Two Mechanisms of H^+/OH^- Transport Across Phospholipid Vesicular Membrane Facilitated by Gramicidin A

B. S. Prabhananda and Mamata H. Kombrabail
Chemical Physics Group, Tata Institute of Fundamental Research, Mumbai 400 005, India

ABSTRACT Two rate-limiting mechanisms have been proposed to explain the gramicidin channel facilitated decay of the pH difference across vesicular membrane (ΔpH) in the pH region 6–8 and salt (MCl, $M^+ = K^+, Na^+$) concentration range 50–300 mM. 1) At low pH conditions (~6), H^+ transport through the gramicidin channel predominantly limits the ΔpH decay rate. 2) At higher pH conditions (~7.5), transport of a deprotonated species (but not through the channel) predominantly limits the rate. The second mechanism has been suggested to be the hydroxyl ion propogation through water chains across the bilayer by hydrogen bond exchange. In both mechanisms alkali metal ion transport providing the compensating flux takes place through the gramicidin channels. Such an identification has been made from a detailed study of the ΔpH decay rate as a function of 1) gramicidin concentration, 2) alkali metal ion concentration, 3) pH, 4) temperature, and 5) changes in the membrane order (by adding small amounts of chloroform to vesicle solutions). The apparent activation energy associated with the second mechanism (~3.2 kcal/mol) is smaller than that associated with the first mechanism (~12 kcal/mol). In these experiments, ΔpH was created by temperature jump, and vesicles were prepared using soybean phospholipid or a mixture of 94% egg phosphatidylcholine and 6% phosphatidic acid.

INTRODUCTION

The peptide gramicidin A has received considerable attention as a model for ion channel proteins (Woolley and Wallace, 1992, and references cited therein). The gramicidin channel is known to be permeable to small monovalent cations, protons, and water but impermeant to divalent cations and anions (Woolley and Wallace, 1992; Wang et al., 1995, and references cited therein). However, there can be a secondary binding of anions near cation-loaded channels (Eisenman et al., 1978). Experiments by Ahmed and Krishnamoorthy (1990) have shown that the transmembrane transport of anions such as $CCCP^-$ (carbonyl cyanide *m*-chlorophenylhydrazone) is also facilitated by gramicidin in vesicles. In these cases it is believed that the transport of the anion does not take place through the channels and that the alkali metal cation transport through the channels provides the compensating charge flux that facilitates the anion transport. Because of such properties gramicidin has been used to facilitate the decay of pH difference (ΔpH) across phospholipid vesicular membrane (Ahmed and Krishnamoorthy, 1990). The importance of ΔpH in energy transduction is well known (Mitchell, 1961). The decay of ΔpH requires H^+/OH^- transport across the membrane. In liposomes, a compensating charge flux such as that of alkali metal ions M^+ or electrons is necessary to abolish the electric potential generated by H^+/OH^- transport, which can oppose further conduction (Henderson et al., 1969; Yamaguchi and Anraku, 1978; Prabhananda and Ugrankar, 1991). Thus the rate-limiting step of ΔpH decay can be either the H^+/OH^- transport step or the M^+ transport step. The purpose of the present work is to identify the rate-limiting steps of gramicidin-facilitated ΔpH decay in the biologically relevant pH range 6–8 to determine whether the same transport mechanism limits the ΔpH decay rate throughout this pH range. In the experiments we have used soybean phospholipid (SBPL) vesicles as the model membrane system, generated ΔpH by temperature jump (T-jump), and measured the ΔpH decay rate as described elsewhere (Krishnamoorthy, 1986; Prabhananda and Ugrankar, 1991; Prabhananda and Kombrabail, 1992).

