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Valinomycin: a very effective inhibitor of the cyanide-insensitive alternative pathway in plant mitochondria

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The effect of valinomycin on the respiration of potato tuber callus mitochondria (*Solanum tuberosum L.* cv. Bintje) was investigated. The rate of electron transfer via the cytochrome pathway can be stimulated by abolishing membrane potential with valinomycin. However, valinomycin was found to inhibit CN-resistant respiration in potato tuber callus mitochondria. The inhibition is independent of the substrate used (external NADH, succinate and malate). The inhibition was enhanced by increasing the K^+ concentration in the medium from 0 to 125 mM and was also observed when valinomycin was added in the presence of carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP). In the presence of 125 mM K^+, 1 μM valinomycin inhibited electron flow, mediated by the alternative pathway, for approx. 65% and 5 μM valinomycin for approx. 85%. In mitochondria prepared from sweet potato (*Ipomea batatas*) valinomycin also inhibited CN-resistant respiration.

Introduction

In the energized state, mitochondria generate a proton electrochemical potential difference ($\Delta\bar{\mu}_{H^+}$) that can be used for ATP synthesis [1,2]. This $\Delta\bar{\mu}_{H^+}$ develops when electrons are transferred via the NADH-dehydrogenase complex (site 1), the bc_1 complex (site 2) or the cytochrome oxidase complex (site 3). In plant mitochondria, as in animal mitochondria, both in state 3 and state 4 this $\Delta\bar{\mu}_{H^+}$ mainly consists of a membrane potential ($\Delta\Psi$) [3,4].

In addition to cytochrome oxidase, a second terminal oxidase is often present in plant mitochondria. This cyanide-resistant, alternative oxidase transfers electrons directly from ubiquinol to oxygen and this electron transfer is not coupled to phosphorylation of ATP (see Refs. 5 and 6 for reviews).

By measuring $\Delta\Psi$ and pH gradient (ΔpH) using a distribution method, Moore et al. [4] showed that the oxidation of succinate by the alternative pathway was not coupled to the building up of a $\Delta\bar{\mu}_{H^+}$. Similar conclusions have been reached using safranine as an optical probe: no potentials were developed during NADH-supported cyanide-insensitive respiration [7]. Ishida and Palmer showed that there is no pH change in the medium associated with electron flow along the cyanide-resistant pathway [8]. In contrast to this, the oxidation of malate via the alternative pathway generates $\Delta\Psi$ through the operation of site 1 [4,7].

The ionophore valinomycin may be used to adjust $\Delta\Psi$ to a value determined by the K^+ gradient (e.g., $\Delta\Psi = 0$ when the external and internal $[K^+]$ are equal). As a consequence of lowering $\Delta\Psi$ in this way, the rate of electron transfer via the cytochrome pathway will be stimulated with all types of substrate. Such a stimulation is not expected for electron transfer through the alternative pathway with succinate or (external) NADH as the substrate, because this electron transfer is not coupled to the generation of $\Delta\Psi$.

In this paper we describe an (unexpected) inhibitory effect of valinomycin on alternative pathway-mediated electron transfer.

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Abbreviations: BSA, bovine serum albumine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone.

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Material and Methods

Plant material

Potato tubers (*S. tuberosum* L. cv. Bintje) were harvested in October and stored at 8 °C in a ventilated room. Experiments with these tubers were performed from November until June. Between July and October experiments were performed with potatoes either bought at local stores or freshly harvested. Sweet potato tubers (*I. batatas*) were bought at local stores.

Treatment of the tissue

Callus-forming potato tuber discs were cultured at 28 °C as described by Hemrika-Wagner et al. [9]. Mitochondria were isolated from this callus between the 7th and the 24th day of cultivation.

