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Role of metal ion free valinomycin-carbonyl cyanide m-chlorophenylhydrazone complex in the enhancement of the rates of gramicidin facilitated net H^+, Li^+ and Na^+ transport across phospholipid vesicular membrane

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Abstract

The studies on the decay of the pH difference, ΔpH, across soyabean phospholipid vesicular membrane have shown that the rates of net proton transport and the associated Li^+ and Na^+ ion transport across the membrane can be enhanced by the combined action of gramicidin, valinomycin and carbonyl cyanide m-chlorophenylhydrazone (CCCP) in K^+-free vesicle solutions. The data obtained under different experimental conditions suggest that this enhancement is a consequence of facilitation of $CCCP^-$ transport (1) by complexing $CCCP^-$ with the highly membrane permeant valinomycin without the metal ion bound to it and (2) by the associated Li^+ or Na^+ transport through the gramicidin channel such that no net charge is transported across the membrane. The dissociation constant of the weak valinomycin-$CCCP^-$ complex has been estimated to be > 200 mM in the membrane. The ΔpH in these experiments were created by temperature jump.

Keywords: Gramicidin; Valinomycin; Carbonyl cyanide m-chlorophenylhydrazone; Liposome; Membrane; Ion transport; Temperature jump

1. Introduction

Interfering with biological processes is a key strategy employed in the control of diseases with the help of antibiotics. In many situations, a combination of antibiotics in small doses is more potent than when they are administered separately in larger doses. Understanding the reason for the increased potency is an important objective in basic research. According to Mitchell's hypothesis [1], a proton concentration gradient across the membrane (ΔpH), drives oxidative and photo phosphorylation, which are important processes in biological systems. Therefore, mechanisms which can cause the ΔpH to decay efficiently and inhibit adenosine triphosphate synthesis in different environments are of interest.

Weak acids such as carbonyl cyanide m-chlorophenylhydrazone (CCCP) can facilitate ΔpH decay

Abbreviations: ACES, N-(acetamido)-2-aminoethanesulphonic acid; CCCP, carbonyl cyanide m-chlorophenylhydrazone; Lip, lipid; SBPL, soyabean phospholipid; VAL, valinomycin; Val, neutral valinomycin; Val-M^+, metal ion bound valinomycin; ΔpH, pH difference across vesicular membrane; $1/\tau$, ΔpH relaxation rate.

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by transporting H^+ across the membrane (say from inside a vesicle to outside): after a fast transfer of H^+ from the aqueous medium inside the vesicle to the anion $CCCP^-$ in the inner membrane layer, the electrically neutral CCCPH is rapidly transported across the membrane and H^+ is released to the aqueous medium outside the vesicle. In liposomes, continued H^+ conduction driven by a $[H^+]$ gradient and leading to ΔpH decay requires (a) abolishment of the electric potential generated by the H^+ transport (which opposes further H^+ transport) [2,3] and (b) restoring the concentration of CCCP in the inner layer by the back transport of $CCCP^-$. Thus, ΔpH decay by CCCP is facilitated by a net transport of alkali metal ion M^+ and the anion $CCCP^-$ in a direction opposite to that of net H^+ transport along with fast transfers of M^+ between the aqueous medium and the membrane at the interfaces. The ΔpH decay by the combined action of CCCP and valinomycin (VAL) in vesicle solutions containing KCl [3–5] and NaCl [5] has shown [5] that both the above objectives are achieved by the formation and transport of the electrically neutral ternary complex Val-M^+-$CCCP^-$ ($M^+ = K^+$, Na^+). For similar salt concentrations the ΔpH decay rate observed with NaCl is considerably lower than that with KCl. With LiCl it was slower than even the lower limit of our instrument ($< 0.1\ s^{-1}$) [5]. This is because the yield of the ternary complex with Na^+ or Li^+ is substantially less than that with K^+ and the rate-limiting step is the transport of the ternary complex. Therefore, to enhance the ΔpH decay rate further in K^+-free solutions containing Na^+ or Li^+ ions and at a given concentration of VAL and CCCP, we require another strategy which facilitates both Na^+ or Li^+ and $CCCP^-$ transport. Addition of gramicidin A to liposomes is one such strategy [4].

