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Effects of calcium-channel blockers and activator on electron transport in pea chloroplasts

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The effect of calcium-channel blockers on electron transport in pea chloroplasts has been investigated. Verapamil (approx. 10^{-3} M) and 1,4-dihydropyridine derivatives (nifedipine, nicardipine, riodipine) (approx. 10^{-4} M) inhibit the photosynthetic reduction of 2,6-dichlorophenolindophenol (DCIP). The inhibiting potency of the dihydropyridine-type compounds increases with pH, with nicardipine (possessing a tertiary nitrogen atom in the side-chain) showing the strongest pH dependence. CGP-28392, a calcium-channel activator of the 1,4-dihydropyridine type, produces virtually no effect on electron-transport activity, when used at concentrations at which calcium-entry blockers cause inhibition. The influence of riodipine is much stronger on the $H_2O \rightarrow$ DCIP than on the diphenylcarbazide $\rightarrow$ DCIP pathway, and on long-lived fluorescence than on millisecond delayed fluorescence, indicating that the blocker interacts with the donor side of PS II. Divalent cations like univalent cations (Na^+, K^+, 100 mM) increase the potency of the blockers in inhibiting chloroplast activity in order $Mg^{2+} \approx Ca^{2+} \ll Ba^{2+}$ (10 mM). With the activator and blockers applied simultaneously the inhibiting power of the blocker is enhanced. The same effect was observed when the blockers were used with the local anesthetic, tetracaine.

Introduction

The mechanism of molecular oxygen evolution in photosynthesis is least studied among the photosynthetic energy transduction phenomena in higher plants and algae. Data that have become available in recent years indicate the involvement of calcium in the operation of PS II, a system which performs water photolysis. The results obtained may be broken down into two groups, one dealing with the reconstitution of PS II activity and demonstrating the involvement of calcium [1,2], and the other concerned with effects of different agents on Ca^{2+} and with Ca^{2+}-binding components. For instance, calmodulin antagonists [3] and local anesthetics [4] were found to be potent inhibitors of electron transport on the oxidizing side of PS II. Ca^{2+} withdrawal was suggested as a mechanism of action of local anesthetics [5]. Although acting on different sites of Ca^{2+}-dependent processes, they can interact with a common target, the calcium channel [6,7]. In calcium channel studies, verapamil and 1,4-dihydropyridine derivatives are widely used. If a channel-like structure does exist in PS II, there are good reasons to expect the inactivation of PS II-driven electron transport by those compounds. As for the effect of calcium channel blockers on photosynthetic electron transport, the inhibition by nifedipine of oxygen evolution has been observed [8]. The mechanisms of calcium involvement in PS II activity is, however, unclear and more detailed investigations along these lines are needed.

In the present work, we investigated the effects of a number of calcium channel blockers of different chemical types and the effect of dihydropyridine-type calcium-channel activator on electron transport in chloroplasts.

Materials and Methods

Compounds used in the experiments were the 1,4-dihydropyridine derivatives riodipine and nifedipine (Institute of Organic Synthesis, Latvian Academy of Sciences), nicardipine (Omiya Research Laboratory, Nikken

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PS II, Photosystem II; DPC, diphenylcarbazide.

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Co., Japan), a calcium agonist CGP-28392 (Ciba-Geigy, Basel, Switzerland) and verapamil (Sigma). Chloroplasts type C were isolated from leaves of 12–14-day old pea seedlings. Leaves were homogenized in a medium containing 0.3 M NaCl, 30 mM Tris-HCl buffer (pH 7.8), 2 mM $MgCl_2$ and 0.5 mM EDTA. The homogenate was filtered through cheesecloth. The debris was removed by centrifugation at $300 \times g$ for 1 min. Chloroplasts were spun down at $2500 \times g$ in 7 min. The pellet was washed by a medium containing 0.2 M sucrose, 50 mM phosphate buffer (pH 7.4), 10 mM KCl, 2 mM $MgCl_2$ and 0.5 mM EDTA. Chloroplasts obtained were resuspended in a small amount of a medium containing 10% (v/v) glycerol and stored prior to use at −196 °C. In the control, the DCIP reduction rate is 70–100 μM/mg Chl per h. Chlorophyll content was determined by the method of Arnon [9]. Electron-transport activity was estimated spectrally by the reduction of DCIP, used as an artificial electron acceptor, and polarographically by measuring the rate of oxygen evolution. The intensity of millisecond delayed fluorescence was measured in the stationary induction phase and the long-lived delayed fluorescence 2 s after cessation of light. Measurements were made on a phosphoroscope as described elsewhere [10]. The chloroplast oxygen-evolving complex was inactivated by 15 min treatment with 0.8 M Tris-HCl buffer (pH 8.0) [11]. The incubation mixture and contents of the constituent agents are presented in the legends to the figures and tables.