MATERIALS AND METHODS

The SBPL vesicle solutions with 2 mM pyranine and phosphate buffer inside vesicles and *N*-(acetamido)-2-aminoethanesulfonic acid (ACES) buffer outside vesicles at concentrations given in the figure legends were prepared from asolectin (Sigma) or from a 94% egg phosphatidylcholine (Sigma) + 6% phosphatidic acid (Sigma) mixture by sonication in a bath-type sonicator and passing through a G-50 sephadex column as described earlier (Krishnamoorthy, 1986; Prabhananda and Ugrankar, 1991). The solutions also had KCl and NaCl at concentrations given in figure legends or mentioned in the text. The pH was adjusted using HCl/KOH/NaOH. Microliter amounts of 5 mM or 15 mM gramicidin A (Sigma) in ethanol were added to vesicle solutions with vortex stirring and allowed to stand for ~2 h before the experiments. The ΔpH decay rate was measured at 23°C ± 1.5 by monitoring the fluorescence from the pH indicator pyranine entrapped inside vesicles. ΔpH (~0.02) was created by T-jump with a heating time constant of ~5 μs (Prabhananda and Ugrankar, 1991). The ΔpH decay time constants τ were determined using at least four relaxation traces (Prabhananda and Kombrabail, 1995). The apparent activation energies were determined from observations in the temperature range 280–298 K. All of the observed experimental trends were confirmed by experiments on at least two different vesicle preparations.

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Address reprint requests to Dr. B. S. Prabhananda, Chemical Physics Group, Tata Institute of Fundamental Research, Mumbai 400 005, India. Tel.: 091-22-2152971; Fax: 091-22-2152110; E-mail: bsp@tifrvax.tifr.res.in.

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THEORETICAL EQUATIONS

An expression for the ΔpH relaxation rate, $1/\tau$, in vesicles has been obtained by Grzesiek and Dencher (1986), assuming the H^+/OH^- transport to be the rate-limiting step. Their derivation assumes that the electric potential (created by H^+/OH^- transport across the membrane) is abolished in a fast step (fast compared to $1/\tau$) by the charge-compensating alkali metal ion flux. We have also derived a similar equation in terms of transport rate constants (Prabhananda and Ugrankar, 1991) using the relaxation kinetics approach (Czerlinsky, 1966). Many of the ΔpH decay mechanisms studied (Prabhananda and Ugrankar, 1991; Prabhananda and Kombrabail, 1992, 1995) do not satisfy the above-mentioned assumption. However, in the mechanisms inferred below the compensating charge flux of M^+ through the gramicidin channels is fast compared to the observed $1/\tau$. Therefore we can use either Eq. 1 given by Grzesiek and Dencher (1986) or Eq. A-18 given by us (Prabhananda and Ugrankar, 1991) after including a term corresponding to OH^- transport as given below,

$$1/\tau = (\ln 10)\{1 + (b_i V_i)/b_e V_e\}\{k_H[H^+] + k_{OH}[OH^-]\}/b_i, \quad (1)$$

where k_H and k_{OH} are the effective rate constants for the translocation of H^+ and OH^- across the bilayer membrane. The internal buffer capacity b_i has contributions from all of the groups in fast proton exchange equilibrium with the aqueous medium inside vesicles.

$$b_i = (\ln 10)\{\sum C_j K_{Hj}[H^+](K_{Hj} + [H^+])^2\}, \quad (2)$$

where C_1 and K_{H1} are the concentrations and apparent proton dissociation constant of the buffer entrapped inside vesicles. $C_2 = 30$ mM, $K_{H2} = 10^{-6.9}$ M, $C_3 = 45$ mM, $K_{H3} = 10^{-7.8}$ M are associated with the endogenous groups in SBPL vesicles (Prabhananda and Kombrabail, 1992). In Eq. 1 b_e is the buffer capacity external to the vesicles, and V_i and V_e are the total volumes inside and outside the vesicles.

