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Mutual inactivation of valinomycin and protonophores by complex formation in liposomal membranes

G. Krishnamoorthy

Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India

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The simultaneous presence of a protonophore [3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile or carbonyl cyanide *m*-chlorophenylhydrazone] and valinomycin in a liposome suspension results in time-dependent inactivation of ion transport by both the protonophore and valinomycin. Correlation of the inactivation with spectrophotometric observations on the formation of a complex between the protonophore and valinomycin strongly suggests that the complex observed has no (or very low) activity for the transport of either H^+ or K^+. The stoichiometry of valinomycin and the protonophore in the inactive complex is shown to be 1:1.

Valinomycin; Protonophore; Ion transport inactivation; Proton transport; Temperature-induced pH jump

1. INTRODUCTION

Ionophores such as valinomycin and protonophores [1] are very useful in manipulating the components of the transmembrane electrochemical proton gradient, pH gradient (ΔpH) and membrane potential ($\Delta\psi$). In situations where dissipation of ΔpH is required, protonophores are generally used along with valinomycin in a K^+ medium. Valinomycin-K^+ accelerates the decay of ΔpH by neutralising the $\Delta\psi$ created by transport of H^+/OH^- by the protonophore. Synergistic ion transport involving protonophores, valinomycin-K^+ and lipophilic ions has been suggested by several workers [2–4]. Also, complex formation between valinomycin-K^+ and the anionic form of protonophores and other lipophilic anions has been observed [5–8]. However, the role of these complexes in the transport of ions is unclear.

During studies on rapid relaxation of ΔpH across liposomal membranes [9], I observed a time-dependent and pronounced decrease in the rate constant associated with ΔpH relaxation by a combination of valinomycin-K^+ and protonophores such as SF6847 and CCCP. Here, I show that the ternary complex involving the protonophore, valinomycin and K^+ has no (or very low) activity for the transport of either H^+ or K^+. This conclusion is drawn by comparing the rate of complex formation with the time-dependent decrease in decay rate of ΔpH monitored using the new method [9].

2. EXPERIMENTAL

The preparation and characterization of pyranine-enclosed vesicles, the method of rapidly (5 μs) creating a transmembrane ΔpH using T-jumps and fluorescence detection of the subsequent decay of ΔpH were carried out according to the previous procedures [9]. A home-made T-jump instrument [11] was used in these experiments. Spectrophotometric measurements were made on a Cary 17-D spectrophotometer. Some of the fluorescence measurements (fig.1B) were recorded using a Shimadzu RF 540 spectrofluorophotometer.

Correspondence address: G. Krishnamoorthy, Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India

Abbreviations: SF6847, 3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; T-jump, temperature jump; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid

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The ionophores were added as ethanolic solutions [<0.3% (v/v) final concentration]. Lipids and ionophores (except SF6847) were obtained from Sigma. SF6847 was synthesised according to [12]. Further details of experimental conditions are given in the figure legends.

3. RESULTS AND DISCUSSION

The simultaneous presence of the protonophore SF6847 and valinomycin caused rapid decay of the ΔpH, imposed across liposomal membranes, as expected (fig.1A). However, the rate of decay of ΔpH (at a particular value of ΔpH) showed a pronounced decrease with time after mixing the protonophore and valinomycin in the suspension of liposomes (fig.1A). The new method, using T-jumps coupled with pyranine as the internal pH indicator [9], was used in creating the ΔpH and monitoring its decay. Under the conditions given in fig.1A, the time constant for ΔpH decay increased from ~15 to ~500 ms in approx. 15 min following addition of SF6847 and valinomycin.

That this process of deactivation of the SF6847-valinomycin system was not caused by the T-jumps applied was shown by the following experiments. (a) A suspension of liposomes in which SF6847 and valinomycin were mixed and incubated for 15 min (without repeated application of T-jumps) showed a low rate of ΔpH decay (not shown). This rate was very similar to that observed at 15 min in the experiment (fig.1A) in which repeated T-jumps were applied. (b) Fig.1B depicts an experiment in which the ΔpH was created by addition of acid instead of application of T-jumps. The ΔpH decayed within the mixing time (~2 s) in the sample where SF6847 and valinomycin were added just prior to addition of acid. In contrast, the ΔpH decayed with a half-time of ~5 s when the ionophores were incubated for 50 min in the liposome suspension. This experiment, despite the inability to resolve the kinetics of inactivation, clearly shows that the inactivation was not caused by the T-jump.

