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Enhancement of rates of H^+, Na^+ and K^+ transport across phospholipid vesicular membrane by the combined action of carbonyl cyanide *m*-chlorophenylhydrazone and valinomycin: temperature-jump studies

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Abstract

Enhancement of ΔpH relaxation rate by the combined action of valinomycin (VAL) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) has been studied under a variety of concentration conditions in soyabean phospholipid (SBPL) vesicles after creating a pH gradient across the vesicular membrane ΔpH by temperature jump. After taking note of the changes by VAL and CCCP induced membrane disorder (using nigericin and monensin mediated ΔpH decay as probes) the following could be inferred about the mechanism of enhancement of ΔpH decay rate: (i) in solutions containing KCl, the rate limiting species have been identified to be (a) Val-K^+-$CCCP^-$, at low $[Val]_0$ and $[CCCP]_0$ (with translocation rate constant $k_2 \sim 3.2 \cdot 10^3\ s^{-1}$); (b) CCCPH, at high $[Val]_0$ (with translocation rate constant $k_1 \sim 2 \cdot 10^5\ s^{-1}$); (c) the neutral valinomycin species Val, at high $[CCCP]_0$. (ii) In solutions containing NaCl, in our concentration range, the rate limiting species are Val-Na^+-$CCCP^-$. (iii) The apparent dissociation constant K_M^* of Val-M^+ decreases with pH in SBPL vesicles but is independent of pH in vesicles prepared from PC + 6% PA. (iv) The differences in the ionic strength dependencies of kinetic data shows that the environments of Na^+ and K^+ binding sites on VAL are different. (v) In vesicle solutions containing 100 mM MCl, the cation selectivity of VAL (towards K^+ in preference to Na^+) is reduced when $CCCP^-$ is already bound to it in the membrane. The $CCCP^-$ dissociation constant of Val-M^+-$CCCP^-$ is smaller with $M^+ = Na^+$ ($\sim$ 0.22 mM at 100 mM NaCl) when compared to that with $M^+ = K^+$ ($\sim$ 2 mM at 100 mM KCl). Attributing these differences to the differences in electrostatic interaction between $CCCP^-$ and M^+ in Val-M^+-$CCCP^-$, we can say that $CCCP^-$ binds closer to the Na^+ binding site than to the K^+ binding site on VAL.

Keywords: CCCP; Valinomycin; Liposome; Membrane; Ion transport; Proton transport; Temperature jump

1. Introduction

The correlation between proton conduction and the uncoupling of oxidative phosphorylation [1–3] has been responsible for the interest in the mechanisms of transmembrane H^+ transport and methods for enhancing their rates. In liposomes net transport of H^+ alone would set up a membrane potential which would retard further H^+ transport. Thus, proton conduction driven by a $[H^+]$ difference across membrane, requires a compensating charge flux such as that of alkali metal ion M^+, in a direction opposite to that of net H^+ transport to abolish this potential [4]. Exchange carriers such as nigericin and monensin transport both H^+ and M^+, exchange them at the aqueous medium-membrane interface and enable the ΔpH decay. In the previous studies we have shown how the rates of exchange carrier mediated decay of pH difference across membrane ΔpH, can be varied by a suitable choice of M^+, pH, and $[M^+]$ [5,6]. A weak acid such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in its protonated form CCCPH, can also transport H^+. The compensating charge flux can be provided by the transport of M^+ say by an alkali metal ion carrier such as valinomycin (VAL) [7,8]. For the ΔpH decay across a bilayer membrane by the combined action of VAL and CCCP, there has to be back transport of $CCCP^-$, the deprotonated form, to maintain

Abbreviations: ACES, *N*-(acetamido)-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; lip, lipid; SBPL, soyabean phospholipid; PC, L-α-phosphatidylcholine; PA, L-α-phosphatidic acid; VAL, valinomycin; Val, neutral valinomycin; Val-M^+, metal ion bound valinomycin; Nig, nigericin; Mon, monensin.

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[CCCP] in the two layers. The data reported in the literature for limited experimental conditions show that H^+ transport aided by VAL alone [9] is considerably slow compared to that by the combined action of CCCP and VAL [10,11].

In the present work we have obtained the data in the above system under a variety of experimental conditions to examine (a) the extent to which the transport data is affected by the changes in the lipid order at high concentrations of VAL and CCCP; (b) the rate limiting species under different concentration conditions; (c) pH dependence of metal ion binding to VAL either due to competitive binding of M^+ and H^+ to VAL or due to pH dependent changes in the membrane properties; (d) the differences in ion transport data obtained with K^+ and Na^+ in view of the different binding sites of K^+ and Na^+ on VAL inferred from structural studies [12]; (e) the possibility of reduction in the alkali metal cat ion selectivity of VAL on binding to $CCCP^-$ similar to that inferred from changes in ion permeabilities on adding an organic anion, trinitro-*m*-cresolate [13]; (f) the possible involvement of VAL clusters [14] in the rate limiting steps of ΔpH decay. We have also obtained a theoretical expression for the rate of ΔpH decay ($1/\tau$) by the combined action of a weak acid and VAL, which can account for all the observed behaviours of τ.

Temperature jump (T-jump) relaxation is a convenient technique to study the biologically relevant kinetics of H^+ transport and the associated M^+ transport [5,15–17]. To clarify the above mentioned points we have studied in the present work, the decay of pH difference across vesicular membrane (ΔpH) by the combined action of VAL and CCCP after creating a ΔpH by T-jump [5,17]. Soyabean phospholipid (SBPL) vesicle solutions have been used in the present study because they are stable and convenient model membrane systems for T-jump studies [5].

2. Materials and methods

SBPL vesicles with 2 mM pyranine and 2 mM or 50 mM phosphate or 0.25 mM *N*-(acetamido)-2-aminoethanesulfonic acid (ACES) buffer inside and 25 mM or 5 mM ACES or 25 mM tris(hydroxymethyl)aminomethane (Tris) outside vesicles were prepared at the desired pH and at the chosen [KCl], [NaCl] or [LiCl] from asolectin (Sigma) following the procedure given elsewhere [5,17]. Vesicles were also prepared using a 94:6 (w/w) mixture of L-α-phosphatidylcholine (PC) from frozen egg yolk and L-α-phosphatidic acid (PA) sodium salt from egg yolk lecithin (Sigma) using a similar procedure. The absorbances of vesicle solutions at 700 nm were monitored

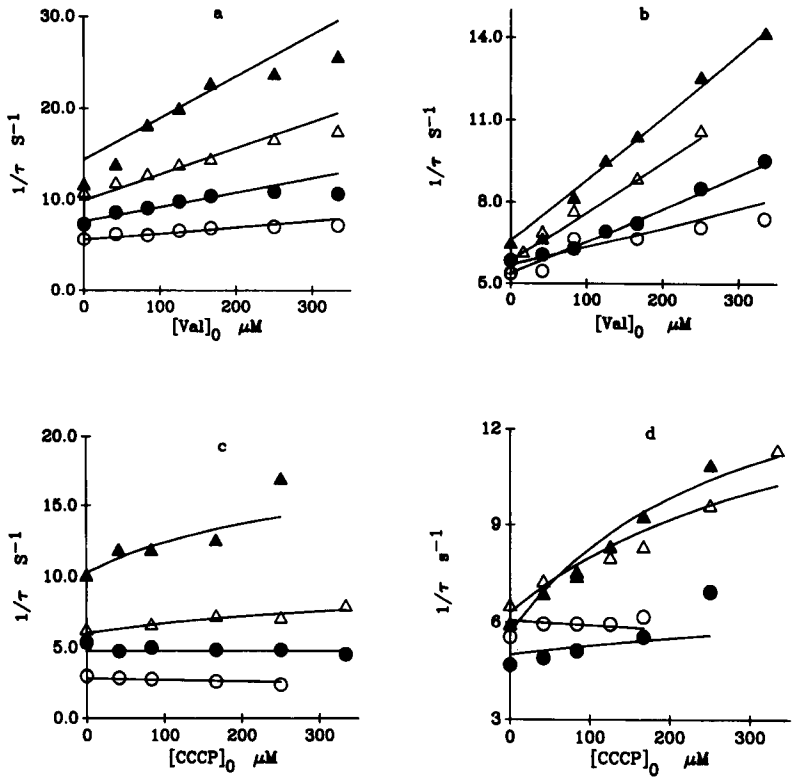

Fig. 1. Changes in nigericin and monensin mediated ΔpH decay rates on increasing [VAL] and [CCCP] in the membrane observed with 25 mM ACES outside and 50 mM phosphate inside SBPL vesicles, [lip] ≈ 5.2 mM, [KCl] = 100 mM. On increasing $[Val]_0$ with (a) $[Nig]_0 \approx 0.9$ μM, (b) $[Mon]_0 \approx 1.8$ μM. On increasing $[CCCP]_0$ with (c) $[Nig]_0 \approx 0.8$ μM and (d) $[Mon]_0 \approx 1.8$ μM. pH 6.2 ▲6.5 △, 7.0 ● 7.5 ○. Solid lines calculated using Eq. 1 and parameters given in the text.

