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CONDITIONS LIMITING THE USE OF IONOPHORE A23187 AS A PROBE OF DIVALENT CATION INVOLVEMENT IN BIOLOGICAL REACTIONS

EVIDENCE FROM THE SLOW FLUORESCENCE QUENCHING OF TYPE A SPINACH CHLOROPLASTS

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Summary

The conditions under which ionophore A23187 can be used as a probe of Mg^{2+} involvement in the reactions of intact (Type A) spinach chloroplasts have been investigated by monitoring ionophore-induced reversal of slow fluorescence quenching. The following observations were made: (1) A23187-dependent reversal of quenching is a strong function of pH. This is consistent with competition between protons and divalent cations for the carboxylic acid moiety of the ionophore. (2) In the presence of exogenous Mg^{2+}, quenching reversal by A23187 is significantly slowed. It is suggested that formation of the dimeric A23187 · Mg^{2+} complex delays action of the ionophore at the thylakoid membrane by slowing equilibration of the ionophore among chloroplast membrane phases. (3) In the absence of Mg^{2+}, significant interaction of A23187 with certain monovalent cations — Li^+ and Na^+, but not K^+ — is observed. Evaluations of the interaction of ionophore A23187 with specific biological systems and inferences of divalent cation involvement, or lack thereof, must take these limitations into account.

Introduction

The ionophore A23187 facilitates H^+/divalent cation exchange across membranes and is of significant biological interest due to its selectivity for divalent over monovalent cations [1]. An A23187-induced effect on a biological process is generally assumed to reflect ionophore-mediated divalent cation/H^+ exchange

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll.

and, consequently, involvement of divalent cations in the process under study. Conversely, if the ionophore fails to alter a process, one infers a lack of divalent cation involvement. However, no investigation of the conditions under which these assumptions can be applied to biological systems has been reported. Several recent findings suggest that such an investigation is warranted. pH has been found to be an important factor in determining the extent of ionophore-divalent cation interaction in a model system [2]. Moreover, doubt has been cast on the extent of A23187 selectivity for divalent cations [2,3].

I have, therefore, examined the effect of experimental conditions on the ability of A23187 to facilitate H^+/cation exchange in Type A [4] chloroplasts from spinach. Particular attention has been paid to the effects of pH and the concentration of both divalent and monovalent cations.

Intact chloroplasts were used in this investigation for several reasons. First, Mg^{2+} appears to be an important cation in photosynthetic regulation at multiple levels [5]. Definition of the conditions under which A23187 can be an effective probe of Mg^{2+} involvement in chloroplast function is therefore of great importance. Second, intact chloroplasts constitute at two-compartment system, with the thylakoid vesicles enclosed in turn by the chloroplast envelope. By utilizing such a system one can assess the relevance of factors such as (a) movement of ionophore from one membrane to another and (b) balance between driving forces for cation movement across different membranes. Finally, the cation/H^+ exchange induced by A23187 can be followed readily in Type A chloroplasts.

The parameter utilized to monitor A23187-induced cation/H^+ exchange is the reversal, by the ionophore, of the slow fluorescence quenching of Type A chloroplasts [6,7]. Slow fluorescence quenching is interpreted as a reflection of cation efflux from the thylakoid in response to light-induced proton uptake [8,9]. Thus, reversal of quenching by A23187 can be assumed to represent the return of cations — presumably divalent cations — to the thylakoid loculus in exchange for protons.

Both pH and the presence of free divalent cations were found to influence A23187-induced cation/H^+ exchange in the chloroplast system significantly. In addition, certain monovalent cations are clearly able to substitute for Mg^{2+} in ionophore-mediated processes.

Materials and Methods

Type A chloroplasts were prepared from greenhouse-grown spinach by several modifications [10,11] of the method of Cockburn et al. [12]. In light of a recent report [13] of improved yields of Type A chloroplasts, the chloroplasts were washed once with 0.33 M sorbitol brought to pH 7.5 with Tris base. The resuspending medium contained 0.33 M sorbitol, 50 mM HEPES, 2 mM EDTA, 1 mM $MgCl_2$, and 0.05 mM K_2HPO_4, adjusted to pH 7.6 with KOH. Chloroplasts were more than 85% intact as judged by the ferricyanide reduction method [14,15] and fixed CO_2 at rates in excess of 135 μmoles $\cdot$ h^{-1} $\cdot$ mg^{-1} Chl [11].

