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AVAILABILITY OF MONOVALENT AND DIVALENT CATIONS WITHIN INTACT CHLOROPLASTS FOR THE ACTION OF IONOPHORES NIGERICIN AND A23187

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SUMMARY

1. A23187 will uncouple electron transport by broken chloroplasts in a divalent cation dependent manner provided that they have been treated with a low concentration of EDTA.

2. A23187 stimulates oxaloacetate-dependent oxygen evolution and inhibits phosphoglycerate reduction by intact chloroplasts isolated in a cation-free medium whereas the full effect of nigericin was dependent on the presence of external K^+.

3. Uncoupling of oxaloacetate reduction by A23187 in intact chloroplasts is inhibited by EDTA and this effect is overcome by excess Mg^{2+}.

4. The results suggest that divalent and not monovalent cations are available for collapsing the light-induced H^+ gradient within the intact organelle.

INTRODUCTION

There have been many attempts to determine the nature of the ions that move across the chloroplast inner membrane (thylakoids) to balance light-induced proton uptake, but with conflicting results. Deamer and Packer [1] showed that Cl^- was taken up in conjuction with H^+ uptake, while Dilley and Vernon [2] suggested that K^+ and Mg^{2+} efflux totally balanced the light-induced H^+ influx. More recently Hind et al. [3] found that a more or less equal Cl^- uptake and Mg^{2+} efflux balanced the H^+ uptake and that Ca^{2+}, Na^+ and K^+ movements did not normally occur.

All the above investigations were carried out using chloroplasts from which the outer membranes had been removed by osmotic shock. As it is possible that the variation in suspending medium chosen by the above investigators may have been responsible for the conflicting results, we have attempted to determine the nature of the counter ions for the H^+ pump by using chloroplasts that retain their outer membranes.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid.

There is evidence that the outer chloroplast membranes act as an efficient barrier to free diffusion of cations [4–7]. Thus, regardless of the external medium, the chloroplast inner membrane should be in contact with an ionic environment similar to that found in the stroma under in vivo conditions. In fact isolated intact chloroplasts suspended in a medium containing no added cations can support oxygen evolution dependent on added mediators such as phosphoglycerate [5], and show cation-induced chlorophyll fluorescence changes similar to those seen with in vivo systems [8, 9]. We have used chloroplasts isolated in this way to investigate the uncoupling action of cation-specific ionophores in an attempt to deduce the nature of the ionic species available within the stroma to act as the counter ion for H^+ pumping. This work complements and throws new light on recent investigations into the effect of ionophores on energy-dependent cation-induced chlorophyll fluorescence quenching observed with isolated intact chloroplasts [10].

MATERIALS AND METHODS

Intact chloroplasts were isolated from spinach (*Spinacea oleracea*) by the method of Stokes and Walker [11] except for the following changes. The chloroplasts were washed and resuspended in a cation-free medium consisting of 0.33 M sorbitol and 10 mM HEPES brought to pH 7.6 with 1 M Tris base. The chloroplasts were shown to be 60–70 % intact by comparing the uncoupled rates of ferricyanide reduction by our chloroplast suspension and the same chloroplasts after they had been subjected to an osmotic shock. 10 mM DL-glyceraldehyde was added to the reaction mixture to prevent interference from intact chloroplast activities. Chlorophyll was determined by the method of Arnon [13].

The chloroplasts showed oxygen evolution which was dependent on the addition of phosphoglycerate or oxaloacetate, though the rates were somewhat inhibited compared to chloroplasts washed and resuspended in Stokes and Walker's medium [11]. Oxygen evolution was measured as described previously [14]. The K^+ and Mg^{2+} levels of the chloroplasts were measured, after digesting the chloroplasts in 20 % HNO_3 for 60 min at 80 °C and centrifuging at 48 000 $\times$ *g* for 10 min, by flame photometry and atomic absorption spectrophotometry respectively. The K^+ and Mg^{2+} levels of the external medium were measured by the same methods after removing the chloroplasts by centrifugation at 48 000 $\times$ *g* for 10 min. The cation levels of the chloroplasts were corrected for levels found in the external medium and the Mg^{2+} content of the chloroplasts was also corrected for the Mg^{2+} content of chlorophyll. Nigericin and A23187 were obtained from Eli Lilly, Indianapolis, and stock solutions were made up in ethanol.