Isolation of mitochondria

For the isolation of mitochondria from potato tuber callus, the procedure described by Hemrika-Wagner [9] was slightly modified. Tissue (8 g) was ground with a mortar and pestle at 4 °C for 1 min with 10–20 ml homogenizing medium, comprising 0.35 M mannitol, 20 mM Hepes, 1 mM EDTA, 2 mM cysteine and 0.1% BSA (pH 7.2), pressed through a double layer of perlon gauze and centrifuged for 5 min at $1000 \times g$ in a SIGMA 3 MK centrifuge. The supernatant fluid was centrifuged for 10 min at $10000 \times g$. The precipitated mitochondria were washed twice, first in a medium comprising 0.35 M mannitol, 20 mM Hepes, 1 mM cysteine and 0.1% BSA (pH 6.9) and after centrifugation (10 min, $10000 \times g$) in a medium comprising 0.35 M mannitol, 20 mM Hepes and 0.1% BSA (pH 6.8). The resulting $10000 \times g$ pellet was suspended in 0.5–1 ml of the second washing medium and this suspension was used for respiratory measurements.

For the isolation of mitochondria from sweet potato [10], tissue (100 g) was homogenized in a Braun multipress MP 50 with about 200 ml chilled isolation medium comprising 0.7 M mannitol, 20 mM Hepes, 5 mM EDTA and 4 mM cysteine (pH 7.5). The homogenate was pressed through a perlon gauze and centrifuged at $1500 \times g$ for 15 min. The supernatant fluid was centrifuged for 15 min at $10000 \times g$. The precipitated mitochondria were washed twice, first in a medium comprising 0.7 M mannitol, 20 mM Hepes and 1 mM cysteine (pH 7.2–7.5) and after centrifugation (15 min $10000 \times g$) in a medium comprising 0.7 M mannitol and 20 mM Hepes (pH 7.2). The resulting $10000 \times g$ pellet was suspended in 1–2 ml of the second washing medium and this suspension was used for respiratory measurements.

Oxygen consumption

Oxygen uptake of mitochondria was recorded at 25 °C with a Clark-type O_2 electrode using a YSI monitor model 53. The incubation volume was 1 ml. Oxygen uptake of potato callus mitochondria was measured in a medium comprising 0.35 M mannitol, 20 mM Hepes, 10 mM Tris-HCl, 0.5 mM EDTA and 0.1% BSA (pH 6.8). 125 mM KCl or LiCl was present when indicated. Respiration of sweet potato mitochondria was measured in a medium comprising 0.7 M mannitol, 20 mM Hepes, 10 mM Tris-HCl, 0.1 mM EDTA and 5 mM $MgCl_2$ (pH 7.2) or in a medium containing 0.45 M mannitol, 10 mM Tris-HCl, 0.1 mM EDTA and 5 mM $MgCl_2$ (pH 7.2) plus 125 mM KCl.

The capacity of the alternative pathway was determined as respiration after the addition of 0.1–0.2 mM NaCN. Cytochromal respiration was measured in the presence of 3.8 mM BHAM. The capacities of the alternative pathway and the cytochromal respiration were corrected for residual respiration measured in the presence of 0.1–0.2 mM NaCN and 12.5–25 mM BHAM.

Swelling experiments

Mitochondrial swelling was measured as a decrease of absorbance at 540 nm with an LKB Ultrospec K Spectrofotometer. Passive swelling of mitochondria was measured in the same media as that used for oxygen consumption, except that for swelling of potato callus mitochondria in the presence of 125 mM KCl the mannitol concentration was reduced to 0.1 M to obtain the same osmolarity as that obtained without KCl. In positive control experiments other media were used, for callus mitochondria medium comprising 70 mM KCl, 20 mM Hepes, 10 mM Tris-HCl, 0.5 mM EDTA, 0.1% BSA (pH 6.8) and 100 mM malate, for sweet potato mitochondria the medium comprising 70 mM KCl, 0.14 M mannitol, 20 mM Hepes, 10 mM Tris-HCl, 0.1 mM EDTA, 5 mM $MgCl_2$ (pH 7.2) and 100 mM malate. The reaction was initiated by addition of mitochondria. All swelling experiments were carried out in the presence of 0.1 mM NaCN.