The gramicidin added to vesicle solutions of volume V_0 at a concentration $[\text{gramicidin}]_0$ is partitioned mainly to the membrane. Therefore the average concentration of gramicidin in the membrane $[G]_m = [\text{gramicidin}]_0 V_0/V_m$, where V_m is the vesicular membrane volume. Using the dimensions of SBPL vesicles (Grzesiek and Dencher, 1986) ($V_m \approx 2\ V_i$) and our estimate of V_e/V_i (= 2.2 when the lipid concentration [lip] = 1 M) (Prabhananda and Ugrankar, 1991), we get $[G]_m \approx ([\text{gramicidin}]_0/[\text{lip}])$ M. Experiments have shown that gramicidin dimers form channels in the membrane (Hladky and Haydon, 1972; Veatch et al., 1975). Using the estimate of the dimerization constant of gramicidin A in the lipid medium (Woolley and Wallace, 1992), we can say that at the concentrations used in our experiments the fraction of gramicidin in the monomeric form is negligible and the gramicidin channel concentration in the membrane $[GC]_m \approx [G]_m/2$. Therefore, to a good approximation we can write

$$[GC]_m = \{[\text{gramicidin}]_0/(2[\text{lip}])\}\ \text{M}. \quad (3)$$

H^+ transport through the channel requires that the H^+ bind to the channel first. If H^+ and M^+ bind competitively to gramicidin channels with apparent dissociation constants K_H and K_M, respectively, and if the unoccupied channel concentration is negligible, the concentration of gramicidin channels to which H^+ are bound, $[GCH]_m$, is given by

$$[GCH]_m = [GC]_m/\{1 + K_H[M^+]/(K_M[H^+])\}. \quad (4)$$

When the dominant H^+ transport is through the channel, we can write the following equation using k_H^*, the rate constant associated with H^+ translocation within the channel:

$$k_H[H^+] = k_H^*[GCH]_m = k_H^*(K_M/K_H)([GC]_m[H^+]/[M^+]), \quad (5)$$

when $(K_H\ [M^+])/(K_M\ [H^+]) \gg 1$. This condition is satisfied even though $K_H < K_M$, because $[M^+] \gg [H^+]$ in our experiments. When two types of metal ions at concentrations $[M1^+]$ and $[M2^+]$ bind competitively to gramicidin channels with apparent dissociation constants K_{M1} and K_{M2}, respectively, and if the unoccupied channel concentration is negligible, $[GCH]_m$ can be expressed as follows instead of as Eq. 4:

$$[GCH]_m = [GC]_m/(1 + \{1 + K_{M1}[M2^+]/(K_{M2}[M1^+])\}\{K_H[M1^+]/(K_{M1}[H^+])\}) \quad (6)$$

Hydroxyl ions are not propagated through the gramicidin channels. Their transport from the inner layer to the outer layer across the vesicular membrane and the transport of M^+ providing the compensating flux can be considered to be equivalent to the following chemical reaction:

$$(M^+)_{im}(OH^-)_{im} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (M^+)_{om}(OH^-)_{im} \xrightarrow{k_3} (M^+)_{om}(OH^-)_{om}, \quad (7)$$

where the subscripts im and om refer to the ions in the inner and outer layers of the vesicular membrane. These are in fast equilibrium with the ions in the aqueous buffer medium inside and outside vesicles by fast exchange at the membrane-water interface. In the steady-state kinetics, the concentration of $(M^+)_{om}(OH^-)_{im}$ can be taken to be constant with respect to time (Amdur and Hammes, 1966). This enables us to write the following for the overall OH^- transport rate, which can be used in Eq. 1:

$$k_{OH}[OH^-] = [(M^+)_{im}(OH^-)_{im}]k_1k_3/\{k_2 + k_3\}. \quad (8)$$

If in the membrane region participating in the OH^- propagation the apparent dissociation constant of water (measured using the $[H^+]$ in the aqueous medium) is

