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Selective interaction of valinomycin/K^+ with the cytochrome *bf* complex of chloroplasts

Synergistic effect with MOA stilbene on extent of cytochrome b_{563} reduction in continuous light

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Valinomycin/K^+ is shown to selectively interact at sub-micromolar concentrations with the cytochrome *bf* complex in thylakoid membranes, inducing a red shift of the ferrohaem *b* absorbance α-band, a slow down of post-illumination *b*-reoxidation and a corresponding increase of *b*-reduction level in continuous light. These effects of valinomycin/K^+ are not related to its field dissipating action, as they are not affected by nonactin. Presence of K^+ is required. Phenomenologically the valinomycin/K^+ effects are similar to those caused by 10 times higher MOA stilbene concentrations. However, synergism is observed between the two inhibitors, suggesting different modes of action. When both inhibitors are combined more than one haem b can be reduced by illumination.

Cytochrome *bf* complex; Q-cycle; Valinomycin; MOA stilbene; Cytochrome b_{563} reduction

1. INTRODUCTION

The cytochrome *bf* complex of the thylakoid membrane is of central importance for photosynthetic electron transport between the two photosystems (see reviews [1–5]). There is general agreement that the oxidation of PQH_2 at the *bf* complex is linked to proton translocation from the stroma into the lumen. On the other hand, there are still uncertainties about the details of electron and proton transfer within the complex, particularly with respect to the in vivo role of various proposed mechanisms of Q-, SQ- and *b*-cycles (for a recent review see [5]).

Specific inhibitors have been playing a decisive role in the elucidation of the functioning of the bc_1 complex in mitochondria [6] and of the *bf* complex in chloroplasts [7]. So far, two basic types of bf inhibitors have been characterised, the DBMIB-type [7–9] at the quinol oxidation site (also called Q_p-; Q_o- or Q_z-site) and the NQNO-type [10,11] at the quinone reduction site (also called Q_n-; Q_i- or Q_c-site). Recently, with MOA stilbene a new inhibitor of the *bf* complex was introduced [12], with properties similar to NQNO, but displaying the advantage of lesser side-effect on PSII activity.

Here we report on the discovery that valinomycin/K^+, which in the past has been routinely used for dissipation of transmembrane electrical fields [13,14], does selectively interact at sub-micromolar concentrations with the *bf* complex and by doing so strongly enhances the inhibitory effect of MOA stilbene. It will be shown that also valinomycin/K^+ by itself is effective in terms of enhancing cyt b_{563} reduction in continuous light at only 1/10 of the concentration required with MOA stilbene for a comparative effect.

2. MATERIALS AND METHODS

Intact chloroplasts were prepared from freshly harvested, greenhouse grown spinach leaves essentially as previously described [15]. If not stated otherwise, the chloroplasts were resuspended isotonically in the standard reaction medium 'C' [15]. Thylakoids were obtained by 30 s hypotonic shock in H_2O and isotonic resuspension by adding an equal amount of double-strength standard medium.

Absorbance changes were measured with a laboratory-built kinetic spectrophotometer based on 16 individual light-emitting-diode (LED) measuring light sources equipped with narrow-band interference filters [16]. The half-band width was 2–3 nm, depending on individual wavelength. Measuring wavelengths were scanned repetitively, with 12.5 μs for an individual measurement and 200 μs for a full cycle of 16 wavelengths. The integrated measuring-light intensity amounted to 20 nmol quanta $m^{-2} \cdot s^{-1}$. The data were processed by laboratory-developed software to provide time-resolved difference spectra and the deconvoluted contributions of cytochrome b_{563} and cytochrome *f* redox-changes [16]. Deconvolution was based on previously determined 'standard spectra' for the various components contributing to absorbance changes in the 530–590 nm range [16]. The following differential molar extinction coefficients were assumed, essentially

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; MOA stilbene, E-β-methoxyacrylat-stilbene; NQNO, 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide; Q_p-site, plastoquinol binding site at electrically positive, lumen oriented side of the thylakoid membrane; Q_n-site, plastoquinone binding site at electrically negative, stroma oriented side of the thylakoid membrane.