From activation energy considerations we can say that in liposomes electroneutral transports will be faster than that of the charged species [6]. With sufficient gramicidin channels in the membrane there will be fast conduction of M^+ through the channels. Since no net electrical charge is transferred across the membrane by the combination of a $CCCP^-$ translocation and a M^+ transport through the gramicidin channel in the same direction, gramicidin can facilitate $CCCP^-$ transport. If this hypothesis is true, gramicidin will facilitate CCCP mediated ΔpH decay when $CCCP^-$ translocation limits its rate. Experimental observations given below and in the literature [4] are consistent with such an expectation. We can increase the rate further by reducing the translocation energy of activation by reducing the electrostatic interaction between $CCCP^-$ and the polar regions of the bilayer. Presumably, this can be done by complexing $CCCP^-$ with a hydrophobic molecule such that the charge on the anion is shielded by nonpolar groups (similar to that in valinomycin-M^+ complex). In the work described below we show that VAL itself can be used for such purposes and give kinetic evidence for the existence of a hitherto undetected complex of VAL and CCCP, Val-$CCCP^-$ (without the metal ion in the complex).

2. Materials and methods

Vesicle solutions with 2 mM pyranine inside and other concentration conditions as given in the figure legends were prepared from asolectin (Sigma) following the procedure described elsewhere [7,8]. Microlitre amounts of stock solutions of 5 mM or 15 mM gramicidin, 1 mM or 10 mM valinomycin and 1 mM, 10 mM or 40 mM CCCP (Sigma) in ethanol were added to vesicle solutions with vortex stirring. ΔpH was created by temperature jump (T-jump) and ΔpH decay at $23°C \pm 1.5$ was observed by monitoring the fluorescence from the pH indicator pyranine entrapped inside vesicles, as described elsewhere [7–9]. The ΔpH relaxation times τ were measured from at least four traces obtained from the same sample by comparing the observed trace with those obtained from a calibrated exponential generator [5,7,9].

3. Results and discussion

3.1. ΔpH decay by the combined action of CCCP and gramicidin

Fig. 1a–d shows the experimentally observed CCCP-mediated ΔpH relaxation rates ($1/\tau$) in SBPL vesicle solutions containing gramicidin, under different pH conditions in the pH range 6 to 8. $1/\tau$ is proportional to the concentration of the rate-limiting species. The following two arguments favor the deprotonated form, $CCCP^-$, as the rate-limiting species

(rather than the protonated form CCCPH) in these experiments. (1) Activation energy considerations suggest that the transport of the charged species across the membrane is much slower than that of the electrically neutral species [6]. (2) The increase in the concentration [CCCPH] with decrease in pH can be estimated from a knowledge of the proton dissociation constant pK of CCCP ($\sim$ 6.5) in the membrane. These estimates show that if CCCPH was the rate-limiting species the dependence of $1/\tau$ on $[CCCP]_0$ (indicated by the slopes of the plots in Fig. 1) should have increased with decrease in pH (especially when the increase in F/b_i, discussed below, is also noted). This is not seen in the data of Fig. 1.

The CCCP facilitated ΔpH decay in SBPL vesicles was observed in the time range of our instrument ($\tau < 10$ s) only when sufficient gramicidin had been added. Gramicidin channels are known to transport cations and not anions. Therefore, the fast metal ion (M^+) transport through the channels must be facilitating the translocation of the rate limiting species, $CCCP^-$, in the membrane as expected from the hypothesis mentioned above. The transport scheme