Results

The tertiary nitrogen atom of verapamil and of blockers of different chemical types is known to play an important role in the effect they exert. An electrically charged nitrogen atom (pK 8.5–8.6) decreases the antagonistic potency of verapamil (by two orders) [12]. Data are also available from a study of the inhibitory effect of local anesthetics of the procaine type (tertiary nitrogen compounds), on the electron transport in chloroplasts, indicating that they are potent in inhibiting when in the neutral form [13]. On account of this, experiments were conducted in the alkaline pH region. It is seen from Fig. 1 that verapamil and the 1,4-dihydropyridine-type compounds – riodipine, nifedipine and nicardipine – inhibit electron transport somewhere in the $H_2O \rightarrow$ DCIP pathway. The dihydropyridine derivatives are, however, more potent than verapamil. CGP-28392, a calcium-channel activator, appears to be virtually without effect on electron transport when used at concentrations at which the dihydropyridine-type blockers were used. The concentration-related inhibition of DCIP reduction (measured for nicardipine) is similar in behavior pattern to the concentration-dependent inactivation of O_2 evolution, with ferricyanide used as an electron acceptor. The extent of inhibition of chloroplast activity by verapamil increases with incubation time, the greatest inhibition by riodipine occurs as early as 30–60 s after its addition. The inhibiting potency of riodipine, nicardipine and nifedipine reduces, as the pH of the incubation medium is decreased toward neutral values (Fig. 2). Nicardipine shows the greatest dependence on pH, suggesting the significant influence of the charge across the tertiary nitrogen on the efficiency of its interaction with the chloroplast membrane. A similar pH dependence of the inhibiting potency has been observed with local anesthetics whose tertiary nitrogen atoms have pK within the range 8–8.5 [13].

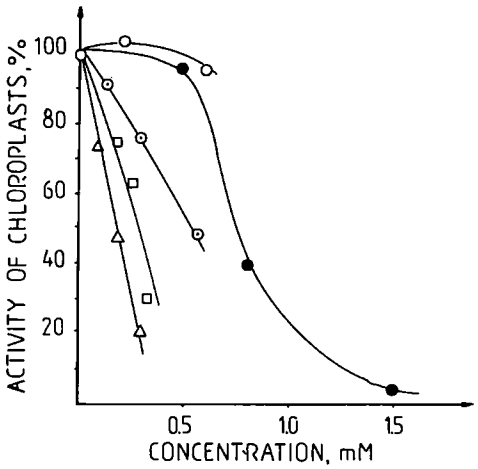

Fig. 1. Effect of calcium antagonists and agonist on the photoreduction of DCIP by chloroplasts. △, nicardipine; □, nifedipine; ⊙, riodipine; ○, CGP-28392; ●, verapamil. Incubation medium: 0.2 M sucrose, 20 mM Tris-HCl (pH 8.2) 10 mM KCl, 23 μg Chl/ml chloroplasts. Incubation time, 3 min.

To locate the blocker inhibition site, we compared the influence of riodipine on electron transport in the $H_2O \rightarrow$ DCIP and DPC → DCIP pathways in chloroplasts with inactivated oxygen-evolving complex (Table I). As can be seen, riodipine inhibits first of all the

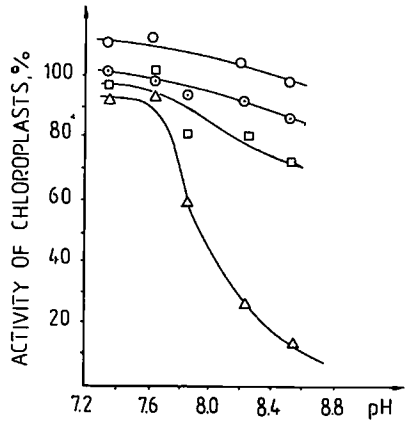

Fig. 2. pH-dependence of the inhibition potency of calcium-channel blockers and activator on the electron transport in chloroplasts. △, nicardipine; □, nifedipine; ⊙, riodipine; ○, CGP-28392. Concentration of blockers and activator, 250 μM. Incubation medium: 0.2 M sucrose 20 mM Tris-HCl (pH 8.2), 10 mM KCl.

