Vol. 205, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1994 Pages 30-37

INVOLVEMENT OF SUPEROXIDE RADICAL IN SIGNAL TRANSDUCTION REGULATING STOMATAL MOVEMENTS

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Received September 28, 1994

Stomatal movements are regulated by alternate phase transition in the lipophilic domains of membrane initiated by blue light through activation of plasmalemma redox system. ABA blocks the blue light activated redox reactions leading to the accumulation of superoxide ($O_2^{\cdot -}$) radical inducing melting of the membrane and increased fluidity. Phenolic compounds which reverse the ABA effects reduce $O_2^{\cdot -}$ radical and thereby reverse phase transition in lipophilic domains of membrane. Stomatal movements thus appear to be regulated by blue light activated redox reactions involving formation of $O_2^{\cdot -}$ radical and its reduction by the reducing equivalents available in the GCPs. © 1994 Academic Press, Inc.

Stomatal movement is dependent on photosynthesis, respiration and blue light sensitive system for energy to maintain the ionic gradients involved in the process [1, 2]. Blue light stimulates the proton pump at the guard cell plasmalemma but the mechanism is not completely understood though being distinct from the photosynthesis [3]. On blue light illumination of GCPs, proton extrusion reaches maximum by 2 minutes accompanied by membrane hyperpolarization [4, 5]. The BL-triggered proton secretion is inhibited by ABA [4]. The existence of flavin containing PL-redox

Abbreviations: ABA, abscisic acid; BL, blue light; PL, plasmalemma; PAR, photosynthetic active radiation; GCP, guard cell protoplast; PBN, N-t-butyl phenyl nitron; EPR, electron paramagnetic resonance; SDR, stomatal diffusive resistance.

0006-291X/94 $5.00
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system has been suggested which can pump protons out of the guard cells on illumination of blue light, requiring NAD(P)H [3, 6, 7] and the system needs oxygen [8]. Evidence for its operation in guard cells remains to be established.

Flavins, when excited by blue light have been shown to produce superoxide radical in one second and inhibitors which inhibit BL-mediated reactions also inhibit $O_2^{\cdot -}$ radical generation. It has been suggested that $O_2^{\cdot -}$ radical may be involved in sensory transduction of BL-mediated response [9].

ABA which inhibits blue light mediated proton secretion in GCPs has been shown to induce alterations in the lipophilic domains of the membrane making the membrane fluid [10]. The molecular mechanism underlying the alteration was however unknown. Membrane fluidity has been often correlated with generation of $O_2^{\cdot -}$ radical in the membrane affecting the lipophilic domain of the membrane [11]. In this study we have examined the possibility of generation of superoxide radical in BL-stimulated guard cell protoplasts by ABA and the antagonistic action of various phenolic compounds in order to establish molecular mechanism underlying alternate phase transition in the lipophilic domains of the plasmalemma membrane regulating opening and closure of the stomata.

Materials and Methods

Plants were raised in the growth chamber by placing the seeds of Vicia faba in pots of moistened coarse sand under fluorescent light intensity of 80 W /m² (PAR) at 25° ± 1°C temperature.

Young fully expanded leaves from 4 to 6 weeks old plants were used to isolate GCPs. Guard cell protoplasts were isolated enzymatically from the abaxial epidermis of leaves as described previously [12]. Protoplasts were suspended in mannitol (0.4 M) and $CaCl_2$ (1 mM), pH adjusted to 6.5 at 0°C.

Method of Strain and his co-workers [13] was employed to determine the chlorophyll concentration.

Chemicals:
± ABA, esculetin, coumarin and scopoletin were supplied from Sigma Chemical Co. USA. Umbelliferone and PBN were imported from Aldrich Chemical Co. Milwaukee, WI, USA. Mannitol was purchased from Loba Chemical Co. India and KCl used was of Sarabhai Chemical Co. Baroda, India. $CaCl_2$ was supplied by BDH Chemical Co. Bombay, India.

Protocol for spin trapping of superoxide radical formation by GCPs:
An aliquot of 50 μl GCPs was treated with different phenolic compounds in combination of KCl (1mM) and ABA (10 μM). KCl treated GCPs were used as control. Superoxide anion formed under different condition was trapped by putting 15 μl PBN (500 mM) in each experimental setup, during the incubation. These samples were kept under red light (5 mW /cm^2) in order to maintain photosynthesis at saturation, for 10 min. followed by a blue light treatment (2.5 mW /cm^2) for 2 min. After incubation of about 1 hour the aliquots were transferred to glass capillaries and one end was sealed. Each set of experiment was repeated three times. The superoxide anion-PBN adduct formed during various experimental setups were recorded on EPR spectrometer. The instrument settings were, field set 3237 G, scan range 100 G, temperature 27°C, micro wave power 5 mW, micro wave frequency 9.01 GHz, modulation frequency 100 KHz, receiver gain value $3.2 \times 10^4 \times 10$, modulation 2 x 1, time constant 2 sec. and scan time 8 minutes. EPR integrated absorption intensity is calculated employing the following formula