K (= $[OH^-]_{im}$ $[H^+]/[H_2O]_{im}$) and $[H_2O]_{im}$ + $[OH^-]_{im}$ = C_0, Eq. 8 becomes

$$k_{OH}[OH^-] = C_0 \cdot \{K/(K + [H^+])\} \cdot \{k_1k_3/(k_2 + k_3)\}. \quad (9)$$

In our experiments $b_iV_i \ll b_eV_e$. Therefore, using Eqs. 5 and 9 we can rewrite Eq. 1 as follows, with $[GC]_m$ given by Eq. 3:

$$1/\tau = (\ln 10/b_i)\{k_H^*(K_M/K_H)[GC]_m[H^+]/[M^+] + \{k_1k_3/(k_2 + k_3)\}C_0K/(K + [H^+])\}. \quad (10)$$

RESULTS AND DISCUSSION

Membrane order relevant for transport is not affected by gramicidin

To ensure that the observed changes in τ are not a consequence of changes in the integrity of the membrane on adding gramicidin, we have used nigericin-mediated ΔpH decay as the probe (Prabhananda and Kombrabail, 1995). From the τ estimated using the traces in Fig. 1 we note that $1/\tau$ observed with the combination of nigericin and gramicidin is close to the sum of $1/\tau$ values observed with nigericin alone and gramicidin alone. Such a relation shows that the nigericin-mediated contribution to the ΔpH decay and hence its transport rates are not affected significantly by the presence of gramicidin in the vesicular membrane in the concentration range of our experiments. A similar conclusion can be arrived at from the data at pH ~7.5 given in the literature (figure 1 of Ahmed and Krishnamoorthy, 1990). The changes in the core of the lipid bilayer by gramicidin suggested by Muller et al. (1996) are presumably not large enough to cause significant changes in the transport rates.

τ data that show two prominent mechanisms in the rate-limiting steps of ΔpH decay

Fig. 2 shows that the ΔpH relaxation rate ($1/\tau$) has a contribution proportional to $[gramicidin]_0$. This contribution is dominant in the data obtained at pH ~6 (Fig. 2 *a*) and less dominant in the data obtained at pH ~7.5 (Fig. 2 *b*). Obviously such a contribution is associated with the "first mechanism" in which transport through gramicidin channels limits the rate of ΔpH decay. The presence of a "second

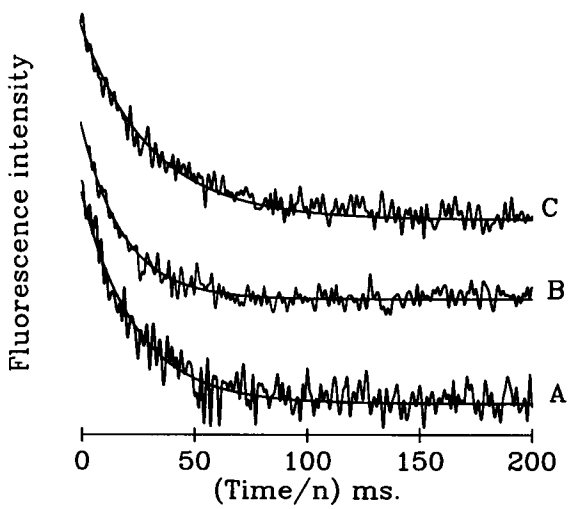

FIGURE 1 ΔpH decay traces observed on adding (*A*) gramicidin at 80 μM, (*B*) nigericin at 1.7 μM, and (*C*) gramicidin at 80 μM + nigericin at 1.7 μM to SBPL vesicle solutions at 24°C. 50 mM phosphate buffer inside and 50 mM ACES buffer outside vesicles at pH ~6.2. [lipid] = 5 mM and [KCl] = 100 mM in solutions. (*A*) n = 4, τ = 94 ms, for (*B*) n = 2, τ = 37 ms and (*C*) n = 0.7 and τ = 23 ms, where τ is the time constant associated with the superposed matching exponential. In the absence of gramicidin-induced membrane disorder, τ = 26 ms was expected in *C*.

