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Enhancement of transmembrane proton conductivity of protonophores by membrane-permeant cations

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The rate of protonophore-mediated decay of pH gradient across lipid vesicular membranes was found to be enhanced by orders of magnitude by valinomycin-K^+. Experiments in the presence of gramicidin have shown that the observed rate enhancement by valinomycin-K^+ is not due to collapse of the diffusion potential alone. The enhancement of the rate showed hyperbolic dependence on the concentration of valinomycin. Rate enhancement was observed in the presence of the membrane permeant cation tetraphenylphosphonium (TTP^+) also. Several factors which might enhance the intrinsic H^+ conductivity of protonophores were analyzed. The level of partitioning of the protonophore into the membrane and the p*K* of membrane-bound protonophores were measured. Valinomycin-K^+ did not alter both these parameters significantly. TPP^+ increased the partitioning of protonophores and decreased the pK values of membrane-bound protonophores. However, these changes were too small to explain the observed rate enhancements. We suggest that valinomycin-K^+ and TPP^+ enhance the H^+ conductivity of protonophores by increasing the permeability of the ionized form of protonophores by forming an ion pair.

Introduction

Ever since Mitchell proposed the chemiosmotic hypothesis of energy transduction [1], there has been increasing use of ionophores such as valinomycin and protonophores [2–4]. These ionophores are used, either individually or in combination, in controlling transmembrane pH gradient (ΔpH) and membrane potential ($\Delta\psi$). Protonophore-aided H^+ flux across vesicular membranes (driven by ΔpH) increased by orders of magnitude by the presence of valinomycin in a K^+ medium. This is generally attributed to the neutralization, by valinomycin-K^+, of H^+ diffusion potential ($\Delta\psi_d$) created by the H^+ flux [4,5]. Further, we have shown earlier that valinomycin and protonophores form an inactive complex with each other by a moderately slow process when present in liposomal membranes [6].

In order to check whether the dramatic enhancement of protonophore-aided H^+ flux by valinomycin is due to neutralization of $\Delta\psi_d$ alone, we have measured the rate of H^+ transport in the presence of protonophore and gramicidin. The gramicidin channel neutralizes the $\Delta\psi_d$ effectively by conducting K^+. However, in this case, the H^+ flux was significantly lower compared to rates observed in the presence of valinomycin. Hence alternative mechanisms have to be sought in order to explain the rate enhancement by valinomycin. Such mechanisms could be categorized into two types: (a) a specific mechanism involving valinomycin and the protonophore and (b) a general mechanism involving a membrane-permeant cation and the protonophore. In this paper, we describe our experiments addressing these questions. Apart from the ionophores, we have used permeant ions such as tetraphenylphosphonium (TPP^+) and tetraphenyl boron (TPB^-). We have measured the fast kinetics of H^+ transport under a variety of conditions. We have also measured the partition coefficient of protonophores into the membrane and their protonation equilibria in the membrane. Our results strongly suggest that an interaction between the deprotonated form of the protonophore and a permeant cation (valinomycin-K^+ or TPP^+) is responsible for the

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalanonitrile; pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid; *T*-jump, temperature jump; TPP^+, tetraphenylphosphonium; TPB^-, tetraphenylboron; $\Delta\psi_d$, diffusion potential created by H^+ flux.

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dramatic increase in the rate of protonophore-aided H^+ flux.

Materials and Methods

Pyranine-trapped soybean phospholipid vesicles were prepared and characterized as described earlier [6,7]. A ΔpH was created rapidly (5 μs) by application of a temperature jump (T-jump) and its decay was monitored by the fluorescent pH indicator pyranine [7]. A home-made *T*-jump instrument [8] interfaced to a home-made transient recorder was used in these experiments. The exponential time constant (τ) of ΔpH relaxation traces [9] were used to get the first-order rate constant $k(=1/\tau)$. In experiments with gramicidin, liposome suspensions were incubated with gramicidin for approx. 15 min at 25°C in order to complete the process of channel formation [7]. In experiments with valinomycin and protonophore together, valinomycin was incubated in the liposome suspension for about 15 min before the addition of the protonophore. Under these conditions, the rate of formation of the inactive complex between valinomycin and the protonophore [6] was very low (unpublished observations). Also, the rates (k) were measured within 1 min after the addition of the protonophore.

The extent of partitioning of protonophores and TPP^+ into the membrane was estimated using an ultrafiltration system (Amicon). The concentrations of these compounds in the initial filtrate through an Amicon YM-30 membrane were estimated from their absorbance in either the ultraviolet region (TPP^+) or the visible region (protonophores).

The pK values of membrane-bound protonophores were estimated from the pH dependence of optical spectra of the protonophores in a suspension of liposomes. The concentration of lipids was high (≥ 5 mM) such that $> 80\%$ of the protonophore was in the membrane as shown by ultrafiltration assays. The absorbances at 460 nm (in the case of SF6847) or 400 nm (in the case of CCCP and FCCP) corresponding to the ionized form of the protonophores were used for the estimation of the pK values.

Gramicidin, CCCP, sodium tetraphenylborate and soybean phospholipids were obtained from Sigma Chemical Co. SF6847 was synthesized according to Ref. 10. FCCP was obtained from Fluka. Tetraphenylphosphonium bromide was purchased from Aldrich.