Assay media contained sorbitol (0.33 M) and Hepes buffer (50 mM). The pH was 8.0, adjusted with KOH, unless otherwise noted. Chlorophyll concentra-

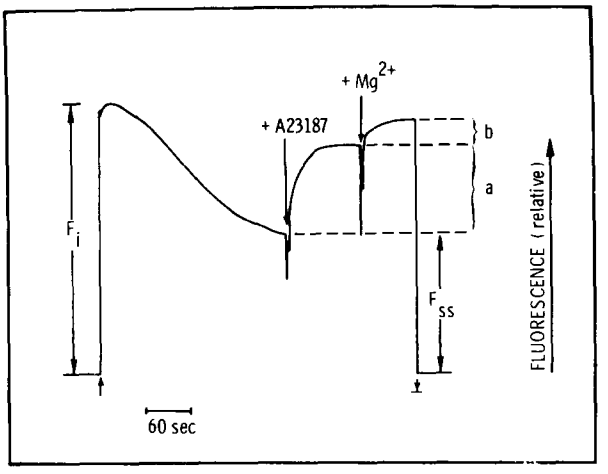

Fig. 1. Protocol for measurement of slow fluorescence quenching in Type A spinach chloroplasts. Chloroplasts were incubated in sorbitol/Hepes medium for one minute in the dark, then illuminated for four minutes. A23187 (2.4 μM) and $MgCl_2$ (7.5 mM) were added as indicated. Actinic light on (↑) and off (↓). Quenching (%) = $[(F_i - F_{ss})/F_i] \times 100$. Ionophore reversible quenching [16] is defined as $[(a + b)/F_i] \times 100$.

tion was 7.5 $\mu g \cdot ml^{-1}$ for fluorescence measurements, 12.8 $\mu g \cdot ml^{-1}$ for polarographic measurements.

Fluorescence was measured as described previously [10]. Broad-band blue actinic illumination (78 $kerg \cdot cm^{-2} \cdot s^{-1}$) was provided by a 1000-watt projector lamp and a Schott KG-3, Corning 4-96 filter combination. Temperature was maintained at 20°C. The protocol for measurement of slow fluorescence quenching and its reversal by A23187 is detailed in Fig. 1. Ionophore-induced reversal of fluorescence quenching was saturated at 0.48 μM A23187 ($\approx 6.4 \cdot 10^{-8}$ μmol ionophore $\cdot mg^{-1}$ Chl; see Ref. 7, Fig. 3). 2.4 μM ionophore (1.25 $\mu g \cdot ml^{-1}$) was added in the experiments reported here unless otherwise noted.

When chloroplasts were to be subjected to osmotic shock, $Na_4P_2O_7$ and EDTA were omitted from the resuspending medium to prevent uncoupling [17,18]. Chloroplasts were suspended in distilled water for 2 min. An equal volume of twice concentrated assay medium was then added.

Results

Effect of pH on A23187-induced quenching reversal

Pfeiffer and Lardy [2] have recently demonstrated that the extraction of divalent cations from an aqueous to an organic phase by A23187 is competitively inhibited by protons. This reflects the fact that A23187 is a carboxylic acid which will complex divalent cations only in its deprotonated, anionic form. The significance of pH in the reversal of slow fluorescence quenching by A23187 was therefore of interest. In Fig. 2, I have plotted the fraction of the total quenching reversal obtained upon A23187 addition ($a/(a + b)$, Fig. 1) as a function of pH. These results demonstrate that the ability of A23187 alone to reverse slow fluorescence quenching is severely reduced as the pH of the

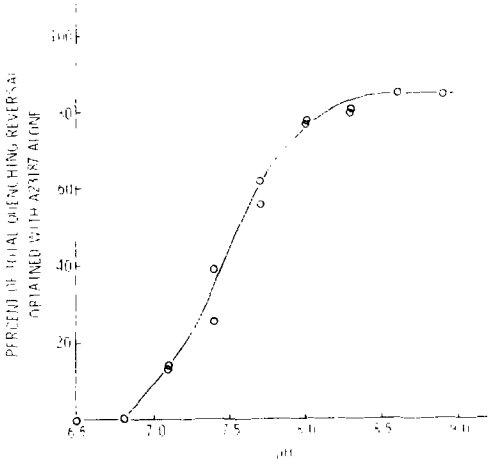

Fig. 2. Variation with pH of the ability of ionophore A23187 alone to reverse fluorescence quenching of Type A chloroplasts. The fraction of the total quenching reversal obtained upon A23187 addition [$a/(a + b)$, Fig. 1] is plotted to eliminate effects due to the large variation in the extent of quenching with pH. At low pH, A23187 has no effect on the fluorescence yield of Type A chloroplasts; reversal is completely dependent on external Mg^{2+}. Note that the position of this curve on the pH axis is dependent on the relative concentrations of Mg^{2+} and H^+ available to the ionophore [2].