RESULTS

Ionophore A23187 has been shown to facilitate an H^+/divalent cation exchange across lipid membranes [15] and has been shown to uncouple ferricyanide reduction by broken chloroplasts in a Mg^{2+}-dependent reaction [6]. Table I shows divalent cation-dependent uncoupling of ferricyanide reduction with a sensitivity: $Mg^{2+} \approx Ca^{2+} > Sr^{2+} > Ba^{2+}$. It should be noted that the divalent cation require-

TABLE I

DIVALENT CATION REQUIREMENT FOR UNCOUPLING BY A23187 OF OSMOTICALLY SHOCKED CHLOROPLAST ELECTRON TRANSPORT

The reaction mixture contained in a volume of 2 ml: 100 μg chlorophyll, 0.33 M sorbitol, 10 mM HEPES brought to pH 7.6 with 1 M Tris base, and 2 mM potassium ferricyanide. 10 μM A23187 and 0.5 mM EDTA were also present. The reaction mixture was maintained at 20 °C and was illuminated with 90 kergs · cm^{-2} · s^{-1} red light which was passed through a heat filter. The divalent cations were added as the chloride salts.

Additions	μatoms oxygen · mg^{-1} chlorophyll · h^{-1}
none	40
+ 2 mM Mg^{2+}	153
+ 2 mM Ca^{2+}	155
+ 2 mM Sr^{2+}	148
+ 2 mM Ba^{2+}	82
+ 5 mM NH_4Cl	135

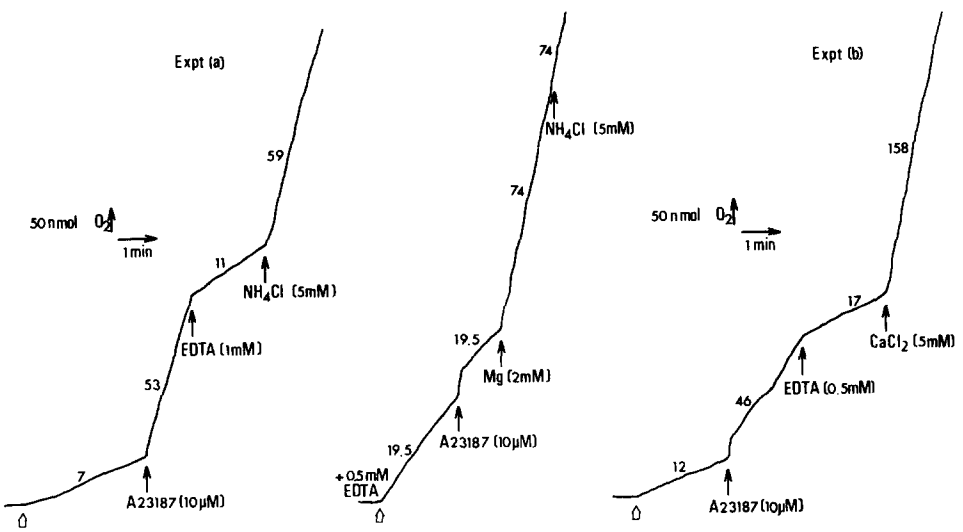

Fig. 1. Light-induced oxygen evolution by osmotically shocked chloroplasts in the presence of ferricyanide showing the effect of A23187, EDTA and divalent cations. Reaction conditions were as described in Table I. Open arrows indicate the onset of illumination. The difference between expts (a) and (b) was simply that the chloroplasts were isolated from different spinach on different days. The figures along the traces are the rates in μatoms oxygen evolved · mg^{-1} chlorophyll · h^{-1}.

ment was seen only after adding 0.5 mM EDTA to the external medium (Fig. 1a). However there was some variability and A23187 did not always fully uncouple unless divalent cations were added (Fig. 1b). The effect was not seen earlier [6] since, although not reported in the data, the chloroplasts had been washed with a medium containing low concentrations of EDTA.

