellular peroxidase [32] was inhibited by CN^- (Fig. 2). Some increase in DCF fluorescence was observed on addition of CCCP, DCMU, and particularly BH. CCCP seems to accelerate hydrolysis of DCFH diacetate. The H_2O_2- and CCCP-dependent (pK value is 5.95 [33]) increase of the dye fluorescence measured in absence of epidermal peels decelerated or stopped at pH 5.0 or 8.0 (data not shown). DCMU enhances singlet oxygen production in PS II, although to less degree than do phenolic inhibitors [34]. Singlet oxygen reacts with NAD(P)H to form $O_2^{\cdot-}$ [35]. Inhibition of alternative oxidase of plant mitochondria also enhances production of $O_2^{\cdot-}$ [36] and H_2O_2 with the participation of superoxide dismutase.

As a cause of ultrastructural changes in guard cells that are characteristic of apoptosis [22], cyanide initiates internucleosomal DNA fragmentation (Fig. 4). Since isolated epidermal peels contain two cell types, namely stoma guard cells and basic epidermal cells, a question arises, whose DNA is responsible for the "ladder"? The nuclei of epidermal cells are destroyed and disappear far faster than the nuclei of guard cells upon the action of cyanide: after 1-1.5 and 20-24 h, respectively [21, 24]. So, the DNA ladder observed after 6 h of incubation and imperceptible after 3 h (Fig. 4) might be attributed to fragmentation of guard cell rather than epidermal cell DNA.

DG and CCCP added separately in the dark, but not in the light, caused destruction of guard cell nuclei when the incubation period was prolonged to two days (Fig. 3). Prolongation of incubation to four days led to destruction of the nuclei in the presence of CCCP both in the dark and in the light, whereas further destruction of nuclei in the presence of DG was observed preferably in the dark and to less degree in the light. The light compared with the dark provides the cells with additional possibility for energy provision – via photosynthetic electron transfer. Suppression of destruction of guard cell nuclei upon combined action of DG and CCCP is most likely explained by switching of death types from PCD to necrosis, in which the nuclei of dead cells – on evidence of light microscopy – remain undestroyed.

In necrosis, the protoplast volume increases, cytoplasm and cell organelles disintegrate, plasma membrane breaks, and cell contents release into the intracellular space [11, 37]. The same pattern is observed with electron microscopy of guard cells of pea leaf epidermis after incubation with DG + CCCP (Fig. 5e). The ultrastructure of guard cells underwent great changes: plasma, vacuolar, and nuclear membranes broke, as well as membranes of mitochondria and chloroplasts that swelled; chromatin structure changed. Being added separately, CCCP (Fig. 5d) and DG did not cause such expansive deterioration, and in this case cell death was accompanied by destruction of nuclei (Fig. 3), as with CN^- as inducer of apoptosis (Fig. 1).

According to the data of light microscopy (Fig. 1), electron microscopy (Fig. 5) has shown that DCMU prevents the CN^--induced apoptosis of guard cells. The novel result obtained from our study is that this effect of DCMU is canceled by the uncoupler CCCP (Fig. 1). According to the previously outlined supposition [23-25], apoptosis of guard cells depends on ROS and is regulated by redox state of plastoquinone at the site *o* of cytochrome b_6f complex of chloroplasts. When plastoquinone at site *o* is oxidized (in the presence of DCMU or due to defect of PS II in pea mutant) or displaced by stigmatellin or DNP-INT, apoptosis of stoma cells is prevented. The reduced state of plastoquinone at site *o* (in the presence of CN^-) is conducive to switching on PCD. The CCCP-induced canceling of the effect of DCMU inhibiting apoptosis of stoma cells in the presence of CN^- might be due to alteration in redox state of plastoquinone; the uncoupler CCCP, which stimulates the cyclic electron transfer with involvement of PS I and cytochrome b_6f complex, obviously influences the components of the cyclic chain, transforms plastoquinone into less oxidized state, and, inhibiting simultaneously both photosynthetic and oxidative phosphorylation (but not substrate phosphorylation in glycolysis), turns on PCD.

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