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Effect of phloretin on ionophore mediated electroneutral transmembrane translocations of H^+, K^+ and Na^+ in phospholipid vesicles

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Abstract

Rates of M^+/H^+ exchange ($M^+ = K^+$, Na^+) across phospholipid membranes by ionophore mediated electroneutral translocations and transports through channels could either increase or decrease or change negligibly on adding the polar molecule phloretin to the membrane. The changes depend on pH, the concentration and choice of M^+ and choice of ionophore/channel. Such diverse behaviours have been inferred from studies on the decay of the pH difference across soybean phospholipid vesicular membrane ($=\Delta$pH). The transporters used in this study are (a) the exchange ionophores: nigericin, monensin; (b) combinations of alkali metal ion carriers, valinomycin or nonactin with weak acids carbonyl cyanide *m*-chlorophenylhydrazone or 2,4-dinitrophenol and (c) channels formed by gramicidin A. All the diverse results can be rationally explained if we take note of the following. (i) The rate limiting steps are associated with the transmembrane translocations involving the rate limiting species identified in the literature. (ii) Phloretin in the membrane decreases the apparent M^+ dissociation constant, K_M, of the M^+ bound ionophores/channels which has the effect of increasing the concentration of these species. (iii) The concentrations of H^+ bound ionophores/channels decrease on adding phloretin. (iv) Phloretin inhibits ternary complex formation (involving valinomycin or nonactin, M^+ and an anion) by forming 1:2 complexes with valinomycin–M^+ or nonactin–M^+. (v) On adding 6-ketocholestanol to the membrane (instead of phloretin) K_M increases. The decreases/increases in K_M mentioned above are consistent with the consequences of a hypothesis in which phloretin decreases and 6-ketocholestanol increases the positive internal membrane dipole potential.

Keywords: Membrane transport; Vesicle; Lipid bilayer; Phloretin; Internal membrane dipole potential; Antibiotic

1. Introduction

Biologically relevant membrane transports are affected by the adsorption/incorporation of polar molecules in the membrane. For example, phloretin, which has an electric dipole moment $\sim$5 D [1], inhibits glucose transport [2], enhances the rate of lipophilic cation transport and reduces the rate of lipophilic anion transport [3–5]. The changes in ionic conductance have been attributed to phloretin in-

Abbreviations: ΔpH, pH difference across the bilayer membrane; SBPL, soybean phospholipid; τ, ΔpH relaxation time; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; VAL, valinomycin; NON, nonactin; GRAM, gramicidin A; NIG, nigericin; MON, monensin; PHL, phloretin; ACES, *N*-(acetamido)-2-aminoethanesulphonic acid; M^+, metal ion; Nig–M, nigericin–metal ion complex; Mon–M, monensin–metal ion complex; Nig–H, protonated nigericin; Mon–H, protonated monensin; T-jump, temperature jump

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duced reduction of the positive internal membrane dipole potential and the consequent changes in the partitioning of ions to the membrane and changes in the free energy profiles [3,5]. It has been suggested that the changes in the transport of neutral molecules on adding phloretin could be due to electrical effects on their translocators in the membrane [3]. Limited data are available in the literature on the effect of phloretin on antibiotic mediated H^+/OH^- conductance and alkali metal ion M^+ ($=K^+$, Na^+) transport by electroneutral translocation mechanisms. In the past, the explanation of such data in terms of reduction in the internal membrane potential had been based on the controversial assumption that the rate limiting step of M^+/H^+ exchange is the ion–antibiotic binding equilibrium at the aqueous medium–membrane interface [6]. The present study was undertaken (a) to see whether the ion transport by different antibiotics could be affected to different extents (a characteristic seen in the resistance to antibiotic action due to mutation of disease causing organisms) by the presence of polar molecules in the membrane, (b) to see whether the magnitudes of such changes depend on the choice of conditions such as pH and M^+ ion concentrations and (c) to examine whether such data could be explained from changes in the internal membrane dipole potential. From the changes in the rate of decay of the pH difference across the membrane ($=\Delta$pH) we have inferred the magnitude of changes in the ion transport rates on adding phloretin to vesicular membranes (see Section 2). Since phloretin is known to permeate across lipid bilayers with permeability $\sim 2\times10^{-4}$–2×10^{-3} cm/s [7–9], we expect the phloretin concentration to have reached an equilibrium distribution in both layers of the vesicular membrane before the start of our experiments (>5 min after adding phloretin).

The ΔpH decay technique used in this work to infer ion transports is described briefly in Section 2 along with a discussion of its validity and the relation between ΔpH relaxation rate ($=1/\tau$) and the concentration of rate limiting species. This technique has been used in previous works also [10,11]. In the present study we have chosen (i) the exchange ionophores nigericin (NIG) and monensin (MON); (ii) the M^+ carriers valinomycin (VAL) and nonactin (NON) in combination with the H^+ uncouplers carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or 2,4-dinitrophenol (DNP) and (iii) the channel forming gramicidin (GRAM). The mechanisms of ΔpH decay by these antibiotics in soybean phospholipid (SBPL) vesicle solutions containing MCl ($M^+=K^+$, Na^+, Li^+) have been established earlier [11–16].

2. Materials and methods

SBPL vesicle solutions with 2 mM pyranine and phosphate buffer entrapped inside and *N*-(acetamido)-2-aminoethanesulphonic acid (ACES) buffer outside vesicles were prepared from asolectin (Sigma) by sonication in a bath-type sonicator and passing through a G-50 Sephadex column as described earlier [10,11]. The buffer concentration, salt concentration and pH used for the experiments are given in the text or figure legends. The small unilamellar vesicles prepared by the above procedure had an average outer diameter of $\sim$300 Å [10]. Stock solutions with 1 mM NIG, 1 or 3 mM MON, 1 or 10 mM VAL, 1, 10 or 40 mM CCCP, 0.5 or 2 mM NON, 5 or 15 mM GRAM, 250 mM phloretin (Sigma) and 250 mM 6-ketocholestanol (Sigma) in ethanol were added to vesicle solutions in microlitre amounts with vortex stirring.