$I = KW^2h$ where
K = line shape constant (6.5×10^{-10})
I = integrated line intensity of first derivative signal
W = width of line
h = height of line

Results

Figure 1 shows the spectra of PBN-$O_2^{\cdot -}$ adduct formed under various experimental conditions. Abscisic acid induces an increase in the superoxide radical generation which is depicted in the spectra (fig. 1-C). Phenolic compounds reverse the ABA effect by reducing superoxide radical generation which is clear from the low profile of $O_2^{\cdot -}$ in the spectra (fig. 1 D-M).

Derivatives of cinnamic acid, t-cinnamic acid, p-coumaric acid and caffeic acid which are shown to reverse the ABA effect have shown a potency to reduce superoxide radical levels in GCPs (fig. 1 D-F).

Among compounds of coumarin series, coumarin and esculetin fail to inhibit ABA-induced superoxide radical formation while sco-

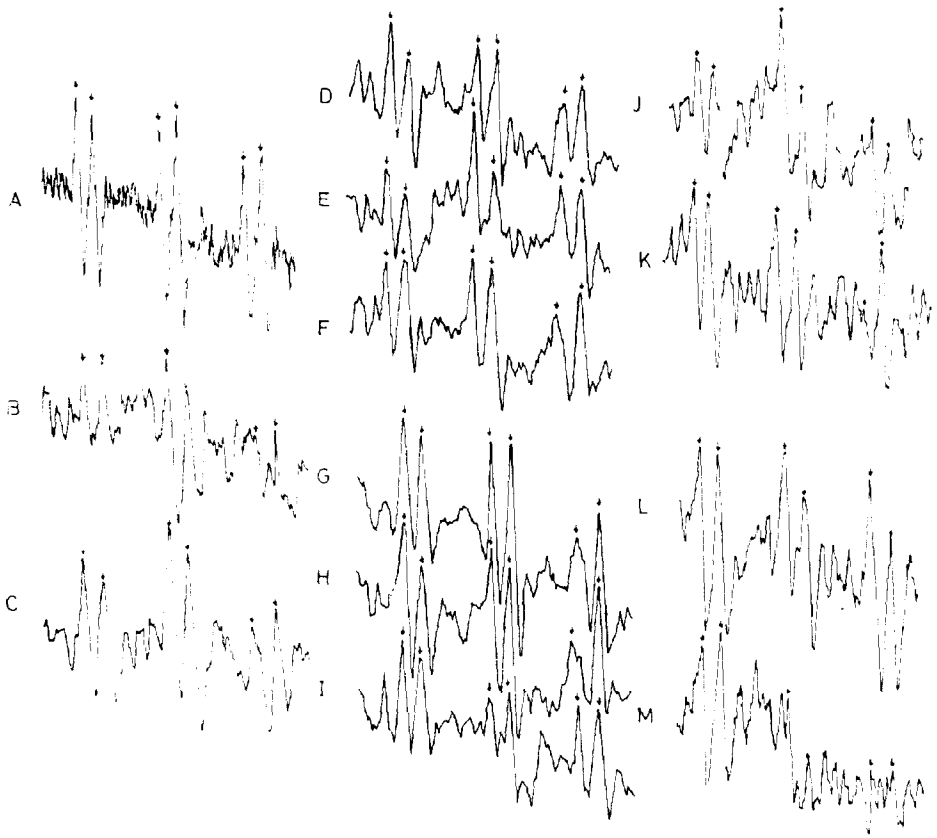

Fig. 1. Shows the EPR spectra of PBN-superoxide adduct recorded (A) using pyragallol autoxidation system and when superoxide radical was generated from the GCPs after (B) KCl, (C) KCl + ABA, and (D) PCA, (E) TCA, (F) caffeic acid, (G) quercetine, (H) rutin, (I) morin, (J) umbelliferone, (K) esculetin, (L) coumarin, (M) scopoletin treatments in presence of KCl and ABA.

poletin and umbelliferone restrict its formation (fig. 1 G-J).

Among flavonoids tested quercetine and rutin do not affect ABA induced $O_2^{\cdot -}$ production but morin significantly suppresses superoxide radical generation (fig. 1 K-M).

