DIVALENT-CATION IONOPHORES AND Ca^{2+} TRANSPORT IN SPINACH CHLOROPLASTS

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1. Introduction

Processes such as ion transport and energy coupling have been studied with the aid of ionophores in several biological systems including chloroplasts [1–10]. One group of ionophores used is that of the monocarboxylic polyethers of which nigericin, monesins, X-206 and X-537A are examples [3]. They act as potent uncouplers of photophosphorylation depending on the presence of suitable alkali metal cations [4–7].

Valinomycin is representative of another group of neutral antibiotics that includes gramicidin, enniatins and macrotetrolides [2,9,10]. Among this group, only gramacidin is a strong uncoupler of photophosphorylation (for a review see [10]). The actions of valinomycin in chloroplasts are more complex [11,12].

We have recently shown [13] that the divalent-cation ionophore A23187 uncouples photophosphorylation specially in the presence of Ca^{2+}. One of the carboxylic ionophores, X-537A has been demonstrated to be different from others because it can also transport divalent cations across an organic solvent [1] or biological membranes such as sarcoplasmic reticulum vesicles [8,14].

Beauvericin, a cyclic hexadepsipeptide ionophore of the enniatin family [15], has been found to induce transport of monovalent cations across the mitochondrial membrane [10,16]. Recently Roeske et al. [17] found that beauvericin also has a high affinity for calcium and barium ions and Prince et al. [18] found that beauvericin but not anniatin transport calcium ions in liposomes and bacterial chromatophores.

In this paper we report the effects of beauvericin and X-537A on proton and calcium transport and on photosynthetic reactions in spinach chloroplasts and extend our previous observations with A23187 [13]. The three antibiotics induce a light-dependent calcium uptake by chloroplasts and inhibit proton uptake and photophosphorylation although they differ in their effects on electron transport.

2. Experimental

Chloroplasts were isolated from market spinach leaves (*Spinacea olearacea L*) as previously described [19] except that the chloroplasts were finally suspended in 0.4 M sucrose.

The pH change of chloroplasts suspensions was determined as described [20]. The reaction medium (2 ml) was 100 mM choline chloride, 20 μM pyocyanine and chloroplasts containing 80 μg of chlorophyll. The initial pH was adjusted to 6.50 with Tris base (0.2 μmoles).

Calcium uptake was determined in the dark or in saturating actinic light in the same reaction medium (1 ml) described for pH change with the addition of $CaCl_2$ (between 0.01 and 1 mM) containing 1 μCi of ^{45}Ca. Temperature was 25°C. After the incubation the chloroplasts were collected by rapid filtration with negative pressure on a 0.8 μ Millipore filter and washed with 3 ml of 100 mM choline chloride. The radioactivity trapped by the filter alone (less than 1.5% of the total counts) was corrected. The filters were put into vials containing 5 ml of scintillation fluid (2,5-dephenyl oxazole (5g/l) and naphtalene (100 g/l) in dioxan), and the radioactivity was counted in a Beckman LS-233 liquid scintillation spectrometer.

Photophosphorylation, electron transport from water to ferricyanide and the light-triggered ATPase were determined as described [19,20].

The antibiotics were the generous gifts of Dr Donald R. Brannon, The Lilly Research Laboratories, Indianapolis (A23187 and beauvericin), Dr Roger W. Roeske, Department of Biochemistry, School of Medicine, Indiana University (beauvericin) and Dr Julius Berger, Department of Chemical Research, Hoffmann-La Roche Inc., New Jersey (X-537A and X-206).

Antibiotics solutions were prepared in ethanol or dimethylsulphoxide. The solvents at the final concentrations used (less than 2%) were without effect in control experiments.

3. Results

3.1. *Actions of ionophores on the light-dependent proton and calcium uptake by chloroplasts*

Table 1 shows that the ionophores beauvericin and X-537A strongly inhibited the light-dependent proton uptake by chloroplasts suspended in an unbuffered medium devoid of alkali metal cations. The presence of 1 or 5 mM $CaCl_2$ in the reaction medium did not significantly affect the proton uptake by itself but greatly enhanced the inhibition by both antibiotics.

X-537A was a better inhibitor than beauvericin in the absence or in the presence of $CaCl_2 \cdot MgCl_2$ (5 mM) was not able to substitute for $CaCl_2$ in enhancing the inhibition of proton uptake by these antibiotics or by A23187 [13]. The inhibition by beauvericin was not affected by 50 mM KCl, NaCl, CsCl or LiCl. On the other hand, Shavit et al. [7] have shown that the inhibition of the pH rise by low concentrations of X-537A (0.08 μM) depended on the presence of potassium ions. We observed that NaCl also potentiated the inhibition by X-537A but at higher concentrations of the ionophore.