The SBPL vesicle solutions in a suitable cell were subjected to a temperature jump (T-jump) of $\sim$1.5°C by Joule heating (with heating time constant $\sim$5 μs) using the T-jump instrument described elsewhere [17]. The subsequent changes in the pyranine fluorescence were recorded as a function of time at the resulting temperature ($\sim 23\pm1$°C) using a kinetic spectrophotometer and a home built transient recorder [18]. The relaxation times τ were determined by comparing the observed traces with those from a calibrated exponential generator.

2.1. Creation of ΔpH and requirement for its decay

When a buffered solution is subjected to T-jump the H^+ dissociation constant K_j of the buffer groups increases and because of the fast protonation equilibria there will be an increase in $[H^+]$ in the solution (equation A9 in [11]). In our vesicle solutions, the temperature dependence of K_j of buffer species inside vesicles is different from those outside vesicles. Therefore, the magnitude of the pH jump (associated

with the T-jump) inside vesicles will be different from that outside vesicles. With ACES buffer outside and phosphate buffer inside vesicles such pH jumps cause $[H^+]$ outside (C_o) to have a value higher than that inside vesicles (C_i) [11].

The $[H^+]$ gradient ($\Delta pH \sim 0.025$) drives the transport of H^+/OH^- causing the ΔpH to decay. However, H^+/OH^- transport generates a membrane potential V. The ΔpH decay stops once the free energy change $\Delta G = -RT \ln (C_o/C_i) + FV$ associated with the transport ceases to be negative. Therefore, in liposomes for continued H^+/OH^- conduction leading to ΔpH decay it is necessary to have a mechanism which collapses V, for example by metal ion transport in a direction opposite to that of H^+ [19–21]. Since the $[M^+]$ used in our experiments are relatively large, the $[M^+]$ gradient created by such a compensating charge flux makes a negligibly small contribution to ΔG (see Fig. 2 in [20] for a demonstration of such coupled H^+ and K^+ transports). Thus, both H^+ and M^+ transports are essential for ΔpH decay. The slowest of these transports determines the rate of ΔpH decay. Tables 1 and 2 list the species identified to be the carriers of H^+ or M^+ in the rate limiting steps [11–16].

2.2. Relation between $1/\tau$ and rate limiting species

In our systems the transfer of H^+ and M^+ between the aqueous medium and the membrane bound ionophores/weak acids at the interfaces leading to the formation/H^+ dissociation of the species Nig–H, Mon–H, CCCPH and DNPH as well as formation/M^+ dissociation of the species Nig–M, Mon–M and VAL–M^+ are fast compared to $1/\tau$. Furthermore, the formation and dissociation of ternary complexes VAL–M^+–$CCCP^-$ and VAL–M^+–DNP^- in the membrane are also fast compared to $1/\tau$. The rate limiting steps are associated with the translocation of H^+/M^+ across the membrane in the form of electroneutral complexes mentioned above [11–16]. The ΔpH decay requires H^+/M^+ exchange by a combination of these steps. The expressions for the time constant ($=\tau$) associated with the exponential ΔpH decay for such transport schemes show that if the ΔpH decay is limited by the translocations of two species in the membrane with translocation rate constants k_{r1} and k_{r2},

$$1/\tau = (\ln 10/b_i)\, k_{r1}\, k_{r2}\, [\text{species 1}]_m\, [\text{species 2}]_m / (k_{r1}\, [\text{species 1}]_m + k_{r2}\, [\text{species 2}]_m) \quad (1)$$

When $k_{r1}\,[\text{species 1}]_m \ll k_{r2}\,[\text{species 2}]_m$, 'species 1' become the rate limiting species and the expression for $1/\tau$ can be written as follows.

$$1/\tau = (\ln 10/b_i)\, k_{r1}\, [\text{species 1}]_m \quad (2)$$

The subscripts 'm' in the above expressions refer to concentrations in the membrane. In this paper we have given the concentrations with respect to total

Table 1
Phloretin induced changes in τ explainable by an increase in M^+ binding and a decrease in H^+ binding to ionophores

Transporter[a]	M^+	Rate limiting species[b]	pH	τ in ms with $[PHL]_0$ in mM =		
				0	0.4	0.8
NIG	Na^+	Nig–Na	6.23	232	196	168
1.7 μM		Nig–Na+Nig–H	7.47	84	84	87
NIG	K^+	Nig–H	6.22	30	45	59
1.0 μM		Nig–H	7.48	110	186	345
MON	Na^+	Mon-H	6.23	12	21	34
3.3 μM		Mon-H	7.47	144	213	315
MON	K^+	Mon–K	6.22	60	59	53
2.0 μM		Mon–K+Mon–H	7.48	112	112	144
GRAM	Li^+	H^+ bound	6.22	88	120	110
75 μM		channels				

[MCl] = 100 mM. [lip] = 5.2 mM for experiments with Na^+ and Li^+ ions, and 4.4 mM with K^+ ions. Inside buffer = 50 mM phosphate with K^+ and Na^+ ions and 0.25 mM ACES with Li^+ ions. Outside buffer = 50 mM ACES.

[a]The concentrations of the vesicle solutions calculated with respect to the total volume are given below the transporters.

[b]The rate limiting species have been identified and are given in the literature [11,12,15].

