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Reactive Oxygen Species in Programmed Death of Pea Guard Cells

V. D. Samuilov*, D. B. Kiselevsky, A. A. Shestak, A. V. Nesov, and L. A. Vasil'ev

Department of Physiology of Microorganisms, Biological Faculty, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-3807; E-mail: vdsamuilov@mail.ru

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Abstract—Hydrogen peroxide potentiates CN^--induced apoptosis of guard cells recorded as destruction of cell nuclei in the epidermis from pea leaves. A still stronger effect was exerted by the addition of H_2O_2 and NADH, which are the substrates of the plant cell wall peroxidase producing $O_2^{\cdot-}$ coupled to the oxidation of NADH. The CN^-- or (CN^- + H_2O_2)-induced destruction of guard cell nuclei was completely removed by nitroblue tetrazolium (NBT) oxidizing $O_2^{\cdot-}$ and preventing thereby the subsequent generation of H_2O_2. The reduced NBT was deposited in the cells as formazan crystals. Cyanide-induced apoptosis was diminished by mannitol and ethanol, which are $OH^{\cdot}$ traps. The dyes Rose Bengal (RB) and tetramethylrhodamine ethyl ester (TMRE) photosensitizing singlet oxygen production suppressed the CN^--induced destruction of the cell nuclei in the light. This suppression was removed by exogenous NADH, which reacts with $^1O_2$ yielding $O_2^{\cdot-}$. Incubation of leaf slices with RB in the light lowered the photosynthetic O_2 evolution rate and induced the permeability of guard cells for propidium iodide, which cannot pass across intact membranes. Inhibition of photosynthetic O_2 evolution by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea or bromoxynil prevented CN^--induced apoptosis of guard cells in the light but not in the dark. RB in combination with exogenous NADH caused H_2O_2 production that was sensitive to NBT and estimated from dichlorofluorescein (DCF) fluorescence. Data on NBT reduction and DCF and TMRE fluorescence obtained using a confocal microscope and data on the NADH-dependent H_2O_2 production are indicative of generation of reactive oxygen species in the chloroplasts, mitochondria, and nuclear region of guard cells as well as with participation of apoplastic peroxidase. Cyanide inhibited generation of reactive oxygen species in mitochondria and induced their generation in chloroplasts. The results show that H_2O_2, $OH^{\cdot}$, and $O_2^{\cdot-}$ resources utilized for H_2O_2 production are involved in apoptosis of guard cells. It is likely that singlet oxygen generated by RB in the light, judging from the permeability of the plasmatic membrane for propidium iodide, makes Photosystem II of chloroplasts inoperative and induces necrosis of the guard cells.

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Cyanide causes programmed cell death (PCD) in plants [1, 2] with characteristic features of apoptosis: condensation and margination of chromatin and fragmentation of nuclei followed by disappearance of nuclear fragments in stoma guard cells of epidermis isolated from pea leaves [3, 4]. Illumination accelerates the CN^--induced death of pea leaf guard cells (containing both chloroplasts and mitochondria) in contrast to epidermal cells (containing mitochondria but not chloroplasts) [3]. Cyanide-induced decay of guard and epidermal cell nuclei depends on reactive oxygen species (ROS): it is prevented by antioxidants, such as α-tocopherol, butylated hydroxytoluene, and mannitol, and under anaerobic conditions [5, 6]. The process is controlled by the redox state of plastoquinone at the site *o* of b_6f-cytochrome complex of the chloroplast photosynthetic electron transport chain [5, 6], follows a combined scenario including apoptosis and autophagy, and depends on proteins synthesized both in the cytoplasm and in chloroplasts and mitochondria [7].

ROS produced in the cells are singlet oxygen $^1O_2$ and the products of one-, two-, and three-electron reduction

Abbreviations: CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; DCF) 2′,7′-dichlorofluorescein; DCFH-DA) 2′,7′-dichlorofluorescin diacetate; DCMU) 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DPI) diphenyleneiodonium; NBT) nitroblue tetrazolium; PCD) programmed cell death; RB) Rose Bengal; ROS) reactive oxygen species; SOD) superoxide dismutase; TMRE) tetramethylrhodamine ethyl ester.

* To whom correspondence should be addressed.

of oxygen: $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$. Oxygen in the ground state is a triplet biradical. Despite high reactive capability of $^3O_2$, its oxidative reactions are kinetically hindered because of spin constraints.

Singlet oxygen is produced from $^3O_2$ due to intermolecular energy transfer from a triplet sensitizer. Its oxidative capability is significantly higher than that of $^3O_2$ because of the absence of spin constraints. Excessive illumination during photosynthesis causes a transition of singlet excited chlorophyll into the triplet excited state, and the interaction of triplet chlorophyll with $^3O_2$ leads to formation of $^1O_2$. The quenching of excessive electron excitation and neutralization of triplet chlorophyll and singlet oxygen in phototrophic organisms occurs via thermal deactivation [8]. A special role in this process belongs to carotenoids, which de-excite both the triplet chlorophyll and $^1O_2$.

