Valinomycin as a tool for the reversal of the effect of protonophoric uncouplers

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1. INTRODUCTION

Transitions in the energy-state of biological systems are most often elicited by oxygen pulses, light pulses or uncouplers. The study of the changes accompanying such transitions greatly contributed to the understanding of the mechanism of energy-transduction. Transitions induced by light or oxygen pulses are reversible. In contrast, the reversal of the de-energization evoked by uncouplers is, as a rule, troublesome. We encountered this difficulty while using the powerful protonophoric uncouplers [2] FCCP or S-13 for de-energization in a study of the respiration-driven ion fluxes in a halotolerant bacterium, *Ba*$_1$. Anions of protonophoric uncouplers form stable, ternary complexes with valinomycin-K^+ [3–6]. Thus, we have tried to utilize complex formation as a tool for the reversal of the uncoupler-induced perturbation. Here, we show that in bacterium *Ba*$_1$, the interference of FCCP or of S-13 with energy-linked functions can be abolished with the aid of valinomycin. The applicability of this method of 'recoupling' may be extended to systems, which are relatively insensitive to valinomycin per se, as is the case with the bacterium, *Ba*$_1$ [7].

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; *S*-13,2,5-dichloro-3-(*tert*-butyl)-4′nitrosalicylanilide; 9-AA, 9-aminoacridine; Dis-C_3-(5), 3,3′-dipropylthiodicarbocyanine iodide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; *Q*-9AA, % change in the fluorescence of 9-aminoacridine; *Q*-Dis, % change in the fluorescence of Dis-C_3-(5); ΔpH, trans-membrane pH-gradient; ΔΨ, membrane potential; $\Delta I_{600\ nm}$, initial rate of change in the light scattering at 600 nm (arbitrary units)

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2. MATERIALS AND METHODS

Hepes, valinomycin and FCCP were products of Sigma Chemical Co. (St Louis MO); Dis-C_3-(5) and S-13 were generous gifts from Professor L. Ernster, University of Stockholm (Stockholm).

Bacterium, *Ba*$_1$ was grown and harvested as in [1] for the 'high salt-grown' organism. 'KCl-loaded' cells were prepared by washing the harvested cells repeatedly with 0.8 M KCl at 0°C. To measure the rate of volume changes elicited by energy-dependent extrusion of KCl from the cells, the light scattering method in [1] was used. The cross-membranal ΔpH and ΔΨ were assessed with the aid of the fluorescent dyes 9-AA and Dis-C_3-(5), respectively [8,9].

Light scattering and fluorescence were measured in a Perkin-Elmer fluorescence spectrophotometer, model MPF-44B. Absorption spectra were taken with a DW-2aUV–Vis spectrophotometer. Oxygen was assayed polarographically, ATP as in [10] and protein as in [11]. For other experimental details see figure and table legends.

3. RESULTS AND DISCUSSION

According to [3] complex formation between FCCP and valinomycin-K^+ is reflected by a shift in the absorption peak of the uncoupler. In the

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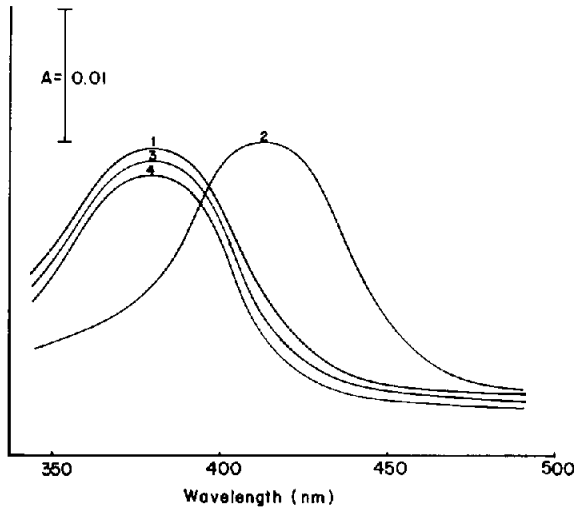