Nigericin is well known to facilitate an H^+/K^+ neutral exchange [12]. We found that $1.3 \cdot 10^{-7}$ M nigericin did not uncouple electron flow to methyl viologen

by osmotically shocked chloroplasts either in the presence or absence of EDTA unless 2 mM K^+ was also present in the medium.

Before investigating the effect of A23187 and nigericin on intact chloroplasts, we measured the K^+ and Mg^{2+} content of our intact chloroplasts. Table II shows that the chloroplasts contained about 1 μmol $K^+ \cdot mg^{-1}$ chlorophyll and 0.2 – 0.3 μmol $Mg^{2+} \cdot mg^{-1}$ chlorophyll. These values are in the same order as those previously reported for intact chloroplasts [17–19]. We also measured the K^+ and Mg^{2+} concentrations in the supernatant after removing the chloroplasts by centrifugation, and found that the medium contained measurable levels of K^+ and Mg^{2+} which were carried over from the chloroplast suspensions.

TABLE II

K^+ AND Mg^{2+} LEVELS IN INTACT CHLOROPLASTS ISOLATED IN A CATION-FREE BUFFER AND OF THE MEDIUM IN WHICH THE CHLOROPLASTS WERE SUSPENDED

Cation	Intra-chloroplast level (μmol · mg^{-1} chlorophyll)	Inter-chloroplast level	
		Concn for 100 μg chlorophyll · ml^{-1}	μmol · mg^{-1} chlorophyll
K^+	~ 1.0	0.1– 0.2 mM	1.0 –2.0
Mg^{2+}	0.2–0.3	10 –15 μM	0.10–0.15

Table II shows not only the K^+ and Mg^{2+} levels of the external medium expressed per mg chlorophyll but also the concentrations found under our experimental conditions (i.e. 100 μg chlorophyll · ml^{-1}). We assume that this K^+ and Mg^{2+} leaks from the chloroplasts which are not biochemically intact (30–40 %) and that the 60–70 % intact chloroplasts retain their K^+ and Mg^{2+}. The K^+ and Mg^{2+} content of the biochemically intact chloroplasts would therefore have been proportionately higher than the values shown in Table II.

We then investigated the action of A23187 and nigericin on oxaloacetate-mediated oxygen evolution by intact chloroplasts. As oxaloacetate is converted to malate by intact chloroplasts in a reaction requiring NADPH but not ATP the rate of electron transport is increased by an uncoupler, unlike the inhibition of electron flow which is seen when CO_2 or phosphoglycerate are used as electron acceptors (see below). Fig. 2a shows that nigericin does not uncouple unless 2 mM KCl is added to the external medium. The figure shows the rates determined in the presence and absence of K^+ but also shows that stimulation to the uncoupled rate was brought about by the addition of 2 mM KCl after a few minutes' illumination in the presence of nigericin alone.

When we compared the action of nigericin and A23187 on oxaloacetate-dependent oxygen evolution we found that A23187 alone was an equally effective uncoupler as nigericin plus K^+.

Fig. 2b shows that the rate of oxygen evolution was stimulated in the absence of any added divalent cation, and that the presence of 2 mM $MgCl_2$ inhibited electron flow even in the absence of A23187. As Mg^{2+} added after uncoupling by either A23187 or nigericin plus K^+ partially inhibited oxygen evolution, we conclude that

the inhibitory effect of Mg^{2+} is a secondary effect unrelated to the ability of A23187 to uncouple. It is probably related to the inhibitory effect of Mg^{2+} on intact chloroplast activities which has been reported previously and which was ascribed to its action on the chloroplast outer membrane [5, 16].