Table 2
Phloretin induced changes in τ explainable by an increase in M^+ binding to ionophores plus ionophore–M^+–phloretin$_n$ complex formation

Transporter[a]	M^+	Rate limiting species[b]	pH	τ in ms with $[PHL]_0$ in mM =			
				0	0.21	0.41	0.82
VAL+CCCP	Na^+	VAL–Na^+–$CCCP^-$	6.23	280	135	58	–
20 μM, 53 μM		VAL–Na^+–$CCCP^-$	7.47	161	95	51	–
VAL+CCCP	K^+	VAL–K^+–$CCCP^-$	6.22	100	320	855	–
2 μM, 2 μM		VAL–K^+–$CCCP^-$	7.48	68	240	750	–
VAL+CCCP	K^+	VAL–K^+–$CCCP^-$	7.0[c]	25	28	26	–
125 μM, 1.7 μM							
VAL+DNP	Na^+	VAL–Na^+–DNP^-	7.0	75	62	51	–
25 μM, 833 μM							
VAL+DNP	K^+	VAL–K^+–DNP^-	7.0[c]	120	495	1700	–
2.5 μM, 250 μM							
NON+CCCP	Na^+	NON–Na^+–$CCCP^-$	6.23	72	–	66	60
13 μM, 267 μM		NON–Na^+–$CCCP^-$	7.47	48	–	43	36
NON+CCCP	K^+	NON–K^+–$CCCP^-$	6.22	64	–	150	510
13 μM, 17 μM		NON–K^+–$CCCP^-$	7.48	30	–	66	245

Other conditions as in Table 1.
[a]The concentrations of the vesicle solutions calculated with respect to the total volume are given below the transporters.
[b]The rate limiting species have been identified and are given in the literature [13,14,25].
[c]In these experiments the vesicles had 2 mM phosphate inside instead of 50 mM phosphate.

volume of the solutions (with subscripts '0'). In SBPL vesicle solutions, when the carrier species 'CR' is mainly partitioned to the membrane (as with NIG, MON, VAL and CCCP) we can use $[CR]_m = 0.95\ [CR]_0/[lip]$ M to get the concentrations in the membrane [11]. However, only a small fraction of DNP is partitioned to the membrane and in this case we have to use the partition coefficient and the expressions given in [14]. In Eqs. 1 and 2 b_i is the internal buffer capacity of vesicles.

$$b_i = (\ln 10) \sum C_j K_j [H^+]/(K_j + [H^+])^2 \qquad (3)$$

where C_j and K_j are the concentrations and H^+ dissociation constants of species 'j' which are in fast protonation equilibrium with the aqueous medium inside vesicles. Thus, $C_1 = 30$ mM, $K_1 = 10^{-6.9}$ M and $C_2 = 45$ mM, $K_2 = 10^{-7.8}$ M are associated with the endogenous groups; $C_3 = 2$ mM, $K_3 = 10^{-7.25}$ M are associated with pyranine trapped inside, C_4 and $K_4 = 10^{-6.9}$ M are associated with the phosphate buffer trapped inside; C_j and K_j (for $j > 4$) are associated with membrane permeant species in fast protonation equilibrium with the aqueous medium inside.

2.3. Fluorescence changes are from ΔpH decay

Pyranine is a good fluorescent probe of pH inside vesicles [22]. The fluorescence detected at >500 nm after excitation at 466 nm is mainly from the unprotonated form of pyranine [22]. In our experiments, on giving a T-jump the concentration of the unprotonated form of pyranine inside vesicles changes due to (a) temperature dependence of the pyranine pK, (b) the pH jump coming from the changes in the pK of the buffering species and (c) pH change associated with H^+ transport across the membrane. (a) and (b) are fast processes and manifest themselves as fast fluorescence changes. (c) is responsible for the slower fluorescence change (with time constant τ) and is associated with ΔpH decay [11]. That this interpretation is correct can be seen from the following. (i) In our experiments the net H^+ transport is from outside to inside vesicles and this should be seen as a decrease in the fluorescence if pyranine is inside and as an increase in the fluorescence if pyranine is outside vesicles. Such a reversal in the direction of slower fluorescence change has been observed (Fig. 3 in [11]). (ii) If the K_j of the buffer species inside has a larger temperature dependence compared to that

outside vesicles, on T-jump the net H^+ transport will be from inside to outside which should be manifested as an increase in the fluorescence from pyranine. This has been observed [10]. (iii) The amplitudes of both fast and slow fluorescence changes under different experimental conditions show agreement with theoretical expressions based on the above interpretation of fluorescence changes [11]. (iv) The increase of $1/\tau$ on adding appropriate ion carriers [11–16] is consistent with the interpretation of the slow fluorescence change given above. The rate constants which determine $1/\tau$ are for the temperature after the T-jump.

The observed fluorescence changes cannot be assigned to changes in the binding of pyranine to vesicles as a consequence of T-jump since then we do not expect the reversal of direction of fluorescence changes mentioned in (i) and (ii) above.

The amplitudes and τ depend on the constitution and concentrations of vesicles and buffers used in the experiments (see equations given in the appendices in [11,13] and discussion (iv) on page 486 in [11]). If there had been disruption of vesicles on T-jump the relaxation traces should have changed with successive T-jumps. We observed reproducible relaxation traces (both amplitudes and τ) even after several T-jumps on the same sample, thus discounting the possibility of disruption of vesicles by T-jump. If there had been disruption of vesicles on adding phloretin we should have observed similar phloretin induced changes in the relaxation traces in all the