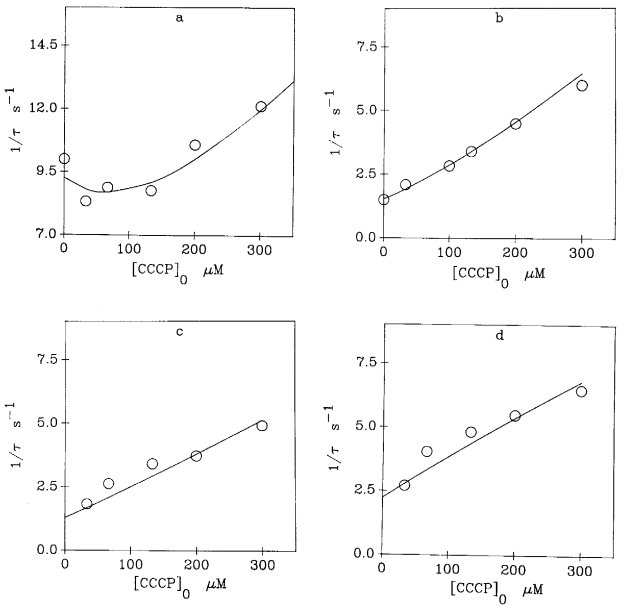

Fig. 1. Dependence of ΔpH relaxation rate, $1/\tau$, on [CCCP] at (a) pH $\sim$ 6, (b) pH $\sim$ 6.55, (c) pH $\sim$ 7 and (d) pH $\sim$ 7.65 in 5 mM SBPL vesicle solutions containing 100 mM KCl, 25 mM ACES out side and 50 mM phosphate inside vesicles. Solid lines are theoretical (see text).

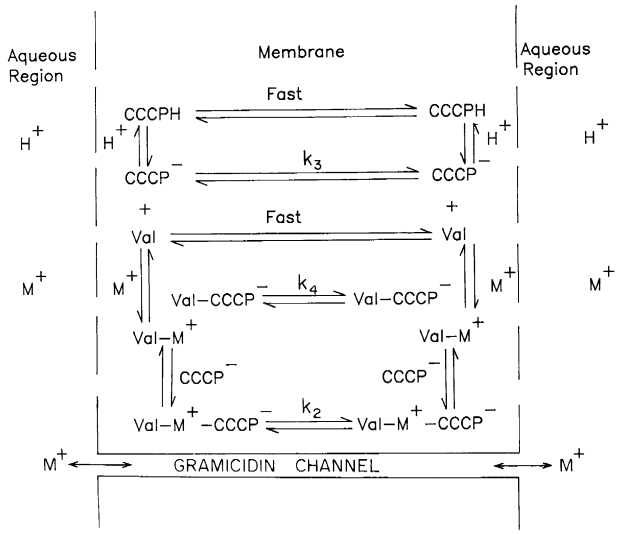

Fig. 2. Suggested transport scheme for the ΔpH decay in which gramicidin and VAL aided $CCCP^-$ translocation is the rate-limiting step.

which takes note of these observations is included in Fig. 2.

Eq. (A3) (with [VAL] = 0) gives an expression for the contribution to $1/\tau$ from this mechanism. This is derived using the theoretical approach of relaxation kinetics. In Eq. (A3), Eq. (A6) and Eq. (A7) we have included the following additional factors which also affect the rate of ΔpH decay: (a) Membrane permeant species at sufficiently high concentrations affect the membrane order, which in turn changes the transport rate constants by a factor F. Using nigericin-mediated ΔpH decay as the probe [5], we determined the extent of these changes on adding CCCP and VAL to the SBPL vesicle solutions (see Eq. (A4)). Due to F, $1/\tau$ increases on lowering the pH and on increasing $[CCCP]_0$. (b) Theoretical expressions and experimental observations [5,7,10] have shown that $1/\tau$ is proportional to $1/b_i$ where b_i is the internal buffer capacity of the vesicles. In CCCP loaded SBPL vesicles b_i decreases on lowering the pH and increases due to contribution from CCCP in the inner layer of the vesicles, on increasing $[CCCP]_0$ [5]. (c) Since gramicidin had been added to vesicle solutions used for obtaining the data of Fig. 1a–d, we should also take note of the contribution to $1/\tau$ from H^+ transport through the gramicidin channels. An estimate of this contribution can be obtained using Eq.

(A6). At the gramicidin concentrations used, this contribution is small and becomes significant only at low pH conditions. The discussions given above help us understand qualitatively the following 'finer details' of the data in Fig. 1.

(1) Calculations using the estimates of parameters given in Ref. [5] show that the factor F/b_i occurring in Eq. (A3) changes less than 20% with increase in $[CCCP]_0$ in our concentration range. Because of this reason the plots of $1/\tau$ against $[CCCP]_0$ remain nearly linear as seen in Fig. 1. (2) Calculations also show that F/b_i increases on lowering the pH. Because of this reason the decrease of $1/\tau$ on lowering the pH is not as much as can be expected from the decrease of $[CCCP^-]_{il}$ (Fig. 1). (3) At the pH corresponding to Fig. 1a (pH ~ 6) the concentration of H^+ bound gramicidin channel and hence its contribution to $1/\tau$ becomes prominent (Eq. (A6)). This contribution decreases on increasing $[CCCP]_0$ because of the increase of internal buffer capacity b_i due to contribution from the CCCP in the inner layer of the vesicular membrane. Because of this reason $1/\tau$ shows a small decrease with increase in $[CCCP]_0$ in the small $[CCCP]_0$ region of Fig. 1a.