Discussion

Blue light mediated opening of the stomata is associated with membrane hyperpolarization, potassium influx and proton efflux [4, 5] and these processes are strongly inhibited by abscisic

acid [4, 5, 14]. Studies with guard cell protoplasts of Vicia faba have shown that GCPs treated with ABA are characterized by profound melting of the membrane resulting in increased membrane fluidity of membrane [10]. How ABA brought about increased membrane fluidity however remained unknown. Recent work in animal system have shown that increased fluidity of the membrane was often associated with increased formation of superoxide radical [11]. Our results with GCPs clearly show increased formation of superoxide radical in ABA treated GCPs which explain earlier reported membrane melting and increased fluidity in plasmalemma. Scandalios [15] has shown that under stress conditions which lead to stomatal closure, excessive superoxide radical is produced by leaves. Drought conditions are known to result in marked increase in ABA levels which would cause increased superoxide radical generation.

The ABA effects on stomatal closure, potassium efflux and fluidity changes are shown to be reversed by phenolic compounds and a perfect structure activity relationship has been established in this antagonistic action [16]. The same structure activity relationship is observed in modulating superoxide radical. Phenolic compounds which antagonize ABA mediated stomatal closure also suppress formation of superoxide radical and those which are inactive do not affect superoxide radical generation [fig. 2].

Thus trans-cinnamic acid, p-coumaric acid and caffeic acid which antagonize ABA-induced stomatal closure reduce superoxide generation by ABA. In the coumarin series coumarin and esculetin which do not reverse the ABA mediated stomatal closure also do not affect the superoxide radical generation while scopoletin and umbelliferone which reverse the ABA affect initiating the stomatal opening suppress the generation of superoxide radical. The

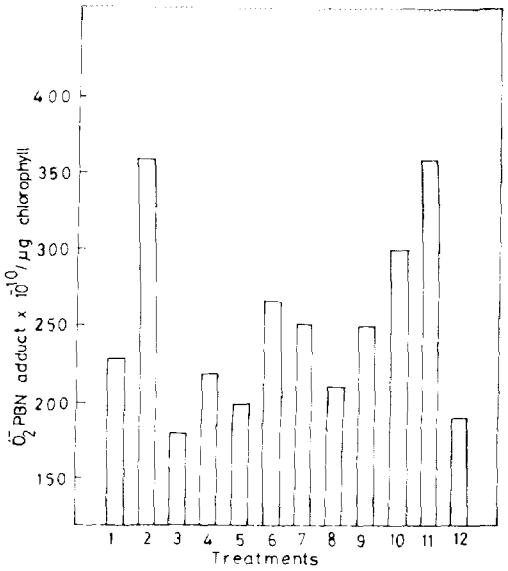

Fig.2. Histographical representation of quantity of PBN-.O_2^- adduct formed under different treatments of phenolics, (1) KCl, (2) KCl + ABA, and (3) PCA, (4) TCA, (5) caffeic acid, (6) quercetine, (7) rutin, (8) morin, (9) umbelliferone, (10) esculetin, (11) coumarin, (12) scopoletin treatments in presence of KCl and ABA.

same is true for flavonoid compounds. Quercetine and rutin fail to suppress superoxide radical generation, while morin significantly depresses it. Flavonoid compounds differ in their action in whole leaves and epidermal peels. Quercetine and rutin fail to restore the ABA induced changes in SDR, while morin almost quantitatively reverse the SDR rates to control value [17]. Thus superoxide radical generation seems to play an important role in initiating stomatal closure and other membrane changes associated with it, while reduction of superoxide radical by hydrogen donors seems to regulate stomatal opening.

Thus alternate phase transition in the lipophilic domains of the membrane appears to revolve around the generation of superoxide radical and its coupling with NADPH oxidation regulating the proton secretion.

That plasmalemma of the guard cells of stomata possess a redox system distinct from the proton translocating ATPase system has been suggested [18] and evidence for the existence of the redox system in the plasmalemma has been provided recently [19]. A suggestion has been made that blue light activation of proton pump located at the plasmalemma may utilize O_2 as terminal electron acceptor.

Blue light pulse of 30 seconds has been shown to enhance oxygen consumption in GCPs during activated proton secretion without any increase in CO_2 production. The utilization of oxygen therefore appears to be coupled to proton secretion. Flavins when excited with blue light have been shown to reduce molecular oxygen to $O_2^{\cdot -}$ radical within a second and this is inhibited by substances known to inhibit blue responses in the plants [9]. A suggestion has been made that $O_2^{\cdot -}$ radical may be involved in the transduction of blue light. ABA thus would seem to uncouple the plasmalemma redox system regulating $O_2^{\cdot -}$ radical generation and proton secretion.

Acknowledgment

The financial support provided by CSIR, New Delhi [grant no. 9/301(21)/91-EMR-1] is gratefully acknowledged.

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