The ΔpH-relaxation traces (fig.1A) could be fitted well to single exponentials (with time constant τ) in agreement with the exponential decay of ΔpH derived for low values of ΔpH [10]. Relaxation rates ($k_{app} = 1/\tau$) associated with the traces in fig.1A are plotted in fig.2 (trace c). It can be seen (fig.2) that a time-dependent decrease in k_{app} was observed not only with SF6847 but also with

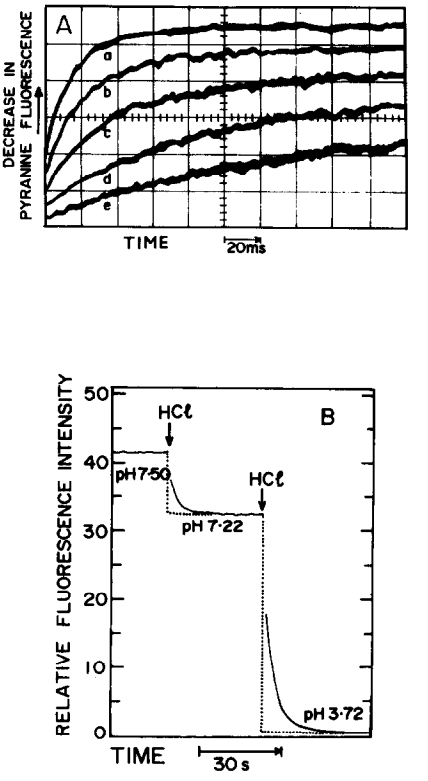

Fig.1. (A) Oscilloscope traces showing time-dependent rate of relaxation of ΔpH by SF6847 and valinomycin. The sample was 1.1 ml sonicated soybean phospholipid vesicles at 2.1 mg/ml suspended in 150 mM KCl and 30 mM Tris-HCl at pH 7.5 and thermostatted at 25°C. The vesicular internal medium comprised 150 mM KCl, 30 mM KH_2PO_4 and 1 mM pyranine at pH 7.5. ΔpH (0.065 unit) was created in about 5 μs by the application of a T-jump (2.5°C) and the decay of ΔpH was monitored by the fluorescence of the internal pH indicator, pyranine (for details see [10]). SF6847 and valinomycin (each at 4.5 μM final aqueous concentration) were added to the vesicle suspension (at time zero). Application of T-jump and subsequent monitoring of relaxation of ΔpH were performed at 0.6 min (a), 3.0 min (b), 7.0 min (c), 15 min (d) and 18.9 min (e). (B) Relaxation of ΔpH created by the addition of acid to a suspension of vesicles. The sample (1.1 ml) was identical to that in A. Excitation was at 455 nm and emission at 520 nm. SF6847 and valinomycin were added to a final concentration of 4.5 μM each. In the two samples, HCl (10 and 15 μl of a 200 mM solution) was added at 0.5 min (···) or 50 min (——) after the addition of ionophores.

CCCP. Further, this was also observed in liposomes prepared from egg phosphatidylcholine (fig.2). Since k_{app} is a linear function of the concentration of both valinomycin and protonophore [9], the decrease could have been caused by inac-