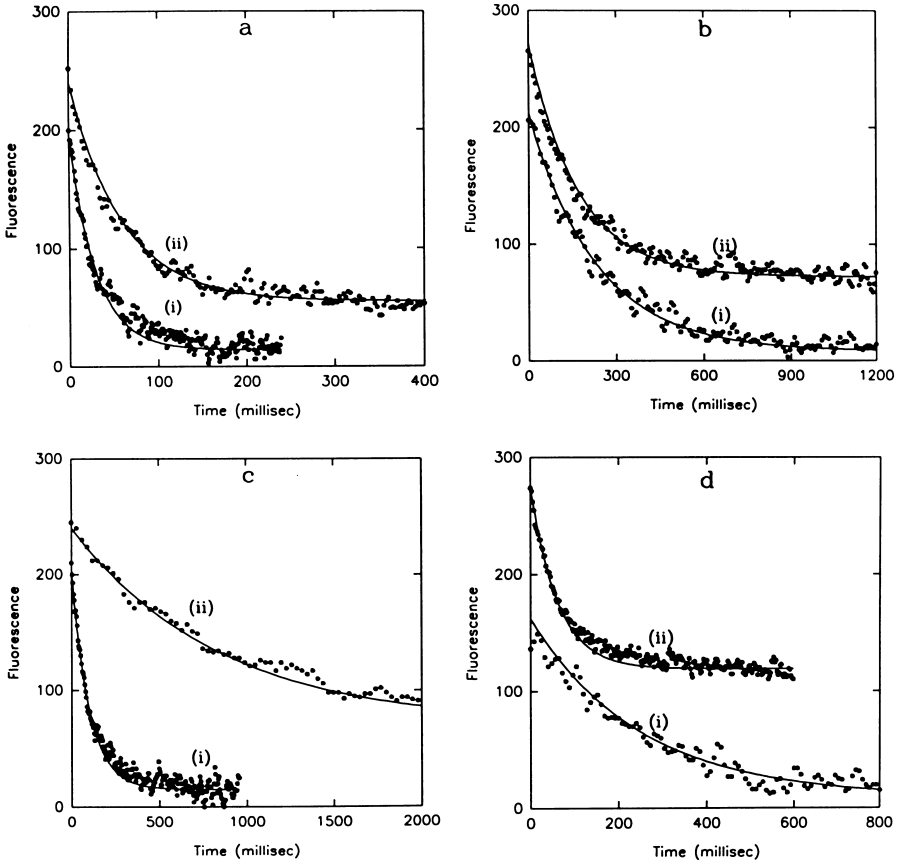

Fig. 1. Changes in ΔpH relaxation traces observed at pH 6.2, on adding phloretin to SBPL vesicle solutions with 50 mM phosphate buffer inside and 50 mM ACES buffer outside vesicles. In NIG mediated ΔpH decay with (a) [lip] = 4.4 mM, $[NIG]_0 = 1$ μM, [KCl] = 100 mM, (b) [lip] = 5.2 mM, $[NIG]_0 = 1.67$ μM, [NaCl] = 100 mM and (i) $[PHL]_0 = 0$, (ii) $[PHL]_0 = 820$ μM. In ΔpH decay mediated by the combined action of VAL and CCCP with (c) [lip] = 4.4 mM, $[VAL]_0 = 2$ μM, $[CCCP]_0 = 2$ μM, [KCl] = 100 mM, (d) [lip] = 5.2 mM, $[VAL]_0 = 20$ μM, $[CCCP]_0 = 53$ μM, [NaCl] = 100 mM and (i) $[PHL]_0 = 0$, (ii) $[PHL]_0 = 410$ μM. Solid lines correspond to single exponentials with time constant τ = (a) 30 ms, 60 ms, (b) 230 ms, 170 ms, (c) 100 ms, 850 ms, (d) 250 ms, 62 ms. The subscripts '0' refer to concentrations calculated with respect to the total volume of vesicle solution.