using a Cary 17 D spectrophotometer to ensure near uniformity of different preparations. Stock solutions of 1 mM nigericin (Nig) and 3 mM monensin (Mon) (Sigma) in ethanol were used in the studies on ΔpH decay mediated by these exchange ionophores. Microlitre amounts of stock solutions of 1 mM or 10 mM VAL (Sigma) in ethanol were first added to the vesicle solutions in 0.25 mM ACES with vortex mixing and then mixing microlitre amounts of 1 mM or 10 mM or 40 mM CCCP (Sigma) stock solutions in ethanol. The solutions were then diluted with ACES buffer to get the required concentrations and pH. This procedure minimises the probability of 'inactivation' of carriers [18]. Only in a few experiments, at very high concentrations of VAL we observed 'inactivation' as a time dependent relaxation rate [18]. In such a situation $1/\tau$ extrapolated to zero time was used as data. The T-jump instrument used in our earlier studies [5,6] was used in the present work also. In our experiments ΔpH was ≈ 0.02. ΔpH relaxation times τ, were determined by comparing the observed relaxation traces with traces obtained by a calibrated exponential generator [5,6]. A particular set of data was obtained using the same vesicle preparation to avoid scatter of data due to differences in preparations. At least four T-jump relaxation traces observed at 24° C $\pm$ 1.5 on the same sample were used to obtain τ.

3. Results and discussion

By adding VAL and CCCP at suitable concentrations ($=[\mathrm{Val}]_0$ and $[\mathrm{CCCP}]_0$ in vesicle solutions containing KCl or NaCl), ΔpH relaxations with time constants τ in the

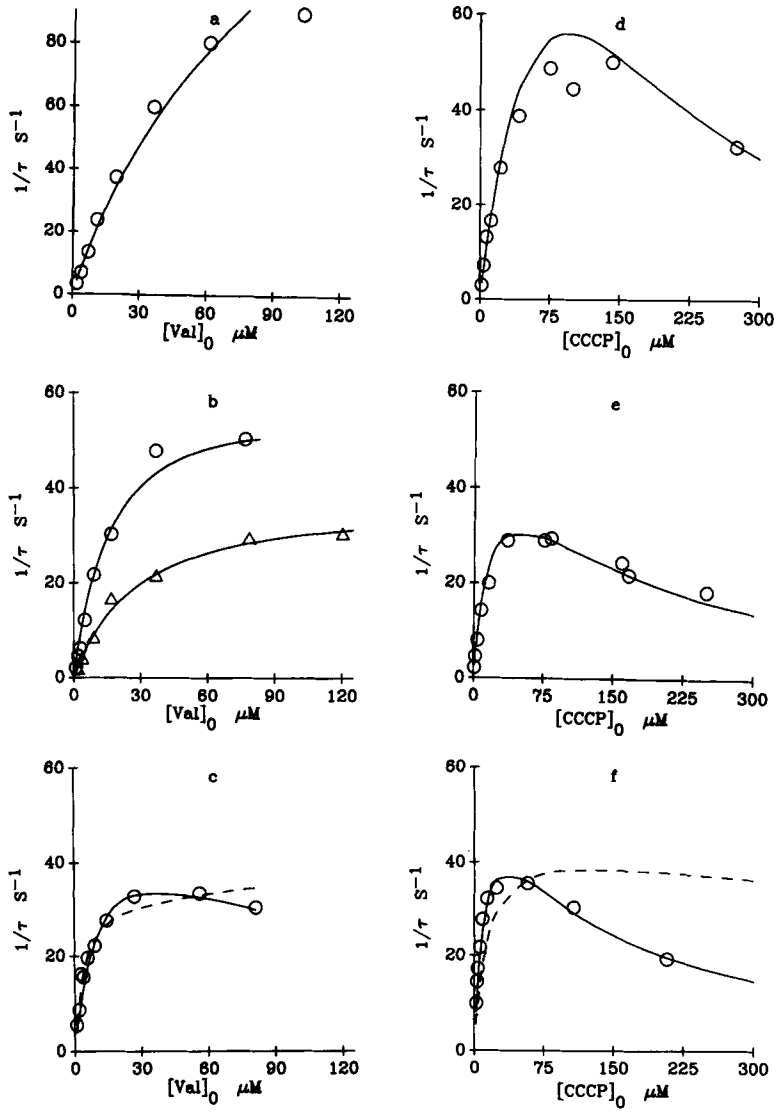

Fig. 2. Dependence of $1/\tau$ on $[\mathrm{Val}]_0$ keeping $[\mathrm{CCCP}]_0 = 2$ μM ((a)–(c)); on $[\mathrm{CCCP}]_0$ keeping $[\mathrm{Val}]_0 = 2$ μM ((d)–(f)). [KCl] = 100 mM, 50 mM ACES outside and 50 mM phosphate inside vesicles. (a) and (d) at pH 6.2; (b) and (e) at pH 6.95; (c) and (f) at pH 7.5. In (a)–(f) [lip] = 5.1 mM, O and in (b) [lip] = 9.1 mM, $\triangle$. Solid lines in (a)–(f) calculated using Eq. 4, Table 1 and using $f = 1.0$, 1.1, 1.0, 0.8, 0.9, 0.95, respectively. Broken line in (c) calculated neglecting the term with $[\mathrm{Val\text{-}M^+}]_{il}$ in the denominator of Eq. (4) and using $K_1 = 1$ mM and with $f = 0.4$. Broken line in (f) calculated neglecting the term with $[\mathrm{CCCP^-}]_{il}$ in the denominator of Eq. (4) and with $f = 0.5$. (See Table 1 for the definition of 'f'.)

second to millisecond range could be observed. In these experiments, when the metal ions were replaced by Li^+, $1/\tau$ was negligible ($< 0.1\ s^{-1}$). The amplitudes [5] and the reproducibility of relaxation traces on giving successive T-jumps to the same sample showed that the vesicular membranes were intact at all the concentrations used in our experiments.

3.1. Data which show that VAL and CCCP induced changes in membrane enhance and not block the diffusion of ionophores in our concentration range

In addition to participating in the H^+ and M^+ transports, CCCP or VAL in the membrane can affect rate of ΔpH decay by altering the diffusion rate constants of rate limiting species by (a) increasing the disorder and fluidity in the membrane or by (b) physically blocking their transport. For species of size similar to nigericin or monensin the magnitudes of such changes can be probed by monitoring the changes in $1/\tau$, when $1/\tau$ is dominantly determined by these exchange carriers. Fig. 1. shows typical data obtained in such studies in solutions containing KCl. Studies at a typical pH (≈ 7) showed that changes of similar magnitudes are observed even in solutions containing NaCl. The exchange carrier mediated ΔpH relaxation rate data in Fig. 1 could be fitted to the following empirical relation.

$$1/\tau = (A_0/b_i)(1 + B_0([H^+]/(K_s + [H^+]))([H^+]/(K_c + [H^+]))([VC]_0/[lip])) \quad (1)$$

In the above equation $pK_s = 7$, $K_c = 0$ and $B_0 = B_v = 20$ for VC ≡ VAL; $pK_s = 7$, $pK_c = 6.5$ and $B_0 = B_c = 60$–90 for VC ≡ CCCP. Also [lip] = lipid concentration in vesicle solution. The contribution from CCCP in the membrane to the internal buffer capacity of vesicles b_i (Eq. A-6), makes the $1/\tau$ against $[CCCP]_0$ plots nonlinear (and show a decrease of $1/\tau$ with $[CCCP]_0$ at higher pH). A_0 depends on pH, [lip] and $[Nig]_0$ or $[Mon]_0$. $A_0/b_i = 1/\tau$ in the absence of VAL or CCCP. B_0 ($= B_v$ and B_c) are parameters reflecting VAL and CCCP induced changes respectively in the diffusion rates of Nig or Mon. In obtaining the data of Fig. 1, $[Nig]_0$, $[Mon]_0$ and [lip] were slightly varied for different choices of pH to avoid overlap of data and to confirm the observed trends under slightly different conditions. For example, the data of Fig. 1d were obtained using slightly higher $[Mon]_0$/[lip] at pH 6.5 and 7.5 than that at pH 6.2 and 7.0.

From a study at different temperatures we could infer that the activation energy associated with nigericin mediated ΔpH decay (and hence that associated with diffusion rate constant of the ionophore) decreases from 15 kcal/mol to 12 kcal/mol indicating an increased disorder and hence increased fluidity on adding $[Val]_0 \approx 170\ \mu M$ to vesicles ([lip] ≈ 5 mM) in a medium containing 100 mM KCl.