The electron transport chains of mitochondria and chloroplasts, NADPH-oxidase associated with the plasmatic membrane, and cell-wall peroxidase (apoplastic peroxidase) are loci of $O_2^{\cdot-}$ production. $O_2^{\cdot-}$ is transformed into H_2O_2 enzymatically by superoxide dismutase (SOD) or non-enzymatically: $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$. Reduction of H_2O_2 with involvement of multivalent metals leads to $OH^{\cdot}$ formation in Fenton ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$) or Haber–Weiss ($O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$) reactions. The E_0' values for $O_2/O_2^{\cdot-}$, $HO_2^{\cdot}/H_2O_2$, $H_2O_2/OH^{\cdot}$, and H_2O and $OH^{\cdot}/H_2O$ are –0.33, 1.07, 0.32, and 2.31 V, respectively [9]. The lifetimes of $O_2^{\cdot-}$ and H_2O_2 are determined by their catalytic transformations with involvement of SOD, catalases, and peroxidases. The lifetimes of $OH^{\cdot}$ and singlet oxygen are 10^{-9} and 10^{-5} sec, respectively [10].

The main loci of $O_2^{\cdot-}$ production in mitochondria of animals are respiratory chain complexes III and I [11]. However, the relative input of plant mitochondria to ROS production is small. An alternative oxidase competes with complex III [12], which catalyzes the CN^--resistant oxidation of ubiquinone by oxygen with formation of H_2O [13]. Hydrogen peroxide is an inducer of expression of the alternative oxidase [14]. The ROS production in the rotenone-sensitive complex I of plant mitochondria is restricted by alternative NADH- and NADPH-dehydrogenases interacting with the respiratory chain both on the inner and outer surfaces of the mitochondrial membrane [15].

Addition of H_2O_2 potentiated the CN^--induced apoptosis of guard cells in pea leaf epidermis [16]. Under physiological conditions, both $O_2^{\cdot-}$ and H_2O_2 are neutralized to form H_2O with involvement of SOD and ascorbate peroxidase, as well as via ferredoxin-dependent reduction of monodehydroascorbate to ascorbate, which is characterized by the highest reaction rate constants: $2.4 \cdot 10^8$, 10^7, and 10^7 $M^{-1} \cdot sec^{-1}$, respectively [17]. The chloroplast levels of ascorbate and glutathione (which is necessary for regeneration of ascorbate from dehydroascorbate) comprising 25 mM and more are significantly higher than the saturating concentrations for ascorbate peroxidase and dehydroascorbate reductase reactions [18].

Quinacrine and diphenyleneiodonium, known as inhibitors of NADPH-oxidase associated with the plasmatic membrane, had no effect on respiration and photosynthetic evolution of O_2 by leaf slices but prevented the CN^--induced death of guard cells. This suggested that NADPH-oxidase is a source of ROS causing CN^--induced death of stoma guard cells [16].

NADPH-oxidases (NOX) are well studied in animals [19, 20]. They are structurally divided into three groups: 1) NOX1-4, transmembrane protein gp91phox (glycoprotein with molecular mass 91 kD of phagocytic oxidase), which binds NADPH and FAD on the cytoplasmic surface and contains two hemes of cytochrome b_{558} in the transmembrane area, which are localized in the inner and outer membrane monolayers, and generates $O_2^{\cdot-}$ on the outer surface; 2) NOX5, the protein gp91phox, which is bound with a calmodulin-like domain with four Ca^{2+}-binding sites on the cytoplasmic surface; 3) dual oxidases (DUOX) composed of NOX5 and peroxidase domain on the outer surface of the membrane. It looks like plants also have oxidases resembling DUOX [21].

In plants, peroxidases playing an important role in ROS metabolism are localized in peroxisomes and cell walls (apoplastic peroxidase). The reaction of H_2O_2 with an electron donor DH_2 represented as the equation

$$H_2O_2 + DH_2 \rightarrow 2H_2O + D$$

is far from exhausting the possibilities of these heme proteins. As for isolated horseradish peroxidase and plant cell wall peroxidases, there are demonstrated [22-24] reactions with phenolic compounds (phenyl propane derivatives, such as coniferyl alcohol) utilized in lignin synthesis. Phenolic substances can be substituted by non-phenolic ones, including the added NADH. The apoplastic peroxidase is characterized by low affinity to NADH, which is required at millimolar concentrations [24, 25]. So the natural substrates of the enzyme are apparently phenolic substances, rather than NADH, which should be maintained at high concentration outside cells. The plant cell wall contains malate dehydrogenase reducing NAD^+ [26]. Reactions catalyzed by peroxidase with involvement of NADH comprise three cycles [22-24].

A. Peroxidase cycle:

1) E (Fe^{3+}) + H_2O_2 + $2H^+$ → Compound I (Fe^{5+}) + $2H_2O$;

2) Compound I (Fe^{5+}) + NADH → Compound II (Fe^{4+}) + $NAD^{\cdot}$ + H^+;

3) Compound II (Fe^{4+}) + NADH → E (Fe^{3+}) + $NAD^{\cdot}$ + H^+,

where E is peroxidase and compounds I and II are peroxidase with indicated valence of the heme iron.