Results

The rates of relaxation of ΔpH by protonophores SF 6847, FCCP and CCCP were very low in the absence of either valinomycin or gramicidin, as expected (Table I, lines 1, 5 and 9). Addition of either valinomycin or gramicidin enhanced the rate, presumably by collapsing $\Delta\psi_d$. Moreover, the rates observed in the presence of valinomycin were one or two orders of magnitude higher than those observed with gramicidin. In these measurements, ΔpH was created rapidly (5 μs) by *T*-jump and its decay was monitored using the fluorescent dye pyranine as internal pH indicator [7]. The relaxation of ΔpH, in these systems, depends upon (i) the intrinsic H^+ conductance of the protonophore and (ii) the counterion (K^+) flux through either the gramicidin channel or valinomycin. The observed rate (k) of relaxation of ΔpH will reflect the kinetics of the slower of the above two processes. The proton permeability of the bare membrane (in the absence of any ionophore) was very low (line 13 in Table I and Ref. 11). Hence it does not interfere in the results obtained in the presence of protonophores. Also, the flux of protons through gramicidin channel alone was very much lower (line 15 in Table I and Ref. 7) compared tothe flux values (k) in

TABLE I

Enhancement of proton conductivity of protonophores by valinomycin

The rates of decay of ΔpH k were obtained from *T*-jump experiments. ΔpH (0.07 unit) was created by *T*-jump and its decay was monitored by the internal pH indicator, pyranine [7]. Liposome suspensions contained 2.5 mM soybean phospholipids with 150 mM KCl, 30 mM Tris (pH 7.5) as the external medium and 150 mM KCl, 30 mM KH_2PO_4, 1 mM pyranine (pH 7.5) as the internal medium. The temperature jump was from 23°C to 25.5°C. When the rates were very low ($k < 0.3\ s^{-1}$), ΔpH was created by the addition of HCl (samples 1, 5, 9, 13 and 14). $k = 1/\tau$ where τ is the exponential time constant of the relaxation trace.

	Additions to the liposome suspension		Rate of decay of ΔpH, k [a] (s^{-1})
1.	18 μM	FCCP	0.017
2.	18 μM	FCCP + 4.5 μM gramicidin	2.5
3.	18 μM	FCCP + 1.8 μM valinomycin	50.5
4.	18 μM	FCCP + 4.5 μM gramicidin + 1.8 μM valinomycin	184.0
5.	36 μM	CCCP	0.015
6.	36 μM	CCCP + 4.5 μM gramicidin	2.3
7.	36 μM	CCCP + 1.8 μM valinomycin	56.3
8.	36 μM	CCCP + 4.5 μM gramicidin + 1.8 μM valinomycin	274.7
9.	8.1 μM	SF6847	0.016
10.	8.1 μM	SF6847 + 4.5 μM gramicidin	28.1
11.	8.1 μM	SF6847 + 1.8 μM valinomycin	39.6
12.	8.1 μM	SF6847 + 4.5 μM gramicidin + 1.8 μM valinomycin	79.5
13.	None		0.010
14.	1.8 μM	valinomycin	0.025
15.	4.5 μM	gramicidin	1.12
16.	4.5 μM	gramicidin + 1.8 μM valinomycin	1.17

[a] Average of four or more relaxation traces. Errors were less than 10%.

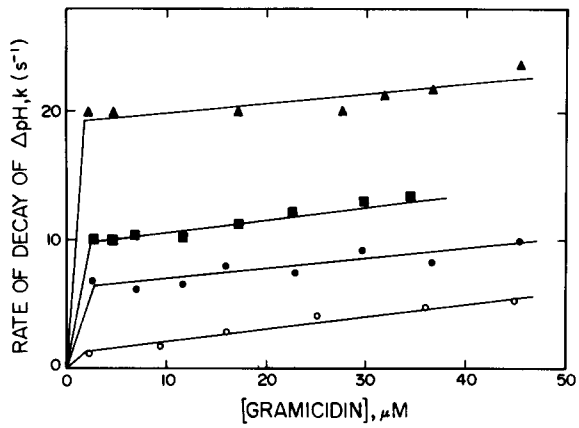


Fig. 1. Dependence of the rate of decay of ΔpH (k) on the concentration of gramicidin in protonophore-gramicidin systems. The suspension of liposomes (described in the legend to Table I) was thermostatted at 23±1°C. A ΔpH of 0.07 unit was created by a T-jump of 2.5°C [7]. $k = 1/\tau$ where τ is the exponential time constant of the ΔpH relaxation trace. ○, in the absence of any protonophore; ●, 109 μM FCCP; ■, 218 μM CCCP and ▲, 9.1 μM SF6847. When [gramicidin] = 0, $k < 0.02\ s^{-1}$ in all the cases.

the presence of protonophores and either valinomycin or gramicidin.

The observation of lower rates (Table I, lines, 2, 6 and 10) in the presence of gramicidin (when compared to samples with valinomycin) can be explained if the K^+ conductance through gramicidin was rate-limiting in these samples. This possibility was ruled out based on the following three observations. (a) It was shown by one of us earlier [7] that gramicdin, at the concentration used in our experiments (4.5 μM), can conduct K^+ effectively with time-averaged single-channel conductance greater than 10^{-12} S. This had given rise to ΔpH relaxation rates (k) higher than $5 \cdot 10^3\ s^{-1}$ in the presence of high concentrations of protonophore. (b) The values of k observed in the presence of both gramicidin and valinomycin (Table I, lines 4, 8 and 12) were very much higher than the additive values of k obtained with either gramicidin or valinomycin only (lines 2 + 3, 6 + 7 and 10 + 11). This also shows that the enhancement of k by valinomycin cannot be due to neutralization of $\Delta\psi_d$ alone. (c) When the concentration of the protono-