TABLE I

The inhibitory effect of riodipine on electron transport (DPC → DCIP, H_2O → DCIP) and delayed fluorescence in chloroplasts

Incubation medium: (a) 0.2 M sucrose, 40 mM Tris-HCl (pH 8.3), 10 mM KCl, 15 μg/ml chlorophyll content, 0.5 mM DPC; (b) 0.2 M sucrose, 50 mM Tris-HCl (pH 8.3), 10 mM KCl, 5 mM $MgCl_2$, 20 μg/ml chlorophyll content, 2 mM methylamine.

Addition	[a] Chloroplast activity (%)		[b] Delayed fluorescence intensity (%)	
	H_2O → DCIP	DPC → DCIP	millisecond component	long-lived component
None	100	100	100	100
Riodipine (250 μM)	20	72	48.3	14.3

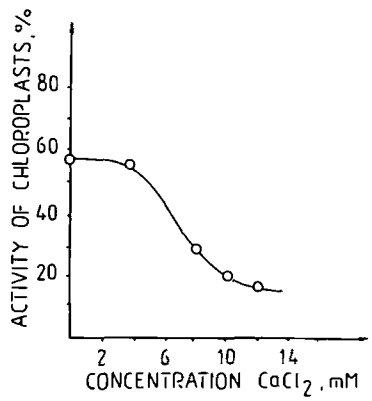

Fig. 3. Influence of calcium on the inhibition potency of nicardipine. Incubation medium: 0.2 M sucrose, 40 mM Tris-HCl (pH 8.4), 10 mM KCl, 15 μg Chl/ml chloroplasts, 25 μM nicardipine.

pathway ahead of the site of envolvement of DPC. That is, the donor side of PS II is inhibited. These observations are in agreement with the observed effect of riodipine on chloroplast delayed fluorescence. It is known that the inhibition of the acceptor side of PS II is accompanied by the suppression of millisecond delayed fluorescence and the inhibition of the donor side is accompanied by the suppression of long-lived delayed fluorescence with a decay time of 2 s [14,15]. Fluorescence was measured in the presence of methylamine (Table I), a protonophore that abolishes the influence of pH on millisecond delayed fluorescence. As seen from Table I, riodipine affects mainly those electron carriers responsible for the long-lived fluorescence. The data indicate that the inhibitory effect of blockers is due primarily to interaction with the donor side of PS II.

It is known that the interaction of blockers with the calcium channel is under a strong influence of divalent cations. The mechanism of their action may be different, for instance, by antagonizing the cation-binding group of the calcium channel, or by changing the extramembrane concentration of the blocker as a consequence of the significant cation-induced change in the surface potential. To elucidate this point, we investigated the effect of calcium-entry blockers on photosynthetic electron transport in the presence of univalent and bivalent cations (Table II). It appears from the data that the inhibitory power of blockers increases in the presence of ions of metals. Moreover, in the presence of cations (except for Na^+) chloroplast activity was inactivated to some extent by the agonist. The cause of this is probably a change in the electrostatic potential of the membrane, caused by the cations, since (i) the effect of cations was the strongest in the presence of a charged blocker, nicardipine, and (ii) the potentiation of the blocker effect was observed with fairly high cation concentrations (Fig. 3). Note that the influence of Ba^{2+} is much stronger in combination with a blocker than with Ca^{2+} or Mg^{2+}. In the absence of the blocker, different bivalent cations inhibit chloroplast activity to nearly the same extent. This suggests that the observed effect of Ba^{2+} cations is a manifestation of some specific property of Ba^{2+} in the presence of Ca^{2+}-entry blockers, It is interesting that in animal cells, Ba^{2+} easily enters the calcium channel, causing an irreversible lesion in it [16].

TABLE II

Effect of cations on the influence of calcium-channel blockers and CGP-28392 on the DCIP photoreduction in chloroplasts

Incubation medium: 0.2 M sucrose, 40 mM Tris-HCl (pH 8.4), 10 mM KCl, 15 μ/ml chlorophyll content.

Addition (mM)	Activity of chloroplasts (H_2O → DCIP) (%)			
	control	riodipine [a] (200 μM)	nicardipine [a] (25 μM)	CGP-28392 [a] (100 μM)
None	100	72.3	53.3	96.7
$MgCl_2$ 10	81.1	41.1	12.3	72.6
$CaCl_2$ 10	77.8	54.3	15.7	82.8
$BaCl_2$ 10	67.8	4.9	1.6	37.7
KCl 100	80	33.3	13.9	73.6
NaCl 100	75.6	55.9	33.7	104

[a] 100% is chloroplast activity in the presence of a salt.