B. Oxidase cycle:

4) $NAD^{\cdot} + O_2 \rightarrow NAD^+ + O_2^{\cdot-}$;

5) $NADH + O_2^{\cdot-} + H^+ \rightarrow NAD^{\cdot} + H_2O_2$,

where reaction (4) proceeds spontaneously and reaction (5) with involvement of peroxidase. Since reaction (5) has a low rate [23], the extracellular SOD comes into force. It was found in the secondary cell wall of higher plants [27, 28] and in plant nectar, in which H_2O_2 prevents the development of microorganisms [29, 30].

C. Hydroxyl cycle:

6) $E\ (Fe^{3+}) + O_2^{\cdot-} \rightarrow$ Compound III,

in which the heme iron is in equilibrium $Fe^{3+}–O_2^{\cdot-} \leftrightarrow Fe^{2+}–O_2$.

7) Compound III + $H_2O_2 \rightarrow E\ (Fe^{3+}) + OH^{\cdot} + OH^- + O_2$.

The goal of present work was to estimate the role of various ROS in CN^--induced apoptosis of pea stoma guard cells and to reveal possible loci of ROS production.

MATERIALS AND METHODS

The experiments were carried out on lower leaf epidermis of pea (*Pisum sativum* L. Alpha) seedlings grown for 7-15 days under 16 h illumination/8 h darkness at 20-24°C [3]. The epidermis was separated with forceps and placed into distilled water. The epidermal peel was infiltrated by incubation in vacuum for 1-2 min for rapid influx of added reagents into the cells. The samples were placed into polystyrene plates and incubated in distilled water with reagents (the composition is given in the figure legends) at room temperature either in dark or under illumination with a lamp at the light intensity of ~100 μE·m^{-2}·sec^{-1}.

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 15 min for removal of the fixative, incubated for 5 min in water, and stained with nuclear dye, Carazzi's hematoxylin, for 30 min. The stained epidermal peels were washed with tap water and inspected using a light microscope. The part of cells with destroyed nuclei and lack of nuclei was determined from 300-500 inspected cells [3]. Experiments were repeated 3-4 times. The data of typical experiments are given.

Release and absorption of O_2 by pea leaf slices (20-25 mg/ml) was measured using a closed Clark Pt-electrode in medium containing 10 mM Hepes-NaOH, pH 7.2, and 25 mM KCl. White light of saturating intensity (~600 μE·m^{-2}·sec^{-1}) was used in the experiments.

The production of H_2O_2 in epidermal peels was determined by fluorometry [16]. The epidermal peels were submerged in 50 μM solution of a vital dye (2′,7′-dichlorofluorescin diacetate (DCFH-DA)) and incubated in the dark for 10 min. When penetrated into the cell, the dye is subjected to deacetylation by intracellular esterases to form 2′,7′-dichlorofluorescin (DCFH). In the cells DCFH is oxidized to intensively fluorescing 2′,7′-dichlorofluorescein (DCF) with fluorescence excitation and emission maxima at 495 and 523 nm, respectively. The dye is oxidized in the cell by H_2O_2 with involvement of intracellular peroxidases [31]. DCFH is not oxidized by $O_2^{\cdot-}$. Hydroxyl radical is a good oxidizer for DCFH, but because it has a short half-life and high oxidative potential, and the DCFH concentration is low, it is doubtable that DCFH in the cell can compete with other substrates for $OH^{\cdot}$. Following the washing free of excess of the dye, the samples were assayed on a VersaFluor spectrofluorometer (Bio-Rad, USA). The peel was fixed using Silica gel on a transparent polystyrene plate and placed into a sample cell containing 25 mM Hepes-NaOH solution, pH 7.2. The excitation wavelength for DCF fluorescence was 485-495 nm, and the emission was registered at 515-525 nm.

Oxidation of NADH catalyzed by horseradish peroxidase was measured spectrophotometrically at 340 nm.

Confocal microscopy was used to determine the structures generating ROS in the cells. The preparations were incubated with 20 μM DCFH-DA immediately before fluorometry and investigated under an AXIOVERT 200M microscope with LSM 510Meta confocal adapter (Carl Zeiss, Germany). Fluorescence of DCF was excited at 488 nm and registered at 500-530 nm. Fluorescence of chlorophyll was excited at 633 nm and registered at 650-710 nm. Fluorescence of TMRE was excited at 543 nm and registered at 565-615 nm.

RESULTS AND DISCUSSION

Programmed cell death in the presence of substances influencing ROS production. Nitroblue tetrazolium (NBT) is reduced by $O_2^{\cdot-}$ to formazan [32, 33]. Incubation of pea leaf epidermis with NBT led to massive formation of formazan crystals in guard cells (Fig. 1), thus indicating effective competition of NBT with SOD for $O_2^{\cdot-}$. Formazan was mainly deposited in chloroplasts and cell nuclei. The amount of formazan was significantly less in epidermal cells than in guard cells.