medium is lowered. At low pH (e.g. pH $\leqslant$ 6.8) quenching reversal is completely dependent on added Mg^{2+}.

Heldt et al. [19] have demonstrated that stromal pH in the light is a linear function of the pH of the medium. Thus, as medium pH is lowered, there should be a drop in stromal pH and a consequent decrease in formation of the Mg^{2+} · A23187 complex in the stroma. Under such circumstances, the ability of A23187 to facilitate Mg^{2+} entry into the thylakoid, and thereby to reverse quenching, will be reduced.

Effect of Mg^{2+} on reversal of slow fluorescence quenching

Because A23187 facilitates H^+/divalent cation exchange, it was expected that addition of Mg^{2+} to the assay medium prior to A23187 addition would speed quenching reversal. On the contrary, at pH 8.0 the presence of $MgCl_2$, in the concentration range 1 to 50 mM was found to slow quenching reversal (Fig. 3B). $CaCl_2$ and $MnCl_2$ produced results similar to those obtained with $MgCl_2$, whereas addition of the salts of monovalent cations (NaCl, KCl, LiCl) in concentrations up to 150 mM failed to slow A23187-induced quenching reversal in sorbitol/Hepes medium. An increase in the concentration of A23187 used to elicit quenching reversal partially counteracted the delaying effect of Mg^{2+} (Fig. 3B, broken trace). The dependence of the half-time of A23187-induced quenching reversal on external Mg^{2+} concentration is plotted in Fig. 3 for several concentrations of A23187.

It is important to note that the delaying effect of Mg^{2+} on A23187-induced quenching reversal is observed only if the cation is added prior to the ionophore. A recent observation by Telfer and Barber [20] that the ability of A23187 plus Mg^{2+} to uncouple electron flow in intact chloroplasts is depen-