Assays and reagents

Protein was determined by the method of Bradford [11], using BSA as a standard. Valinomycin was purchased from Boehringer, FCCP from Fluka, nigericin was from Sigma and monensin was from Eli Lilly & Company. All ionophores were dissolved in absolute ethanol. Glutamine oxaloacetate transaminase was from Boehringer.

Results

Fig. 1 shows the stimulation of the external NADH-oxidation via the cytochrome pathway by valinomycin in the presence of K^+. Because the internal K^+ concentration of the mitochondria was estimated to be 125 mM [7], an external K^+ concentration of 125 mM was

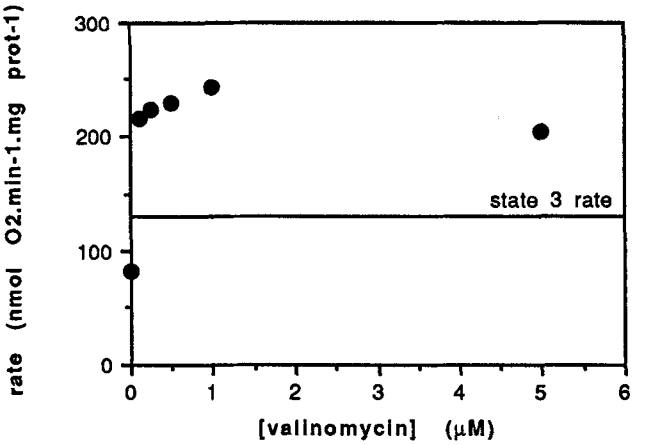

Fig. 1. Stimulation of the cytochrome pathway in potato callus mitochondria by valinomycin. Cytochromal respiration was measured with 2.8 mM NADH as a substrate in the presence of 3.8 mM BHAM. The medium contained 125 mM KCl. The mitochondrial protein concentration during the measurements was 0.32 mg·ml^{-1}. The horizontal line indicates state 3 respiration rate measured in the absence of valinomycin and with 2.4 or 4 mM NaH_2PO_4 and 0.07 or 0.12 mM ADP present. Reaction rates were corrected for residual respiration. For further details see Material and Methods.

also used. In the presence of valinomycin the respiration rate was much higher than the state 3 rate. With 0.1 μM valinomycin, the stimulation was already almost complete; higher valinomycin concentrations did not lead to a significant increase of the respiration rate. With 5 μM valinomycin the stimulation of the cytochromal respiration even decreased.

Addition of valinomycin in the presence of cyanide yielded strikingly different results. NADH-oxidation by callus mitochondria showed a considerable cyanide resistance due to electron transfer via the alternative oxidase. Valinomycin appeared to inhibit this CN-resistant NADH oxidation (Fig. 2). Maximal inhibition of the CN-resistant pathway by valinomycin was achieved in the presence of K^+ (Fig. 2A). The inhibition was lower but still significant in the absence of KCl. To determine whether the enhanced inhibition in the presence of KCl was caused by a higher ionic strength the titrations were also performed with 125 mM LiCl in the reaction medium. The ionic strength did not seem to have any effect on the inhibition of the CN-resistant respiration by valinomycin (Fig. 2A). Electron transfer from external NADH to oxygen does not generate a membrane potential. As a consequence the addition of an uncoupler, such as FCCP, should not affect the inhibition of the alternative pathway-mediated respiration by valinomycin. However, this inhibition was somewhat lower in the presence of FCCP (Fig. 2B; 125 mM K^+ added).

During oxidation of both (external) NADH and succinate by plant mitochondria, phosphorylation site 1 is not active; as expected the same inhibition characteristics were found (Fig. 3). With malate a more complex situation is expected: due to the generation of a membrane potential at site 1, a stimulation of the electron transport might interfere with the inhibitory effect. However, the inhibition by valinomycin of malate-dependent CN-resistant electron transfer was not significantly different from that with succinate or NADH.

The experiments in Fig. 3 were carried out in the absence of added KCl. Similarly to external NADH, with malate or succinate as substrate in the presence of 125 mM KCl a larger inhibition was found (not shown).