Nitroblue tetrazolium itself had no effect on guard cell nuclei, but it prevented their CN^--induced decom-

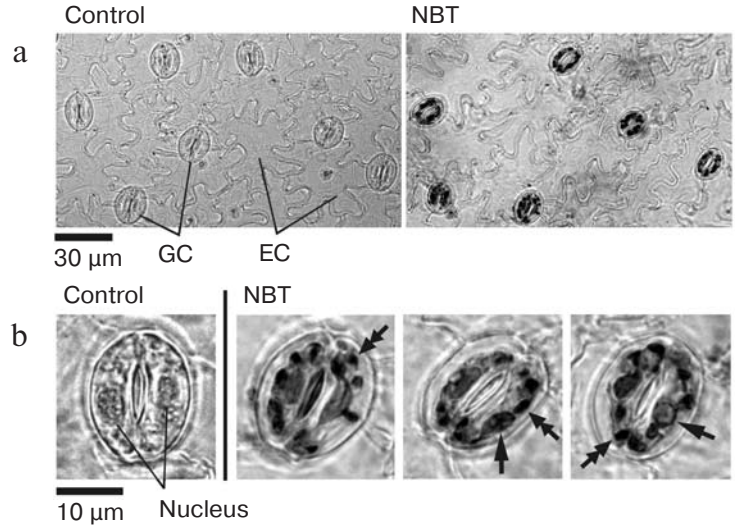

Fig. 1. Light microscopy of guard cells (GC) and epidermal cells (EC) in epidermis isolated from pea leaves: a) GC and EC in isolated epidermis; b) GC. Incubation with 0.5 mM NBT for 24 h in the light. Single arrows, formazan crystals in GC nuclei; double arrows, formazan crystals in chloroplasts.

position (Table 1). Moreover, NBT cancelled the effect of H_2O_2 potentiating the CN^--induced decomposition of nuclei (compare rows 1-2 and 3-4 in Table 1). Still greater decomposition of nuclei was induced by combination of H_2O_2 and NADH, substrates of plant cell wall peroxidase causing formation of $O_2^{\cdot-}$ and H_2O_2 [22-24].

Mannitol was used as a trap for hydroxyl radical [34, 35]. It decreased the CN^--induced decomposition of guard cell nuclei (Table 1). Ethyl alcohol is also a trap for $OH^{\cdot}$ [36]. It also decreased decomposition of nuclei caused by CN^-.

The dye Rose Bengal (RB) generates $^1O_2$ in the light [37]. RB caused a slight decomposition of nuclei in the dark, but not in the light (Table 1). In the dark, RB had no effect on the CN^--induced decomposition of nuclei and in the light prevented it. Tetramethylrhodamine ethyl ester (TMRE) had similar but less prominent effect in the light. Like RB, photoexcited rhodamine dyes interact with triplet oxygen converting it into the excited singlet state [38-40].

Prevention of cyanide-induced apoptosis by RB in the light was cancelled by NADH (Table 1). NADH alone (without RB) caused slight decomposition of guard cell nuclei.

Both NADH and NADPH are oxidized by $^1O_2$ in a one-electron reaction without enzymes to the form radicals NAD(P)$^{\cdot}$ and $O_2^{\cdot-}$ (see [41] and the literature cited there). Both NAD$^{\cdot}$ and NADP$^{\cdot}$ non-enzymatically reduce triplet oxygen to $O_2^{\cdot-}$ (see introductory part, Eq. (4)). Since membranes are impermeable to NAD(P)H [42], both reactions occur outside the cells. A question arises, how does NADH cancel the effect of RB against the CN^--induced decomposition of cell nuclei if membranes are also impermeable to $O_2^{\cdot-}$, although permeable to $HO_2^{\cdot}$ ($pK_a = 4.8$) and to $O_2^{\cdot-}$ via anion channels [43-46]? Diffusion of H_2O_2 across membranes in animals and plants is facilitated by specific aquaporins (see [47] and literature cited there). It is most probable that H_2O_2 and $OH^{\cdot}$ produced with involvement of apoplastic peroxidase and other enzymes including extracellular SOD (introductory part, Eqs. (5)-(7)) impair functions of the plasma membrane and initiate the guard cell death.

Why do RB and TMRE generating singlet oxygen prevent decomposition of cell nuclei? The data of oximetry has shown that preincubation of pea leaf slices with RB for 1 h in the light decelerates the photosynthetic release of O_2 (Fig. 2). This may be because $^1O_2$ generated by RB cleaves D1 and D2 proteins of chloroplast Photosystem II [37], thus inhibiting photosynthesis, like DCMU and bromoxynil do (Fig. 2). Suppression of electron transport in Photosystem II by DCMU prevented the CN^--induced decomposition of guard cell nuclei in the light [5, 6]. Bromoxynil had the same effect on PCD of guard cells as DCMU (Fig. 3a). Their effect was present in the light and absent in the dark. Thus, RB impairs chloroplast Photosystem II responsible for pea guard cell death in the light [48]. This leads to suppression of PCD.