Fig.1. Spectral evidence for the interaction between FCCP and valinomycin. The sample cell contained in a 3.0 ml total vol. 30 mM Hepes (pH 7.0), 0.67 μM FCCP and 0.8 M KCl or NaCl as indicated. FCCP was omitted from the reference cell. When indicated, 1.52 μM valinomycin was also added both to the sample cell and the reference cell. After 5 min incubation the difference spectra were recorded. Curves: KCl + FCCP (1); KCl + FCCP + valinomycin (2); NaCl + FCCP (3); NaCl + FCCP + valinomycin (4).

presence of 0.8 M KCl, which was the major salt constituent of the standard assay medium, valinomycin caused a shift in the absorption peak of FCCP from 381–415 nm (fig.1). No shift was observed when NaCl replaced the potassium salt. Under conditions similar to fig.1, the protonophore S-13 also interacted with valinomycin-K^+. Here the shift in the absorption peak was from 382–428 nm (not shown).

The feasibility of 'recoupling' by valinomycin was first tested on energy-dependent extrusion of salt from cells of Ba_1, pre-loaded with KCl. Details about this pump activity leading to contraction of the cytoplasm were given in [1]. It can be most conveniently followed by monitoring the changes in light scattering. The pump became functional when the cells were introduced in the assay medium containing the respiratory substrate (fig.2). FCCP nearly completely suppressed the volume contraction, whereas valinomycin by itself had no effect on the kinetics of changes in light scattering. However, when valinomycin was added subsequently to the assay system containing the uncoupler, a gradual reactivation of the pump activity occurred. Valinomycin was also found to abolish the effect of the uncoupler S-13 under conditions which were analogous to that in fig.2 (not shown).

To assess the connection between the restoration of the pump activity and the formation of the uncoupler–valinomycin-K^+ complex the titration in fig.3 was done: [FCCP] was constant while [valinomycin] varied. The absorbancy at 415 nm (indicating the formation of the complex) and the light scattering (reflecting the pump activity) changed in a near-parallel manner, thus suggesting

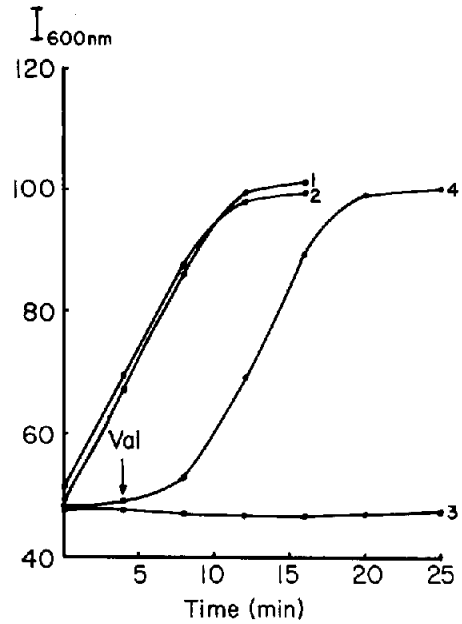

Fig.2. Effect of FCCP and of valinomycin on salt extrusion from cells of Ba_1, preloaded with 0.8 M KCl. The optical cell contained in 3.0 ml total vol. 30 mM Hepes (pH 7.0), 0.8 M KCl and 50 mM ethanol at 37°C. When indicated also 0.67 μM FCCP and/or 1.52 μM valinomycin were added. The reaction was started by adding the bacterial cells (0.1 mg protein/ml). The kinetics of the change in light scattering ($I_{600\ nm}$) was monitored. Curves: no addition (1); valinomycin (2); FCCP (3); FCCP followed by valinomycin at the time indicated by arrow (4).