in agreement with [17]: cytochrome b_{563}, $\Delta\varepsilon(564\text{–}593) = 21\ mM^{-1}\cdot cm^{-1}$; cytochrome *f*, $\Delta\varepsilon(554\text{–}570) = 21\ mM^{-1}\cdot cm^{-1}$. In agreement with this assumption the concentration ratio of cytochrome b_{563}/cytochrome *f* was found to be 2, as determined by complete chemical oxidation and reduction of the two cytochromes. In order to obtain the relative extent of cytochrome b_{563} reduction, its concentration, as derived by deconvolution, was divided by the total concentration of cytochrome *f*. At a chlorophyll concentration of 40 $\mu g\cdot ml^{-1}$ the cytochrome *f* concentration amounted to 55 nM.

Valinomycin, nigericin and nonactin were purchased from Sigma (Deisenhofen, Germany). Catalase was obtained from Boehringer (Mannheim, Germany).

3. RESULTS AND DISCUSSION

3.1. *Valinomycin/K⁺ induced spectral bandshift of ferrohaem* b

Fig. 1 shows the effect of the addition of 0.6 μM valinomycin to a stirred suspension of dark spinach thylakoids on absorbance properties of chemically prereduced cytochrome b_{563}. In panel A the kinetics of the valinomycin-induced difference signal, $\Delta I/I$ (568.5–560.3), is displayed for high and low concentrations of K^+ in the suspension medium. Panel B shows the time-resolved difference spectra in the 540–590 nm range, which indicate a shift of the ferrohaem *b* α-absorbance band. Under the assumption that only one haem is involved, computer-simulation on the basis of our cytochrome b_{563} 'standard-spectrum' [16] leads to the conclusion that an absorbance band at 564.0 nm is shifted by 0.5 nm to longer wavelengths (data not shown).

It should be noted that under the conditions given with the experiment of Fig. 1 no significant transmembrane potential can be involved and, hence, valinomycin/K^+ does not act on the basis of its well known field dissipating action. Actually, essentially the same spectral shift is observed when 1 μM nonactin is present which would prevent any potential transmembrane field formation (not shown) (see [18]). However, the data also show that the spectral shift is not simply caused by valinomycin binding. Rather the K^+-carrier function of valinomycin [13,14] appears to be essential. This points to the intriguing possibility of local electrostatic interaction between the ferrohaem and the K^+-ion, with corresponding consequences on redox potential and properties of low-potential chain electron transport.

The observed valinomycin/K^+ effect is almost identical to that recently reported for MOA stilbene on a reduced purified cytochrome *bf* preparation [12]. However, with valinomycin the concentration required for the spectral shift is distinctly lower than with MOA stilbene. The valinomycin concentration dependency is depicted in Fig. 2, revealing a half-maximal effect at 0.05 μM and saturation around 1 μM, with the concentration of the *bf* complex being 0.11 μM. For equivalent effects of MOA stilbene 20 μM and 75 μM were reported at a concentration of isolated *bf* complex amounting to 5 μM. Obviously, despite the presence of an extended lipid phase and various other proteins associated with the thylakoid membrane, approximately one valinomycin/K^+ per *bf* complex is sufficient for a pronounced effect on ferrohaem *b*. Hence, this interaction may be considered very specific and equivalent to selective binding. There remains, however, the possibility that the decisive effect is exerted by K^+ binding while the valinomycin only plays the role of carrying this ion to its binding site.