Thus, Fig. 1 is consistent with the conclusions based on the hypothesis that the translocation of $CCCP^-$ across the vesicular membrane is facilitated by the concerted transport of M^+ through the gramicidin channels. $1/\tau$ calculated using Eq. (A7), on the basis of the mechanism given in Fig. 2 (without VAL and associated species), taking note of all the above-mentioned factors and using $k_3 \sim 2\ s^{-1}$ and $[Val]_{il} = 0$ in Eq. (A3) (solid lines of Fig. 1), show good agreement with the observed data. Similar behavior was observed in vesicle solutions containing NaCl also.

3.2. ΔpH decay by the combined action of gramicidin, CCCP and VAL in SBPL vesicle solutions containing LiCl or NaCl

As mentioned in Section 1, $CCCP^-$ translocation can be made faster by complexing it with a suitable molecule which reduces the free energy of activation for the translocation process. The efficacy of VAL as such a complexing agent (Fig. 2) can be conveniently tested if we minimise the contributions to the ΔpH decay from a mechanism involving the metal ion

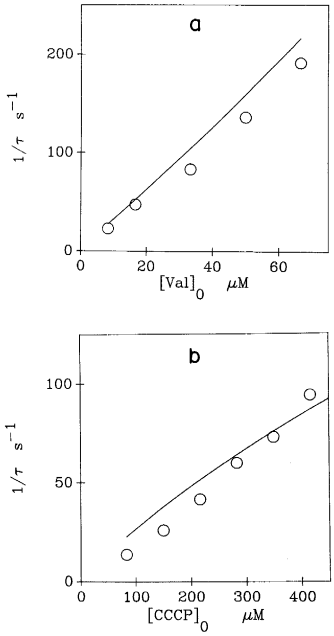

Fig. 3. Dependence of $1/\tau$ on (a) $[Val]_0$ keeping $[CCCP]_0 = 84$ μM with [lip] = 5.3 mM and on (b) $[CCCP]_0$ keeping $[Val]_0 = 12.5$ μM with [lip] = 6.5 mM in SBPL vesicle solutions containing 100 mM LiCl, $[gramicidin]_0 = 25$ μM, 10 mM ACES outside vesicles, and 0.25 mM ACES inside vesicles at pH ~ 6.9. Solid lines are theoretical (see text).

bound ternary complex Val-M^+-$CCCP^-$ [5]. Because of this reason we use K^+-free vesicle solutions. The compensating charge flux required for the ΔpH decay can be provided by using Li^+ or Na^+ as the alkali metal ions which form the above-mentioned ternary complexes at negligible concentrations and are transported across the membrane through gramicidin channels.

In vesicle solutions containing CCCP and Li^+, $1/\tau$ remains below the lower limit of our instrument ($< 0.1\ s^{-1}$) even after adding VAL if gramicidin is not added. (This can also be seen in Fig. 6 of Ref. [5] by extrapolating the $1/\tau$ data.) However, when gramicidin is also present in such vesicle solutions containing CCCP, there is a significant enhancement in the ΔpH relaxation rate: $1/\tau$ increases linearly with $[VAL]_0$ (Fig. 3a). From this observation we conclude that the rate of ΔpH decay and the rate of the rate-limiting step of $CCCP^-$ translocation are enhanced by VAL in the membrane. Similar conclusions can also be drawn from the higher slope of the