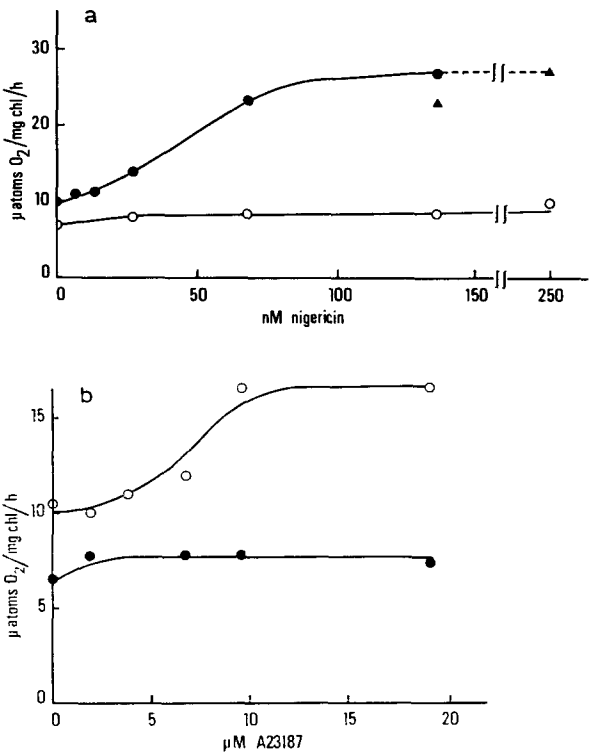

Fig. 2. Effect of (a) nigericin and (b) A23187 on oxaloacetate-dependent oxygen evolution by intact chloroplasts isolated in a cation free buffer. The reaction mixture contained in a volume of 2 ml: 200 µg chlorophyll, 0.33 M sorbitol, 1 mM oxaloacetate, 10 mM HEPES brought to pH 7.6 with 1 M Tris base. (a) ○, no additions; ●, +2 mM KCl; ▲, 2 mM KCl injected after 3 minutes illumination, and (b) ○, no additions; ●, +2 mM $MgCl_2$. Other conditions were as described in Table I.

Figs 3a and 3b show the effect of nigericin and A23187 respectively on oxygen evolution dependent on added phosphoglycerate. The assimilation of phosphoglycerate requires both NADPH and ATP so that the rate of oxygen evolution is higher than that seen with oxaloacetate (less restriction by the high energy state on electron flow). In this case, however, uncoupling brings about an inhibition of electron flow because of the cessation of ATP synthesis. It can be seen in Fig. 3a that inhibition by nigericin occurs even in the absence of K^+ but that its action is greatly enhanced by the presence of 2 mM KCl. On the other hand although A23187 severely inhibits oxygen evolution when added alone (Fig. 3b), the presence of Mg^{2+} reduces its effectiveness.