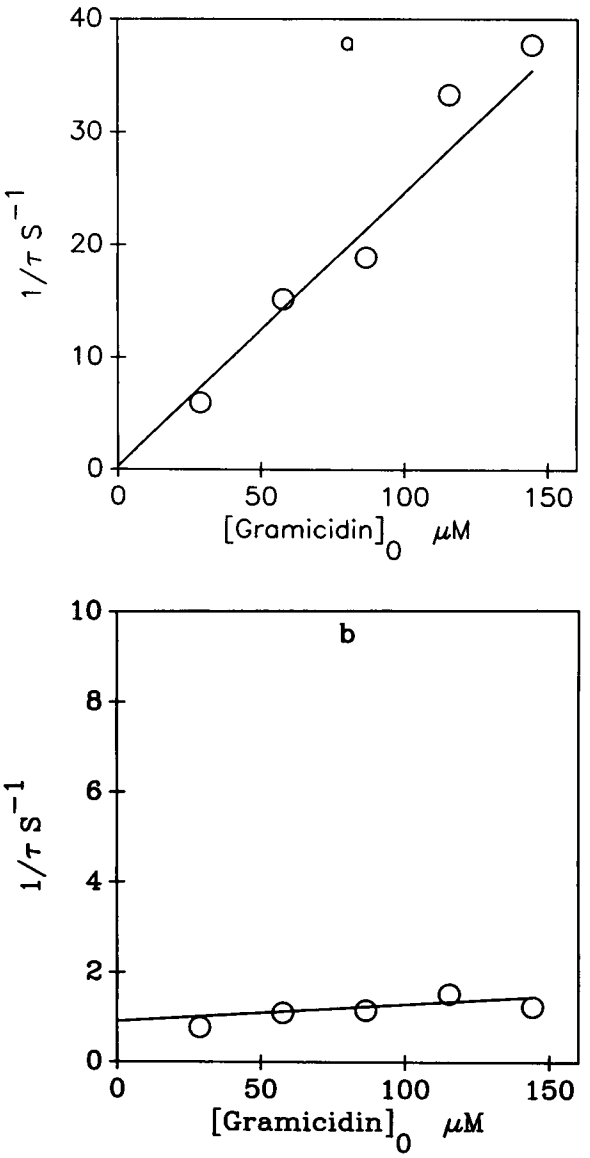

FIGURE 2 Dependence of ΔpH decay rate $1/\tau$ on the concentration $[gramicidin]_0$ in SBPL vesicle solutions at pH (*a*) ~6.1 and (*b*) ~7.6. 50 mM phosphate inside and 25 mM ACES ourside vesicles with [KCl] = 100 mM and [lip] = 3.9 mM. The solid lines were calculated using Eq. 10 as described in the text.

mechanism" that does not vary with $[gramicidin]_0$, but which requires sufficient gramicidin to be present in the membrane, can be seen in the data obtained at pH 7.5. Such a mechanism can be inferred by noting that at $[gramicidin]_0 = 0$, the extrapolated $1/\tau$ is significantly higher than the negligible $1/\tau$ ($<0.1\ s^{-1}$) actually observed (Fig. 2 *b*). Similar behavior can also be seen in the data given in the literature (Ahmed and Krishnamoorthy, 1990). The nature of these two mechanisms can be inferred using the data given in Figs. 3 and 4.