The inhibition of the CN-resistant pathway by valinomycin is not a specific property of potato callus mitochondria. Valinomycin also inhibits the CN-resistant pathway of mitochondria from sweet potato (*I. batatas*) (Fig. 4). Again, with mitochondria from sweet potato the inhibition is independent of the substrate used (Fig. 4A) and the inhibition is enhanced by in-

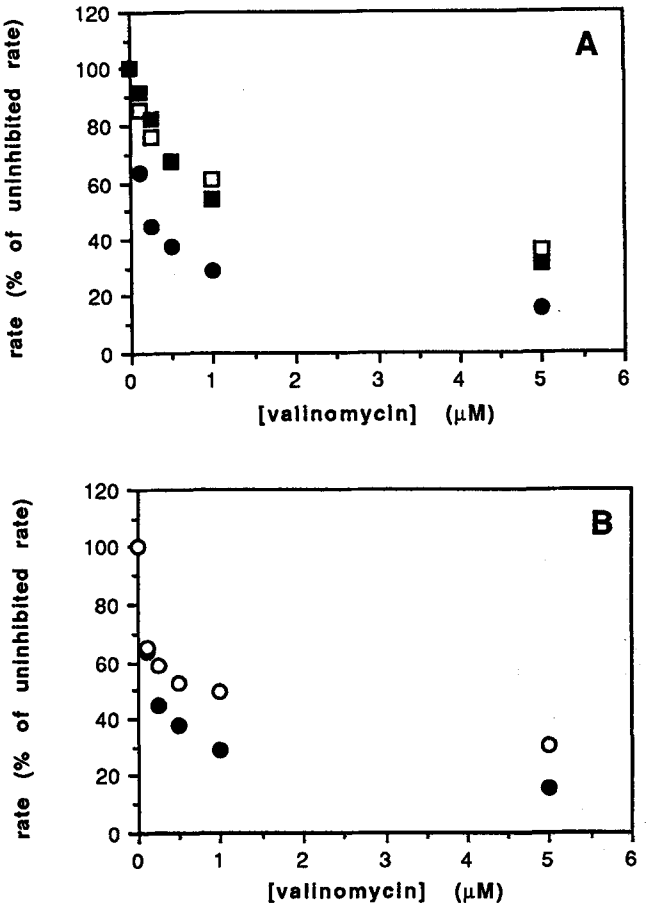

Fig. 2. Inhibition of the CN-resistant oxidation of external NADH in potato callus mitochondria by valinomycin. CN-resistant respiration was measured with 2.8 mM NADH as a substrate in the presence of 0.2 mM NaCN. The mitochondrial protein concentration during the measurements was 0.54 mg·ml^{-1}. Further conditions are as described in Fig. 1. (A) □, 0 mM KCl, ●, 125 mM KCl; ■, 125 mM LiCl. (B) ●, 125 mM KCl; ○, 125 mM KCl+2 μM FCCP. 100% activity of CN-resistant respiration was 67, 54, 51 and 30 nmol O_2·min^{-1}·mg protein^{-1} for 0 mM KCl, 125 mM KCl, 125 mM LiCl and 125 mM KCl+2 μM FCCP, respectively.

creasing the K^+ concentration in the medium from 0 to 125 mM (Fig. 4B). In the experiment with malate as substrate, ADP and phosphate were added to stimulate electron transfer through site 1.

Besides the effect of valinomycin also the effects of the ionophores nigericin and monensin were investigated. With these ionophores no inhibition of the CN-resistant pathway was observed (results not shown).

Inhibition by valinomycin is enhanced by the presence of K^+. The data in Fig. 2A show that Li^+ is not able to replace K^+ in this respect. With Rb^+ the inhibition by valinomycin was even higher than that in the presence of K^+ (not shown). The effectivity of the cations is: $Rb^+ > K^+ > Li^+$, which is similar to the effectivity in forming complexes with valinomycin [13].