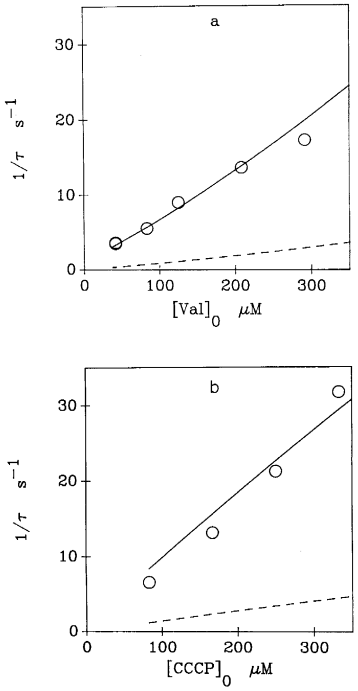

Fig. 4. Dependence of $1/\tau$ on (a) $[Val]_0$ keeping $[CCCP]_0 = 4$ μM and on (b) $[CCCP]_0$ keeping $[Val]_0 = 6$ μM in 5.4 mM SBPL vesicle solutions containing $[gramicidin]_0 = 25$ μM, 100 mM NaCl, 25 mM ACES outside, and 50 mM phosphate inside vesicles at pH ~ 7. Solid lines are theoretical (see text). Broken lines correspond to data in the absence of gramicidin.

$1/\tau$ against $[CCCP]_0$ plot obtained in vesicle solutions containing VAL (Fig. 3b) when compared to that obtained without VAL (Fig. 1c). We attribute this enhancement to the formation of a 1:1 complex, Val-$CCCP^-$, which enhances the translocation rate of $CCCP^-$.

It follows from the above assignment that in vesicle solutions with Na^+ as the alkali metal ion, in addition to Val-Na^+-$CCCP^-$, we should have Val-$CCCP^-$ also contributing to $1/\tau$ when gramicidin is added. The latter contribution will be larger than the former, since the yield of the ternary complex Val-Na^+-$CCCP^-$ is less than that of the binary complex Val-$CCCP^-$, even though the dissociation constant $K_1 < K_2$ (see Eq. (A1)). This is because $[Val\text{-}Na^+] \ll [Val]$. Contributions to $1/\tau$ from both the mechanisms vary linearly with $[VAL]_0$ when $[CCCP]_0$ is kept constant and with $[CCCP]_0$ when $[VAL]_0$ is kept constant. Experimental data in Fig. 4 are consistent with these expectations. (Broken lines in Fig. 4 correspond to the first contribution which is present even in the absence of gramicidin and hence calculated using the parameters determined in Ref. [5] and including the contribution from VAL alone seen in Fig. 3b of Ref. [5].) The 'finer details' which ensure linearity of the plots in Figs. 3 and 4 have already been discussed in Section 3.1 above.

3.3. Tests which favor participation of the complex Val-$CCCP^-$ in the ΔpH decay by the combined action of gramicidin, CCCP and VAL in SBPL vesicle solutions containing Li^+ or Na^+

In our hypothesis, VAL enhances the ΔpH decay rate by forming the complex Val-$CCCP^-$ which enables rapid translocation of $CCCP^-$ across the membrane in the above-mentioned systems. The predictions of this hypothesis have been verified as follows.

(a) If the complex Val-$CCCP^-$ is the dominant rate-limiting species, then $1/\tau$ should be proportional to $[Val\text{-}CCCP^-]_{il}$. If the concentrations $[Val]_{il}$ and $[CCCP^-]_{il}$ are smaller than the dissociation constant of the complex ($= K_2$, see Eq. (A1)) then $[Val\text{-}CCCP^-]_{il}$ (and hence $1/\tau$) will be nearly proportional to the products $[Val]_{il} \times [CCCP^-]_{il}$ and $[VAL]_0 \times [CCCP]_0$ in view of Eq. (A1) and Eq. (A2). The linear nature of the plots in Figs. 3 and 4, and the near constancy of the ratio $(1/\tau)/\{[Val]_0 \times [CCCP]_0\}$ in these plots (evaluated using the concentrations given in the figure legends and after subtracting the contribution from the translocation of Val-Na^+-$CCCP^-$ in Fig. 4) are consistent with this prediction. The small deviations from the constancy are due to contributions to $1/\tau$ from other mechanisms and contributions to b_i from CCCP in the inner layer of the vesicular membrane, discussed above.