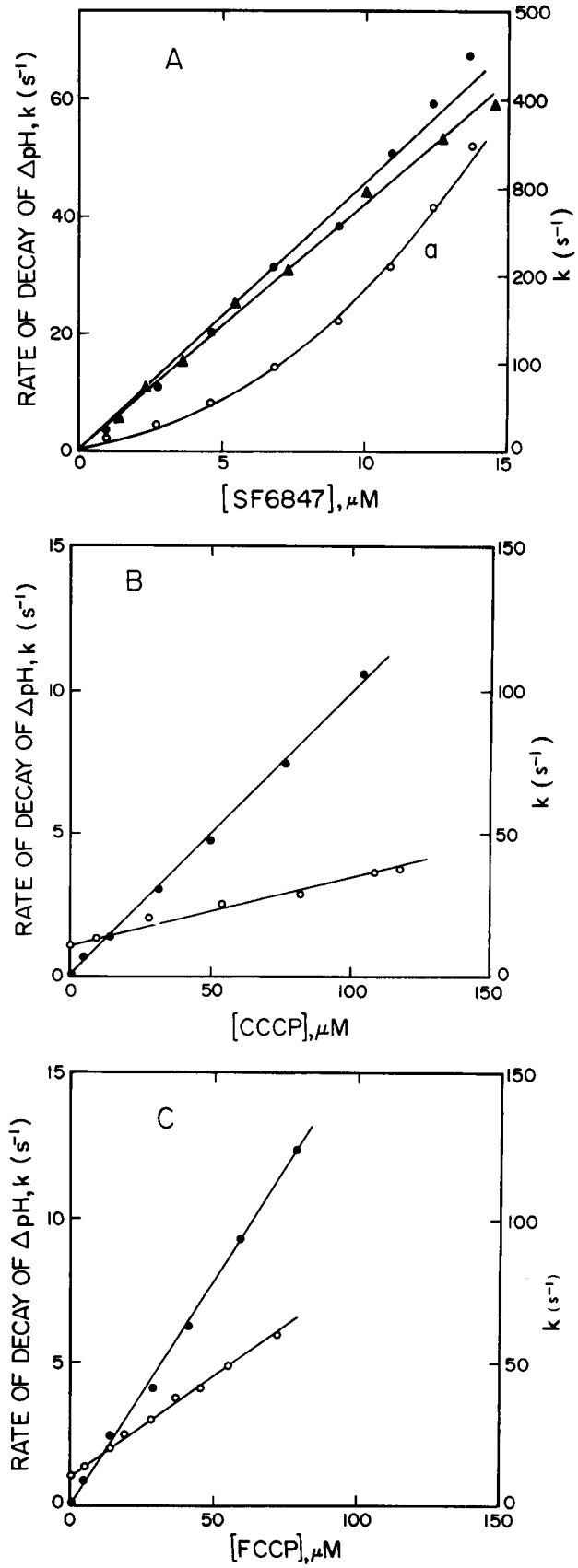


Fig. 2. Dependence of the rate of decay of ΔpH on the concentration of SF6847 (A), CCCP (B) and FCCP (C). All the samples contained 4.5 μM gramicidin. ○, protonophore and gramicidin only (left scale on the Y-axis). ●, protonophore, gramicidin and either 636 μM (A) or 364 μM (B and C) TPP^+ (right scale on the Y-axis). ▲, SF6847, gramicidin and 9.1 μM valinomycin (right scale on the Y-axis). The superlinear increase of k with the total concentration of SF6847 (curve a) was fitted to the equation $k = 3 \cdot 10^5\ [P] + 8 \cdot 10^8\ [P_2]$ with $[P_2]/[P]^2 = 3 \cdot 10^2$ M. [P] and $[P_2]$ are equilibrium concentrations of monomer and dimer forms of SF6847. $[P] + 2[P_2]$ = total concentration of SF6847. The continuous line is simulation of the above equation.

phore was kept constant, the rates (k) depended insignificantly on the concentration of gramicidin in the range 2–45 μM (Fig. 1, filled symbols). The small increase observed was due to H^+ conductance through gramicidin as shown in Fig. 1 (open symbols). Alternatively, when the concentration of gramicidin was kept constant, the rate increased linearly with the concentration of either CCCP or FCCP and superlinearly with the concentration of SF6847 (Fig. 2). (The superlinear dependence will be explained later). These data (Figs. 1 and 2) show clearly that the observed rates of relaxation of ΔpH (Table I, lines 2, 6 and 10) are rate-limited by the intrinsic H^+ conductance of the protonophore and not by the K^+ flux through gramicidin. Hence, the rate enhancement by valinomycin (Table I, lines 4, 8 and 12) should be due to some factor(s) other than neutralization of $\Delta\psi_d$.

Fig. 3A shows the increase of k with the concentration of valinomycin in the presence of gramicidin. The increase was monotonous and non-saturating upto 30 μM of valinomycin in the case of SF 6847. In the cases of CCCP and FCCP, the increase was hyperbolic with saturation around 10 μM of valinomycin. Double-reciprocal plots of the rate versus concentration of valinomycin showed linear dependence (Fig. 3A, inset). This suggests an interaction between valinomycin and protonphores as the cause of the rate enhancement (see Appendix and Discussion). However, the visible absorption spectra of the protonophores were not significantly altered under these conditions (data not shown). Valinomycin did not cause any rate enhancement in a K^+-free Na^+ medium (data not shown).

In order to model the rate enhancement by valinomycin, another membrane-permeant cation TPP^+ was used. Addition of TPP^+ also resulted in a dramatic increase in the values of k (Figs. 3B and 2). However, in contrast to observations with valinomycin, the increase was linear upto 800 μM of TPP^+ for all the three protonophores. That these rate enhancements were not due to any protonophoric activity of TPP^+, was shown as below. The values of k obtained in the absence of any protonophore were in the range of 0.8–1.2 s^{-1} only.