The term with pK_s ($= 7$) is presumably associated with SBPL vesicles since it is present in the data obtained with both VAL and CCCP. We note that in SBPL vesicles a similar pK had been inferred in the endogeneous contribution to buffer capacity also [5]. Thus, the vesicular membrane appears to be more susceptible to disorder at lower pH. The term with pK_c ($= 6.5$, close to the pK of CCCP in SBPL vesicles [11]) is present only in the data obtained with CCCP (Fig. 1). Thus, Eq. (1) suggests that it is $[CCCPH]_0$ ($= [CCCP]_0[H^+]/(K_c + [H^+])$) rather than $[CCCP^-]_0$ which causes significant membrane disorder. This may be because the permeability of membrane to CCCPH is nearly 10^4 times more than that to $CCCP^-$ [19]. Irrespective of the validity of this explanation, Fig. 1 shows that because of VAL or CCCP induced changes in the membrane there is an enhancement in diffusion rates of ionophores by a factor of 2 in our concentration range when their sizes are similar to Nig and Mon (and not blocking of their transport).

3.2. Data which identify the rate-limiting species of ΔpH decay

Rate-limiting species of ΔpH decay can be identified by noting that the changes in their concentrations should be correlated with the changes in $1/\tau$. Fig. 2 shows the variations of $1/\tau$ at three pH conditions observed (i) on varying $[Val]_0$ keeping $[CCCP]_0$ small and constant; (ii) on varying $[CCCP]_0$ keeping $[Val]_0$ small and constant. The factor by which τ changes on increasing $[Val]_0$ or $[CCCP]_0$ in these figures are orders of magnitude larger than those seen in Fig. 1 for similar concentration changes (attributed to increase in membrane disorder). Furthermore, at higher concentrations the changes are in a direction opposite to that seen in Fig. 1. Therefore, these changes in τ are not due to VAL or CCCP induced changes in membrane order.

Fig. 2 shows that the linear increase in $1/\tau$ with $[Val]_0$ and $[CCCP]_0$ have slopes of similar magnitudes for small $[Val]_0$ and $[CCCP]_0$ in solutions containing KCl. A similar behaviour could be inferred in our concentration range in solutions containing NaCl also (Fig. 3) when the changes in $1/\tau$ due to changes in the membrane order, ΔpH decay by VAL alone and contribution to b_i from CCCP are factored out. Only the concentration of a 1:1 complex involving VAL and CCCP can show such a behaviour. Therefore, in the above situations such a complex can be identified as the rate-limiting species of ΔpH decay. We can also say that a M^+ is bound in such a complex since the concentration of the rate-limiting species depended on the choice of M^+ in the medium: changing the metal ion from K^+ to Na^+ required us to use higher $[Val]_0$ or $[CCCP]_0$ to get $1/\tau$ of comparable magnitude (Figs. 2 and 3). In view of the increased stability from electrostatic interactions we can expect the deprotonated form, $CCCP^-$, to be involved in the formation of the rate-limiting complex. We can combine the above three inferences and conclude that for small $[Val]_0$ and $[CCCP]_0$ in KCl medium

and in NaCl medium in our concentration range, the rate-limiting species of ΔpH decay is the 1:1:1 complex Val-M^+-$CCCP^-$ and write,

$$1/\tau = A[\text{Val-M}^+][\text{CCCP}^-]/b_i, \text{ or}$$

$$\tau = (b_i/A)((K_H + [H^+])/K_H)((K_M^*/[M^+]) + 1) \times (1/([\text{Val}]_0[\text{CCCP}]_0)) \quad (2)$$

where K_M^* and K_H are the apparent metal ion dissociation constant of Val-M^+ and apparent H^+ dissociation constant of CCCPH respectively. The parameter 'A' depends on the rate constant of translocation by diffusion and formation constant ($1/K_1$) of Val-M^+-$CCCP^-$ complex.

Yamaguchi and Anraku [10] had explained the hyperbolic dependence of initial proton uptake in liposomes on $[\text{Val}]_0$ and $[K^+]$ in the presence of limiting amounts of CCCP, assuming the rate-limiting species to be the ternary complex Val-K^+-$CCCP^-$. If this is the rate-limiting species of ΔpH decay at all $[\text{Val}]_0$ and $[\text{CCCP}]_0$, $1/\tau$ should tend towards a saturating value, since [Val-K^+-$CCCP^-$] is limited by the small $[\text{CCCP}]_0$ in Fig. 2a–c and the small $[\text{Val}]_0$ in Fig. 2d–f. Ahmed and Krishnamoorthy [11] had suggested such a possibility from their $[\text{Val}]_0$ dependent data at pH 7.5.

However, when [CCCPH] $\ll$ [Val-K^+-$CCCP^-$] the H^+ transporting translocation of CCCPH across the membrane could also contribute to the limiting of ΔpH decay rate, since the diffusion rate constant of CCCPH is finite. In such a situation $1/\tau$ should decrease on increasing $[\text{Val}]_0$ since [CCCPH] tends towards smaller values on increasing $[\text{Val}]_0$ due to the increased formation of the ternary complex. Such a decrease is expected to be more prominent at higher pH conditions, since $[\text{CCCPH}]/[\text{CCCP}]_0$ decreases with increase in pH. The data of Fig. 2, shows such behaviour. Therefore, we can say that [CCCPH] also limits the rate of ΔpH decay at sufficiently high $[\text{Val}]_0$.

Fig. 2d–f also shows that at sufficiently high $[\text{CCCP}]_0$, $1/\tau$ decreases on increasing $[\text{CCCP}]_0$. Similar behaviour seen in conductance data obtained with substituted benzimidazoles as weak acids had been attributed to the blocking of ion transports [20,21]. In view of Fig. 1 discussed above such a situation is unlikely with CCCP. From a knowledge of different contributions to b_i (see Eq. A-6) we can also say that the magnitude of increase in b_i on increasing $[\text{CCCP}]_0$ does not account for the observed decrease in $1/\tau$. Also, Fig. 2d–f could not be explained quantitatively assuming the rate-limiting species to be Val-K^+-$CCCP^-$ and attributing the reduction in $1/\tau$ with $[\text{CCCP}]_0$ to the formation of a 1:2 complex (which does not contribute to the ΔpH decay but reduces [Val-K^+-$CCCP^-$]). The decrease of $1/\tau$ with increase in $[\text{CCCP}]_0$ at high $[\text{CCCP}]_0$ can be understood if [Val] or [Val-K^+] (which also decrease with increase in $[\text{CCCP}]_0$) contribute to limiting the rate. Increasing [KCl] from 50 mM to 300 mM at constant ionic strength (using [LiCl] + [KCl] = 300 mM) increases [Val-K^+] at the expense of [Val]. Therefore, Fig. 4e which shows a decrease in $1/\tau$ on increasing $[K^+]$ suggests that [Val] limits the rate at high $[\text{CCCP}]_0$, contrary to the speculations in the literature that [Val-K^+] limits the rate [11].

3.3. Confirmation of the rate-limiting species and rate-limiting steps

If Val-K^+-$CCCP^-$ and CCCPH limit the ΔpH decay rate in vesicle solutions containing KCl at high $[\text{Val}]_0$ and limiting $[\text{CCCP}]_0$ (as inferred above), $1/\tau$ should increase linearly with $[\text{CCCP}]_0$ since the concentrations of these species vary linearly with $[\text{CCCP}]_0$. Similarly $1/\tau$ should vary linearly with $[\text{Val}]_0$ if Val-K^+-$CCCP^-$ and Val limit the rate in the experiments at high $[\text{CCCP}]_0$ and limiting $[\text{Val}]_0$. The τ data of Fig. 4a and b are consistent with these expectations.

At high $[\text{Val}]_0$ or $[\text{CCCP}]_0$, [Val-K^+-$CCCP^-$] $\approx$ limiting $[\text{CCCP}]_0$ or limiting $[\text{Val}]_0$ respectively. They do not depend on pH. Therefore, if Val-K^+-$CCCP^-$ was the only rate-limiting species, the dependence of $1/\tau$ on pH should have been similar with both limiting $[\text{CCCP}]_0$ and limiting $[\text{Val}]_0$. Fig. 4c shows that this is not true. However, if [CCCPH] and [Val] contribute to limiting the rate at high $[\text{Val}]_0$ and $[\text{CCCP}]_0$, respectively (inferred above), the increase of τ with pH should be more prominent with high $[\text{Val}]_0$ than with high $[\text{CCCP}]_0$ since on increasing pH > 7,

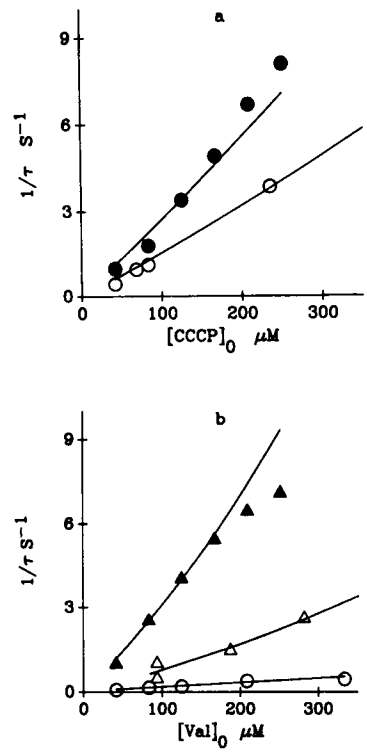

Fig. 3. Dependence of τ on carrier concentration in solutions with 25 mM ACES outside and 50 mM phosphate inside vesicles, pH = 7 and 100 mM NaCl. (a) On $[\text{CCCP}]]_0$ with $[\text{Val}]_0 = 6$ μM, ([lip] = 5.3 mM) ○; $[\text{Val}]_0 = 8$ μM, ([lip] = 5 mM) ●; (b) On $[\text{Val}]_0$ with $[\text{CCCP}]_0 = 0$ ○, $[\text{CCCP}]_0 = 4$ μM, ([lip] = 5.4 mM), △; $[\text{CCCP}]_0 = 8$ μM ([lip] = 5.2 mM), ▲ Solid lines obtained using Eq. 4 Table 1 and $f = 1.25$ and 1.1.