(b) When the alkali metal ion in the solution is Na^+ translocation of Val-Na^+-$CCCP^-$ facilitates ΔpH decay in the absence of gramicidin and in this case τ decreases on increasing pH (see Figs. 5c and 5d of Ref. [5]). A main contributor for such a behavior is the pH dependence of metal ion binding to VAL in SBPL vesicles [5]. (The apparent metal ion dissociation constant of Val-M^+ can be expressed as $K_m^* = K_m\,(1 + [H^+]/10^{-6.6}$ (Eq. A-5 of Ref. [5]) in our pH range). When gramicidin is added, the translocation of Val-$CCCP^-$ dominantly decides the

ΔpH decay rate. Since the formation of these rate-limiting species does not involve metal ion binding, the pH dependence of K_m^* does not occur dominantly in the expression for τ. Therefore, our above-mentioned hypothesis predicts that on adding gramicidin, τ should decrease to a lesser extent on increasing pH than that observed in the absence of gramicidin. The pH dependent data of Fig. 5 are consistent with this prediction.

(c) In the experiments with NaCl in vesicle solutions Val-Na^+-$CCCP^-$ is the rate-limiting species in the absence of gramicidin. On increasing [NaCl], [Val-Na^+-$CCCP^-$]$_{il}$ increases due to increased [Na^+] in spite of increased ionic strength. This is reflected in the τ data of Fig. 6. According to our hypothesis, on adding gramicidin to these vesicle solutions, Val-$CCCP^-$ becomes the dominant rate-limiting species. Since Na^+ is not involved in the formation of this complex, [Val-$CCCP^-$]$_{il}$ is not expected to increase with [NaCl]. On the other hand, increased ionic strength should favor the dissociation of this complex, thereby reducing [Val-$CCCP^-$]. Thus, our hypothesis predicts a decrease in $1/\tau$ with increase in [NaCl] in the experiments with gramicidin in vesicle solutions. The data in Fig. 6 are consistent with this prediction.

(d) The transport scheme in Fig. 2 is based on our hypothesis. Eq. (A7) has been derived on the basis of this scheme and includes all the known factors which can affect $1/\tau$. Therefore, if our hypothesis is valid, it should be possible to reproduce the data given in Figs. 3–5 using a reasonable set of parameters in Eq. (A7). With $k_4 \sim k_2 \sim 3 \times 10^3$ s^{-1}, the experimental data can be reproduced within the limits of experimental errors if the dissociation constant for Val-$CCCP^-$, K_2 is chosen to be $\sim$200 mM. (Other parameters needed for such a calculation can be found above and in Ref. [5].) By noting that the translocation of $CCCP^-$ as Val-$CCCP^-$ across the main barrier may not require traversing the entire thickness of the membrane layer (along with a concerted transport of M^+ through the channel) and from considerations of size, we can expect $k_4 > k_2$, which gives $K_2 > 200$ mM. This estimate is consistent with our requirement given in (a) above, that [$CCCP^-$]$_{il}$ and [Val]$_{il}$ should be less than K_2. The concentrations required for such a comparison can be obtained from [CCCP]$_0$ and [VAL]$_0$ using Eq. (A2). The solid lines of Figs. 3–5 which agree with the data have been obtained using the above parameters with an error limit of $\sim$30%.

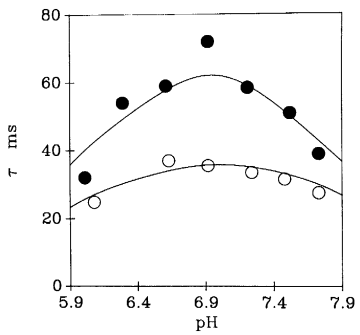

Fig. 5. Dependence of $1/\tau$ on pH in 5.4 mM SBPL vesicle solutions containing 100 mM NaCl, [gramicidin]$_0 = 42$ μM, [Val]$_0 = 5.2$ μM and [CCCP]$_0 = 208$ μM. Outside vesicles 25 mM ACES; inside vesicles 2 mM phosphate buffer, ○; or 50 mM phosphate buffer ●. Solid lines are theoretical (see text).

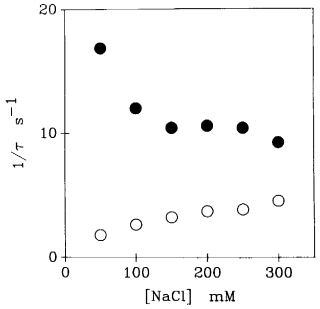

Fig. 6. Dependence of $1/\tau$ on [NaCl] in 6.3 mM SBPL vesicle solutions with 50 mM phosphate buffer inside and 25 mM ACES outside at pH $\sim$ 7, [Val]$_0 = 6$ μM, [CCCP]$_0 = 190$ μM and with (a) [gramicidin]$_0 = 0$, ○ and (b) [gramicidin]$_0 = 40$ μM, ●.