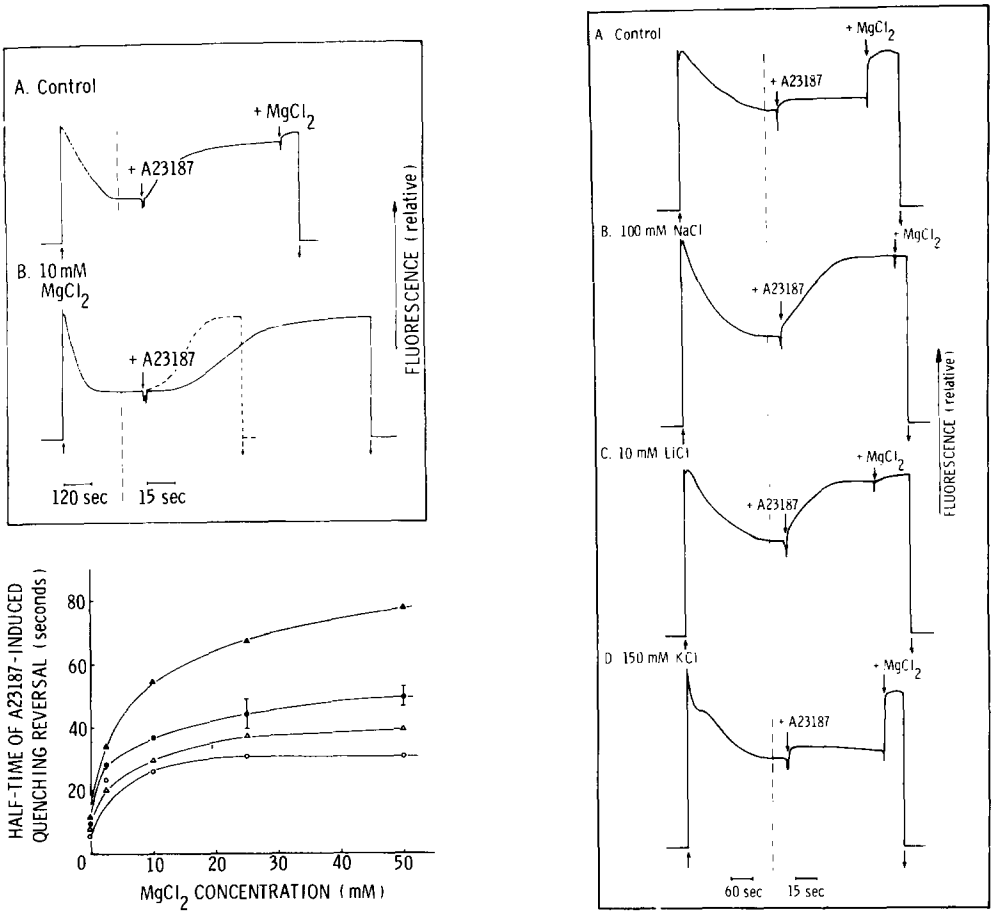

Fig. 3. Above. The effect of exogenous Mg^{2+} on reversal of slow fluorescence quenching by A23187. Ionophore is added as indicated: 1.25 $\mu g \cdot ml^{-1}$ for A and the solid trace in B; 7.5 $\mu g \cdot ml^{-1}$ for the broken trace in B. For B the sorbitol/Hepes medium is supplemented with 10 mM $MgCl_2$. Other details as in Fig. 1. Note that after 4 min illumination the time scale is expanded. Below. Effect of $MgCl_2$ concentration on the half-time of quenching reversal at several concentrations of A23187 (μM) 2.4 (▲); 4.8 (●); 7.2 (△); 14 (○).

Fig. 4. Effect of monovalent cations on quenching reversal by ionophore A23187 in sorbitol/Hepes medium supplemented with 2 mM EDTA. Details as in Fig. 1. Note that after 4 min illumination the time scale is expanded.

dent upon the order in which ionophore and cation are added may be a manifestation of this same phenomenon.

Measurements of A23187-induced quenching reversal made in the absence of exogenous Mg^{2+} (e.g. Fig. 1) suggest that these chloroplasts contain sufficient Mg^{2+} for substantial, rapid reveral of quenching at this pH (pH 8.0). Exogenous Mg^{2+} must therefore alter A23187 interaction with or access to the thylakoid membrane. The fact that increased A23187 concentrations can overcome the delaying effects of high exogenous Mg^{2+} concentrations is consistent with this proposal.

Effect of monovalent cations on quenching reversal by A23187

In Sorbitol/Hepes medium, addition of monovalent cations in the 10 to 200 mM concentration range is without effect on quenching reversal. In contrast, addition of Na^+ or Li^+ to Sorbitol/Hepes medium containing EDTA profoundly alters the time course of A23187-induced quenching reversal.

As has been reported previously [7], EDTA (2 mM) inhibits quenching reversal by A23187 (Fig. 4A). The inhibition presumably reflects the finding that, upon A23187 addition in the presence of EDTA, Mg^{2+} is lost to the medium [7,21,22], rather than returning to the thylakoid. Subsequent addition of Mg^{2+} (>2 mM), as shown, returns the fluorescence to a high level.

A small, rapidly completed reversal phase is induced by A23187 addition in the presence of EDTA. (This phase can be equated with the larger A23187-induced reversal observed in the absence of EDTA and thus with re-entry of Mg^{2+} into the thylakoids. Its magnitude appears to be determined by competition between the driving forces for Mg^{2+} movement across the envelope and the thylakoid membrane.) In the presence of NaCl or LiCl, but not KCl or sorbitol, (Fig. 4, B—D), rapid reversal is followed by a second, slower component, such that the total reversal obtained is equivalent to that observed with $MgCl_2$. Subsequent addition of $MgCl_2$ has little or no effect (Fig. 4, B and C). Addition of NaCl or LiCl, but not KCl, after A23187 also results in an increase in fluorescence equivalent to that obtained with $MgCl_2$. In other words, certain monovalent cations can replace Mg^{2+} in supporting A23187-induced quenching reversal. The effect is independent of anion: NaCl, Na_2SO_4, and sodium gluconate give identical results. Choline-Cl is equivalent to KCl.