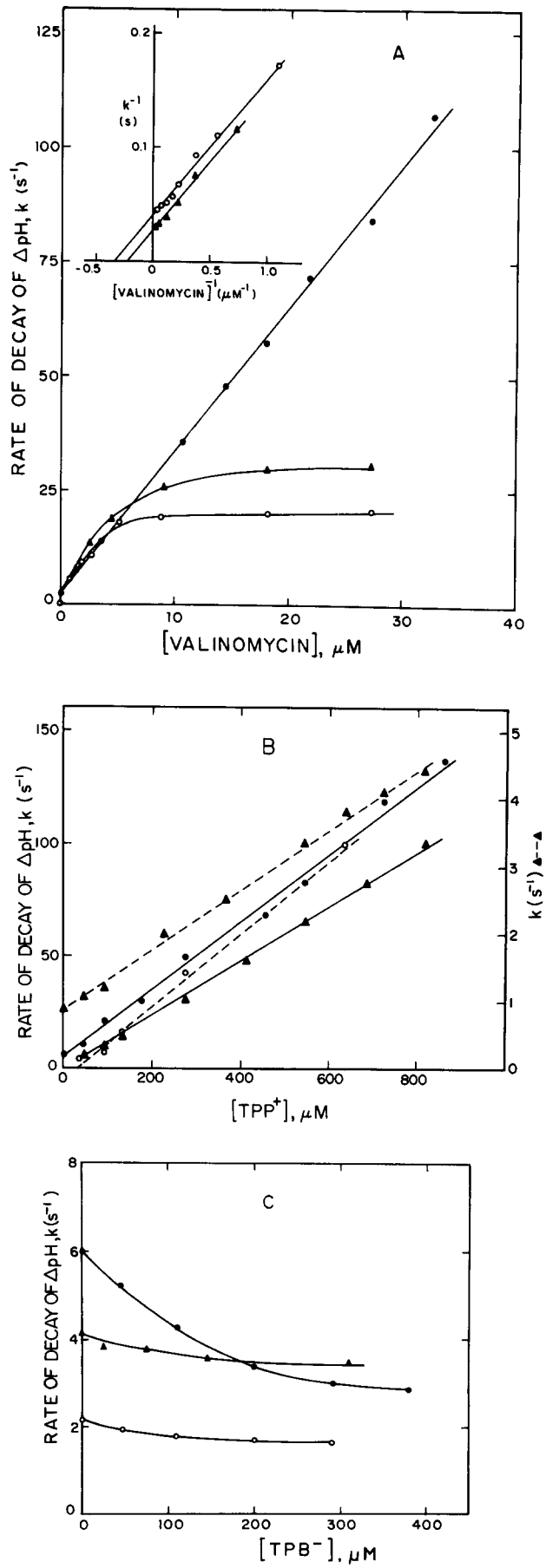


Fig. 3. Dependence of the rate of decay of ΔpH on the concentration of valinomycin (A). TPP^+ (B) and TPB^- (C). All the samples contained 4.5 μM gramicidin. The concentation of protonophores were as follows: (A) 0.91 μM SF6847 (●), 0.91 μM FCCP (▲), 0.91 μM CCCP (○). (B) and (C) 3.6 μM SF6847 (●), 0.91 μM FCCP (▲------▲), 36 μM FCCP (▲———▲); 36 μM CCCP (○). In (B), the right scale is for 0.91 μM FCCP (▲------▲) and the left scale is for all others. The inset in (A) shows the plots of $1/k$ versus 1/[valinomycin] in the cases of FCCP (▲) and CCCP (○). The dissociation constants K_d for the complex between the protonophore and valinomycin (see Appendix) were estimated from these plots. They are 4.7 μM and 2.9 μM for FCCP and CCCP, respectively, at lipid concentrations of 2.5 mM.

TABLE II

Partitioning of protonophores into the membrane

10 ml of liposome suspension (2.5 mM lipid) in 150 mM KCl, 30 mM Tris (pH 7.5) with the ionophores mentioned below was filtered through an Amicon YM-30 ultrafiltration membrane. Initial 1.0 ml of the filtrate was assayed for the protonophore spectrophotometrically.

	Additions to the liposome suspension	Concentration of the protonophore in the initial filtrate (μM)
1.	36.3 μM CCCP	11.3
2.	36.3 μM CCCP + 20.0 μM valinomycin	10.5
3.	36.3 μM CCCP + 800 μM TPP^+	2.3
4.	36.3 μM CCCP + 200 μM TPB^-	12.0
5.	18.1 μM FCCP	3.0
6.	18.1 μM FCCP + 5.0 μM valinomycin	3.1
7.	18.1 μM FCCP + 800 μM TPP^+	0.3
8.	18.1 μM FCCP + 200 μM TPB^-	3.7
9.	5.0 μM SF6847	1.3
10.	5.0 μM SF6847 + 5.0 μM valinomycin	1.5
11.	5.0 μM SF6847 + 800 μM TPP^+	1.0
12.	5.0 μM SF6847 + 200 μM TPB^-	2.1

Also, experiments with only a protonophore and TPP^+ together showed that TPP^+ did not collapse $\Delta\psi_d$ effectively ($k < 0.1\ s^{-1}$). Although TPP^+ enhanced the rate similar to valinomycin-K^+, it did not cause inactivation of the protonophore, unlike valinomycin-K^+ [6]. Also TPP^+ did not perturb the visible spectra of protonophores significantly (data not shown). In contrast to rate enhancements by TPP^+, the permeant anion TPB^- caused a small but definite decrease in the rates (Fig. 3C). TPP^+ partitioned into the membrane by approx. 25% as shown by our ultrafiltration assays. This agrees with earlier estimation by others [12]. In comparison, > 99% of TPB^- and valinomycin is expected to be in the membrane phase [13,14].