The inhibition of the CN-resistant pathway by valinomycin was not correlated with swelling of mitochondria. In agreement with Zoglowek et al. [14] swelling of mitochondria in the presence of KCl, malate and valinomycin occurred (Fig. 5 experiment A and D). Swelling of potato callus mitochondria was markedly less (Fig. 5A) than in sweet potato mitochondria (Fig. 5D). In the absence of KCl and in the presence of valinomycin no passive swelling of mitochondria was found with neither potato callus mitochondria (Fig. 5B) or with sweet potato mitochondria (Fig. 5E). In the presence of 125 mM KCl and valinomycin passive swelling was found with both types of mitochondrion (Fig. 5C and 5F). Jung and Brierley previously reported passive swelling of potato tuber mitochondria in the presence of 100 mM KCl and valinomycin [15].

In potato callus mitochondria the inhibition of the CN-resistant oxidation of external NADH with 5 μM valinomycin in the absence of KCl was 65% and in the presence of KCl it was 85% (Fig. 2A). In sweet potato mitochondria the inhibition was, respectively, 70% and 80% (Fig. 4). While in the presence of valinomycin and KCl both inhibition of the CN-resistant respiration and swelling were found, in the absence of KCl, valinomycin inhibited the CN-resistant respiration but no swelling was measured. With succinate as respiratory substrate, a similar result was obtained (not shown).

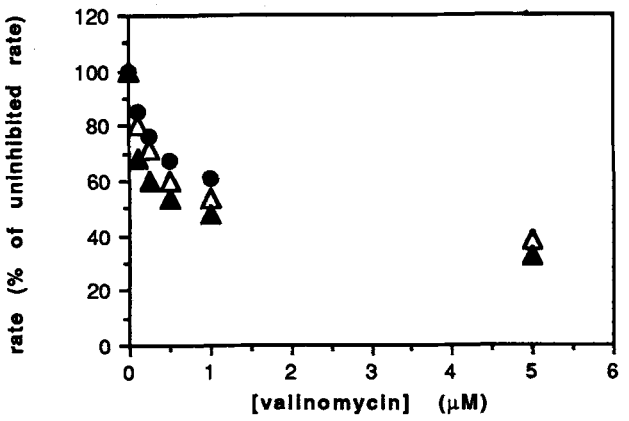

Fig. 3. Inhibition of the CN-resistant oxidation of various substrates in potato callus mitochondria. Respiration was measured in the presence of 0.2 mM NaCN. The medium contained no KCl. The mitochondrial protein concentration during the measurements was 0.54 mg·ml^{-1}. •, 2.8 mM NADH; △, 20 mM succinate; ▲, 40 mM malate. 100% activity of CN-resistant respiration was 67, 41 and 74 nmol $O_2 \cdot min^{-1} \cdot mg$ protein^{-1} for NADH, succinate and malate, respectively. When malate was used as a substrate, 0.15 mM NAD^+ was added as cofactor [12] and 24 mM glutamate and 10 μl glutamine oxaloacetate transaminase were added.