[CCCPH] decreases, whereas [Val] tends towards a constant value. Fig. 4c shows data consistent with this expectation.

The translocation of the rate-limiting species by diffusion across the membrane is the rate-limiting step of ΔpH decay. The data cannot be explained by the alternate possibilities: (a) If proton exchange at the interface limits the rate we should observe an increase in $1/\tau$ with increase in buffer concentration (which can be dominantly involved in the proton exchange [22] at the interface) Figs. 4d, 5c and 5d show a smaller $1/\tau$ at higher buffer concentration inside vesicles throughout our pH range. (b) If metal ion binding at the interface limits the rate $1/\tau$ should increase with increase in $[K^+]$. Fig. 4e shows a smaller $1/\tau$ at higher [KCl] for various choices of $[CCCP]_0$. (c) If the transfers of CCCP and VAL between the aqueous medium and the membrane at the interface limit the rate, τ should decrease with increase in [lip] whereas Fig. 4f shows an increase.

3.4. Data which show that the binding of M^+ to VAL is pH dependent in SBPL

The apparent metal ion dissociation constant K_M^* of Val-M^+ can be estimated from the yield of [Val-M^+] as a

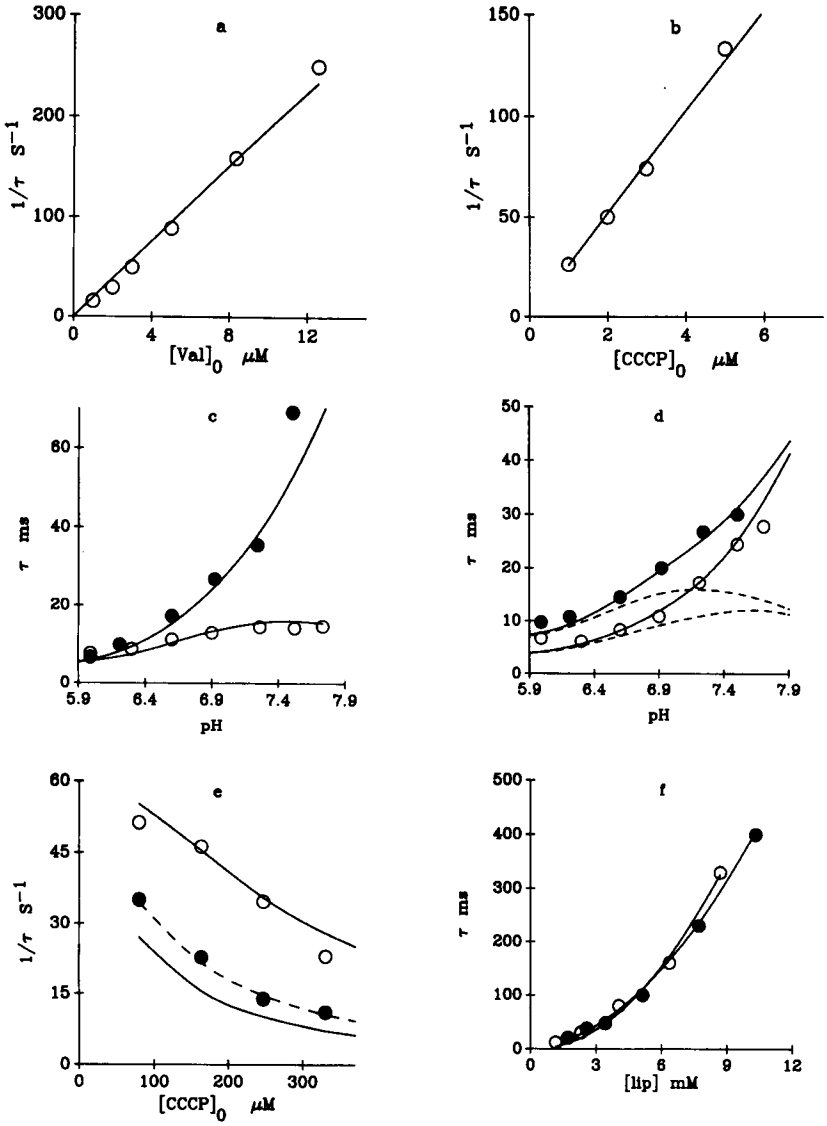

Fig. 4. Data which confirm the identification of rate limiting species and rate limiting steps. (a) Dependence on $[Val]_0$ with $[CCCP]_0 = 85$ μM. (b) Dependence on $[CCCP]_0$ with $[Val]_0 = 85$ μM. [lip] = 5.2 mM, outside 25 mM ACES and inside 50 mM phosphate, [KCl] = 100 mM, pH = 7 in both (a) and (b). pH dependence of τ observed with (c) $[CCCP]_0 = 85$ μM, $[Val]_0 = 3.3$ μM, and inside 2 mM phosphate ○; $[CCCP]_0 = 2.2$ μM, $[Val]_0 = 254$ μM and inside 50 mM phosphate ●. (d) $[CCCP]_0 = 2.2$ μM, $[Val]_0 = 127$ μM, and inside 2 mM phosphate, ○, or 50 mM phosphate buffer, ●. [lip] = 5.6 mM, [KCl] = 100 mM, outside 25 mM ACES in (c) and (d). (e) Decrease of $1/\tau$ on increasing [KCl] from 50 mM ○; to 300 mM ●, keeping [LiCl] + [KCl] = 300 mM, $[Val]_0 = 2$ μM, [lip] = 5.3 mM and pH 7 for different values of $[CCCP]_0$. (f) Dependence of τ on [lip] at pH 7 in NaCl medium $[Val]_0 = 25$ μM and $[CCCP]_0 = 170$ μM, ○; in KCl medium $[Val]_0 = 2$ μM and $[CCCP]_0 = 2$ μM, ●. Inside 50 mM phosphate and outside 25 mM ACES. Solid lines calculated using Eq. 4, Table 1 and $f = 0.75$, 0.8, 0.95 (and 1.0), 1.1 (and 1.25), 1.5, 1.1 (and 1.0) in (a)–(f) respectively. Broken lines in (d) calculated neglecting the term with $[Val\text{-}M^+]_{il}$ in the denominator of Eq. 4 and in (e) calculated assuming [KCl] = 220 mM.

function of $[M^+]$ using τ data corresponding to the experimental conditions of Eq. (2) (Fig. 5). Fig. 5a had been obtained at constant ionic strength using mixtures of LiCl and KCl and varying [KCl]. The results of Fig. 5a discussed below have been confirmed using mixtures of NaCl and KCl also. In these experiments, because of the ion selectivity of VAL, $[Val\text{-}Li^+]$ and $[Val\text{-}Na^+]$ are negligibly small compared to $[Val\text{-}K^+]$. The intercepts in Fig. 5a show that $1/\tau$ at pH ≈ 6 and ≈ 7 have similar magnitudes in SBPL vesicles in the limit of infinite $[K^+]$ (when all the VAL are expected to have K^+ bound to them). This observation can be explained if the decrease of $1/\tau$ with pH due to the pH dependence of b_i in Eq. (2) (Eq. A-6 and Ref. [5] and [9]) is compensated by the increase in $[CCCP^-]$. Such a requirement gives an estimate of pK_H of CCCP (≈ 6.5) in SBPL vesicles. The agreement of this estimate and the pK_H determined directly from spectrophotometric observations (≈ 6.7) [11], shows that 'A' and $A\,[CCCP^-]/b_i$ in Eq. (2) do not vary significantly in the pH range ≈ 6 to ≈ 7. Therefore, the variation of $1/\tau$