Is there any correlation between the enhancement of intrinsic H^+ conductivity of the protonophore and the concentration of the ionized form (P^-) of the protonophore in the membrane? To answer this question, the following were estimated: (i) the partitioning of the protonophore into the membrane and (ii) the pK of the protonophore in the membrane. The extents of perturbation of these quantities by valinomycin, TPP^+ and TPB^- were also estimated.

The extent of partitioning of protonophores into the membrane was determined by ultrafiltration as described in Materials and Methods. Table II lists the concentration of free (aqueous phase) protonophores under a variety of conditions. It is seen that more than 80% of the protonophore is already in the membrane phase in the absence of any perturbant (lines 1, 5 and 9 of Table II). Hence any rate enhancement arising from increased partitioning of the protonophore into the membrane cannot be more than approx. 1.3-fold. While the presence of valinomycin did not cause any significant change in partitioning, TPB^- cuased a slight decrease in partitioning into the membrane. However, TPP^+ favoured the partitioning into the membrane significantly (lines 3, 7 and 11 in Table II).

The pK values of the protonophores were estimated from their pH-dependent absorption spectra [15]. Estimations of the pK values of membrane-bound protonophores were carried out under lipid concentrations such that > 80% of protonophores were membrane-bound. Typical pH-titration curves obtained in the case of SF6847 are shown in Fig. 4. pK values obtained by fitting such data to single ionization are given in Table III for all the three protonophores. Similar to the effect on membrane partitioning, valinomycin and TPB^- did not alter the pK values significantly. Presence of TPP^+ decreased the pK values significantly. This would lead to a substantial increase in the concentration of membrane-bound P^- (by approx. 3-fold in the case of SF6847) by TPP^+ (800 μM).

Apart from the abovementioned ways, both the partition coefficient and the pK of membrane-bound protonophore were simultaneously estimated in a single experiment described by Yamaguchi et al. [15]. In this experiment, the concentration of the liposome was varied keeping the concentration of the protonophore constant. The ratio of the concentration of ionized and neutral forms of the protonophore K_{obs} ($= \{[P_b^-] +$

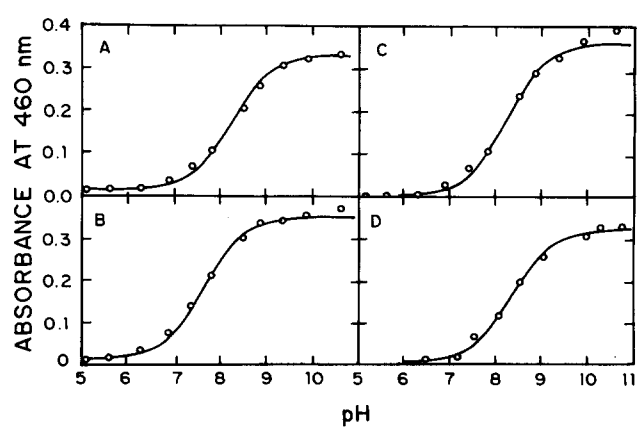


Fig. 4. pH titration of the absorbance (at 460 nm) of SF6847 (5 μM) in a suspension of liposomes (5.0 mM lipid). The medium had 150 mM KCl and 30 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 4.5–7.0) or tris(hydroxymethyl)aminomethane (pH 7.0–9.0) or 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (pH 9.0–11.0). (A) SF6874 only; (B) with 800 μM TPP^+; (C) with 5 μM valinomycin and (D) with 200 μM TPB^-. Continuous lines were simulated with the pK values listed in Table III.

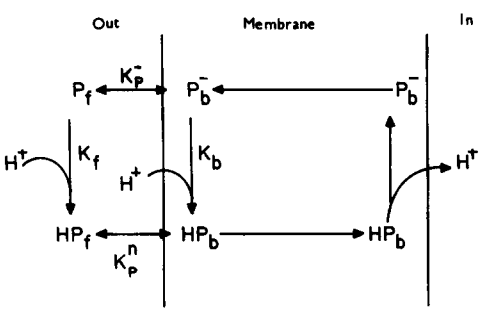
TABLE III

pK values of membrane-bound protonophores

pK values were obtained by fitting the pH dependence of absorbance of the protonophores in a suspension of liposomes (Fig. 4).

	Conditions	pK
1.	CCCP only	6.65
2.	CCCP + 4.0 μM valinomycin	6.65
3.	CCCP + 400 μM TPP^+	6.50
4.	CCCP + 200 μM TPB^-	6.65
5.	FCCP only	6.50
6.	FCCP + 5.0 μM valinomycin	6.55
7.	FCCP + 800 μM TPP^+	5.70
8.	FCCP + 200 μM TPB^-	6.75
9.	SF6847 only	8.25
10.	SF6847 + 5.0 μM valinomycin	8.23
11.	SF6847 + 800 μM TPP^+	7.65
12.	SF6847 + 200 μM TPB^-	8.30