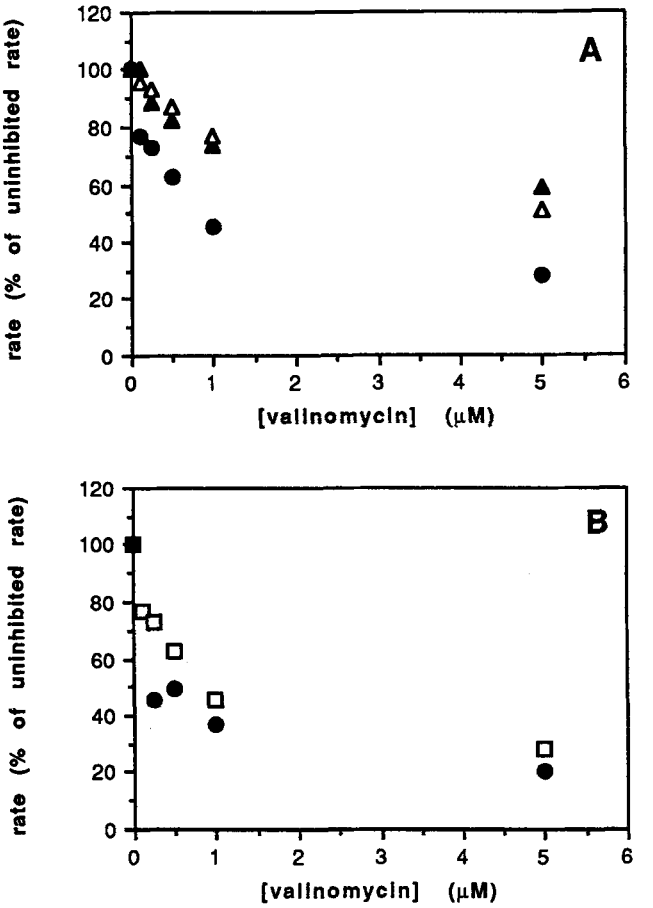

Fig. 4. Inhibition of the CN-resistant oxidation in sweet potato mitochondria. Conditions as described in Material and Methods. The mitochondrial protein concentration during the measurements was 0.36 mg·ml^{-1}. (A) Effect of various substrates: •, 2.8 mM NADH; △, 20 mM succinate, ▲, 40 mM malate. (B) The effect of KCl with 2.8 mM NADH as a substrate. □, 0 mM KCl; •, 125 mM KCl. CN-resistant oxidation of malate was measured after addition of 4 or 8 mM NaH_2PO_4 and 0.12 or 0.24 mM ADP, CN-resistant oxidation of external NADH and succinate was measured in the absence of ADP and phosphate. 100% activity of CN-resistant respiration was 18, 74, 81 and 24 nmol $O_2 \cdot min^{-1} \cdot mg$ protein^{-1} for NADH (0 mM KCl), succinate, malate and NADH (125 mM KCl), respectively.

Discussion

In this paper the ionophore valinomycin is shown to inhibit the CN-resistant pathway. The inhibition of the CN-resistant pathway by valinomycin was not related

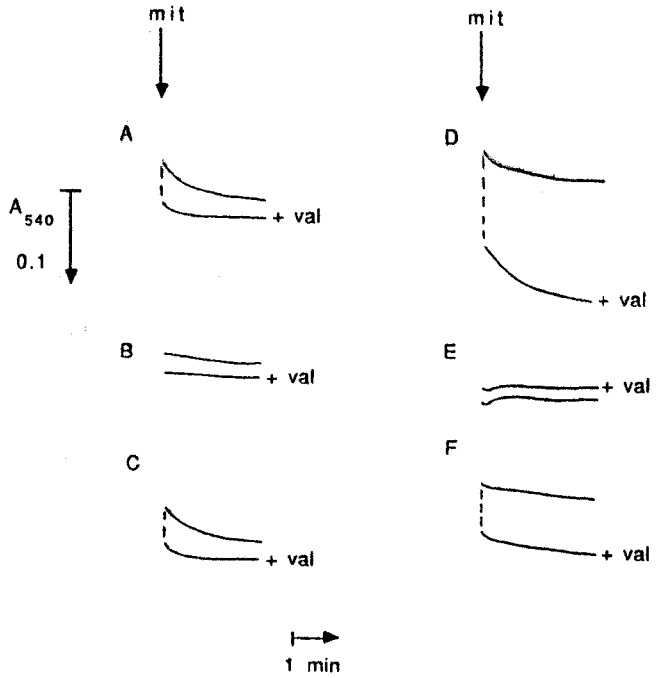

Fig. 5. Swelling of mitochondria. Swelling was measured as described in Material and Methods, in the presence of 0.1 mM NaCN. Each experiment shows a trace obtained in the absence and a trace obtained in the presence (+val) of valinomycin. (A-C) 0.19 mg protein·ml^{-1} potato callus mitochondria. (D–F) 0.18 mg protein·ml^{-1} sweet potato mitochondria. (A, D) Positive controls (swelling in the presence of K^+ and 100 mM malate). (B, E) Experiments under respiratory conditions (+ 2.8 mM NADH) in the absence of K^+. (C, F) Experiments under respiratory conditions (+2.8 mM NADH) in the presence of 125 mM KCl. Valinomycin concentration used was 10 μM in A and D and 5 μM in B, C, E and F.

to generation of $\Delta\Psi$, because the inhibition was also seen in the absence of a potassium gradient (125 mM KCl Fig. 2A), even when additional FCCP was present (Fig. 2B).