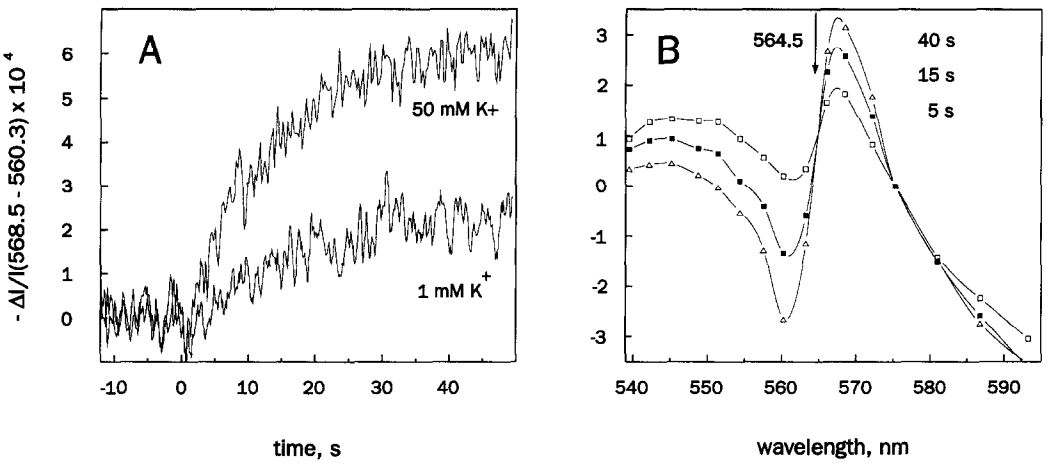

Fig. 1. Characterization of the ferrohaem *b* α-band absorbance shift induced by addition of 0.6 μM valinomycin to a stirred suspension of spinach thylakoids. (A) Kinetics of the valinomycin-induced absorbance changes in the presence of 50 mM K^+ and 1 mM K^+. The difference signal (568.5 nm and 560.3 nm) relates to the wavelengths of maximum and minimum in the difference spectrum. (B) Time resolved difference spectra of the valinomycin-induced absorbance change. The various data points are associated with the 16 wavelengths determined by the combination of 16 individual light-emitting-diodes and narrow-band interference filters on which the applied kinetic spectrophotometer is based (see section 2). Reference wavelength was 575.3 nm. Data from 10 runs were averaged. Before valinomycin-addition, the suspension was incubated for 10 min in the presence of 15 μM Na-dithionite, catalase (650 units ml^{-1}) and 0.5 μM benzylviologen, in order to assure full reduction of cytochrome b_{563} (see [19]). Chlorophyll concentration was 80 $\mu g\ ml^{-1}$ corresponding to approximately 0.11 μM cytochrome *bf* complex.

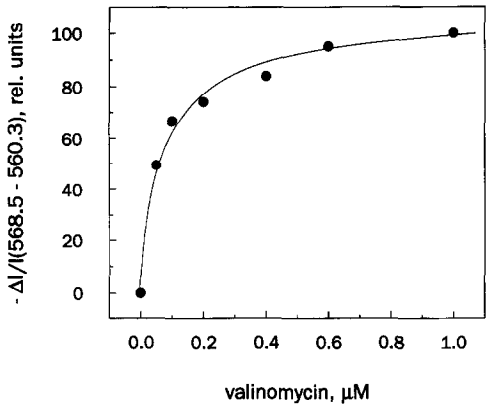

Fig. 2. Relationship between the relative amplitude of the valinomycin-induced difference signal and the concentration of the ionophore in the presence of 50 mM K^+. The maximal difference signal, $\Delta I/I$(568.5–560.3), was evaluated 60 s after valinomycin-addition. Conditions as for Fig. 1.

3.2. *Inhibitory effect on post-illumination* b-*oxidation and* f-*reduction*

In Fig. 3, relaxation kinetics of post-illumination cytochrome b_{563} re-oxidation and cytochrome *f* re-reduction are depicted. Valinomycin/K^+ induces similar effects as MOA stilbene, as it causes lag phases preceding *b*-reoxidation and *f*-reduction after light-off and also increases the half-times and amplitudes of the observed changes. This indicates that valinomycin/K^+ just like MOA stilbene increases the extents of *b*-reduction and *f*-oxidation during continuous illuminaton by slowing down *b*-oxidation and *f*-reduction. This experiment was carried out in the presence of nonactin, which caused full dissipation of the light-induced membrane potential, as confirmed by P515 measurements (not shown) (see [18]). The observed effects seem to be in agreement with an inhibitory effect of valinomycin/K^+ at the Q_n-site, in analogy to the interpretation of the MOA stilbene effect [12]. However, other interpretations appear possible. For example, it cannot be excluded that valinomycin/K^+ shifts the redox potential of one or both *b*-haems or that the K^+ interferes with a redox-linked proton pump [20]. The following results suggest that the effect of valinomycin/K^+ indeed is not identical to that of MOA stilbene.