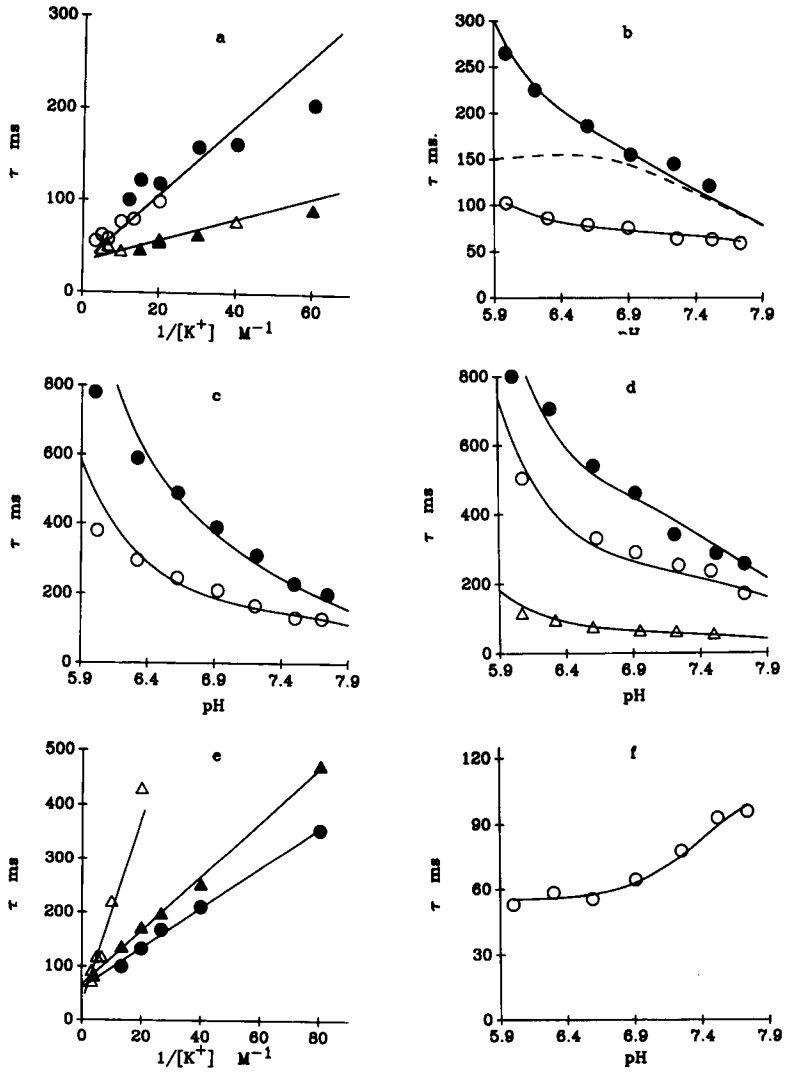

Fig. 5. Dependence of τ on metal ion concentration and pH in SBPL vesicles ((a)–(d)) and in 94% PC + 6% PA lipid vesicles ((e) and (f)). (a) [lip] = 5.3 mM, 5 mM ACES outside, 0.25 mM ACES inside vesicles, $[Val]_0 = 3.8\ \mu M$, $[CCCP]_0 = 2.5\ \mu M$, [LiCl] + [KCl] = 100 mM at pH 6.1, ● and pH 7.0 ▲; [LiCl] + [KCl] = 300 mM at pH 6.1, ○ and pH 7.0 △. (b) pH dependence of τ observed with [KCl] = 100 mM, $[Val]_0 = 3.3\ \mu M$, $[CCCP]_0 = 2.2\ \mu M$, [lip] = 5.6 mM, 25 mM ACES outside, and 2 mM ○ or 50 mM ● phosphate inside vesicles. pH dependence of τ in NaCl medium with 25 mM ACES outside and (c) [NaCl] = 100 mM, [lip] = 5.5 mM, $[Val]_0 = 30\ \mu M$, $[CCCP]_0 = 40\ \mu M$. Phosphate buffer inside 2 mM ○, and 50 mM ●; (d) [NaCl] = 100 mM, [lip] = 5.5 mM, $[CCCP]_0 = 200\ \mu M$, $[Val]_0 = 5\ \mu M$, phosphate buffer inside 2 mM ○, and 50 mM ●. [NaCl] = 300 mM, [lip] = 5.5 mM, $[CCCP]_0 = 200\ \mu M$, $[Val]_0 = 10\ \mu M$ and phosphate buffer inside 2 mM Δ. Dependence of τ on $1/[K^+]$ and pH in 94% PC + 6% PA observed with (e) [KCl] + [NaCl] = 100 mM, [lip] = 5.3 mM, 25 mM ACES outside and 2 mM phosphate inside vesicles, $[Val]_0 = [CCCP]_0 = 10\ \mu M$, pH 6.1 ●, pH 7.0 ▲; with [KCl] + [LiCl] = 300 mM, [lip] = 2.6 mM, 25 mM Tris outside and 0.25 mM ACES inside vesicles, $[Val]_0 = [CCCP]_0 = 5\ \mu M$, pH = 7.0 △; (f) with [KCl] = 100 mM, [lip] = 5.3 mM, 25 mM ACES outside and 2 mM phosphate inside vesicles, $[Val]_0 = [CCCP]_0 = 10\ \mu M$. Solid lines obtained using Eq. 4, Table 1 and $f = 1.0$ and 1.3 in (a), 1.1 in (b), 1.0 in (c), 0.9, 0.9 and 1.15 in (d), 0.8, 0.8 and 1.0 in (e) and 1.05 in (f). Broken line in (b) calculated assuming $K_M^* = K_M$.

Table 1
Parameters in the mechanism of Fig. 7 obtained by fitting the τ data at [MCl] = 100 mM ($M^+ = K^+$, Na^+) and 24° to Eq. 4 [a]

Parameter	Lipid	Value	Source
pK_H	SBPL	6.5	Fig. 5
	PC+6% PA	6.9	Fig. 5
pK_{LH}	SBPL	6.6 [b]	Fig. 5
K_M (K^+), mM [c]	SBPL	25	Fig. 5
	PC+6% PA	70	Fig. 5
K_M (Na^+), M [c]	SBPL	425	[d]
K_1 (K^+), mM	SBPL	2	Figs. 2, 4, 5
	PC+6% PA	54	Fig. 5
K_1 (Na^+), mM	SBPL	0.22 [e]	Fig. 6
k_1, s^{-1}		$2.6 \cdot 10^5$	Figs. 2a–2c
		$1.6 \cdot 10^5$	Figs. 2d–2f
k_2, s^{-1}		$3.2 \cdot 10^3$	Figs. 2, 4, 5

Estimates in PC+6% PA obtained assuming k_2 to be similar to that in SBPL.

[a] The parameter 'f' given in the figure legends had been obtained by calculating τ (solid lines in the figures) using $k_2 = 3.2 \cdot 10^3 \times f\ s^{-1}$ in Eq. 4 such that a good fit with the experimental data is obtained. Thus, 'f' in the figure legends reflect the errors in k_2 estimate. Estimates of K_M and k_1 in SBPL correspond to high pH conditions.

[b] The τ data in PC+6% PA vesicles could be explained without pK_{LH}.

[c] $K_M(K^+)$ does not depend on ionic strength in SBPL but increases to ≈ 500 mM in PC+6% PA on increasing [MCl] to 300 mM. $(K_1 K_M)(Na^+)$ increases by a factor ≈ 1.7 on increasing [MCl] from 100 mM to 300 mM.

[d] Assuming relative cation selectivity of VAL to be 17000 [23].

[e] Using $k_2 = 3.2 \cdot 10^3\ s^{-1}$. With $K_2 = 400$ mM we get $K_1(Na^+) = 0.3$ mM.

with pH at constant $[K^+]$ will reflect the change in the yield of [Val-K^+] on changing $[H^+]$ in the above pH range. τ data for different [KCl] at the two pH conditions in Fig. 5a and the pH dependence of τ in Fig. 5b obtained keeping [KCl] = 100 mM show that the yield of [Val-K^+] decreases with increase in $[H^+]$. The data obtained in NaCl medium (Fig. 5c and d) show that in spite of the increase in membrane fluidity with $[H^+]$ (which causes a decrease in τ due to increase in $[CCCPH]_0$, see Eq. (1)) there is an increase in τ. Using Eq. (2) we therefore conclude that [Val-Na^+] also decreases on lowering pH in SBPL vesicles. Eq. (2) shows that the slope/intercept of the linear plots in Fig. 5a can be used to get K_M^* at pH ≈ 6 and 7 in KCl medium. Using this estimate and by fitting Eq. (2) to the data of Fig. 5a–d, the dependence of K_M^* on pH has been found to be

$$K_M^* \approx K_M(1 + [H^+]/K_{LH}) \quad (3)$$

K_M and K_{LH} estimated using Eq. (3) are given in Table 1.