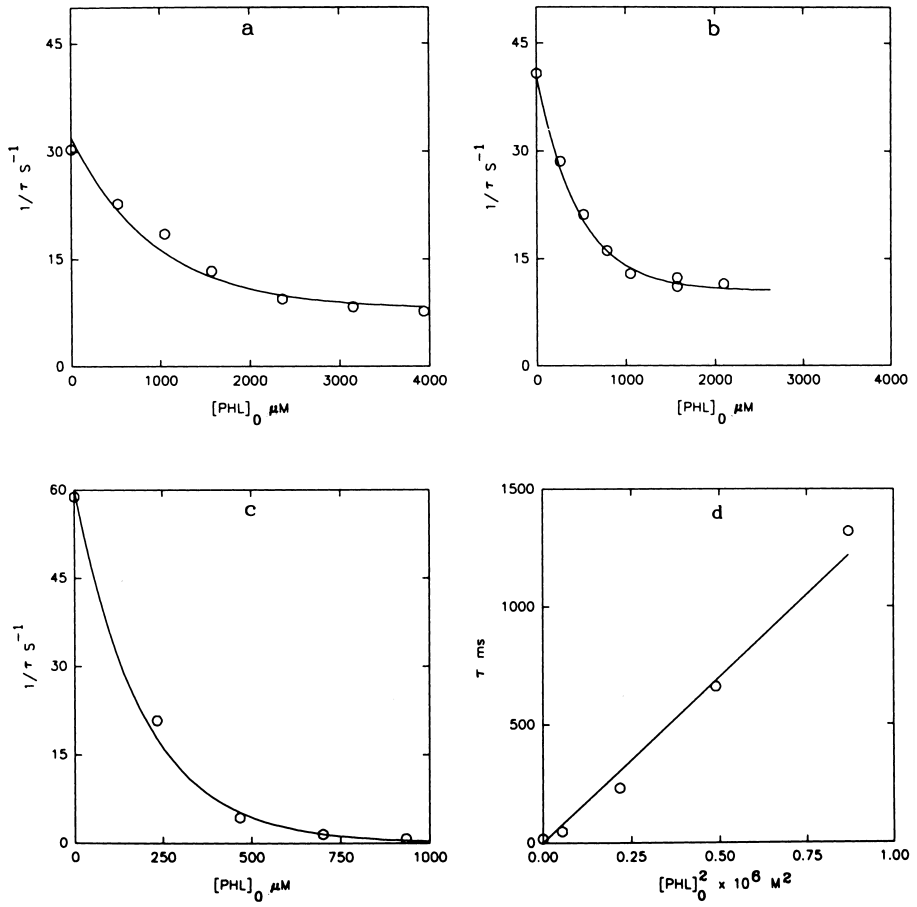

Fig. 2. Dependence of $1/\tau$ on phloretin concentration ($[PHL]_0$), in SBPL vesicle solutions observed with [KCl] = 100 mM, 2 mM phosphate buffer inside and 25 mM ACES buffer outside vesicles at pH ~ 7. (a) $[NIG]_0 = 0.83$ μM, [lip] = 4.45 mM; (b) $[NIG]_0 = 0.52$ μM, [lip] = 2.22 mM; (c) $[VAL]_0 = 5$ μM, $[CCCP]_0 = 5$ μM, [lip] = 5.2 mM; (d) data of (c) replotted as τ against $[PHL]_0^2$. The subscripts '0' refer to concentrations calculated with respect to the total volume of vesicle solution.

experiments. Fig. 1 or Tables 1 and 2 show that this is not the case. The stability of small unilamellar SBPL vesicles used in our experiments and the short duration of T-jump (heating time constant ~5 μs) could have ensured the absence of disruption of vesicles in our experiments. This conclusion is supported by the success of other research groups also in studying transport in vesicles using similar T-jump instruments [23,24].

3. Results

Fig. 1 shows typical changes in ΔpH relaxation traces on adding phloretin to vesicle solutions containing the ionophores of interest. Tables 1 and 2 give the measured τ without and with phloretin in vesicle solutions under different experimental conditions. Fig. 2 shows variations of $1/\tau$ on increasing the phloretin concentration ($[PHL]_0$, estimated with respect to the total volume of the solution). The following are the salient features of the diverse changes in τ seen on adding phloretin. (i) With the same M^+ in vesicle solutions whether the addition of phloretin increases or decreases τ depends on the choice of the ionophore. For example, at pH ~ 6.2 with $M^+ = Na^+$, τ decreases when the exchange ionophore is NIG, increases when the exchange ionophore is MON and decreases when the ΔpH decay is by the combined action of VAL (or NON) and a weak acid (such as CCCP or DNP). (ii) On changing M^+ from Na^+ to K^+, the direction of τ changes is altered. For example, at pH ~ 6.2 with $M^+ = K^+$ τ increases when the ionophore is NIG, decreases when the ionophore

is MON and increases when the ΔpH decay is by the combined action of VAL (or NON) and a weak acid. (iii) When the M^+ chosen is such that its binding to the exchange ionophore is relatively weak (as with NIG and Na^+ or MON and K^+) increasing the pH (say to ~7.5) changes the magnitude of τ change. However, in other cases the pH dependence of τ changes is less prominent. (iv) In the experiments on ΔpH decay by the combined action of VAL and CCCP with K^+ ions in the vesicle solutions, use of a high concentration of VAL reduces the magnitude of phloretin induced τ change. (v) τ changes by a larger factor on adding phloretin in the experiments with VAL+CCCP+K^+ when compared to that in the experiments with exchange ionophores (compare Fig. 2a and c). (vi) Fig. 2c shows that in the case of ΔpH decay by the combined action of VAL+CCCP+K^+ increasing $[PHL]_0$ causes $1/\tau$ to tend towards zero unlike with exchange ionophore mediated ΔpH decays where $1/\tau$ tends towards a non-zero value (Fig. 2a,b). (vii) From the replotted Fig. 2c data (shown in Fig. 2d) we note that when the ΔpH decay is by the combined action of VAL, CCCP and K^+, τ increases nearly linearly with $[PHL]_0^2$. (viii) Fig. 3 shows the variation of τ on changing the concentration of K^+ ions in the experiments on ΔpH decay by the combined action of VAL and CCCP. In these experiments the ionic strength was maintained at a constant value by adding LiCl to solutions. (At the concentrations used the binding of Li^+ to VAL is negligible.) From Fig. 3 we note that the slope of τ against $1/[K^+]$ decreases and the intercept on the τ axis increases on adding phloretin to vesicle solutions. (ix) Comparing Fig. 2a and b we note that the ratio $[PHL]_0/[lip]$ determines the magnitude of τ change. This is expected if almost all the phloretin added to vesicle solutions is bound to the membrane and τ change is due to the presence of phloretin in the membrane.

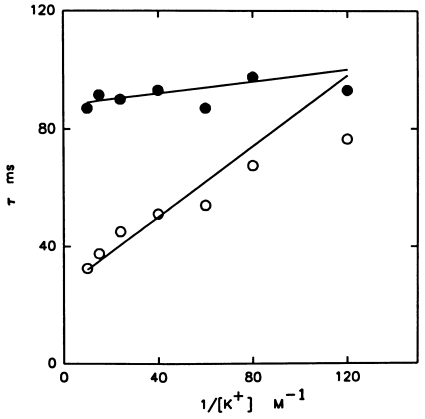

Fig. 3. Variation of τ with $1/[K^+]$ in the ΔpH decay by the combined action of VAL and CCCP ($[VAL]_0 = 2.5$ μM and $[CCCP]_0 = 2.5$ μM) keeping [KCl]+[LiCl] = 0.1 M in 5.2 mM SBPL vesicle solutions with $[PHL]_0 = 0$ (○) or $[PHL]_0 = 200$ μM (●). Inside buffer 0.25 mM ACES and outside buffer 5 mM ACES at pH ~7. The subscripts '0' refer to concentrations calculated with respect to the total volume of vesicle solution.