Experiments analogous to those of Fig. 4 have demonstrated that (1) increasing the concentration of a monovalent cation increases the rate of A23187-induced quenching reversal. (Increasing the concentration of a divalent cation, slows quenching reversal by the ionophore in the presence, as well as in the absence, of EDTA.) (2) For all cation concentrations the ionophore-induced reversal obtained with Li^+ is more rapid than that seen with Na^+. (3) The rate of A23187-induced reversal is maximal at 30 mM LiCl, but requires >150 mM NaCl for saturation *. (Complete, albeit slow quenching reversal is observed for Na^+ concentrations as low as 30 mM). (4) For a given cation concentration, increased proton concentration (lower medium pH) slows reversal.

Several factors suggest that monovalent cation-dependent reversal of slow fluorescence quenching reflects interaction of A23187 with the monovalent cations. First, the effect is clearly not osmotic; neither sorbitol nor K^+ will support it. Second, the relative activities of the various cations — Li^+ is more effective than Na^+; K^+ is without effect — reflect the known affinity of A23187 for monovalent cations [2,3]. Third, the effects of monovalent cations are sensitive to pH, with high concentrations of protons inhibitory. Finally, monovalent cations have significant effects on quenching reversal only in the absence of Mg^{2+}. This is consistent with the finding that the extraction of Li^+ from an

* Monovalent cation-dependent quenching reversal cannot be attributed to interaction of the cations with broken chloroplasts in the preparation. Salt-induced increases in the fluorescence of broken chloroplasts [23,24] are characterized by a lack of cation specificity [25] and by a requirement for monovalent cation concentrations on the order of 100 mM [6,8,9].

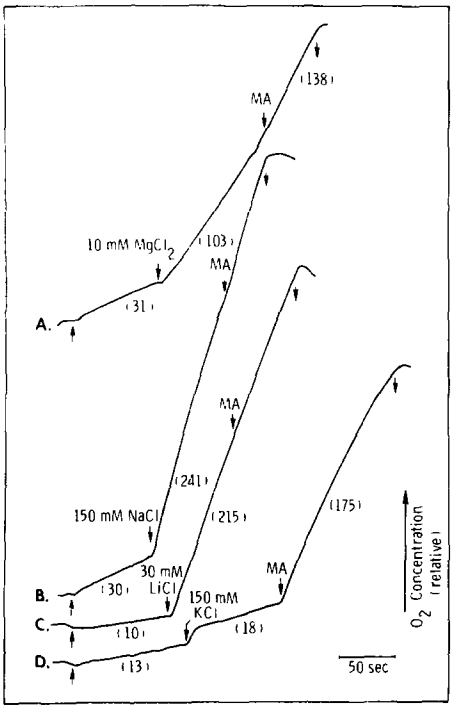

Fig. 5. Effect of cations on uncoupling by A23187 of electron flow to ferricyanide in osmotically shocked chloroplasts. O_2 evolution is monitored in saturating light in Sorbitol-Hepes medium containing 1.7 mM $K_3Fe(CN)_6$, 8.5 μM A23187, and 0.5 mM EDTA (to ensure reproducible cation-dependence of uncoupling [26]). Salts and methylamine (30 mM) were added as designated by the arrows above the traces. Onset and termination of illumination are indicated by ↑ and ↓ respectively. Rates of O_2 evolution (in $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl) are shown in parentheses below the traces. We regularly observe that the uncoupled rate of electron flow obtained with A23187 and either Li^+ or Na^+ is greater than that with A23187 and Mg^{2+}.

aqueous to an organic phase by A23187 occurs only when divalent cations are excluded [3].

If A23187 is, in fact, able to interact with Na^+ and Li^+ in the absence of Mg^{2+}, one would predict that these monovalent cations should facilitate uncoupling of electron transport by A23187. The data of Fig. 5 show that A23187 does, in fact, uncouple electron flow to ferricyanide in osmotically shocked chloroplasts in the presence of Na^+ or Li^+ (or Mg^{2+}), but not K^+. (In previous work [6] only K^+ and Mg^{2+} have been examined for their ability to support uncoupling by A23187.)