Changes in the characteristics of guard cell plasmatic membrane when epidermis was treated with RB are demonstrative. We tested the fluorescent dye propidium iodide, which increases in fluorescence yield when bound with nucleic acids. It is used for staining DNA. It only penetrates into cells with damaged plasma membrane, so it does not stain viable or apoptotic cells. Figure 3b demonstrates that the guard cell nuclei are stained with propidium iodide when epidermis is incubated with RB in

Table 1. Cyanide-induced decomposition of guard cell (GC) nuclei in pea leaf epidermis under the action of reagents influencing ROS production

Tested ROS	Added reagents	Experimental conditions	GC with decomposed nuclei (%) in presence of:		
			reagents	KCN or KCN + H_2O_2*	reagents + KCN or KCN + H_2O_2*
$O_2^{\cdot-}$	NBT	Dark, 18 h	0	35.0 ± 3.0	0
		Light, 18 h	0	68.8 ± 3.9	0
	NBT + H_2O_2	Dark, 18 h	0	87.0 ± 3.0*	0*
		Light, 18 h	0	99.0 ± 1.0*	0*
H_2O_2	H_2O_2	Dark, 18 h	0	35.0 ± 3.0	87.0 ± 3.0
		Light, 18 h	0	68.8 ± 3.9	99.0 ± 1.0
	H_2O_2 + NADH	Dark, 20 h	54.3 ± 8.6	25.3 ± 1.5	100
		Light, 20 h	44.2 ± 2.9	79.0 ± 2.0	100
$OH^{\cdot}$	mannitol	Dark, 20 h	0	42.5 ± 3.0	14.7 ± 2.7
		Light, 20 h	0	75.7 ± 5.0	42.0 ± 4.5
	ethanol	Light, 24 h	0	81.7 ± 4.4	24.5 ± 4.9
$^1O_2$	RB	Dark, 16 h	2.8 ± 1.50	21.2 ± 2.3	20.6 ± 2.8
		Light, 23 h	0	94.2 ± 4.0	1.0 ± 1.0
	TMRE	Light, 19 h	0	64.9 ± 4.3	41.8 ± 6.3
	RB + NADH	Light, 23 h	0	94.2 ± 4.0	75.6 ± 3.2
	NADH	Dark, 20 h	21.5 ± 7.6		
		Light, 20 h	23.6 ± 8.6		

Note: Epidermal peels were treated with 0.5 mM NBT, 100 μM H_2O_2, 1 mM NADH, 20 μM RB, 125 mM mannitol, 5% ethanol, or 1 μM TMRE, and then infiltrated for 1 min, preincubated for 30 min, supplemented with 2.5 mM KCN, infiltrated for 1 min, supplemented with 100 μM H_2O_2, and incubated for 16-24 h either in the dark or in the light. Decomposition of guard cell nuclei in the control experiment (without additives) was absent.

the light. The portion of fluorescing nuclei in the dark was significantly lower than in the light and did not differ from the control value (without additives). The decomposition of nuclei and fluorescence of the volume within the cell wall due to the fluorescent DNA fragments were observed in the presence of KCN as inducer of apoptosis. The CN^--induced decomposition of nuclei was prevented by RB in the light. Dysfunction of Photosystem II involved in PCD [48] apparently led to the switch of cell death from PCD to necrosis. In necrosis, the plasma membrane breaks, whereas the nucleus remains unbroken or undergoes subtle morphological changes in the first stages [49, 50]. The nucleus also remains unchanged for a long time in autophagy [51], as well as in paraptosis, a specific PCD form similar to autophagy [52]. The type of cell death through apoptosis or necrosis is regulated by the energy state of the cells [49, 50, 52-54].

ROS production in epidermal peels. Hydrogen peroxide in epidermal peels from pea leaves was determined by fluorescence of intracellular DCF. Oxidation of dichlorofluorescin to fluorescing DCF in cells depends on peroxidases [31], whose activity is blocked by CN^-. Earlier [16] it was shown that H_2O_2 and menadione increase the yield of DCF fluorescence. Catalase inhibited the H_2O_2-dependent increase in the DCF fluorescence. NBT suppressed the increase in fluorescence stimulated by menadione [16].

RB (tetrachlorotetraiodofluorescein) caused some quenching of DCF fluorescence, which may be associated with the effect of heavy iodine atoms that are present in RB. Subsequent addition of NADH caused increase in DCF fluorescence (Fig. 4, a), thus indicating NADH-dependent H_2O_2 production with involvement of apoplastic peroxidase. NBT suppressed the growth of DCF fluorescence in response to addition of NADH. Diphenyleneiodonium (DPI) and quinacrine suppressed the RB- and NADH-dependent increase in DCF fluorescence (Fig. 4, b and c). In the absence of RB, NADH had no effect on fluorescence of DCF and menadione-induced increase in its fluorescence (Fig. 4, d and e). DCMU and bromoxynil increased DCF fluorescence, which was sensitive to NBT and resistant to added catalase (Fig. 4, f-i). The herbicides DCMU and bromoxynil are known inducers of $^1O_2$ production in Photosystem II [55],