(e) According to our hypothesis, the observed enhancement is specific to the combination of VAL and $CCCP^-$ with gramicidin channels aiding the metal ion transport. The discussion given below leads to such a conclusion. Comparing Fig. 1c and Fig. 3b, we can say that [VAL]$_0 = 12.5$ μM in 6.5 mM lipid (corresponding to [Val]$_{il} \sim 2$ mM) enhances the $CCCP^-$ translocation rate by a factor of $\sim$20 at pH $\sim$ 7. An alternate explanation for this observation, such as being due to VAL-induced unspecific effect (presumably due to a change in the membrane property) is unreasonable. This can be seen from the following arguments. (1) The linear increase of

nigericin or monensin-mediated ΔpH decay rate with $[VAL]_0$ seen in Fig. 1a and 1b of Ref. [5] (attributed to changes in the membrane property) shows that changes in membrane order due to membrane permeant species are additive in nature (unlike that in cooperative phenomena). Therefore, F given by Eq. (A4) is appropriate to determine the factor by which the transport rate constants are enhanced due to changes in the membrane order by VAL and CCCP. The observed enhancement of $CCCP^-$ transport rate for $[VAL]_0 \sim 12.5$ μM in Fig. 3b (~ 20) is too large compared to that predicted by F (< 1.02) associated with the changes in membrane properties. (2) To account for the large enhancement, if we assume that the presence of gramicidin in vesicular membrane increases B_v (Eq. (A4)) by a large factor, then Fig. 1 and Fig. 3b lead to contradictory conclusions about the enhancement of B_c in Eq. (A4): To get a linear plot consistent with the data in Fig. 1c, F/b_i should not significantly vary with $[CCCP]_0$ (see Section 3.1). This is possible with $B_c \sim 60$ to 90 (i.e., without enhancement). However, to get a linear plot of enhanced $1/\tau$ (Fig. 3b) with B_v increased by a large factor (as assumed above), B_c also has to be increased by a large factor so that F/b_i remains nearly constant with respect to changes in $[CCCP]_0$. (3) The data obtained with Na^+ ions (Fig. 4) has contribution $1/\tau_{Na}$ to $1/\tau$, where $1/\tau_{Na}$ is associated with the translocation of the ternary complex Val-Na^+-$CCCP^-$. The change in membrane order or an unspecific effect which enhances the $CCCP^-$ transport rate by a factor of ~ 10 on adding 6 μM of VAL (Fig. 4b) should enhance the Val-Na^+-$CCCP^-$ transport rate also by a similar factor. However, the observed data in Fig. 4b can be accounted for by assuming that only the $CCCP^-$ transport rate is enhanced by this factor. Similar conclusions can be drawn from an analysis of Fig. 4a also. From these observations we conclude that the enhancement is specific to the combination of VAL and $CCCP^-$.

As mentioned earlier, in liposomes a net transport of H^+ in one direction is accompanied by a net M^+ transport in the opposite direction. Therefore, along with the enhancement of net H^+ transport across the membrane, we have enhancement of net Li^+ and Na^+ transport through the gramicidin channels when VAL and CCCP are also present in the vesicular membrane.

Appendix A

The equilibria and apparent dissociation constants relevant for the discussion of data obtained in this work are given below. These equilibria have been considered in Ref. [5] also.

$$CCCPH \rightleftharpoons CCCP^- + H^+;\ K_H.$$

$$Val - M^+ \rightleftharpoons Val + M^+;\ K_m^*.$$

$$Val - M^+ - CCCP^- \rightleftharpoons Val - M^+ + CCCP^-;\ K_1.$$

$$Val - CCCP^- \rightleftharpoons Val + CCCP^-;\ K_2. \qquad (A1)$$

In this paper the concentrations with subscript 'i' refer to those in the aqueous medium inside vesicles and the concentrations with subscript 'il' refer to those in the inner layer of the bilayer membrane. The concentrations estimated with respect to the volume of the vesicle solutions are indicated by the subscript '0'. If $[CR]_0$ is the concentration estimated with respect to the volume of the vesicle solution and if $[CR]_{il}$ is its average concentration in the inner layer of the SBPL vesicular membrane volume, we can show the following using the dimensions of SBPL vesicles [10], if the species 'CR' is mainly partitioned to the membrane [5,7].