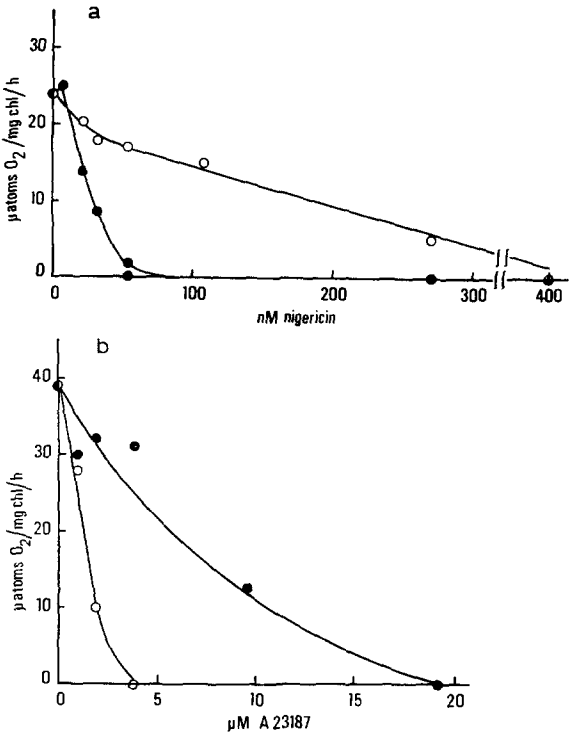

Fig. 3. Effect of (a) nigericin and (b) A23187 on phosphoglycerate-dependent oxygen evolution by intact chloroplasts isolated in a cation-free buffer. The reaction conditions were as described in Fig. 2, except that 1 mM oxaloacetate was replaced by 1 mM phosphoglycerate (a) ○, no additions; ●, +2 mM KCl, and (b) ○, no additions; ●, +3 mM $MgCl_2$.

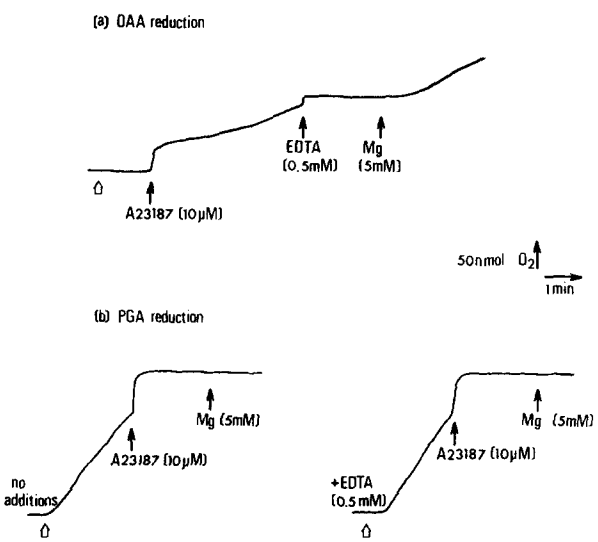

Fig. 4. The effect of EDTA on A23187 uncoupling of (a) oxaloacetate (OAA)- and (b) phosphoglycerate (PGA)-dependent oxygen evolution by intact chloroplasts isolated in a cation-free buffer. Reaction conditions were as described in Figs 2 and 3. A23187, EDTA and $MgCl_2$ were added as indicated. Open arrows indicate the onset of illumination.

We then examined the effect of EDTA on A23187 uncoupling of intact chloroplasts. Fig. 4a shows that EDTA, added after A23187, inhibited the uncoupled rate of oxaloacetate reduction and that addition of excess Mg^{2+} relieved this inhibition. In the case of phosphoglycerate reduction (Fig. 4b), A23187 inhibited both in the presence and absence of EDTA and addition of excess Mg^{2+} did not relieve this inhibition.

DISCUSSION

We have confirmed that A23187 brings about uncoupling of broken chloroplasts in a divalent cation-dependent reaction. Its ability to facilitate an H^+/divalent cation exchange presumably collapses the H^+ gradient, thus releasing control of electron transport. The fact that a low concentration of EDTA was required before divalent cation sensitivity could be demonstrated suggests that there may be some divalent cations associated with the outer surface of the thylakoid membranes even after the chloroplasts have been washed in a divalent cation-free medium. This could account for the results of Andreo and Vallejos [19] who reported divalent cation-insensitive uncoupling of electron transport of broken chloroplasts by A23187.

Comparison of the effect of nigericin and A23187 on intact chloroplast activities suggests that although divalent cations are available within the chloroplast (i.e. within the stroma) for exchange with H^+, monovalent cations are not available. This is a surprising result as measurement of K^+ and Mg^{2+} levels within the chloroplast show that there is more K^+ present than Mg^{2+}. However these results parallel the recently reported effects of these ionophores on energy-dependent cation-induced chlorophyll quenching observed with intact chloroplasts [10]. In this report it was concluded that in intact chloroplasts divalent rather than monovalent cations act as the counter ion to H^+ and it was shown by use of the Ca^{2+}-specific ionophore, beauvaricin [20], that the divalent cation is probably Mg^{2+} and not Ca^{2+}.