It is known from literature that the agonist-binding site (BAY K6644) and the site of blockage by dihydropyridine compounds (nifedipine) are alike [17]. One may therefore expect the overall effect of the activator and blocker applied simultaneously to be different from that when they are added one after the other. Presented in Table III are the results of an experiment in which we examined the inhibition of DCIP reduction chloroplasts by the 1,4-dihydropyridine derivatives, verapamil, the local anesthetic tetracaine and chlorpromazine, the calmodulin antagonist, supplemented with CGP-28392, a calcium channel activator. It is seen that CGP-28392, at a concentration at which it exerts no influence on electron transport activity, stimulates the action of those compounds, the strongest stimulation being with tetracaine. In the case of riodipine, the effect of CGP28392

TABLE III

Effect of calcium channel blockers, tetracaine and chlorpromazine, supplemented with CGP-28392, on the electron transport ($H_2O \rightarrow DCIP$)

Incubation medium: 0.2 M sucrose, 40 mM Tris-HCl (pH 8.2), 10 mM KCl 5 mM $MgCl_2$, 17 μg Chl/ml chloroplasts.

Addition	Activity of chloroplasts (%)		
	−CGP-28392	+CGP-28392 (200 μM)	
		before inhibitor	after inhibitor
None	100	87.5	
Riodipine (200 μM)	62.5	33.7	51.2
Nicardipine (75 μM)	66.7	36	37.5
Rifedipine (150 μM)	87.6	60.8	58
Chlorpromazine (50 μM)	76.2	36.2	32.5
Tetracaine (1.5 mM)	76.1	9.3	5.3
Verapamil (750 μM)	61.1	27.7	22.2

depends on the sequence of adding the compounds. Interestingly, both CGP-28392 and riodipine have similar fluorine-containing groups.

In view of the cooperative effect observed, we conducted an experiment to see whether the effect exist when two different inhibitors are added simultaneously. The results of the experiment are shown in Table IV. It appears that the inhibitory power of the blockers and chlorpromazine is potentiated in the presence of tetracaine, the effect of the latter being more pronounced than that of the activator. When a blocker was used together with chlorpromazine, or different combinations of blockers were used, little or no effect of this kind was seen.

TABLE IV

Effect of the combinations of inhibitors on the electron transport in chloroplasts ($H_2O \rightarrow DCIP$)

Incubation medium: 0.2 M sucrose, 40 mM Tris-HCl (pH 8.4), 10 mM KCl, 5 mM $MgCl_2$, 21 μg/Chl/ml chloroplasts, 50 M riodipine, 15 μM nicardipine, 75 μM nifedipine, 20 μM chlorpromazine, 1 mM tetracaine, 500 μM verapamil.

Addition	Activity of chloroplasts (%)					
	tetracaine	chlorpromazine	riodipine	nicardipine	nifedipine	verapamil
None	95	96	80	80	80	77.5
Tetracaine		36	10	8	25	0
		(91.2) [a]	(76)	(76)	(72)	(62)
Chlorpromazine			74.3	60	76.9	47.5
			(76.8)	(73.5)	(76.8)	(81.3)
Riodipine				46.7	66.7	42.5
				(64)	(64)	(58.1)
Nicardipine					64.1	47.5
					(64)	(54.2)
Nifedipine						45
						(65.8)

[a] Activity of chloroplasts (%) calculated for independent effect of inhibitors.

The observations suggest that the interaction of the calcium channel activator with the chloroplast membrane potentiates the efficiency of the calcium-entry inhibitors. The effect is due either to activator-induced nonspecific structural/functional changes in the membrane, or to the availability of specific activator and blocker binding sites located in close proximity, the interaction of which produces a cooperative effect. The latter seems more probable, because among the 1,4-dihydropyridine derivatives with different blocking and activating properties the stimulation was observed only when the compounds used were different in influence on the calcium channel. Evidence in favor of the latter mechanism is the dependence of the inhibiting effect on the sequence of adding CGP-28392 and riodipine. Presumably, the interaction between the activator and membrane components somehow affects the Ca^{2+}-binding sites. An indication of this is that tetracaine and CGP-28392 produce similar effects. The inhibition of the donor part of PS II by the tetracaine is accompanied by the extrusion of Ca^{2+} from the thylakoid membrane [18].