4. Discussion

The above data show that the rate of ΔpH decay mediated by the ionophores of interest could either increase or decrease or show negligible change on adding phloretin to vesicle solutions. At first sight, these diverse τ changes do not appear to show any systematic pattern. However, when they are examined along with the rate limiting species identified in the literature for the specific experimental conditions [11–16] (see Tables 1 and 2) a definite pattern emerges.

4.1. Phloretin induced increase in M^+ bound species

When the sole rate limiting species has M^+ bound to it τ decreases on adding phloretin when $M^+ = Na^+$ and K^+ (as with Nig–Na, Mon–K) in Table 1 or when $M^+ = Na^+$ only (as with VAL–Na^+–$CCCP^-$, VAL–Na^+–DNP^- and NON–Na^+–$CCCP^-$) in Table 2. Since such a prominent decrease is not seen when the species Nig–H and Mon–H determine the magnitude of $1/\tau$, we can exclude the possibility of phloretin induced increase in the fluidity of the membrane (which is common to the translocation of all the species) and the associated increase in translocation rate constants (k_{rl} in Eq. 2) as the dominant source of changes in τ. Therefore, Eq. 2 can be used to suggest that on adding phloretin to the membrane the concentration of the M^+ bound rate limiting species increases and consequently τ decreases in these systems. Such a change in concentration can come about by a decrease in the apparent metal ion dissociation constants (K_M) of M^+ bound

rate limiting species. (K_M refers to the dissociation constant expressed using $[M^+]$ in the aqueous medium and other concentrations in the membrane.)

4.2. Phloretin induced decrease in H^+ bound species

The increase in τ when the rate limiting species has H^+ bound to it requires us to suggest that concentrations of such species decrease on adding phloretin to the membrane as with Nig–H and Mon–H or H^+ bound gramicidin channels in the membrane. Apart from a possible increase in K_j, a major reason for such a decrease in the concentration of these species is the increase in the M^+ bound species due to increased competitive M^+ binding and conservation of the total concentration of the ionophore or gramicidin channel [11,12,15]. When the translocations of both M^+ bound and H^+ bound species are important in the rate determining step, the net change in τ is expected to be smaller (see Eq. 1). This can also be seen in the data given in Table 1.

4.3. Complexing of phloretin with VAL–M^+

The increase in τ observed on adding phloretin in the experiments using the combination of the metal ion carrier VAL and a weak acid (such as CCCP) (Table 2) with $M^+ = K^+$ does not fit the above pattern. We note that $1/\tau$ tending to zero for high concentrations of phloretin (Fig. 2c) suggests unavailability of metal ion carriers or weak acids in the transport scheme. If phloretin had made CCCP unavailable for transport, the addition of phloretin should have increased τ for both $M^+ = K^+$ and Na^+. Table 2 shows τ increase only for $M^+ = K^+$. The behaviour seen in Fig. 2c is expected if phloretin forms 1:2 complexes with VAL–K^+ making it unavailable for the formation of the rate limiting species.

The equilibria to be considered in this case are the metal ion dissociation with apparent dissociation constant K_M, dissociation of the rate limiting complex with dissociation constant K_d and the 1:2 phloretin complex dissociation with dissociation constant K_p.

$$\mathrm{VAL - M^+\ (m) \rightleftharpoons VAL\ (m) + M^+\ (aq);\ } K_M \qquad (4)$$

$$\mathrm{VAL - M^+ - CCCP^-\ (m) \rightleftharpoons VAL - M^+\ (m) + CCCP^-\ (m);\ } K_d \qquad (5)$$

$$\mathrm{VAL - M^+ - (PHL)_2\ (m) \rightleftharpoons VAL - M^+\ (m) + 2\ PHL\ (m);\ } K_p \qquad (6)$$

The expression for the concentration of the rate limiting complex can be given in terms $[VAL]_t$ (= total concentration of VAL species in the membrane) using the conservation relation.

$$[\mathrm{VAL - M^+ - CCCP^-}]_m = [VAL]_t\ ([M^+]/K_M)\ ([\mathrm{CCCP^-}]_m/K_d) \times 1/\{1 + ([M^+]/K_M)\ (1 + [\mathrm{CCCP^-}]_m/K_d + [PHL]_m{}^2/K_p)\} \qquad (7)$$

The data in Fig. 2c were obtained with $[VAL]_0$ and $[CCCP]_0$ sufficiently small such that the concentration of the rate limiting species in the membrane $[\mathrm{VAL–K^+–CCCP^-}]_m$ is negligible compared to the concentrations of uncomplexed VAL or CCCP. Also for $M^+ = K^+$, the magnitude of K_M is sufficiently small such that the right hand side of Eq. 7 is nearly proportional to $1/[PHL]_m^2$ and hence the observed τ should be nearly proportional to $[PHL]_m^2$ or to $[PHL]_0^2$ (since almost all the phloretin is in the membrane, see (ix) in Section 3). The near linearity of the plot in Fig. 2d is consistent with this prediction. We get $K_p \approx 0.002$ M^2 using Fig. 2d and $[PHL]_m = 0.95\ [PHL]_0/[lip]$ M (equation A27 in [11]).

Because of experimental limitations we have not been able to make direct observations on the complex formed by phloretin with VAL–M^+. However, in view of the direct evidence for the formation of a complex between organic anions and VAL–M^+, such as crystallographic data [26] and spectroscopic observations [27], the above hypothesis of formation of the complex between the polar molecule phloretin (electric dipole moment $\sim$5 D) and VAL–M^+ is reasonable.

4.4. Resolution of three paradoxes in the explanation of Table 2 data

As mentioned above the Table 2 data with $M^+ = Na^+$ can be understood if K_M decreases on adding phloretin (Section 4.1). The Table 2 data

with $M^+ = K^+$ are explained by including the phloretin complex formation (Section 4.3 and Eq. 6). It is difficult to accept that the K_M decrease is restricted to $M^+ = Na^+$ and the phloretin complex formation is restricted to $M^+ = K^+$ situations. Two questions then arise. (1) Why did the complexation of phloretin with VAL–M^+ not increase τ in the experiments with $M^+ = Na^+$? (2) How can one detect a phloretin induced decrease in K_M by a suitable experiment even in the experiments with $M^+ = K^+$?