Table 1

Reversal of uncoupling by valinomycin

Additions[a]	Respiration (natom O/min)		ATP (nmol)		Q-9AA (%)		Q-Dis (%)		$\Delta I_{600\ nm}$ (arb. units)	
	−val.	+val.	−val.	+val.	−val.	+val.	−val.	+val	−val.	+val
None	144.0	130.0	4.0	3.9	7.1	7.5	41.0	41.0	26.5	21.5
FCCP	494.0	219.0	0.9	3.2	1.9	6.6	0.0	30.0	0.0	20.5
S-14	562.0	178.0	–	–	–	–	–	–	0.0	18.4

[a] In all the assays the basic medium contained 30 mM Hepes (pH 7.0), 0.8 M KCl and 50 mM ethanol

Cells of Ba_1 preloaded with 0.8 M KCl were added in the following amounts: 1 mg protein/ml for ATP, Q-9AA and Q-Dis assays; 0.5 mg protein/ml to measure respiration and 0.1 ml protein/ml to assess $\Delta I_{600\ nm}$. When indicated also the following additions were made: FCCP, 0.67 μM; S-13, 4.43 μM; 9AA, 1.60 μM; Dis-C_3-(5), 3.6 μM and valinomycin (val.), 1.52 μM. All the results were calculated for 1 mg protein

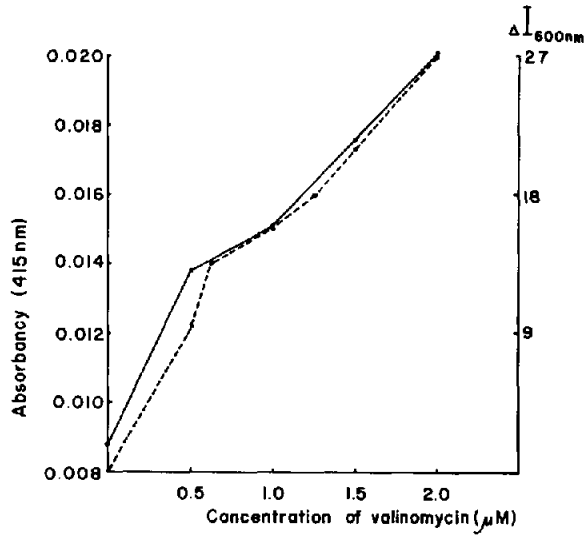

Fig.3. Restoration of pump activity as a function of the valinomycin concentration. Pump activity was measured as in fig.2. The optical cell contained 30 mM Hepes (pH 7.0), 0.8 M KCl, 50 mM ethanol, 0.57 μM FCCP and valinomycin at the concentrations indicated. The reaction was started by adding cells of Ba_1 (0.1 mg protein/ml) pre-loaded with 0.8 M KCl to the assay medium: absorbancy at 415 nm; (---) $\Delta I_{600\ nm}$.

a cause–effect relation between the above two parameters (fig.3).

Additional functions connected with energy transduction which were also tested as to their response to valinomycin-K^+ are shown in table 1. An inspection of the data reveals that valinomycin in presence of K^+ abolished the release of the respiratory control induced by FCCP or S-13, replenished the ATP pool depleted by FCCP, restored ΔpH and $\Delta\Psi$ which were depressed by FCCP.

Valinomycin-K^+, by forming complexes with protonophores, effectively reversed the uncoupling effect of the latter on several energy-linked functions in the halotolerant bacterium, Ba_1. After being sequestered by valinomycin-K^+, the anion of the protonophore appears to be prevented from exerting its action; i.e., to short-circuit the cross-membrane proton current [12]. Gram-negative bacterial cells are known to be relatively insensitive toward valinomycin, unless pretreated with EDTA [13]. It is, thus, likely that valinomycin-K^+ could also be used for recoupling in several other bacterial systems.

In several cases the effectiveness of a protonophore as an uncoupler was found to be poten-

tiated, rather than decreased by the simultaneous presence of a cationophore–cation complex [14]. The phenomenon of potentiation was explained by the assumption that the abolition of $\Delta\Psi$ by electrophoretic counter-flow of the positively charged cationophore–cation complex facilitated the protonophore-mediated passive backflow of protons. It is therefore apparent that neutralization of the net positive charge of the former complex by a direct interaction with the protonophore–anion would prevent the potentiation. Thus, it is reasonable to assume, that it will depend on the properties of the coupling-membrane and/or the conditions of the assay (i.e., the mutual affinity between the 2 types of agents, their stoichiometry, the concentration of the cation) whether the concerted action of a protonophore and a positively charged cationophore will result in an enhancement or a diminution of the extent of uncoupling.

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