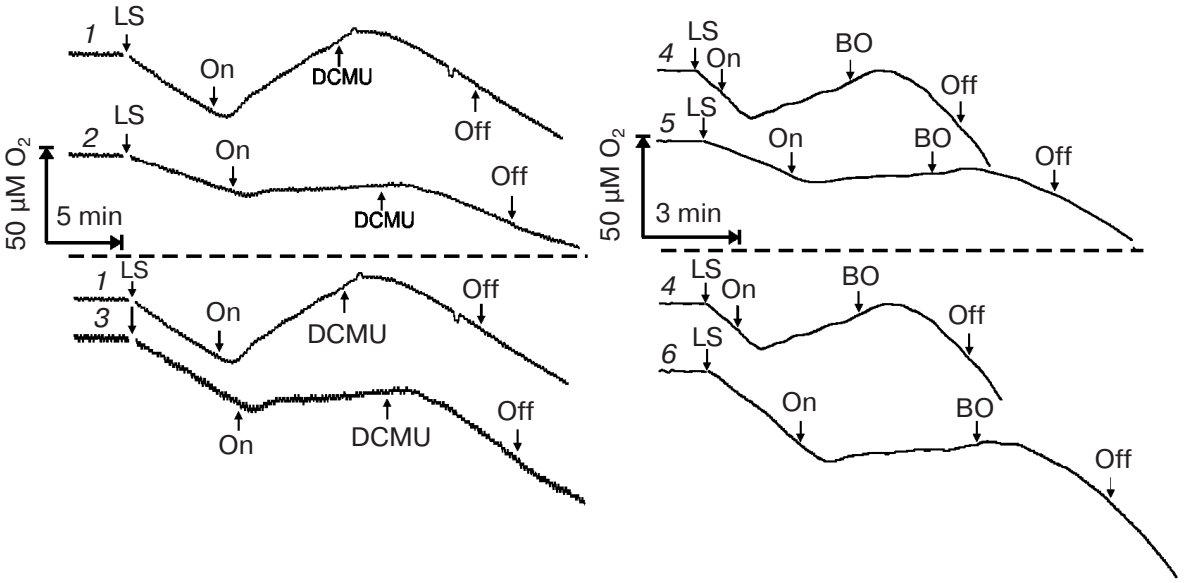

Fig. 2. Effects of Rose Bengal (RB), DCMU, and bromoxynil (BO) on respiration and photosynthetic O_2 release of pea leaf slices (LS). Lines: *1*, *4*) control; *2*, *5*) RB; *3*, *6*) same as lines *2* and *5* normalized to lines *1* and *4* by respiration rate. Leaf slices in distilled water without RB (*1* and *4*) or with 100 μM RB (*2* and *5*) were subjected to vacuum infiltration for 1 min and then incubated in the light for 1 h. "On" and "Off" refer to switching of the light on and off. Additives: LS, 20-25 mg/ml LS; DCMU, 10 μM DCMU; BO, 100 μM bromoxynil. The rates of O_2 uptake (endogenous respiration) and photosynthetic O_2 release in control (lines *1* and *4*) were 400-800 and 280-320 nmol/g LS per min.

which, via interaction with intracellular NAD(P)H, seems to lead to formation of H_2O_2 and fluorescing DCF.

Isolated horseradish peroxidase catalyzes NADH oxidation and H_2O_2 formation (see introductory part, Eqs. (1)-(5)). Horseradish peroxidase-catalyzed NADH oxidation was inhibited by CN^- and stimulated by diphenyleneiodonium (DPI) and quinacrine (Table 2). It was shown [56] that CN^- suppresses H_2O_2 formation and, to a lesser extent, NADH oxidation, whereas DPI stimulates NADH oxidation but inhibits H_2O_2 formation catalyzed by isolated peroxidase. DPI and quinacrine at concentrations below 100 μM had no effect on either respiration or photosynthetic O_2 release by pea leaf slices. Quinacrine at concentrations 30 and 60 μM decreased NAD(P)H-dependent H_2O_2 formation by plasma membranes of rat liver by 45 and 55%, respectively [57]. Inhibition of RB- and NADH-dependent increase in DCF fluorescence by DPI and quinacrine (Fig. 4, b and c) was apparently caused by inhibition of H_2O_2 formation catalyzed by apoplastic peroxidase in the oxidase cycle (see introductory part, Eq. (5)). As it takes place, either the extracellular H_2O_2, when it penetrates into the cell, oxidizes the intracellular deacetylated dichlorofluorescin to form fluorescing DCF, or deacetylated dichlorofluorescin abandons the cell through the plasma membrane damaged by RB and is oxidized by H_2O_2 outside the cell.