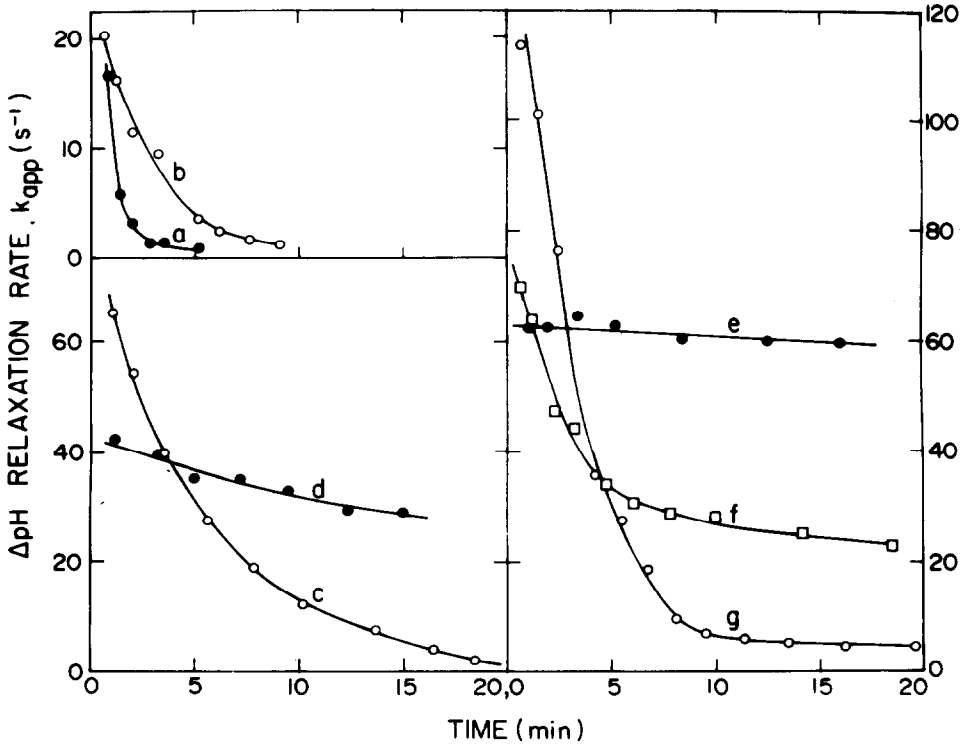

Fig.2. Time-dependent decrease in rate of relaxation of ΔpH (k_{app}). Ionophores were added at time zero and their aqueous concentrations were (a) 0.51 μM SF6847 and 9 μM valinomycin, (b) 0.51 μM SF6847 and 4.5 μM valinomycin, (c) 4.5 μM SF6847 and 4.5 μM valinomycin, (d) 4.5 μM SF6847 and 2.2 μM valinomycin, (e) 4.5 μM CCCP and 4.5 μM valinomycin, (f) 4.5 μM CCCP and 13.5 μM valinomycin and (g) 0.76 μM SF6847 and 13.5 μM valinomycin. (a–f) With soybean phospholipid vesicles (2.1 mg/ml), (g) with egg phosphatidylcholine vesicles (0.7 mg/ml). Other conditions similar to those in fig.1A.

tivation of either of the two ionophores. The experiments described below show that both valinomycin and the protonophore became inactive when present together. In an experiment where valinomycin was mixed with an excess of SF6847 in a suspension of liposomes (4.5 μM valinomycin, 18.0 μM SF6847; other conditions similar to fig.1A), the value of k_{app} decreased from 58 to 5.2 s^{-1} in 36 min. A further addition of 9.0 μM SF6847 brought the value of k_{app} to only 6.0 s^{-1} showing that valinomycin was completely inactivated. Similarly, in another experiment with an excess of valinomycin (1.0 μM SF6847, 4.5 μM valinomycin), k_{app} decreased from 36.1 to 0.8 s^{-1} in 10 min. On further addition of 4.5 μM valinomycin, k_{app} increased to only 1.0 s^{-1} showing, in this case, the inactivation of SF6847 by valinomycin.