Assignment of the "first mechanism" to H^+ transport through the channel

We assign the contribution to $1/\tau$ from the "first mechanism" to the rate-limiting step of H^+ transport through the gramicidin channels. In this case the first term of Eq. 10 can be used to make predictions about the behavior of τ. Our assignment can be confirmed by comparing these predicted behaviors with the observed behaviors as shown below: 1) We should see a reduction in the slope of $1/\tau$ against $[gramicidin]_0$ plots by a factor of $10^{-6.1}/10^{-7.6}$ on increasing pH from 6.1 to 7.6 in view of the factor $[H^+]$ occurring in the first term of Eq. 10. This is indeed observed in Fig. 2 (solid lines in the figure had been calculated using Eq. 10). 2) Because of the competitive binding between K^+ and H^+ to the gramicidin channel, increasing $[K^+]$ should decrease $[GCH]_m$ and hence decrease $1/\tau$. Equation 10 predicts that such a decrease should be by a larger factor at lower pH (~6) because of $[H^+]$ in the first term. At higher pH (~7.5) the decrease has to be smaller not only because of the smaller $[H^+]$ in the first term of Eq. 10, but also because of the dominant contribution from the "second mechanism," which does not depend on $[M^+]$. The pH-dependent τ data obtained with [KCl] = 50 mM and 300 mM (Fig. 3 *a*) are consistent with these expectations. 3) The first term of Eq. 10 also predicts that $1/\tau$ against $[H^+]$ should be linear and that the slope of such a plot should decrease with increase in $[K^+]$. This is not readily seen in the data of Fig. 3 *a*, because in addition to the concentration of rate-limiting species, $1/\tau$ depends on the internal buffer capacity b_i of SBPL vesicles (Eq. 2), which also varies with pH (Grzesiek and Dencher, 1986; Prabhananda and Ugrankar, 1991). By factoring out the latter contribution from the τ data given in Fig. 3 *a* (as in the plots of b_i/τ against $[H^+]$ shown in Fig. 3 *b*), we do get the expected linear increase with $[H^+]$, especially for high $[H^+]$ (i.e., at low pH when the "second mechanism" is less dominant). The observed decrease in the slope of these plots on increasing [KCl] from 50 mM to 300 mM is close to that predicted by the first term of Eq. 10 (i.e., by a factor of ~6). In the "first mechanism," which is dominant at pH ~6, the metal ion transport through the gramicidin channels (providing the compensating charge flux) is fast compared to the rate-limiting step of H^+ transport through the channels even though $K_H < K_M$, because $[K^+] \gg [H^+]$ in our experiments, where K^+ was the metal ion. The apparent activation energy associated with $1/\tau$ (~12 kcal/mol) determined from the temperature dependence of $1/\tau$ (Fig. 4 *b*) at a pH when the "first mechanism" is dominant (pH ~6) includes the free energy difference involved in the competitive binding of H^+ and M^+ in the channel (see Eq. 10).

Assignment of the "second mechanism" to OH^- transport external to the channel

The "second mechanism" is inferred from the prominent intercept in Fig. 2 *b*. Sufficient gramicidin channels are needed to facilitate this mechanism of ΔpH decay, presumably to transport the charge-compensating flux of M^+ in a fast step. However, they are not directly involved in the rate-limiting step. Because a similar behavior is predicted by the second term of Eq. 10, we assign the contribution from the "second mechanism" to the rate-limiting step of OH^- transport external to the gramicidin channels with k_1, $k_2 \gg k_3$. Furthermore, we suggest that such transport is accomplished by the propagation of OH^- across the membrane through associated water molecules in the hydrophobic portion of the bilayer or transient hydrated pores (Nichols and Deamer, 1980; Paula et al., 1996, and references cited therein). Our assignment can be confirmed by comparing the behavior of τ predicted by Eq. 10 with that observed in the experiments (carried out preferably with high $[M^+]$ and low $[H^+]$ when the first term corresponding to the "first mechanism" is less dominant):

1. If OH^- transport is the rate-limiting step in the "second mechanism," it should be possible to show that the M^+ transport does not limit the rate of ΔpH decay. This can be seen from the following argument. In view of our identification of the "first mechanism," we can say that the M^+ transport step is fast compared to $1/\tau$ at pH ~6. On increasing the pH to 7.5, because of the decrease in $[H^+]$ the concentration of the M^+ bound channels and hence the M^+ transport rate will increase, making it much faster than $1/\tau$ at pH ~6. In Fig. 3 *a*, $1/\tau$ at pH ~6 is greater than $1/\tau$ at pH ~7.5. Thus the M^+ transport step is much faster than $1/\tau$ at pH ~7.5 and therefore does not limit the ΔpH decay rate at pH ~7.5 when the "second mechanism" is dominant.