$[P_f^-]\}/\{[HP_b] + [HP_f]\}$) were estimated from the optical spectra. The terms used above are described in Fig. 5. Plots of $(K_f - K_{obs})/[lipid]$ versus K_{obs} were used to obtain the partition constants K_p^- and K_p^n associated with P^- and HP, respectively. K_f is K_{obs} in the absence of liposomes. Fig. 6 shows such plots obtained in the case of SF6847. The partition constants and the pK of membrane-bound SF6847 derived from these plots are given in Table IV. The values of pK thus obtained closely match with those obtained by pH titration (Fig. 4 and Table III). Further, these experiments gave the partition constants for P^- and HP independently. This



$$K_p^- = \frac{[P_b^-]}{[P_f^-][LIPID]} \qquad K_p^n = \frac{[HP_b]}{[HP_f][LIPID]}$$

$$K_f = \frac{[P_f^-]}{[HP_f]} \qquad K_{obs} = \frac{[P_f^-] + [P_b^-]}{[HP_f] + [HP_b]}$$

$$K_b = \frac{[P_b^-]}{[HF_b]} = \frac{K_p^- \cdot K_f}{K_p^n}$$

Fig. 5. Schematic diagram showing the partitioning of ionized (P^-) and neutral (HP) forms of protonophore into the membrane and the H^+-transport cycle. Subscripts b and f denote membrane-bound and free forms, respectively.

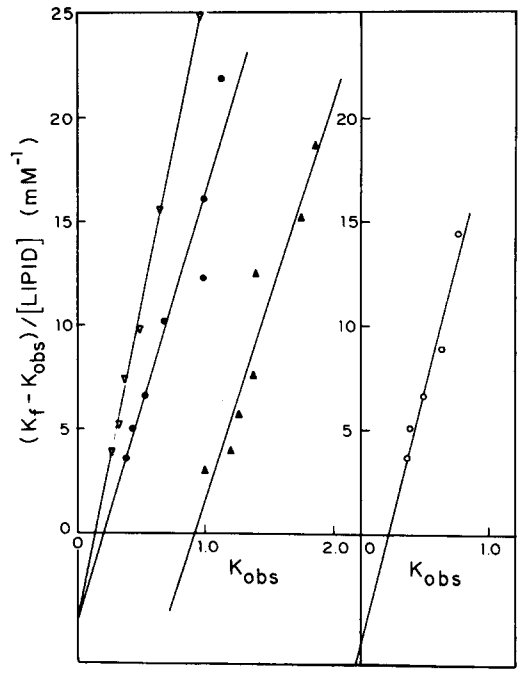


Fig. 6. Plots of $(K_f - K_{obs})/[lipid]$ versus K_{obs} for the estimation of partition constants K_p^- and K_p^n associated with P^- and PH, respectively. The equation used is $(K_f - K_{obs})/[lipid] = K_p^n \cdot K_{obs} - K_f \cdot K_p^-$ [15]. In these experiments with SF6847, the lipid concentration was varied from 0.05 to 0.75 mM keeping [SF6847] constant at 1.0 μM. K_{obs} was estimated as the ratio $A_{7.5}/(A_{11.0} - A_{7.5})$ where $A_{7.5}$ and $A_{11.0}$ are the absorbances (at 460 nm) at pH 7.5 and pH 11.0, respectively. ○, SF6847 only; ●, with 5 μM valinomycin ▽, 200 μM TPB^-; and ▲, 800 μM TPP^+.

is unlike the ultrafiltration assay (Table II) which gave only average values. It can be seen that K_p^n is higher than K_p^- by an order of magnitude. This, which is similar to the observation by Yamaguchi et al. [15], shows the preferential partition of the neutral form over the ionized form. The slight differences seen between our values and theirs could be due to differences in the phospholipids used. The presence of valinomycin did not affect significantly any of these parameters (Table IV). TPP^+ increased the value of K_p^- significantly, in line with the overall increase in membrane partitioning

TABLE IV

Partitioning of ionized and neutral form of SF6847 into the membrane and pK of membrane-bound SF6847

The parameters listed below were estimated from the plots shown in Fig. 6. $K_b = [SF^-]_b/[SFH]_b = (K_p^- \cdot K_f/K_p^n)$. $pK_b = -\log(K_b[H^+])$.

	Conditions	K_p^n (mM^{-1})	K_p^- (mM^{-1})	K_b	pK_b
1.	SF6847 only	21.0	2.05	0.21	8.17
2.	SF6847 + 5.0 μM valinomycin	25.2	2.55	0.22	8.15
3.	SF6847 + 800 μM TPP^+	20.2	5.50	0.89	7.55
4.	SF6847 + 200 μM TPB^-	30.6	2.05	0.15	8.33

seen before (Table II). This results in a significant decrease in the pK of membrane-bound SF6847 (pK_b in Table IV). TPB^- caused a slight increase in K_p^n leading to a slight increase in the value of pK_b. These results are similar to those obtained from pH titrations (Table III).

Taken together, the data on membrane partitioning and pK of the protonophores (Tables II–IV) show that valinomycin did not alter the concentration of membrane-bound P^- significantly. In contrast, TPP^+ caused an increase in the concentration of membrane-bound P^- by approx. 3-fold. However, the magnitude of this increase falls very much short of the observed rate enhancement of approx. 20-fold (Fig. 3B). The marginal decrease in the rates caused by TPB^- could be ascribed to the slight decrease in the concentration of the membrane-bound P^- form of the protonophores. The larger decrease observed in the case of SF6847 (Fig. 3C) is in line with the larger decrease in the concentration of membrane-bound P^- (Tables II and IV).