Discussion

Pfeiffer and Lardy [2,3] have suggested that, in the absence of divalent cations and at elevated pH, the interaction of ionophore A23187 with certain monovalent cations may be appreciable, and A23187-induced influx of Na^+ into red blood cells has recently been observed [27]. The findings reported here demonstrate A23187-mediated transport of Li^+ and Na^+ in the chloroplast system. The pH values at which substantial A23187-monovalent cation inter-

action is observed are well within the physiological pH range. (This contrasts with the elevated pH values used in initial characterizations of the interaction of the ionophore with monovalent cations [28].) These results also demonstrate that the predicted pH-dependence for cation transport by A23187 obtains for chloroplasts, with high H^+ concentrations inhibiting ionophore-Mg^{2+} interaction. Thus, failure of A23187 to mediate cation/H^+ exchange will occur even in the presence of Mg^{2+}, when the H^+ : Mg^{2+} ratio is high. Finally, free divalent cations have been found, under certain conditions, to restrict cation exchange mediated by the ionophore. These factors have been identified in terms of the effect of A23187 on a specific chloroplast phenomenon, namely, slow fluorescence quenching. It is clear, however, that they must be of general concern in the design and interpretation of experiments utilizing the ionophore to probe biological systems.

I have reported that exogenous Mg^{2+} slows quenching reversal by A23187, and have suggested that ionophore interaction with or access to the thylakoid membrane is altered by Mg^{2+}. Two mechanisms for Mg^{2+} action, consistent with the data, can be proposed.

First, Mg^{2+}, by interacting directly with the chloroplast membranes might slow A23187-mediated cation exchange. Significant effects of Mg^{2+} on mitochondrial membrane permeability to cations have been reported [29,30], including a demonstration that Mg^{2+} is a competitive inhibitor of valinomycin-induced K^+ uptake [31]. A Mg^{2+}-induced decrease in membrane fluidity which reduces both intrinsic membrane permeability to cations and the efficacy of exogenous ionophores has been proposed.

Second, it is possible that the ability of A23187 to interact with membranes is altered when the ionophore is complexed with a divalent cation. The Mg^{2+}-A23187 complex is dimeric, i.e. two molecules of the ionophore are liganded to a single cation [2,32]. The data are consistent with the suggestion that the rate at which this complex distributes itself among the various membrane and aqueous phases of the chloroplast is reduced relative to the free ionophore.

The finding that Mg^{2+} delays quenching reversal only when added prior to A23187 leads me to favor the latter hypothesis, as do the recently deduced solution conformations of the free acid and divalent cation complex of the ionophore [32]. The results with monovalent cations also support the contention that participation specifically in a dimeric complex slows ionophore penetration to the thylakoid membrane, since monovalent cations speed, rather than slow, reversal. The data, moreover, indicate that a 1 : 1 ionophore · cation complex is responsible for monovalent cation-dependent reversal. Several observations are consistent with this view: (1) The 1 : 1 complex of A23187 with a monovalent cation should be neutral and therefore theoretically [2] able to facilitate cation exchange. (2) For both Na^+ and Li^+, the concentration of the 1 : 1 complex increases with increasing cation concentration [2]. Increasing concentrations of Li^+ and Na^+ speed A23187-induced quenching reversal when EDTA is present. (3) K^+ does not support A23187-induced quenching reversal; K^+ is unable to form 1 : 1 complexes with the ionophore [2].

Two further comments with regard to fluorescence quenching are warranted. In interpreting the results presented here, I have used the currently prevailing model for slow fluorescence quenching, namely that fluorescence is decreased

when cations are driven from the thylakoid by light-induced proton uptake, and that quenching reversal represents re-entry of the cations. However, several of my conclusions are independent of the specific molecular mechanisms of quenching and its reversal. Whatever, the mechanism by which A23187, in combination with Mg^{2+}, reverses quenching, Na^+ and Li^+ can substitute for Mg^{2+} and protons are inhibitory.

Finally, on the basis of the ability of A23187 in the absence of exogenous cations to reverse the fluorescence quenching of intact chloroplasts, it has been concluded that Mg^{2+} is available in the chloroplast stroma for exchange across the thylakoid membrane [6,33]. The data presented here do not in any way conflict with those conclusions. I have observed interaction of the ionophore with monovalent cations only when divalents are excluded, and then only with Na^+ or Li^+. Direct determinations of the cation content of washed, intact chloroplasts [34] yield an endogenous Mg^{2+} concentration of 37 mM (calculated using the chloroplast volume of Heldt et al. [19]). Furthermore, K^+ is the predominant monovalent cation present in Type A chloroplasts (120 mM vs 3 mM for Na^+). The most plausible explanation for A23187-induced quenching reversal in unsupplemented chloroplasts thus remains ionophore-mediated return of Mg^{2+} to the thylakoids.

Acknowledgments

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