The visible spectra of protonophores SF6847 and CCCP in liposomes showed pronounced changes following the addition of valinomycin (fig.3) similar to those observed previously [5,7]. These spectral changes have been ascribed to the formation of a complex between the protonophore and valinomycin-K^+ [5,7]. In addition, we observed that the spectral changes were time-dependent (fig.3), in contrast to the previous studies [5,7]. The time dependence of the spectral changes was biphasic (fig.4), indicating formation of the complex in two phases. The striking feature of the time dependence (fig.4) was that it was remarkably similar to the time-dependent decrease in k_{app} (fig.2). This similarity was seen in all cases (except trace c in figs 2 and 4, see below) shown. This suggests strongly that the complex formed between the protonophore and valinomycin is inactive in the transport of either H^+ or K^+. In the sample represented by trace c (figs 2,4), k_{app} was

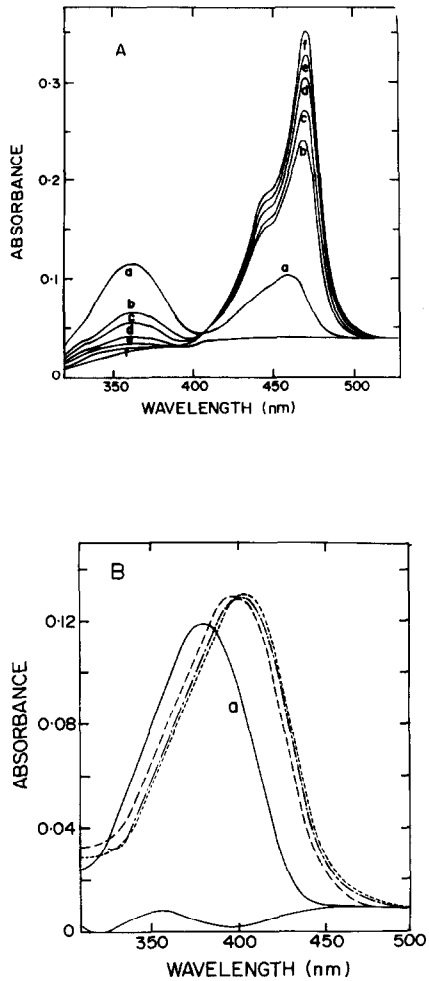

Fig.3. Time-dependent perturbation of visible spectra of SF6847 (A) and CCCP (B) by valinomycin in soybean lipid vesicles. Concentrations of ionophores were (A) 4.5 μM SF6847 and 4.5 μM valinomycin and (B) 4.5 μM CCCP and 13.5 μM valinomycin. Trace a shows the spectra of protonophores in the absence of valinomycin. At time zero, valinomycin was added and the spectra recorded at 0.5 min (b), 2.7 min (c), 7.0 min (d), 11.4 min (e), 17.9 min (f), 0.5 min (– – –), 6.0 min (– – – –) and 21.0 min (– – –). Other conditions similar to those given in fig.1A.

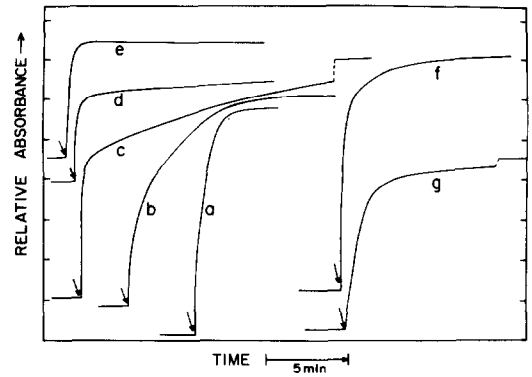

Fig.4. Valinomycin-induced changes in the absorbance of SF6847 (at 470 nm) and CCCP (at 420 nm). Samples (a–f) were identical to those described in fig.2. Arrows represent addition of valinomycin. The absorbance unit was 0.01 per division (a,b,e–g) and 0.05 per division (c,d). The traces are shifted in both axes to avoid congestion.

dependent on the concentration of both valinomycin and SF6847 (4.5 μM each). Hence, the rate of decrease in k_{app} is expected to be double that of complexation. The $t_{1/2}$ associated with inactivation (fig.2c) was indeed half of that associated with the slow phase of complexation (fig.4c), in agreement with this prediction. In other samples, k_{app} was rate-limited by the level of the protonophore only, due to relatively higher concentrations of valinomycin. That even the complex formed during the fast phase of the spectral change (fig.4) is inactive was demonstrated by the following experiment. In samples where the decay of ΔpH was rate-limited by the concentration of protonophore, the k_{app} value at times close to zero, was lower at higher concentrations of valinomycin (fig.2a,b). Concomitantly, the sample with a higher concentration of valinomycin displayed a greater amplitude of the fast spectral change (fig.4a,b). This shows that the decrease in k_{app} is proportional to the increase in the level of complex formed.