Discussion

The results presented here show that the rate of relaxation of ΔpH across vesicular membranes by protonophores can be enhanced by orders of magnitude by membrane-permeant cations such as valiomycin-K^+ or TPP^+. (Table I, Fig. 3). That the rate enhancement by valinomycin-K^+ was not due to a collapse of the diffusion potential $\Delta\psi_d$ was shown clearly (Figs. 1 and 2). Gramicidin, which was present in all these experiments, neutralized the $\Delta\psi_d$ effectively. In samples with protonophore and gramicidin, the rates were proportional to the concentration of the protonophore (Fig. 2) but independent of the concentration of gramicidin (Fig. 1). This shows that the rate-determining step in the relaxation of ΔpH is the intrinsic H^+ conductance of the protonophore and not the neutralization of $\Delta\psi_d$ by K^+ flux through the gramicidin channel. Hence the observed enhancements in the rates of relaxation of ΔpH by permeant cations should arise from enhancement of the intrinsic H^+ conductance of the protonophore.

The mechanism of protonophore-aided H^+ transport has been worked out in detail [3,4,16–18]. Most of this work deals with electrical conductivity measurements across lipid bilayers. These measurements show that the rate constant (at zero membrane potential) associated with transmembrane flux of the ionized form (P^-) of protonophore is lower than that of the neutral form (HP) by one or two orders of magnitude. During the H^+ transport turnover (Fig. 5) driven by ΔpH, P^- does not experience any electrical field. This is different from the situation in electrical conductivity measurements. Hence in our experiments, which involve ΔpH-driven H^+ flux, the rate-determining step would be the movement of P^- across the membrane. (The protonation and deprotonation reactions are expected to be much faster. Although the scheme (Fig. 5) shows direct combination of P_b^- with protons, our results are equally compatible with the model [17] in which the hydrolysis of water deliver the proton to P_b^-). The linear dependence of the rate of relaxation of ΔpH, k on the concentration of either CCCP or FCCP (Figs. 2B and 2C) agrees with the scheme shown in Fig. 5. The quadratic dependence of k on the concentration of SF6847 (Fig. 2A) indicates the involvement of the dimer form HP_2^- in the transport. This is similar to the quadratic dependence observed by Dilger and McLaughlin [18] for the protonophore 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB). HP_2^- is more permeable than P^- [18] (see the legend to Fig. 2A). Any factor which would enhance the flux of either P^- or HP_2^- would then lead to enhancement of the intrinsic H^+ conductance of the protonophore.

One or more of the following factors could lead to enhanced flux of P^- or HP_2^-: (a) an increase in the partition constant of the protonophore in favour of membrane phase, (b) a decrease in the pK value of membrane-bound protonophore, (c) an increase in the formation of the dimer form HP_2^- in the case of SF6847 and (d) an increase in the permeability of P^- or HP_2^-. The first factor cannot account for a rate enhancement more than approx. 1.3-fold as mentioned earlier. The partition constants (Tables II and IV) show that while valinomycin did not have any appreciable effect, TPP^+ increased the partition by approx. 1.2-fold. Obviously, this increase by TPP^+ (800 μM) is negligible when compared to the rate enhancement by approx. 20-fold (Fig. 3B). The second factor, decrease in the pK of membrane-bound protonophore, has a better chance of increasing the concentration of P^- in the membrane. However, as shown in Results, valinomycin did not have any significant effect on the pK values (Tables II and IV). Also, the TPP^+-caused decrease in pK values (Tables III and IV) could account for rate enhancements of approx. 3-fold only in the case of SF6847. (The dimer form HP_2^- was neglected in pK estimations. This is justified by the observation that the pK value did not show significant variation in the concentration range 1–10 μM of SF6847 (data not shown).) Also, the dimerization constant estimated by us (see legend to Fig. 2A) leads to ratios of the concentration of dimer to monomer in the range of 1 : 200 to 1 : 10 000 only). The third possibility, namely, the increase in the concentration of HP_2^- mentioned above, is also not likely. This is because the rate of relaxation of ΔpH increased linearly with the concentration of protonophore in the presence of either valinomycin or TPP^+ (Fig. 2). An increase in the concentration of HP_2^- would have resulted in a quadratic dependence. The linearity was observed even in the case of SF6847 (Fig. 2A) which showed quadratic

dependence in the absence of either valinomycin or TPP^+.

This analysis leaves only the fourth possibility viz. enhancement of the rate by increased permeability of either P^- or HP_2^-. An enhancement in the permeability of these species by either valinomycin-K^+ or TPP^+ could arise from one of the following two mechanisms; (i) a modification of the bilayer membrane properties by these agents and (ii) formation of an ion-pair complex between P^- and valinomycin-K^+ or TPP^+. Modification of membrane characteristics by these agents, especially valinomycin-K^+, seems unlikely due to its very low concentration (the valinomycin/lipid ratio was approx. 1 : 500 in our experiments). However, Hsu and Chan [19] have observed that even low levels of valinomycin (1 : 1000) caused pronounced alterations in the choline methyl proton magnetic resonance signal. The second possibility, namely, a lipophilic complex involving P^- is strongly favoured by our observation of hyperbolic dependence of the rate on the concentration of valinomycin (Fig. 3A). Linear double-reciprocal plots (Fig. 3A, inset) agree with the model of a complex between CCCP or FCCP and valinomycin-K^+ (see Appendix). Linear rate dependences observed in cases of (i) SF6847 and valinomycin, and (ii) all the protonophores and TPP^+ (Fig. 3) could be due to weaker binding in these cases. The transition from quadratic dependence of the rate to linear dependence caused by either valinomycin-K^+ or TPP^+ (Fig. 2A) also suggest the formation of a complex involving P^-. This would lead to a decrease in the level of HP_2^- and hence an approach to linear dependence as seen. Using the data given in Figs. 2 and 3A and Table I, the maximum rate enhancements by valinomycin can be calculated as 800-fold, approx. 400-fold and > 110-fold in the cases of CCCP, FCCP and SF6847, respectively. These enhancements could be the result of increased permeability of the ion pair valinomycin-K^+-P^- compared to P^-. Such ion pair-mediated enhancement of anion permeability has been observed in several systems [20–22]. The enhancement of anion permeability of mitochondrial inner membrane by valinomycin-K^+ [23,24] could also be due to such an ion pair. TPB^- caused slight decreases in the rates (Fig. 3C). These decreases could be accounted for by the decrease in the concentration of membrane-bound P^- (see Results). This also supports our model that the rate enhancements are due to complex formation rather than perturbation of membrane characteristics. The later mechanism would have resulted in a dramatic decrease of the rates by TPB^- especially in the cases of FCCP and CCCP.