ROS production in chloroplasts, mitochondria, and nuclei of guard cells. According to the data of confocal microscopy, in epidermis incubated with DCF-DA, DCF fluoresced in spherical structures and nuclei of guard cells (Fig. 5, upper row; see color insert). Their fluorescence was suppressed by CN^- (Fig. 5, second row). This effect cannot be explained only by cyanide inhibition of peroxidase activity (Table 2) required for formation of fluorescing DCF from its reduced form. Cyanide initiates DCF fluorescence in chloroplasts (Fig. 5, second row): CN^- inhibits ribulose-1,5-bisphosphate carboxylase, thus stimulating ROS production by chloroplast Photosystem

Table 2. Effect of CN^-, DPI, and quinacrine on NADH oxidation catalyzed by isolated horseradish peroxidase (peroxidase activity in control (without additives) was 750-800 pmol oxidized NADH/min per μg peroxidase)

Reagent	Reagent concentration	Activity, %
KCN	1 mM	40
KCN	2.5 mM	24
DPI	20 μM	125
DPI	50 μM	160
Quinacrine	50 μM	130
Quinacrine	100 μM	150

Note: Incubation medium: 10 mM Na (or K)-citrate buffer, pH 6.0, 100 μM NADH, 100 μM H_2O_2, and 20 μg/ml horseradish peroxidase.

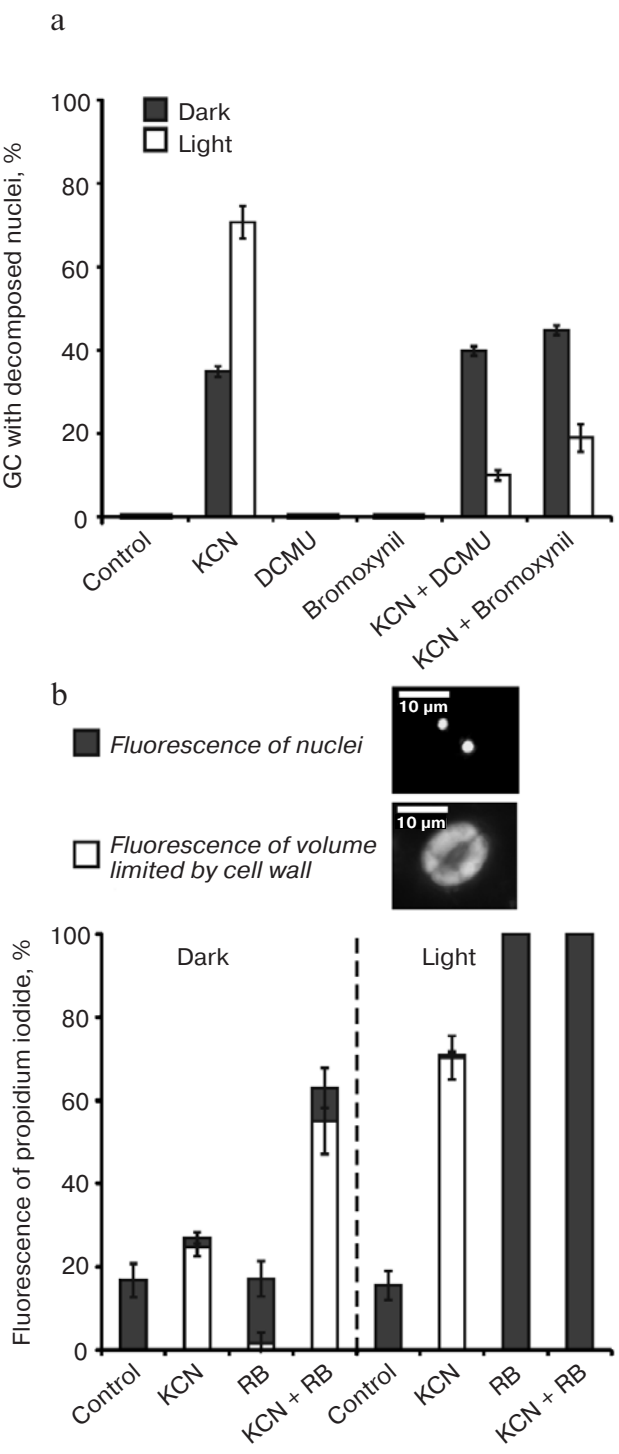

Fig. 3. Effects of the Photosystem II inhibitors DCMU and bromoxynil on CN^--induced decomposition of guard cell (GC) nuclei from pea leaf epidermal peels (a) and effect of Rose Bengal (RB) on permeability of guard cells to the dye propidium iodide (b). Epidermal peels after infiltration with 10 μM DCMU, 100 μM bromoxynil, or 20 μM RB were incubated for 20 min in the dark followed by infiltration with 2.5 mM KCN and incubation for 20 (a) or 23 h (b) in the dark or in the light.