It is worth mentioning the spatial proximity of the sites of action of calcium and chloride [19]. They may influence each other, as the observation of the stabilizing effect of Ca^{2+} in the event of Cl^- deficiency suggests [19]. Consistent with the above suggestion about the proximity of the Ca^{2+} and blocker-binding sites, one may expect that Cl^- influences the inhibiting power of blockers. Fig. 4 shows the KCl concentration dependence of the inhibiting potency of riodipine and nicardipine in the absence of other chloride anions. It is

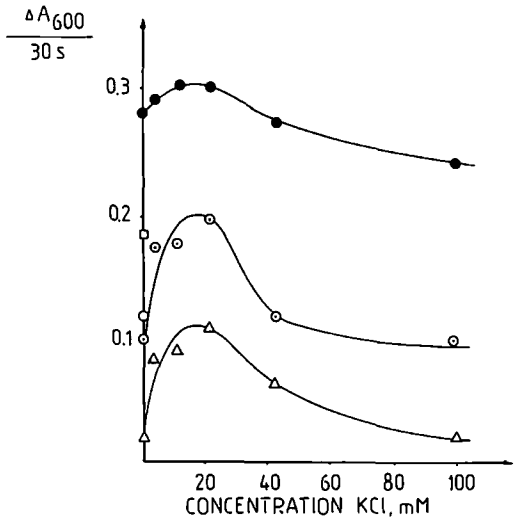

Fig. 4. Effect of Cl^- on the inhibition potency of riodipine and nicardipine. ●, chloroplasts without additions; ⊙, riodipine (125 μM); △, nicardipine (250 μM); □, effect of riodipine (○, nicardipine) in the incubation medium: 0.2 M sucrose, 40 mM Tris-HCl (pH 8.37). Incubation medium: 0.2 M sucrose, 40 mM Tris-H_2SO_4 (pH 8.37), 1.25 mM sodium/potassium phosphate, 0.25 mM KCl, 0.05 mM $MgCl_2$, 21 μg Chl/ml chloroplasts.

seem that with a low Cl^- content the effect of the blockers is the greatest. With a low Cl^- content the effect of the blockers is the greatest. As the Cl^- content is increased, the effect becomes smaller, the smallest effect being with 20 mM Cl^-. With further increase in KCl content, the inactivation effect becomes stronger because of increasing ionic strength (Table II). The change in the inhibiting potency of the inhibitor at low KCl concentrations is not related to the change in the K^+ content, because in a medium containing Cl^-, but free of K^+, the effect was similar to that seen in the presence of 20 mM KCl. These data provide support for the view that the inactivation of PS II activity by calcium-channel blockers is due to their binding sites being located close to the sites of action of Cl^- and Ca^{2+}.

Discussion

The data demonstrate that calcium-channel blockers of two different types inhibit electron transport in chloroplasts at the donor side of PS II. This raises the question of how the observed effect is related to the specific property of those compounds to act as channel blockers. The concentrations at which verapamil and 1,4-dihydropyridine derivatives inactivate electron transport are higher than those which block the calcium channel in animal cells (for verapamil, for instance, it is 5–6-times lower). However, the inhibitors of chloroplast activity show specific features which are inherent in calcium-entry blockers. For instance, the 1,4-dihydropyridine-type compounds exert stronger effects on the calcium channel than does verapamil. The same trends exist with respect to electron transport inactivation. Acting on the calcium channel in different ways (as blockers and an activator), the 1,4-dihydropyridine derivatives also show different potencies toward chloroplast electron transport. The experiment in which the potentiation of the influence of the antagonist was seen in the presence of the agonist also provides arguments for the specific interaction between blockers and membrane. The conclusion drawn from our results is that the inhibition of electron transport is probably via interactions with a structure that resembles a calcium channel (a part of it).

These days, the involvement of Ca^{2+} in the operation of PS II is a matter of fact. It is also well established that two calcium atoms exist per reaction center [20]. However, the binding site and functional role of this cofactor are still unclear. Data on the inhibition of PS II activity by calmodulin antagonists [3], local anesthetics [4,13] and calcium-entry blockers, the common property of which is blockage of calcium influx, suggest the existence of a molecular channel-like structure within PS II, at least partly, which binds calcium. In studying structural organization of calcium-containing sites this fact may be of much help. This also suggests that calcium is functionally involved in the operation of PS II. Of course, the hypothesized 'channel-like' structure, the existence of which is suggested by the above data for PS II, is not coupled to transmembrane Ca^{2+} transport activity. One may suggest, based on the mechanism of Ca^{2+} transport via the Ca^{2+}-channel in animal cells (where changes in the Ca^{2+}-binding affinity depend on the functional state of the channel) that the 'channel-like' structure acts to bind Ca^{2+} and, possibly, to modulate the efficiency of this process during PS II functioning. There is some evidence in favor of this proposition. This is the necessity of light (light 'opens the channel-like structure' by generating electric potential) to reconstitute PS II activity in the presence of Ca^{2+} [21] and the binding of Ca^{2+} by PS II particles in the light, coupled to O_2 evolution, and the release of Ca^{2+} upon cessation of lighting [22].

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