To explain the lack of effect of nigericin in the absence of K^+ it is necessary either to assume that the thermodynamic activity of this cation is low within the chloroplast stroma or that the antibiotic is unable to enter the organelle until K^+ is added to the suspending medium. Neither explanation is easy to accept. The requirement for 2 mM K^+ in the external medium before nigericin entry can occur is difficult to reconcile with its lipophilic nature and with the low concentrations used ($5 \cdot 10^{-8}$ M), bearing in mind that the K^+ level, even in the low salt medium, was well in excess of this (see Table II). Nevertheless, it is conceivable that the entry of this antibiotic into intact chloroplasts is inhibited by low external salt concentration. It is interesting that, as explained below, this does not seem to be the case for A23187.

The differential effect of EDTA on A23187 uncoupling of oxaloacetate reduction and inhibition of phosphoglycerate reduction may be interpreted as the result of the action of A23187 on both the thylakoid membrane and the outer limiting membrane. We assume that Mg^{2+} is normally retained within the chloroplast because of the low cation permeability of the outer membrane [5]. Illumination induces electrogenic H^+ uptake from the stroma into the thylakoid space, and we presume that a part of the electric potential created is balanced by displacement of Mg^{2+} from binding sites within the thylakoid, and that Mg^{2+} effluxes into the stroma. Addition of A23187 alone would thus be expected to uncouple oxaloacetate-dependent oxygen

evolution because it would catalyse a rapid H^+/Mg^{2+} exchange across the thylakoid membrane driven by the H^+ gradient. A23187 might also be expected to induce a slow efflux of Mg^{2+} across the outer chloroplast membrane, as was seen in mitochondria [15]. In the presence of EDTA, the A23187-facilitated Mg^{2+} efflux would be expected to increase due to rapid chelation of the cation in the external medium, thus creating a maximum diffusion gradient. A rapid rate of efflux would leach not only the stroma but also the thylakoids of Mg^{2+}. Uncoupling brought about by A23187 would therefore be expected to be inhibited, due to the removal of the counter ion necessary for the collapse of the H^+ gradient followed by the reimposition of the high-energy-state control of electron transport. A strong inhibition of electron transport by EDTA in the presence of oxaloacetate and A23187 was seen in our experiments (Fig. 4). Addition of Mg^{2+} to the external medium in excess of the EDTA level would be expected to allow uptake of Mg^{2+}, collapse of the H^+ gradient and uncoupling to re-occur. This was also observed in our experiments. In the case of phosphoglycerate-dependent oxygen evolution, A23187 would be expected to cause an inhibition of electron transport both in the presence and absence of EDTA. In the absence of EDTA, A23187 would uncouple because of its action on the thylakoid membrane, thus inhibiting ATP synthesis and consequently phosphoglycerate reduction. In the presence of EDTA, A23187 would cause Mg^{2+} efflux across the outer chloroplast membrane and would consequently inhibit all Mg^{2+}-requiring reactions associated with phosphoglycerate reduction, e.g. Mg-ATPase activity. Again these predictions are borne out by the experimental data.

The results presented in this communication clearly indicate that in a medium containing low levels of monovalent and divalent cations A23187 enters the intact chloroplasts. Because of this, and for the reasons stated above, it seems likely that nigericin can also enter. Accepting this, we must conclude that for some reason the K^+ present in the chloroplast is not available to act as the counter ion for nigericin-induced uncoupling. On the other hand, Mg^{2+} is available for the uncoupling action of A23187 and presumably is available as the exchange ion for the light-induced pump.

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