3.5. Origin of pH dependence of K_M^* in SBPL vesicles

Lev and Buzhinsky [23] and Andreoli et al. [24] have inferred the binding of H^+ to VAL from electrochemical data in black lipid membranes. Therefore, it is possible to suggest that the pH dependence seen in Eq. (3) is due to the competitive binding of H^+ and M^+ to VAL. If this explanation is valid we should get significant increase in the buffer capacity of vesicle solutions ([lip] = 3.2 mM in 125 μM ACES at pH ≈ 6.8 with 100 mM NaCl, 0.5 μM Nig, and 3 μM pyranine) on adding $[Val]_0$ (= 200 μM). The pH changes in the above solutions, on adding 5–15 μl of 0.01 M NaOH (monitored using changes in pyranine fluorescence) showed that the contribution to buffer capacity from VAL is negligible. Thus, we are constrained to assign the pH dependence of Eq. (3) to pH dependent changes in the SBPL membrane which could be changing the effective $[M^+]$ in the membrane and thereby altering the apparent dissociation constant K_M^*. We have confirmed this possibility by examining the ΔpH decay data in vesicles prepared from PC + 6% PA. Such data obtained in KCl medium and given in Fig. 5e and f do not show significant pH dependence of K_M^* (slope/intercepts in Fig. 5e obtained at pH ≈ 6 and 7 have similar magnitudes). Furthermore, K_M and its dependence on ionic strength are higher in PC + 6% PA than in SBPL vesicles showing again that K_M is determined by the nature of lipid. We also note that pK_{LH} estimated in SBPL vesicles (Table 1) is close to the pK_{H2} associated with an endogeneous contribution to buffer capacity in SBPL vesicles (≈ 6.9).

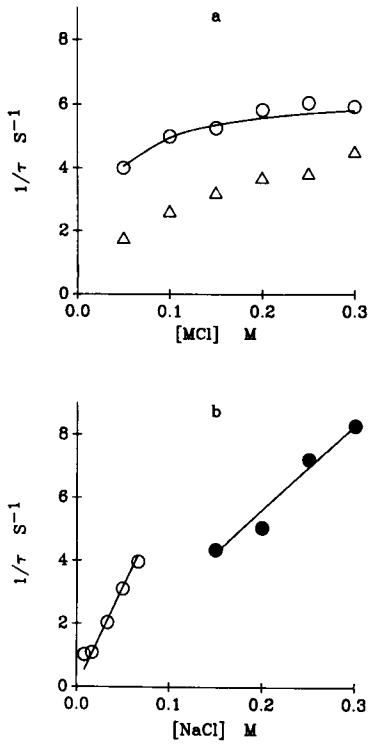

Fig. 6. Dependence of $1/\tau$ on alkali metal ion concentration. (a) Data obtained with 25 mM ACES outside and 50 mM phosphate buffer inside and by varying [KCl], ○, with $[Val]_0 = 2.1$ μM, $[CCCP]_0 = 2.1$ μM and [lip] = 5.5 mM; and by varying [NaCl], △, with $[Val]_0 = 6$ μM, $[CCCP]_0 = 190$ μM and [lip] = 6.3 mM. (b) On [NaCl] at constant ionic strength with 5 mM ACES outside and 0.25 mM ACES inside vesicles. $[Val]_0 = 6.3$ μM, [CCCP] = 150 μM, pH 7.0, [LiCl] + [NaCl] = 100 mM, ○ ([lip] = 4.7 mM), and with [LiCl] + [NaCl] = 300 mM, ● ([lip] = 5.4 mM). Solid lines calculated using Eq. 4, Table 1 and $f = 1.15$.

Such a pK_{H2} had not been inferred in the buffer capacity data of PC + PA lipid vesicles [5]. Presumably the dependence of K_M^* on pH in SBPL vesicles is due to changes in the membrane on protonation of this ionisable group. The small difference between pK_{H2} and pK_{LH} has been explained in the Appendix (Eq. A-5).

3.6. Data showing a reduction in cation selectivity of VAL due to $CCCP^-$

When the anion $CCCP^-$ is bound to VAL, the relative cation selectivity of VAL is reflected in the relative yields of Val-M^+-$CCCP^-$ for different choices of M^+. Using $(1/\tau)/([Val]_0[CCCP]_0)$ at [MCl] = 100 mM, pH 7.5 and concentration conditions pertinent to Eq. (2) we conclude from Figs. 2 and 5 that for a constant value of the product $[Val]_0 \times [CCCP]_0$, the ratio [Val-K^+-$CCCP^-$]/[Val-Na^+-$CCCP^-$] ≈ 400. This ratio is less than [Val-K^+]/[Val-Na^+] (≈ 3400) estimated at similar experimental conditions by using the ion selectivity data [25] and K_M given in Table 1. Tosteson and coworkers [13] have also inferred that complexation with anions can reduce cation selectivity of VAL. The origin of this reduction is in the increased stability of Val-M^+-$CCCP^-$ by a factor of ≈ 8 on changing M^+ from K^+ to Na^+. If the binding site of Na^+ on VAL (compared to that of K^+) is closer to the bound $CCCP^-$, the increased electrostatic interaction can cause such an increase.

3.7. Data reflecting the differences in the environments of K^+ and Na^+ binding sites on VAL

Structural studies [12] have shown that the binding sites of K^+ and Na^+ on VAL are different. If this is true the electric dipole moments of Val-M^+-$CCCP^-$ and their interactions with the surrounding charges will depend on the choice of M^+ ($M^+ = K^+$ or Na^+). This should be reflected as differences in the dependence of kinetic data on ionic strength. Since the slopes or intercepts of the plots in Fig. 5a do not change with ionic strength, we can say that in SBPL vesicles K_M and K_1 associated with the binding of K^+ to VAL are not sensitive to ionic strength. This was confirmed in the data of Fig. 6a which could be explained using K_M and K_1 given in Table 1 even though these τ had been obtained without regulating the ionic strength. Fig. 6a shows similar curvatures for the dependence of $1/\tau$ on $[K^+]$ and $1/\tau$ on $[Na^+]$ at pH ≈ 7. This gives an incorrect impression that K_M for Val-K^+ and Val-Na^+ have similar values if (K_M K_1) is assumed to be independent of ionic strength on replacing K^+ by Na^+. However, the data of Fig. 6b obtained using LiCl + NaCl mixtures to keep the ionic strength constant do not show a curvature implying correctly that K_M associated with Val-Na^+ is very large. They also show a decrease in the slope of $1/\tau$ against $[Na^+]$ plot and imply that the product (K_M K_1) increases by a factor of ≈ 1.7 for $M^+ = Na^+$ on increasing [LiCl] + [NaCl] from 100 mM to 300 mM. The pH dependent data in Fig. 5d confirms such an increase in (K_M K_1) with ionic strength. The increase in (K_M K_1) on increasing the ionic strength observed with $M^+ = Na^+$ (in contrast with insignificant change for $M^+ = K^+$) can be understood if the interaction with charges in the lipid environment stabilises Val-Na^+-$CCCP^-$ to a lesser extent than Val-K^+-$CCCP^-$, with respect to the stabilisation of Val-M^+ and $CCCP^-$. This is possible if the binding site of Na^+ on VAL (compared to that of K^+) is nearer the bound $CCCP^-$ making its electric dipole moment relatively smaller. Such a difference in binding sites is similar to that inferred from the changes in cation selectivity.

3.8. Analysis of the data in terms of the mechanism of ΔpH decay

A mechanism for the ΔpH decay by H^+/M^+ transports across membranes in vesicle solutions containing MCl ($M^+ = Na^+$, K^+) which takes note of the results and inferences mentioned above is given in Fig. 7. In this mechanism, after a fast H^+ exchange at the interface, H^+ is translocated across the membrane as CCCPH. To facilitate its back transport Val is also translocated. After the release of H^+ at the second interface, $CCCP^-$ and the compensating M^+ are translocated in a direction opposite to that of H^+ as Val-M^+-$CCCP^-$. The expression for $1/\tau$ using Eq. (A-15) for the mechanism of Fig. 7, after including the corrections due to changes in the membrane order at high $[Val]_0$ and $[CCCP]_0$ (Eq. 1), can be written as follows.

$$1/\tau = A/b_i B$$

where

$$A = (\ln 10)(1 + b_i V_i/(b_e V_e))k_2^*[\text{Val-M}^+ - \text{CCCP}^-]_{il}$$

and

$$\begin{aligned} B = {} & \left(1 + \left([\text{CCCP}^-]_{il}[M^+]k_2^*/(k_1^* K_1 K_M)\right)\right. \\ & \left. + \left([\text{Val-M}^+]_{il} k_2^* K_H/(k_1^* K_1 [H^+])\right)\right) \\ k_m^* = {} & k_m\left(1 + B_v[H^+]/(K_s + [H^+])([Val]_0/[lip])\right) \\ & \times\left(1 + B_c\left([H^+]/(K_s + [H^+])\right)\right. \\ & \left. \times\left([H^+]/(K_H + [H^+])\right)([CCCP]_0/[lip])\right) \qquad (4) \end{aligned}$$

k_m with $m = 1$ and $m = 2$ are the rate constants for the diffusion of CCCPH and Val-M^+-$CCCP^-$ across the membrane respectively. For molecules of sizes comparable to nigericin or monensin (in the present case for Val-M^+-$CCCP^-$ (i.e., for k_2) we can use B_v and B_c estimates given above (see Eq. (1)). However, for the diffusion of smaller molecules such as CCCPH these corrections could be negligible (i.e., $k_1^* \approx k_1$).