2. Moreover, if the compensating charge flux created by the transport of M^+ through the gramicidin channels does not limit the rate in the "second mechanism," b_i/τ at low $[H^+]$ should not increase with $[M^+]$. This is seen in Fig. 3 *b*. Such a conclusion enables us to write $k_1k_3/(k_2 + k_3) \approx k_1k_3/k_2$.

3. We note from Eq. 10 that when the first term becomes less dominant with higher pH, $1/\tau$ should increase with pH because of the dominant contribution from the second term. The τ data given in Fig. 3 *a* in the higher pH region and the b_i/τ plots in Fig. 3 *b* in the small $[H^+]$ region are consistent with this prediction.

4. The OH^- transport can be either by diffusion across the membrane or by propagation through the water chains/hydrated pores in the membrane. The diffusion mechanism would require an activation energy at least comparable to

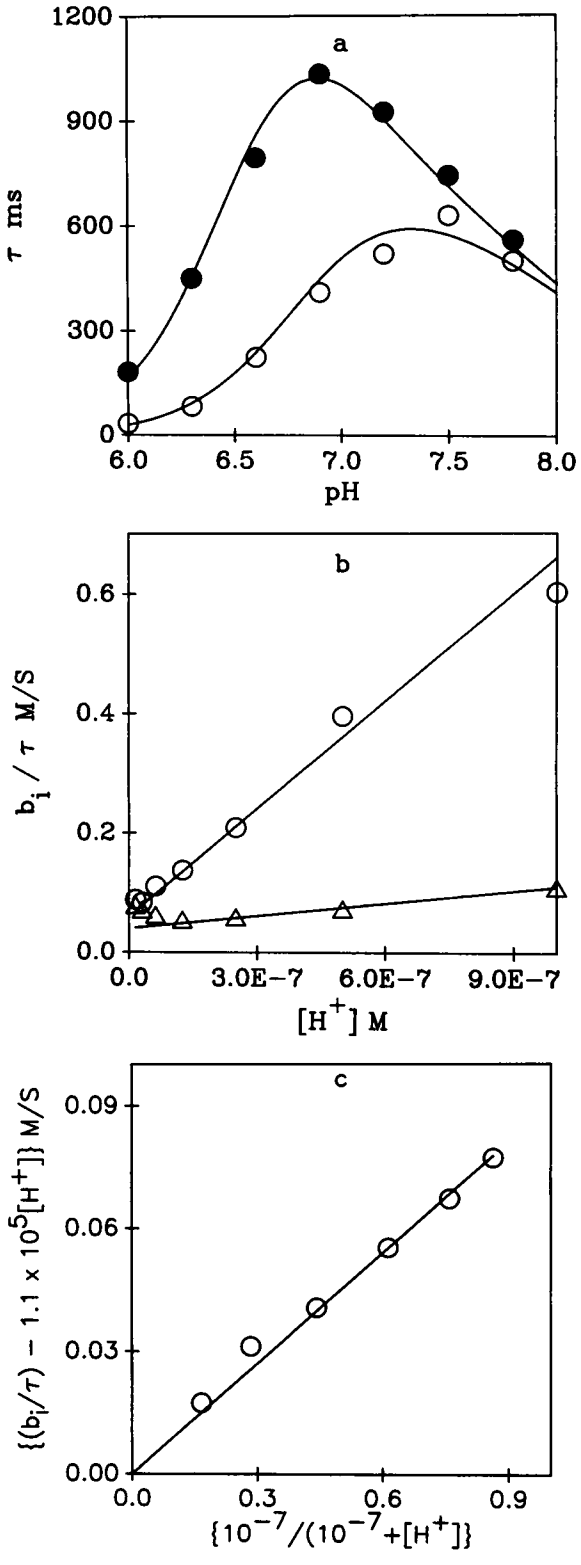

FIGURE 3 (*a*) pH dependence of ΔpH relaxation time τ in SBPL vesicle solutions with gramicidin; [lip] = 5 mM, $[\text{gramicidin}]_0$ = 75 μM, inside vesicles 50 mM phosphate, outside vesicles 25 mM ACES and [KCl] = 50 mM, ○; [KCl] = 300 mM, ●. (*b*) The plots obtained after factoring out the pH dependence of b_i from that of τ in *a*, corresponding to [KCl] = 50 mM, ○; [KCl] = 300 mM, △. (*c*) The plot of the second contribution (obtained by subtracting a contribution linearly varying with $[H^+]$ in b_i/τ in *b*) for [KCl] = 300 mM as a function of $K/(K + [H^+])$, where pK = 7. The solid lines were calculated using Eq. 10 as described in the text.

that required for the translocation of the electrically neutral proton-bound or metal ion-bound nigericin (~15 kcal/mol; Prabhananda and Kombrabail, 1995). On the other hand, OH^- propagation through water chains, similar to the H^+ conduction in ice by hydrogen bond exchange in the water chain (Eigen, 1964), would require much less activation energy. The calculations by Parsegian (1969) show a smaller energy barrier (~5.6 kcal/mol for an ion of radius 2 Å) for the crossing of an ion across low-dielectric membrane through hydrated pores of radius 5 Å than that for its transport by diffusion, which requires partitioning of the ion to the nonpolar region in the membrane. The apparent activation energy determined from the temperature dependence of τ (Fig. 4 *b*) at pH ~7.5-7.8 (when the "second mechanism" is dominant) is ~3.2 kcal/mol and favors the mechanism of OH^- propagation through water chains in the membrane.