3.3. *Synergism between valinomycin/K^+ and MOA stilbene*

In Fig. 4 the relative extent of cyt b_{563} reduction during illumination of uncoupled intact chloroplasts is plotted in dependence of valinomycin/K^+ and MOA stilbene concentrations. Both inhibitors when applied alone cause an increase in *b*-reduction level from 0.4 to 0.9–1.0, with half-maximal effects observed at approximately 1.5 μM valinomycin/K^+ and 15 μM MOA stil-

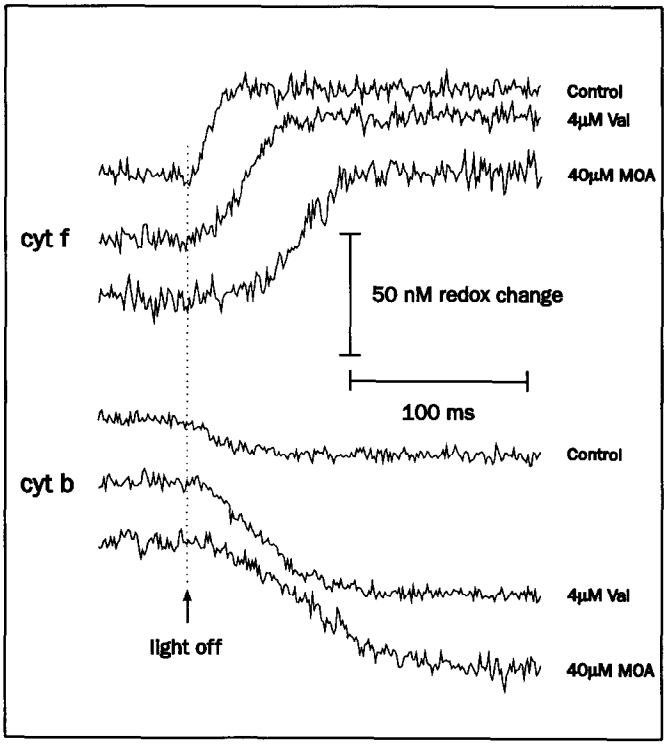

Fig. 3. Comparison of the effects of 4 μM valinomycin/K^+ and 40 μM MOA stilbene on light-off relaxation kinetics reflecting cytochrome b_{563} re-oxidation and cytochrome *f* re-reduction. Intact chloroplasts in the presence of 0.4 mM methyl viologen, catalase (1300 units·ml^{-1}), 0.7 μM nigericin, 0.4 μM nonactin and 50 mM K^+. Chlorophyll concentration, 40 μg·ml^{-1}. Continuous illumination by strong red light (2000 μmol quanta $m^{-2}·s^{-1}$) interrupted every 1.1 s for 400 ms. Averages of 100 runs are depicted. Cytochrome reduction levels were determined from time-resolved difference spectra by computer-assisted deconvolution (see section 2).