We note that Eq. (4) reduces to Eq. (2) for sufficiently small $[Val]_0$ and $[CCCP]_0$ or when K_M is large. Using the relative cation selectivity (≈ 17000) [25] and $K_M = 25$

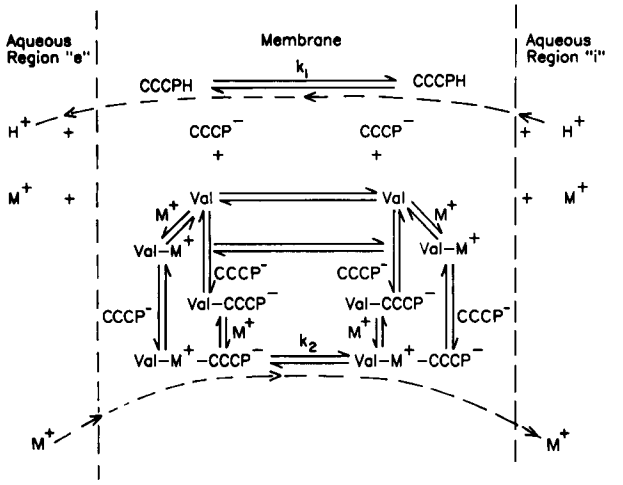

Fig. 7. Suggested mechanism for the ΔpH decay by the combined action of VAL and CCCP.

mM for Val-K^+ (Table 1) K_M for Val-Na^+ was estimated to be $\approx$ 425 M in SBPL vesicles.

K_1, k_2 and k_1 in vesicle solutions containing KCl (Table 1) have been determined using the following criterion: Eq. (4) should reproduce the curvatures, saturating behaviours and dependencies of $1/\tau$ on various parameters seen in Figs. 2 and 4 on using [Val-M^+-CCCP$^-$] obtained with the help of Eq. (A-8). In such calculations, (a) because of the stabilisation associated with Coulombic interaction, [Val-CCCP$^-$] $\ll$ [Val-M^+-CCCP$^-$] in Eq. (A-8) and we have used $K \approx K_{01}$ in Eq. (A-9); (b) in view of our studies on nigericin mediated ion transports [5] and pH independent nature of the parameter 'A' in Eq. (2), k_2 and K_1 were taken to be independent of pH. In solutions containing NaCl and in PC + 6% PA lipid vesicles, K_1 was determined by fitting the data of Figs. 3–6 to Eq. (4) after requiring k_2 associated with Val-K^+-CCCP$^-$ and Val-Na^+-CCCP to have similar magnitudes since they have similar sizes. The parameters thus determined are given in Table 1. The solid lines of Figs. 2–6 have been obtained using Eq. (4) and the parameters given in Table 1. We also note that use of non negligible [Val-CCCP$^-$] (say with $K_{02} \approx 400$ mM) causes only a small change in the estimates of these parameters (Table 1)

The k_2 associated with the translocation of Val-M^+-CCCP$^-$, estimated by us ($\approx 3.2 \cdot 10^3$ s^{-1}), is smaller than that of neutral valinomycin ($\approx 2 \cdot 10^4$ s^{-1}) reported in the literature [26]. Such differences can be understood in terms of differences in their sizes and electric dipole moments. However, k_2 is closer to that of nigericin and monensin ($\approx 6 \cdot 10^3$ s^{-1} and $9 \cdot 10^3$ s^{-1}). Compared to these estimates k_1 associated with CCCPH ($\approx 2 \cdot 10^5$ s^{-1}) is high. This is expected since CCCPH (molecular weight M_r = 204) which is also electroneutral has a smaller size when compared to that of Nig-H or Mon-H (M_r = 725 and 620). However, our k_1 in SBPL is higher than the estimates in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) given in the literature ($1.5 \cdot 10^4$ s^{-1} and $3.0 \cdot 10^4$ s^{-1}) which could be due to differences in the choice of lipid [27]. Dependence of K_1 and K_M on the lipid could also be inferred by analysing the data given in Fig. 5. In vesicles prepared from PC + 6% PA, K_1 was $\approx$ 30-times and K_M was nearly 3-times the values observed in SBPL vesicles. Table 1 shows that on changing M^+ from K^+ to Na^+, K_1 decreases from 2 mM to $\approx$ 0.22 mM, showing additional stabilisation in the sodium complex presumably due to the closer proximity of Na^+ to CCCP$^-$ in the ternary complex. Our estimates of K_1 have been obtained taking note of all the terms of Eq. (4) and using the concentrations in the membrane, unlike that for the potassium complex given in the literature ($\approx$ 2.9 μM) [11].

3.9. Comments on the transport mechanism

(i) Since H^+ flux is compensated by the M^+ ion charge flux, we should observe synergistic transport of K^+ and Na^+ by the VAL and CCCP combination when the dominant mode of net ion transport is by the mechanism of Fig. 7. Such transports have been observed by Yamaguchi and Anraku [10] with K^+ ions, and by Redelmeyer et al. [28] with Na^+ ions. (ii) Fig. 7 is similar to the mechanism proposed by Yamaguchi and Anraku [10]. In this mechanism only electroneutral species are involved in H^+ and Na^+ transports. Preference to ion transports as electroneutral species has been suggested for other systems also [29]. However, Ahmed and Krishnamoorthy [11] have speculated that M^+ is transported as Val-M^+ in a separate cycle in the above system from their observations on the increase in the ΔpH decay rate on adding gramicidin to vesicle solutions containing Na^+ or high $[CCCP]_0$. Our observation that for high $[CCCP]_0$, $1/\tau$ decreases on increasing $[K^+]$ and the possibility of other mechanisms of ion transports in the presence of gramicidin are against such a hypothesis. (iii) Attempts to explain the decrease in $1/\tau$ on increasing $[CCCP]_0$ to the increased formation of 1:2 complexes (with negligible diffusion rates) showed that the parameters which could explain the data at high $[CCCP]_0$ could not account for the $1/\tau$ at low $[CCCP]_0$. In the literature the decrease in ion transport rates on increasing weak acid concentrations has been attributed to the blocking of ion transports by weak acids [20,21]. However, in the mechanism of Fig. 7 such a behaviour is explained as due to a reduction in the rate-limiting Val species due to increased formation of the ternary complex Val-M^+-CCCP$^-$. (iv) Ivanov [14] has speculated on the existence of VAL clusters in bilayers. It is possible to suggest that such clusters may be involved in actual mode of translocation of CCCP$^-$ and M^+ by a 'relay mechanism' instead of by the diffusion of Val-M^+-CCCP. Since the cluster formation is dominantly determined by the interactions between VAL, the disorder in the membrane affects the 'diffusion mechanism' more than it affects the 'relay

mechanism'. Therefore, only in the 'diffusion mechanism' we can expect the B_c and B_v occurring in the expression for k_2^* (see Eqs. 1 and 4) to have values similar to that determined for the diffusion rate constants of nigericin and monensin species. The need to include the membrane disorder factors of such magnitudes in fitting the dependencies of τ on pH, $[CCCP]_0$, and $[Val]_0$ at high concentrations in a medium containing NaCl (Figs. 3 and 5), therefore favours the 'diffusion mechanism' of transports in Fig. 7. (v) Formation of ternary complexes (as required in Fig. 7) is also reasonable since such complexes involving VAL, cation and anion have been detected by NMR also [30].

Appendix 1

Equilibria

We define the apparent dissociation constants K_H and K_M^* for the following equilibria using $[H^+]$ and $[M^+]$ in the aqueous medium and carrier concentrations in the lipid medium.

$$CCCPH \rightleftharpoons CCCP^- + H^+,\ K_H$$

$$Val\text{-}M^+ \rightleftharpoons Val + M^+,\ K_M^* \qquad \text{(A-1)}$$

The carrier concentration in the membrane [CR] is related to its concentration in SBPL vesicle solutions $[CR]_0$ and lipid concentration [lip] by the following relation [5], when the carrier is predominantly in the membrane as with VAL and CCCP [9,11]:

$$[CR] = 0.95[CR]_0/[lip]\,M \qquad \text{(A-2)}$$

If $[Val_f]$ and $[CCCP_f]$ are the total concentrations in the membrane excluding the complexes Val-M^+-$CCCP^-$ and Val-$CCCP^-$ we can write,

$$[CCCPH] = [CCCP_f][H^+]/(K_H + [H^+]);$$

$$[CCCP^-] = [CCCP_f]K_H/(K_H + [H^+])$$

$$[Val\text{-}M^+] = [Val_f][M^+]/([M^+] + K_M^*);$$

$$[Val] = [Val_f]/(1 + [M^+]/K_M^*) \qquad \text{(A-3)}$$

Attributing the pH dependence of K_M^* to pH dependent changes in the lipid and taking $K_M^* = K_M^a$ when an ionisable group on the lipid is protonated and $= K_M$ when it is deprotonated, K_M^* can be expressed as,

$$1/K_M^* = ([H^+](K_{H2} + [H^+]))(1/K_M^a) + (K_{H2}/(K_{H2} + [H^+]))(1/K_M) \qquad \text{(A-4)}$$

where K_{H2} is associated with the ionisable group on the lipid. When $K_M^a \gg K_M$ such that the first term of Eq. (A-4) is negligible,

$$K_M^* = K_M(1 + ([H^+]/K_{LH})) \qquad \text{(A-5)}$$

with $K_{LH} = K_{H2}$. However, if the first term is not negligible Eq. (A-5) can still be used instead of Eq. (A-4) to fit the pH dependence of K_M^* reasonably well for $[H^+] \approx K_{H2}$. But in this case $pK_{LH} < pK_{H2}$. (For example when $K_M^a = 10\ K_M$ we have $pK_{LH} \approx (pK_{H2} - 0.3)$.)