Lipophilic complex between P^- and valinomycin-K^+ has been observed by several workers [6,15,25,26]. Perturbations in the visible spectra of P^- have been used to identify such complexes [6,15,25]. This complex has been implicated in the transport of H^+ and K^+ [4,15,27].

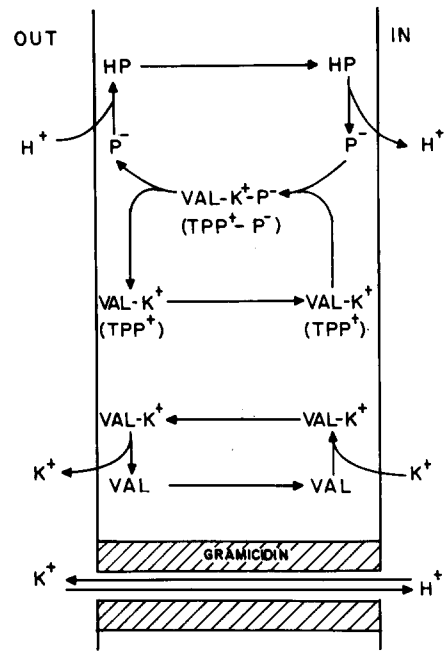


Fig. 7. Schematic diagram showing the direct participation of valinomycin-K^+ (Val-K^+) and TPP^+ in the ΔpH-driven H^+-transport by the protonophore, P. The enhancement of H^+-transport is due to higher permeability of Val-K^+-P^- and TPP^+-P^- compared to that of P^-. In the transport cycle involving the complex (Val-K^+-P^- or TPP^+-P^-), only H^+ is translocated. K^+ is transported independently by either valinomycin or gramicidin. The above complex is active in contrast to the inactive complex which has a distinct visible spectra [6].

However, one of us has shown recently [6] that the complex valinomycin-K^+-P^- with characteristic visible spectra [15,25] is in fact inactive in the transport of either H^+ or K^+. Hence the ion-pair complex inferred from our present studies should be different from the inactive complex seen in the visible spectra [6,15,25].

We present the following scheme as the working hypothesis (Fig. 7). Valinomycin-K^+ or TPP^+ enhances the rate of H^+ transport by protonophores by forming a special ion pair with P^-. The interaction could be weak so as to cause no perturbation of the visible spectra of the protonophore. This active complex, in the case of valinomycin-K^+ could be the precursor to the inactive complex with distinct visible spectra characterized earlier in our laboratory [6]. More work is required to characterize structural differences between the two types of complexes. It is also likely that the difference between the two complexs lie in some differences in their mean positions within the membrane. In our scheme (Fig. 7), the cycle involving the ternary complex transports only H^+ and hence it is electrogenic. The counterion K^+ is transported by valinomycin in an independent cycle. Hence, the presence of gramicidin can enhance the rate of relaxation of ΔpH (k) by increasing the K^+ flux further. The observed enhancement of k in valinomycin-protonophore system

by gramicidin (Table I) strongly faovurs this scheme. Also, the enhancement by TPP^+ can be accounted for by the same scheme. In contrast, in the earlier schemes [27,28] both H^+ and K^+ are transported in a single cycle involving the ternary complex. Due to electroneutral nature of such a scheme, gramicidin is not expected to enhance the rate k. This is similar to H^+/K^+ exchange by nigericin which is not enhanced by gramicidin [7].

Appendix

For the binding between valinomycin-K^+ and protonophore, the dissociation constant K_d is given by

$$K_d = \frac{[V][P]}{[V\text{-}P]} \quad \text{(A-1)}$$

where [V], [P] and [V-P] are the equilibrium concentrations of valinomycin-K^+, the protonophore and the complex, respectively.

When the total concentration of valinomycin is in far excess over that of the protonophore, Eqn. (A-1) can be approximated to

$$K_d = \frac{[V]_o([P]_o - [V\text{-}P])}{[V\text{-}P]} \quad \text{(A-2)}$$

where $[V]_o$ and $[P]_o$ are the total concentrations.

When the counterion flux is not rate-limiting and if both P and V-P can transport H^+, the rate of relaxation of ΔpH is given by

$$k = k_1[P] + k_2[V\text{-}P] \quad \text{(A-3)}$$

where k_1 and k_2 are constants.

When $k_2 \gg k_1$ (see Discussion),

$$k = k_2[V\text{-}P] \quad \text{(A-4)}$$

Combination of Eqns. 2 and 4 and rearrangement gives

$$\frac{1}{k} = \frac{1}{[V]_o} \cdot \frac{K_d}{k_2[P]_o} + \frac{1}{k_2[P]_o} \quad \text{(A-5)}$$

Hence K_d can be estimated from linear plots of $1/k$ versus $1/[V]_o$. These are given in the legend to Fig. 3.

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