On the basis of our previous results with A23187 [13] we suggested that this antibiotic catalyzes a light-dependent calcium for proton exchange. Table 2 shows that X-537A and beauvericin also induced, like A23287, a light-dependent Ca^{2+} uptake by chloroplasts. Again, X-537A was more effective than beauvericin, but less effective than A23187 in inducing the calcium uptake. The latter induced a light (minus dark) uptake of about 400 nmoles $^{45}Ca^{2+}$ per mg chlorophyll. In our experimental conditions (1 mM $CaCl_2$ and pyocyanine present) and in the absence of antibiotics there was practically no light-dependent calcium uptake since the same amount of calcium was bound to the chloroplasts in the light or in the dark (table 2). The amount of calcium bound in the light or in the dark increased with the $CaCl_2$ concentration between 0.01 and 1 mM. The

Table 1
Effect of X-537A and beauvericin on the light-dependent proton uptake by chloroplasts: influence of $CaCl_2$

Additions (μM)	Proton uptake (% of control) $CaCl_2$ (mM) 0	1	5
None	100	100	95
Beauvericin (5)	96	86	80
Beauvericin (10)	73	63	49
Beauvericin (25)	37	23	16
X-537A (8)	67	50	29
X-537A (16)	43	17	3
X-537A (41)	12	4	–

Experimental conditions were as described in the text. The control value measured in the steady-state condition was 732 nmoles H^+/mg chlorophyll.

Table 2
Effect of X-537A, beauvericin and A23187 on calcium uptake by chloroplasts

Additions (μM)	Calcium uptake (nmoles $^{45}Ca^{2+}$/mg chlorophyll) Light	Dark
None	78.7	72.8
X-537A (16)	295.8	78.5
X-537A (41)	390.0	71.8
Beauvericin (10)	151.1	69.2
Beauvericin (25)	181.7	78.6
A23187 (5)	456.8	75.8
A23187 (10)	490.9	74.7

Experimental conditions were as described in the text. $CaCl_2$ was 1 mM and the incubation time, 1 min.

same observation was made in the reaction mixture for the light-triggered ATPase assay [20] that contained ATP, $MgCl_2$ and dithioerythritol (not shown). Our results are in agreement with those reported by Gross et al. [21,22] but not with those of Nobel and Packer [23] who have described a light-dependent calcium uptake by chloroplasts associated with the light-triggered ATPase. The discrepancy between our results and those of Nobel and Packer [23] may be due to differences in the experimental conditions and procedures. We found that the light-triggered ATPase was partially inhibited by A23187, X-537A and beauvericin.

3.2. *Action of beauvericin and X-537A on photophosphorylation and electron transport*

Fig.1 shows that 36 μM beauvericin completely inhibited both cyclic and non-cyclic (ferricyanide associated) photophosphorylation by spinach chloroplasts. The concentration of beauvericin needed for half-maximal inhibition (I_{50}) was 5 μM. The antibiotic did not affect electron transport from water to ferricyanide in the basal or in the uncoupled state but inhibited the rate of ferrocyanide formation in the presence of ADP and P_i (fig.1). It should be noted that the pyocyanine-catalyzed phosphorylation was measured in the absence of alkali metal cations and that the effect of beauvericin was not altered by the addition of 50 mM KCl, NaCl, CsCl or LiCl.

In similar conditions, i.e., in the absence of monovalent cations, X-537A also completely inhibited cyclic photophosphorylation (fig.2) with a I_{50} of 3.5 μM. Table 3 shows the effects of X-537A on electron transport in the absence of alkali metal cations. Electron transport was measured as oxygen uptake (the Mehler reaction [24]). X-537A increased basal and ADP plus P_i-stimulated electron transport and did not affect in the uncoupled state.

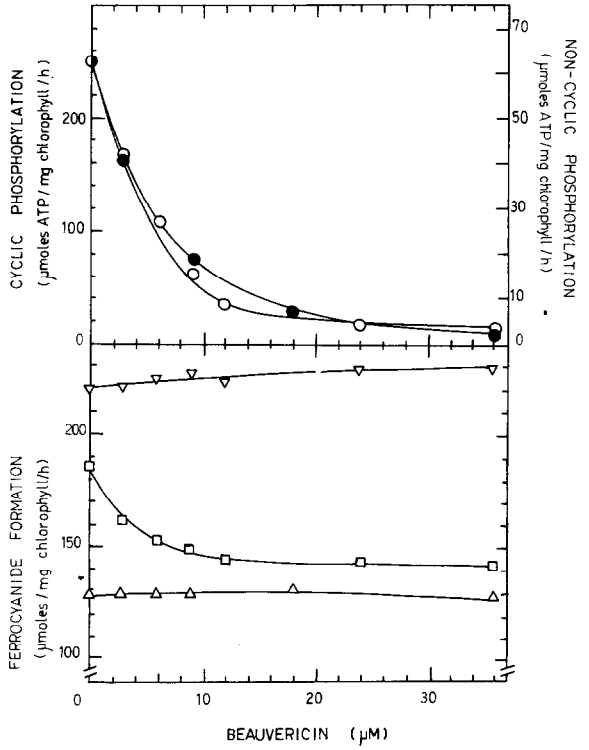

Fig.1. Effect of beauvericin on photophosphorylation and electron transport in spinach chloroplasts. Experimental conditions were as described in the text, except that the reaction medium for cyclic photophosphorylation was devoided of alkali cations (Tris–ADP and Tris–phosphate were used). (o—o), cyclic (pyocyanine-mediated) photophosphorylation; (•—•), non cyclic (ferricyanide associated) photophosphorylation; electron transport from water to ferricyanide was measured in the basal state (Δ—Δ), in the presence of ADP and P_i (□—□) and in the presence of ADP, P_i and 10 mM methylamine hydrochloride (▽—▽).