The concentration dependency of the valinomycin inhibition was completely different from that of stimulation of the cytochromal respiration (Fig. 1). This stimulation, caused by abolishment of $\Delta\Psi$, already reaches a maximum at about 0.1 μM valinomycin (Fig. 1), while half-maximal inhibition of the alternative path only was observed at about 0.2 μM and the inhibition continued to increase with increasing concentrations of valinomycin.

The increased inhibition of the CN-resistant pathway by valinomycin in the presence of 125 mM KCl was not due to changes in ionic strength, because the inhibition also occurred without K^+ and in the presence of 125 mM LiCl (Fig. 2A). Nor was the inhibition dependent on the substrate used; with external NADH, succinate and malate the same inhibition was observed (Fig. 3). The inhibition was also not due to the tissue or plant species used; valinomycin inhibited the induced CN-resistant pathway of potato callus mitochondria as well as the constitutive CN-resistant pathway of mitochondria from sweet potato (Fig. 4).

The inhibition was not correlated with swelling of mitochondria because without KCl and in the presence of 5 μM valinomycin no swelling of potato callus mitochondria and sweet potato mitochondria occurred (Fig. 5B and E), although the inhibition with 5 μM valinomycin is significant (Fig. 2A and 4A). This excludes the possibility that specific structural damage to alternative respiration caused by swelling is responsible for the inhibition of valinomycin.

The inhibition by valinomycin (+125 mM KCl) was lower in the presence of FCCP (Fig. 2B). Although complex formation between valinomycin-K^+ and protonophores has been observed [16], the lower inhibition in the presence of FCCP cannot be explained by this complex formation, because a 1:1 stoichiometry for the complex is described. In that case the minimal inhibition with 5 μM valinomycin +2 μM FCCP would be the same as that obtained with 3 μM valinomycin in the absence of FCCP. However, inhibition by 3 μM valinomycin was larger than with 5 μM valinomycin +2 μM FCCP.

Apparently, valinomycin at a concentration of 1–5 μM is a very effective inhibitor of the alternative pathway. When compared with the widely used hydroxamates, which are inhibitory in the millimolar range, the ionophore is far more effective. The mode of inhibition of valinomycin is still under investigation, but it seems to be unrelated with the effect of valinomycin on the membrane potential. The increased inhibition when external K^+ is added suggests that the effect is caused by the K^+-valinomycin complex; in the absence of added K^+, the potassium ion may be supplied from the mitochondrial matrix. Because stimulation of inhibition by alkali metal ions shows the same specificity as complexation of valinomycin, we conclude that it is very likely that inhibition is not caused specifically by the K^+ complex of valinomycin but generally by the cation-valinomycin complex.

A consequence of this inhibition by valinomycin is that one should be careful when using this ionophore in the study of plant mitochondria. Moore et al. [3] used 0.13 μM valinomycin in their measurements of $\Delta\Psi$ with the $^{86}Rb^+$ distribution method. Our results show that with this concentration there is little inhibition of alternative respiration, but electron flow through the cytochrome pathway may be stimulated (Fig. 1).

In contrast to these results with potato callus mitochondria, Moore et al. found no stimulation of oxygen uptake by 0.13 μM valinomycin in mung bean mitochondria [3]. However, under the conditions employed by these authors (no external K^+, 0.5 mM Rb^+ added) in the presence of valinomycin a $\Delta\Psi$ of about 140 mV is found [3] so that indeed little stimulation of oxygen uptake may be expected.

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