$$[CR]_{il} = 0.95[CR]_0/[lip]M \qquad (A2)$$

The internal buffer capacity b_i of the SBPL vesicles can be calculated including the contribution from the CCCP in the inner layer of the bilayer membrane with the help of Eq. (A6) given in Ref. [5]. Using Eqs. (A2), (A8), (A9) and parameters given in Ref. [5], the concentrations of the complexes $[Val\text{-}Na^+\text{-}CCCP^-]_{il}$ and $[Val\text{-}CCCP^-]_{il}$ can be calculated for a given estimate of K_2. $[CCCP^-]_{il}$ can be calculated by subtracting the concentrations of the complexes from the concentration of CCCP in the membrane in the absence of complexing and using its pK_H (~ 6.5) in the membrane [5].

If in the transport scheme for the ΔpH decay (Fig. 2) the rate-limiting step is the back transport of $CCCP^-$ (as $CCCP^-$ itself with a rate constant k_3 and as Val-$CCCP^-$ with a rate constant k_4), the following expression for the ΔpH relaxation rate $1/\tau$ can be obtained by writing linearised rate equations for small deviations from equilibrium and following a procedure similar to that in our previous work [5].

When the concentration $[\text{Val-CCCP}^-]_{il}$ is small, we can write,

$$1/\tau_1 = (\ln 10/b_i)F\{k_3 + k_4[\text{Val}]_{il}/K_2\}[\text{CCCP}^-]_{il} \quad \text{(A3)}$$

where F is the correction factor which takes note of changes in the transport rate constants due to changes in the membrane properties on adding gramicidin, VAL and CCCP. Ahmed and Krishnamoorthy [4] have shown that, at the concentrations used in our experiments, gramicidin-induced changes are not important. We have also come to a similar conclusion using nigericin-mediated ΔpH decay as the probe. However, studies on nigericin/monensin-mediated ΔpH decay have shown that the ΔpH relaxation rate, $1/\tau$, increases by a pH-dependent factor F (with $B_v \sim 20$ and $B_c \sim 60$–90) at the higher $[\text{VAL}]_0$ and $[\text{CCCP}]_0$ used in our experiments [5].

$$F = 1 + B_v[\text{H}^+]([\text{Val}]_0/[\text{lip}])/(10^{-7} + [\text{H}^+]) + B_c[\text{H}^+]^2([\text{CCCP}]_0/[\text{lip}]) /\{(10^{-7} + [\text{H}^+])(10^{-6.5} + [\text{H}^+])\} \quad \text{(A4)}$$

The contribution $1/\tau_1$ will be present in vesicle solutions containing LiCl or NaCl only if gramicidin is also added to provide compensating charge flux by the transport of alkali metal ions through the gramicidin channels. In vesicle solutions containing NaCl, we have to include a contribution $1/\tau_2$ from the transport of CCCP^- as $\text{Val-Na}^+\text{-CCCP}^-$ with rate constant k_2 [5].

$$1/\tau_2 = (\ln 10/b_i)F k_2[\text{Val} - \text{Na}^+ - \text{CCCP}^-]_{il} \quad \text{(A5)}$$

The contribution from H^+/M^+ transport through gramicidin channels $1/\tau_3$ can also be included in our calculations by using the parameters which fit the observed data in the absence of CCCP and VAL. (This is a small part of the $1/\tau$ observed in our experiments and we have included this term for the sake of completeness, without discussing the origin of this expression.)

$$1/\tau_3 = (\ln 10/b_i)\{(k_G/[\text{MCl}])([\text{Gramicidin}]_0[\text{H}^+] /(2[\text{lip}]) + 0.045\,F10^{-7}/(10^{-7} + [\text{H}^+])\} \quad \text{(A6)}$$

with k_G in the range 1.5×10^6 s^{-1} to 3×10^6 s^{-1}. Similarly we have to include a small contribution $1/\tau_4$ from VAL alone, which can be obtained from Fig. 3b of Ref. [5]. For comparing with the experimental data we can use the sum of these four contributions, since these mechanisms are independent.

$$1/\tau = 1/\tau_1 + 1/\tau_2 + 1/\tau_3 + 1/\tau_4 \quad \text{(A7)}$$

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