It can be shown that the pH dependence of K_M^* is given by Eq. (A-5) when H^+ and M^+ bind to VAL nearly competitively. In this case K_{LH} will be the apparent dissociation constant of Val-H^+ and there will be a contribution from VAL to the buffer capacity at $[H^+] \approx K_{LH}$. This has not been observed in experiments.

The internal buffer capacity b_i of the vesicles is given by

$$b_i = (\ln 10)\left(\Sigma C_j\left(K_{Hj}[H^+]/\left(K_{Hj} + [H^+]\right)^2\right)\right) \qquad \text{(A-6)}$$

where C_1 and K_{H1} are the concentration and dissociation constant of the buffer entrapped inside vesicles and $C_2 = 30$ mM, $K_{H2} = 10^{-6.9}$ M; $C_3 = 45$ mM, $K_{H3} = 10^{-7.8}$ M from the endogeneous groups in SBPL vesicles [6]. C_m, K_{Hm} for $m > 3$ are associated with membrane permeant species such as CCCP in the inner layer which are in fast proton exchange equilibrium with the aqueous medium inside vesicles.

When the dissociation constants K_1 and K_2 associated with the equilibria in the lipid medium

$$Val\text{-}M^+ - CCCP^- \overset{K_1}{\rightleftharpoons} Val\text{-}M^+ + CCCP^-,$$

$$Val\text{-}CCCP^- \overset{K_2}{\rightleftharpoons} Val + CCCP^- \qquad \text{(A-7)}$$

are known, the equilibrium concentrations of VAL-CCCP complexes in the membrane can be determined using

$$[Val\text{-}M^+ - CCCP^-] + [Val\text{-}CCCP^-] = 0.5([Val_t] + [CCCP_t] + K) - 0.5\left(([Val_t] + [CCCP_t] + K)^2 - 4[Val_t][CCCP_t]\right)^{0.5} \qquad \text{(A-8)}$$

$[Val_t]$ and $[CCCP_t]$ are the total concentrations in the membrane and are obtained from $[Val]_0$ and $[CCCP]_0$ using Eq. (A-2). K is calculated using

$$1/K_{01} = [Val\text{-}M^+ - CCCP^-]/([Val_f][CCCP_f]) = [M^+]K_H/(K_1(K_H + [H^+])([M^+] + K_M^*))$$

$$1/K_{02} = [Val\text{-}CCCP^-]/([Val_f][CCCP_f]) = K_H/(K_2(K_H + [H^+])(1 + [M^+]/K_M^*))$$

$$1/K = 1/K_{01} + 1/K_{02} \qquad \text{(A-9)}$$

Kinetics of ΔpH decay from the mechanism of Fig. 7

The physical process of ion transport across membrane can be considered as a chemical reaction in which the ions on one side of the membrane are converted into ions on the other side [5]. When the protonation equilibria are fast,

the rate equation for the H^+ transport across membrane can be written as

$$-\mathrm{d}N_H/\mathrm{d}t = kN_{il} - kN_{el} \quad \text{(A-10)}$$

where N_H = number of H^+ and protonated species 'BH' in the aqueous medium inside vesicles, N_{il} = number of proton carriers 'CH' in the inner layer, N_{el} = number of 'CH' in the outer layer of vesicles and k = translocation rate constant of 'CH'. In terms of concentrations,

$$-V_i\mathrm{d}([H^+]_i + [BH]_i)/\mathrm{d}t = V_{il}k[CH]_i - V_{el}k[CH]_e \quad \text{(A-11)}$$

The volume of aqueous medium inside vesicles $V_i \ll$ the volume of the vesicle solutions V_e. V_{il} is the volume of the inner layer and V_{el} is the volume of the outer layer of vesicles. In our SBPL vesicles [9,17], $V_i \cong V_{il} \cong V_{el}$ and this enables us to write the rate equation without reference to the volume of the vesicular membrane [5,6].

When the translocation steps in Fig. 7 determine the rate of ΔpH decay and the diffusion rates of charged species are negligible compared to those of neutral species, the rate equation for the steady state can be linearised for small deviations from equilibrium as,

$$\begin{aligned}
&(-\mathrm{d}\Delta[H^+]_i/\mathrm{d}t) \\
&\quad = (\ln 10)([H^+]_i/b_i)k_1(\Delta[CCCPH]_{il} - \Delta[CCCPH]_{el}) \\
&(-\mathrm{d}\Delta[CCCP_f]_{il}/\mathrm{d}t) \\
&\quad = k_1(\Delta[CCCPH]_{il} - \Delta[CCCPH]_{el}) \\
&\qquad + k_2(\Delta[\text{Val-}M^+ - CCCP^-]_{il} \\
&\quad - \Delta[\text{Val-}M^+\text{-}CCCP]_{el})
\end{aligned} \quad \text{(A-12)}$$

The subscripts 'il' and 'el' indicate the carrier concentrations in the inner and outer layers of vesicles. The subscripts 'i' and 'e' refer to the aqueous region inside and outside vesicles.

When the equilibration of [Val-M^+] between the inner and outer layers is not fast compared to ΔpH decay we have to take note of the term involving $\Delta[\text{Val-}M^+]_{il}$. With sufficiently large $[M^+]$ in the aqueous medium we can use $\Delta[\text{Val-}M^+]_{il} = (([M^+]/K_M^*)\Delta[Val]_{il})$ and $\Delta[M^+]_{il} = 0$. Furthermore, in the mechanism of Fig. 7, when H^+ is transported from the aqueous medium inside vesicles to the aqueous medium outside by the translocation of a CCCPH, it is necessary that a neutral VAL is also translocated across the membrane to enable the completion of H^+ transport cycle. Thus, $\Delta[Val]_{il} = \Delta[CCCPH]_{il}$ similar to that in bimolecular reactions. In view of the dependence of k_1 on the choice of lipid [27] we can expect k_1 to change with pH in SBPL vesicles. Taking note of such changes, using expressions similar to that for $1/K_M^*$ (Eq. A-5), we can rewrite Eq. (A-12) as follows when the protonation equilibrium associated with CCCP, metal ion binding equilibrium associated with valinomycin and the complex formation equilibria (A-7) are fast compared to the ΔpH decay rate.

$$\begin{aligned}
&-\mathrm{d}(\Delta[H^+]_i)/\mathrm{d}t = a_{11}\Delta[H^+]_i + a_{12}\cdot\Delta[CCCP_f]_{il}, \\
&-\mathrm{d}(\Delta[CCCP_f]_{il})/\mathrm{d}t = a_{21}\Delta[H^+]_i + a_{22}\Delta[CCCP_f]_{il}
\end{aligned} \quad \text{(A-13)}$$

$$\begin{aligned}
a_{11} &= (\ln 10)k_1([H^+]_i/b_i)[CCCP_f]_{il} \\
&\quad \times\left(K_H/(K_H + [H^+]_i)^2\right)(1 + b_iV_i/(b_eV_e)), \\
a_{12} &= 2(\ln 10)k_1([H^+]_i/b_i)([H^+]_i/(K_H + [H^+]_i)), \\
a_{21} &= \Big((k_1 - k_2([\text{Val-}M^+]_{il}/K_1) \\
&\quad + k_2[M^+]_i[CCCP^-]_{il}/(K_1K_M)) \\
&\quad \times\left([CCCP_f]_{il}K_H/(K_H + [H^+]_i)^2\right) \\
&\quad \times(1 + b_iV_i/(b_eV_e))\Big), \\
a_{22} &= \big(2/((K_H + [H^+]_i))([H^+]_i \\
&\quad \times(k_1 + k_2[M^+]_i[CCCP^-]_{il}/(K_1K_M)) \\
&\quad + k_2[\text{Val-}M^+]_{il}K_H/K_1)\big)
\end{aligned} \quad \text{(A-14)}$$

where V_i is the volume inside vesicles in a solution V_e. b_i and b_e are internal and external buffer capacities. In our experiments the term b_iV_i/b_eV_e can be neglected compared to 1.

The solution of Eq. (A-13) is a sum of two exponentials. The faster component of this solution corresponds to the equilibration of the carrier species between the two layers of the membrane such that their distributions correspond to those expected from $[H^+]$ and $[M^+]$ inside and outside vesicles. The slower component is associated with the ΔpH decay by carrier mediated H^+ transport and the associated M^+ transport. Thus, the relaxation rate contribution to ΔpH decay, $1/\tau_1$ from the mechanism of Fig. 7 can be written as Eq. (4) in the text using

$$1/\tau_1 = a_{11} - a_{12}a_{21}/a_{22} \quad \text{(A-15)}$$

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