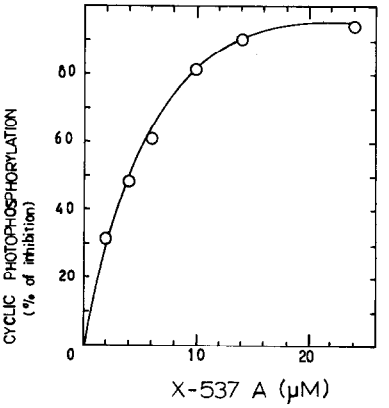

Fig.2. Effect of X-537A on cyclic photophosphorylation in spinach chloroplasts. Experimental conditions were as described in the legend to fig.1.

Table 3
Effect of X-537A on electron transport in spinach chloroplasts

Additions (μM)	Electron transport (μequivalent/mg chlorophyll/hr)		
	−−	+ADP, P_i	+ADP, +P_i, +Methylamine
None	118.6	213.4	260.4
X-537A (14)	225.2	260.8	260.8
X-537A (24)	237.1	260.8	260.8

Light-dependent electron transport was measured using *p*-benzoquinone as electron acceptor and following the reaction as oxygen uptake (the Mehler reaction [24]) in a Gilson Oxygraph with a Teflon covered Clark electrode. The reaction medium was 0.25M sucrose, 20 mM *N*-tris(hydroxymethyl)-aminomethane–HCl buffer (pH 8.0), 3 mM $MgCl_2$, 2 mM dithioerythritol and 100 μM *p*-benzoquinone. The final volume was 1.6 ml and contained an amount of chloroplasts equivalent to 100 μg of chlorophyll. When added Tris-ADP was 2 mM; Tris–phosphate, 2 mM and methylamine hydrochloride, 10 mM. Saturating actinic light was provided by a 150 W proyector.

4. Discussion

The experiments reported in the present paper show that beauvericin inhibits photophosphorylation, coupled electron transport and the light-induced ATPase in spinach chloroplasts (fig.1 and text). Beauvericin, which belongs to the valinomycin group of antibiotics [15] inhibited coupled electron transport like energy transfer inhibitors and valinomycin [11]. However, unlike the former it also inhibited the light-dependent pH rise (table 1).

Estrada-O et al. [25] have shown that the uncoupling of mitochondria by beauvericin depends on the presence of alkali metal cations and that the effects of the ionophore exhibits a variable K^+–Na^+ discrimination depending on the anion present. In contrast to these results, the actions of beauvericin in chloroplasts do not depend on the presence of alkali cations. On the other hand, the inhibition of the light-dependent pH rise and photophosphorylation by beauvericin was enhanced by calcium ions. This effect seems related to the induction by beauvericin of a light-dependent calcium uptake (Table 2) and suggests that beauvericin, like A23187 [13], catalyzes a calcium for proton exchange across the thylakoid membrane. Our results are in agreement with the recent demonstration that beauvericin has a high affinity for calcium [17] and is able to transport calcium but not magnesium in liposomes and bacterial chromatophores [18].

Shavit et al. [7] have shown that low concentrations of the ionophore X-537A uncoupled photophosphorylation and inhibited the light-dependent proton uptake by chloroplasts in the presence of KCl. Higher concentrations of X-537A (5.6 and 28 μM) also induced uptake of potassium and sodium ions by chloroplasts (table I and II of ref. [7]). The present results show that, even in the absence of any alkali metal cations, X-537A uncouples chloroplasts (fig.2 and table 3) and inhibits the light-dependent pH rise (table 1). The uncoupling of chloroplasts and the inhibition of pH rise by X-537A are enhanced not only by potassium ions [7] but also by the presence of sodium and calcium ions, although at higher concentrations of the ionophore.

X-537A like the antibiotic A23187, which is specific for divalent cations, also induces a light-dependent calcium uptake in chloroplasts [13]. However, as has been shown for other biological systems [8,26], A23187 is several times more effective than X-537A (table 2).

In conclusion we have shown that three different antibiotics, A23187, X-537A and beauvericin share the properties of being able to induce a light-dependent calcium uptake by chloroplasts and of inhibiting photophosphorylation and proton uptake. However, they differ in other aspects like their effects on electron transport or their behavior in the presence of alkali cations.

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