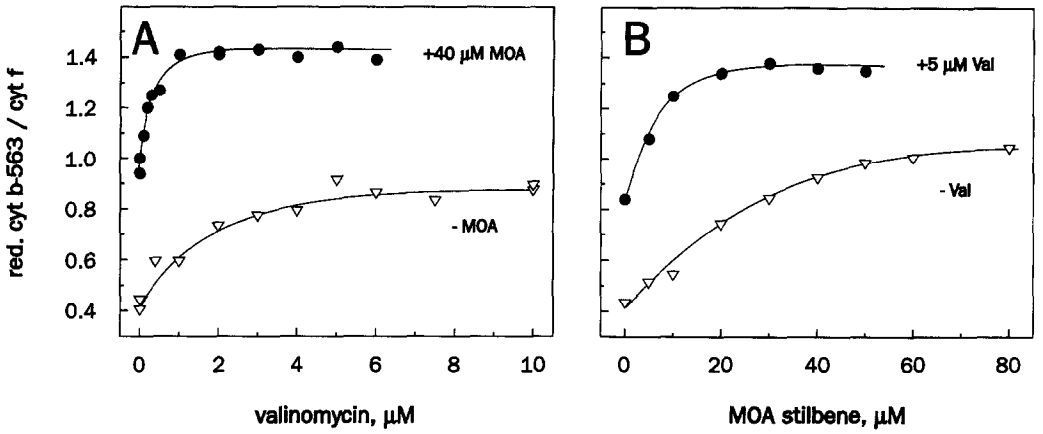

Fig. 4. Relative extent of cytochrome b_{563} reduction in uncoupled intact chloroplasts in dependence of applied concentrations of valinomycin and MOA stilbene. Chloroplasts were illuminated with 2000 μmol quanta $m^{-2} \cdot s^{-1}$ red light in the presence of 0.4 mM methyl viologen, catalase (1300 units $\cdot ml^{-1}$), 1 μM nigericin, 0.4 μM nonactin and 50 mM K^+. Chlorophyll concentration, 40 $\mu g \cdot ml^{-1}$. A fresh sample was used for each data point. The value of reduced cyt b_{563}/cyt *f* was determined 10 s following onset of continuous illumination by a computer-assisted deconvolution method (see section 2). (A) Valinomycin-concentration dependence in presence and absence of a saturating amount of MOA stilbene. (B) MOA stilbene-concentration dependence in presence and absence of a saturating amount of valinomycin.

bene. When applied in the presence of saturating concentrations of the respective other inhibitor, distinctly lower I_{50}-values of the two inhibitors are measured. In the presence of 40 μM MOA stilbene the half-maximal effect of valinomycin/K^+ is found at approximately 0.2 μM, whereas at 5 μM valinomycin/K^+ already 4 μM MOA stilbene give a half-maximal effect. This mutual synergism indicates that despite of the phenomenological similarities expressed in Fig. 3 and apparent by comparison with the data in [12], the modes of action of valinomycin/K^+ and MOA stilbene are not identical.

In the experiment of Fig. 4 the maximal extent of cyt b_{563} reduction amounts to 1.4 haems reduced per *bf* complex. In other, similar experiments we have measured the light-induced reduction of up to 1.9 haems per *bf* complex in the presence of valinomycin/K^+ and MOA stilbene (not shown in the figures). These are the highest light-induced reduction levels so far reported in the literature. Actually, in previous work it has been considered problematic that neither NQNO [11,12] nor MOA stilbene [12] will cause the reduction of more than one haem of cytochrome b_{563} when multiple, closely spaced flashes are applied. This feature, which is in contrast to the effect of Q_n-site inhibitors like antimycin A at the bc_1 complex, where both haems become reduced, has given rise to controversial interpretations [21–24]. Recently, it has been proposed that MOA stilbene and NQNO do not inhibit the rapid catalytic cycle at the Q_n-site but only the slow relaxation of an intermediate state, characterised by maximally one electron per low-potential chain [12]. In line with this interpretation, the combination of valinomycin/K^+ and MOA stilbene could be the first effective means of inhibiting plastoquinone reduction at the Q_n-site.

Extensive further experiments will be required for a more definite assessment of the new valinomycin/K^+ effect and its synergism with MOA stilbene. Experiments with purified cytochrome *bf* complex should allow to distinguish whether the spectral shift applies to both haems or only to one of them. Furthermore, it will be of interest to look for analogous effects on bc_1 complexes of mitochondria and bacteria. Such experiments are presently in preparation.

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