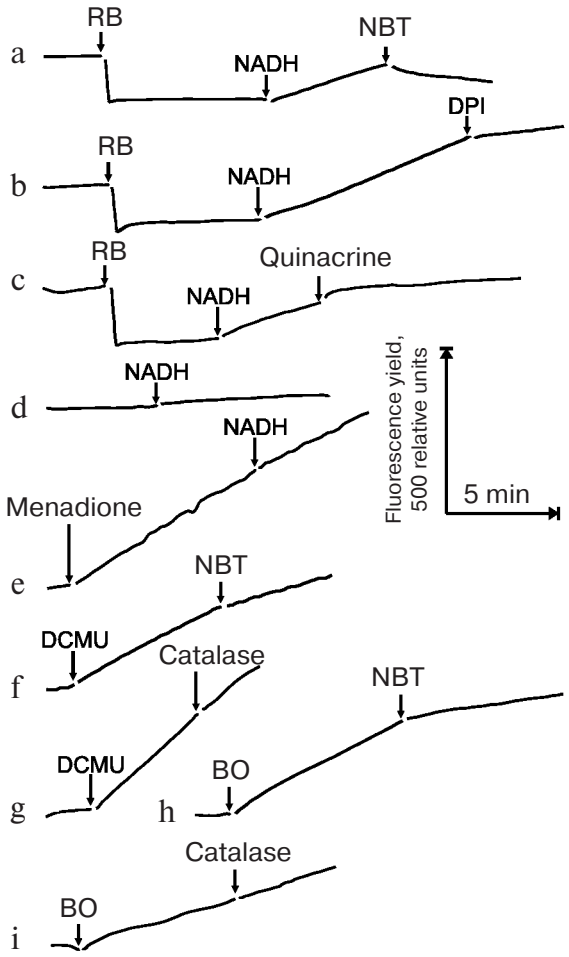

Fig. 4. Formation of DCF from DCFH-DA added to epidermal peels from pea leaves. Additives: RB, 20 μM RB; BO, 100 μM bromoxynil; DPI, 100 μM DPI; Catalase, 2.5 U/ml catalase; Menadione, 100 μM menadione; NBT, 200 μM NBT; Quinacrine, 50 μM quinacrine; DCMU, 10 μM (f) or 50 μM (g) DCMU; NADH, 10 mM NADH.

I (see [16] and the literature cited there). The fungal elicitor cryptogein caused H_2O_2 formation detected from DCF fluorescence in areas of chloroplasts and nuclei in epidermal peels from leaves of *Nicotiana tabacum* [58].

The formazan deposits (Fig. 1) and DCF fluorescence (Fig. 5) in the nucleus can be caused by formation of $O_2^{\cdot-}$ and H_2O_2 in the cell nucleus. Nuclear membranes isolated from ascitic tumor cells generated $O_2^{\cdot-}$ and H_2O_2 [59]. Flavoprotein in hamster liver nuclei generated H_2O_2 in the presence of SOD [60]. Nuclei of endothelial cells contain NAD(P)H-oxidase (NOX4) generating $O_2^{\cdot-}$ [61]. Oxidation of NAD(P)H in nuclear membranes from ascitic cells or hepatoma led to $O_2^{\cdot-}$ formation sensitive to CN^- and N_3^- [62]. However, the activity of NADPH-oxidase isolated from plasma membranes of bean hypocotyl and determined from $O_2^{\cdot-}$ production did not change when KCN was added [63]. Two activities, (i) NADPH-

oxidase generating $O_2^{\cdot-}$ and DPI-sensitive and (ii) NAD(P)H-oxidase/peroxidase generating $O_2^{\cdot-}$ and H_2O_2 and sensitive to CN^- and N_3^-, were found in protoplasts and plasma membranes from tobacco leaf mesophyll [21]. Activity of the second enzyme only was found in mesophyll of grape leaves [21].

To determine the nature of luminous spherical structures (Fig. 5, upper row), TMRE was used, a fluorescing dye, which, being a cation, accumulates in mitochondria generating a transmembrane potential ($\Delta\psi$) with interior negative [64]. TMRE caused bright fluorescence of spherical structures (Fig. 5, third row), which disappeared when epidermal peels were treated with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) dissipating $\Delta\psi$ (Fig. 5, fourth row). The spherical structures generating H_2O_2 according to the data of DCF fluorescence (Fig. 5, upper row) seem to be mitochondria: 1) $\Delta\psi$ dissipation by protonophore quenches the TMRE fluorescence; 2) CN^- inhibiting mitochondrial cytochrome oxidase suppresses ROS production in the respiratory chain complexes III and I and thereby prevents the DCF fluorescence in the spherical structures; 3) TMRE and DCF fluoresce in the same spherical structures. TMRE did not cause fluorescence of chloroplasts. The photosynthetic electron transport chain in chloroplast thylakoids generates interior-positive $\Delta\psi$ [65], so TMRE does not accumulate in chloroplasts and does not cause their fluorescence (Fig. 5).

In general, data on effect of reagents stimulating or suppressing formation of various forms of reactive oxygen on the CN^--induced decomposition of guard cell nuclei indicate the involvement of $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$, but not $^1O_2$, in PCD. $^1O_2$ apparently causes necrotic cell death by affecting the plasma membrane, which becomes permeable, in particular, for propidium iodide. The ROS sources are chloroplasts, mitochondria, and nuclear zone, as indicated by the data on formazan deposits and DCF fluorescence, as well as apoplastic peroxidase from the data on NADH-dependent apoptosis of guard cells and O_2 uptake by leaf slices.

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