Valinomycin-induced changes in the spectra of SF6847 observed in a K^+ medium (figs 3,4) were absent in an Na^+ medium (not shown). Also, SF6847 was not inactivated by valinomycin in the Na^+ medium as assayed in the presence of gramicidin (not shown). Here, presumably, SF6847 and gramicidin transport H^+ and Na^+, respectively. These experiments show the requirement of K^+ in formation of the inactive complex between SF6847 and valinomycin.

The stoichiometry of complexation between valinomycin and SF6847 (in K^+ medium) was obtained by titrating, with valinomycin, the absorbance (at 470 nm) of SF6847 obtained after incubation with valinomycin for sufficiently long periods such that formation of the complex had reached completion (fig.5). The values of A_{470} (at

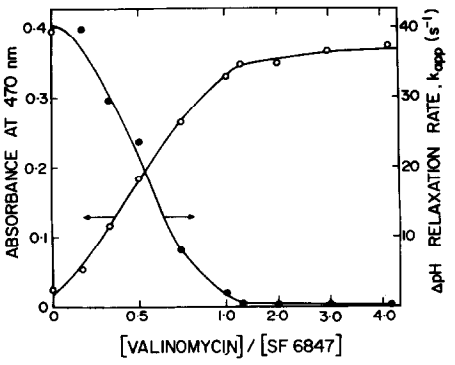

Fig.5. Stoichiometry of the inactive complex involving SF6847 and valinomycin-K^+. In these individual samples, SF6847 (4.5 μM), in a suspension of soybean lipid vesicles, was treated with varying concentrations (0–22 μM) of valinomycin for 2 h at 25°C in order to complete the process of complexation. The level of the complex was obtained from the absorbance at 470 nm (○). The level of uncomplexed SF6847 was assayed by a second addition of 4.5 μM valinomycin and measurement of the transport efficiency parameter k_{app} (●) within 0.5 min. These data were plotted vs the ratio of the initial concentrations of SF6847 and valinomycin.

$t \longrightarrow \infty$) showed saturation at concentrations of valinomycin close to that of SF6847. The level of uncomplexed SF6847 in these samples was assayed by further addition of valinomycin and immediate ($t < 30$ s) measurement of the transport parameter, k_{app}. The value of k_{app} reached a minimum at a concentration of valinomycin equal to that of SF6847. These data indicate 1:1 stoichiometry for the inactive complex.

The present experiments show that the complex formed between valinomycin-K^+ and a protonophore such as SF6847 or CCCP in liposomal membranes has no (or very low) activity in the transport of either K^+ or H^+. Whereas the complex involving SF6847 has virtually no transport activity, the valinomycin-K^+-CCCP complex has a higher and measurable activity. However, this was very much lower than those of uncomplexed ionophores.

The conclusion that valinomycin and the protonophores, by themselves, transport K^+ and H^+ more efficiently compared to the complex seen in visible spectra (fig.2) is in contrast to earlier suggestions that the ternary complex might mediate the movement of these ions [3–5]. Yamaguchi and Anraku [4] invoked the presence of the ternary complex in explaining the dependence of the initial rate of H^+ transport on the concentrations of ionophores. However, they did not show any direct correlation between the level of the complex and the transport efficiency. In contrast, in the present study, such a direct correlation has been used to arrive at the conclusion that the complex observed in spectrophotometric studies (fig.2) has no (or very low) activity for the transport of either H^+ or K^+ (fig.1). However, these results do not rule out the presence of any other type of complex which might mediate the transport of H^+ and/or K^+.

The biphasic nature of complex formation (fig.4) is quite intriguing. It is likely that the biphasic complexation process results from the existence of two populations of valinomycin in the membrane. Detailed characterization of the complexation process requires further experiments which are now in progress.

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