The first question can be answered by noting that the selectivity of VAL to Na^+ is nearly 17 000 times smaller than that for K^+ [25]. Thus, K_M for $M^+ = Na^+$ is so large that the denominator of Eq. 7 reduces to ~ 1 and the concentration of the rate limiting species becomes proportional to $1/K_M$. In this case the complexing with phloretin goes undetected in the τ data. The second question can also be answered with the help of Eq. 7 by noting that a decrease in K_M manifests itself as a decrease in the slope of τ against $1/[M^+]$ since τ is proportional to $1/[VAL–M^+–CCCP^-]_m$. The plots in Fig. 3 are consistent with this expectation, confirming a reduction of K_M by the presence of phloretin in the membrane even when $M^+ = K^+$.

The data in Table 2 also show that with $M^+ = K^+$, small $[CCCP]_0$ and high $[VAL]_0$, the phloretin induced change in τ is small unlike the change in τ with small $[VAL]_0$. This is because, with a large $[VAL]_0$, almost all the CCCP is in the ternary complex form and $1/\tau$ is close to its saturating value (see Fig. 2 in [13]). In this case, free $[VAL–M^+]_m$ is so large that changes in $[VAL–M^+]_m$ by the decrease in K_M and by the phloretin complex formation are not sufficient to cause significant changes in the concentration of the rate limiting ternary complex.

4.5. Differences seen on changing the weak acid/ionophore

When DNP is used instead of CCCP only a small fraction of the DNP is partitioned to the membrane [14]. The expected reduction in the partitioning of DNP^- to the membrane on adding phloretin to the membrane causes an increase in the apparent K_d (estimated using $[DNP]_0$). Eq. 7 predicts that such changes make the phloretin induced increase in τ for $M^+ = K^+$ larger and the phloretin induced decrease in τ for $M^+ = Na^+$ smaller in the experiments with DNP when compared to that with CCCP. The data in Table 2 are consistent with this prediction.

The ΔpH decay data obtained with the combination NON–CCCP are similar to those by the combination VAL–CCCP [16]. However, we need more phloretin to cause a change in τ by the same factor if we use NON instead of VAL (see Table 2). Thus, the magnitudes of the phloretin induced decrease in K_M and the complex formation on adding phloretin are relatively smaller.

4.6. Requirements for the validity of the correlation

Phloretin binds to artificial membranes mainly in the non-ionised form [28] making its apparent proton dissociation constant in the membrane very large. Thus, the change in the internal buffer capacity (b_i) (see Eq. 3) on adding phloretin is negligible and need not be invoked to explain phloretin induced changes in τ. This is the condition for the validity of the correlation between the changes in the concentration of the rate limiting species and changes in τ on adding phloretin used above. We have verified this condition with the help of an experiment on NIG mediated ΔpH decay. On increasing the concentration of the phosphate buffer inside SBPL vesicles from 2 to 50 mM, τ increased from ~ 23 to ~ 45 ms at pH ~ 6.35 and from ~ 116 to ~ 160 ms at pH ~ 7.7. In a similar experiment with phloretin in vesicle solutions at concentrations as high as $[PHL]_0 = 2.3$ mM, τ changed by a similar factor, from ~ 45 to ~ 81 ms at pH ~ 6.35 and from ~ 315 to ~ 410 ms at pH ~ 7.7. This is expected from Eq. 2 if phloretin makes a negligible contribution to b_i.

Since phloretin in the membrane is predominantly non-ionised in the pH range of our experiments, membrane perturbations due to changes in the ionisation state of phloretin need not be invoked to explain the pH dependences of τ changes seen in Tables 1 and 2.

4.7. Comparison of conclusions from ΔpH decay data with other studies given in the literature

Experiments on vesicles prepared from phosphatidylcholine have shown that phloretin binds to the

membrane with a dissociation constant of ~8 μM [8,9]. For a similar dissociation constant with SBPL vesicles and at the concentrations of our experiments almost all the phloretin added is expected to bind to the membrane. We have also come to a similar conclusion by comparing the data in Fig. 2a and b. In phosphatidylcholine vesicles the lipid/phloretin binding site ratio was ~4 [8]. However, the data in Fig. 2 on SBPL vesicles do not appear to be restricted to this value.

From steady state measurements on the electric potential on planar bilayer membrane with smaller K^+ ion concentrations in the aqueous medium, Antonenko and Bulychev [6] inferred that phloretin in the membrane increases the rate of NIG mediated K^+/H^+ exchange across the membrane. Such a situation should decrease τ in the ΔpH decay experiments. Consistent with this expectation we have observed a decrease in τ from 56 to 50 ms on adding 2.3 mM phloretin to 5.4 mM SBPL vesicle solutions at pH ~6 (0.25 mM ACES buffer inside and 5 mM ACES buffer outside vesicles, $[NIG]_0 = 0.8$ μM, [KCl] = 20 mM, [KCl]+[CsCl] = 105 mM). However, the τ obtained under similar conditions at pH ~7.5 increased from 50 to 90 ms and the τ obtained with higher $[K^+]$ (100 mM) also increased (Table 1) on adding phloretin.

Antonenko and Bulychev [6] explained their results assuming the rate limiting step of net transport to be the K^+/H^+ exchange at the aqueous medium–membrane interface rather than the electroneutral translocations of ion carriers. In their explanation the phloretin induced reduction in the positive internal membrane dipole potential enhances the K^+/H^+ exchange rate involving cations and hence enhances the net transport. However, their assumption about the rate limiting step has no experimental support [11]. Also, the phloretin induced increases in τ at higher pH or higher $[M^+]$ (mentioned above and reflecting reduction in transport rates) cannot be explained on the basis of such a model. In our explanation, for small [KCl] and low pH the concentrations may satisfy the condition $[Nig–K]_m < [Nig–H]_m$ making Nig–K the rate limiting species. In this case, phloretin is expected to increase $[Nig–K]_m$ and consequently decrease τ, as observed. However, for higher [KCl] and higher pH such that $[Nig–K]_m > [Nig–H]_m$, Nig–H will be the rate limiting species. In this case, phloretin is expected to decrease $[Nig–H]_m$ and consequently increase τ, as observed.

4.8. Phloretin induced perturbations in the membrane

In spite of the large $[PHL]_0$/[lipid] ratio used in our experiments the vesicles were not disrupted. In some cases even the changes in τ were small. Such observations suggest that phloretin does not affect the close packing in the 'non-polar core region' of the bilayer, presumably because it is adsorbed on lipid surfaces [29]. Also, nuclear magnetic resonance (NMR) studies have shown that incorporation of phloretin leaves the bilayer structure intact, induces only a small disordering of hydrocarbon chains, has no significant effect on head group dynamics but shows distinct structural changes for the phosphocholine head group consistent with the binding of phloretin mainly in the 'polar region' as suggested above [30]. Such a conclusion is not surprising since phloretin is practically insoluble in water or ether, very sparingly soluble in chloroform and freely soluble in alcohol. However, it permeates through the bilayer with time constant ~1 s in phosphatidylcholine vesicles [8]. Since the main barrier to the translocation of electroneutral species comes from the 'close packing' in the 'non-polar core region' of the bilayer [31,32], and since this is not affected by phloretin (see above), we do not expect significant changes in the translocation rate constants k_r on adding phloretin to vesicle solutions in our experiments. Our data also suggest insignificant changes in k_r on adding phloretin (see Section 4.1). Thus, the main perturbation of phloretin adsorbed into the membrane appears to be in the 'polar region'. By suitably orienting itself phloretin reduces the internal membrane dipole potential [3,5,33–35].

4.9. Changes in K_M are from changes in internal membrane potential

The permeability of phospholipid bilayers is considerably greater for anions than for cations [36]. This has been explained by postulating the existence of a positive internal membrane dipole potential. The partitioning to the membrane from the aqueous medium and the translocation rates of cations in the bilayer membrane increase on adding phloretin.

However, they decrease with anions. Such behaviours of charged species have been attributed to a decrease in the internal membrane potential which changes the free energy profiles in membranes [3,5].

From ΔpH decay studies we have inferred phloretin induced changes in the concentrations of electroneutral species. If such changes are a consequence of the decrease in the internal membrane potential it can only come from the changes in the concentrations of their electrically charged precursors. Thus, on the basis of the above hypothesis, increased partitioning of the cations M^+ from the aqueous medium to the membrane (due to a phloretin induced decrease in the internal membrane potential) should increase the concentrations of the M^+ bound species or should decrease K_M. This has been observed in our data (Section 4.1).

However, a change in the apparent H^+ dissociation constant, K_j, may not follow the same pattern. NMR studies [30] as well as direct water absorption and X-ray diffraction studies [37] have shown a decrease in the amount of water adsorbed to the vesicular membrane on adding phloretin. In the literature, the internal dipole potential has been attributed to oriented lipid polar head groups as well as oriented adsorbed water dipoles [33–35]. The oriented phloretin dipole interacts/changes the orientation of these dipoles reducing the internal membrane potential. Such an interaction could reduce the ability of adsorbed water to accept H^+ from the aqueous medium. In addition, the reduction of adsorbed water also reduces the partitioning of H^+ to the membrane. Thus, unlike with M^+, the partitioning of H^+ to the membrane may actually decrease on adding phloretin to the membrane. Such a situation increases K_j. This is reasonable since it is known that an altered environment in the membrane could lead to changes in K_j [38].

A further test of the above hypothesis can be carried out using another dipolar molecule, 6-ketocholestanol, instead of phloretin since it is expected to increase the internal membrane potential (unlike phloretin) [5]. In this case, we should see an increase in the apparent metal ion dissociation constants K_M unlike the decrease seen on adding phloretin. However, the apparent H^+ dissociation constant K_j could still increase from reasons similar to those given above. Therefore, in systems where the concentration changes are dominantly affected by the competitive binding of H^+ and M^+ to ionophores/channels (as with NIG, MON and GRAM channels) the effect of the increase in K_M may be cancelled by the increase in K_j. Presumably because of this reason, the changes in τ observed in these systems on adding 0.41 mM 6-ketocholestanol were within the limits of errors and no definite conclusions could be drawn from these data. We expect a more definitive conclusion from tests in SBPL vesicle solutions containing NaCl with ΔpH decay by a combination of VAL (or NON) and the weak acid CCCP. In the experiments with 50 mM phosphate inside SBPL vesicles, pH ∼ 7, [NaCl] = 100 mM, $[VAL]_0 = 25$ μM and $[CCCP]_0 = 133$ μM, τ increased from 55 to 87 ms on adding 0.41 mM 6-ketocholestanol unlike the decrease in τ observed on adding phloretin (Table 2). In the experiments with 2 mM phosphate inside ∼3 mM SBPL vesicles, pH ∼ 7, [NaCl] = 100 mM, $[NON]_0 = 6.6$ μM and $[CCCP]_0 = 133$ μM, τ increased from 46 to 57 ms on adding 0.41 mM 6-ketocholestanol whereas it decreased to 33 ms on adding 0.41 mM phloretin. These observations support our assignment of the dominant changes in K_M to changes in the internal membrane potential.

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