5. If the OH^- propagating water chain is close to a positively charged group on the lipid (presumably choline), $[H^+]_m$ (as $[H_3O^+]_m$) in its proximity will be small because of electrostatic repulsion. Furthermore, because the vesicles are suspended in the aqueous medium, the concentrations of water and OH^- partitioned to the relevant membrane region can be taken to be a constant for a given vesicle preparation. Therefore it should be possible to use a constant value for C_0 in Eq. 10 when accounting for the $1/\tau$ observed in a given vesicle preparation. We also note that the dissociation constants of ionizable groups can change in the membrane environment (Haines, 1983). The apparent pK of a water molecule close to a positive charge of the metal ion at the active site of carbonic anhydrase has been assigned a value of ~7 (Lindskog et al., 1971). In view of this, we can expect a pK close to this value in Eq. 10, for the water near the positively charged choline. In this case, the second term of Eq. 10 predicts that the contribution to $1/\tau$ from the "second mechanism" should vary linearly with $K/(K + [H^+])$, where the pK is not much different from ~7. The $1/\tau$ data for [KCl] = 300 mM in Fig. 3 *a*, when plotted against $K/(K + [H^+])$ for various values of K (after subtracting the contribution from the "first mechanism" estimated from the data at pH ~6 and after factoring out the term b_i), were linear for pK = 7 (Fig. 3 *c*).

6. Adding small amounts of chloroform to vesicle solutions can change the membrane order and thereby change C_0. However, such an addition is unlikely to affect the gramicidin channels already formed. Therefore, changes in τ at pH ~7.5 (when the "second mechanism" is dominant) on adding a small amount of chloroform should be larger than that at pH ~6 (when the "first mechanism" is dominant). This prediction was tested by adding 10 μl chloroform to vesicle solutions having [lip] = 5 mM. Consistent with the prediction, τ changed by a smaller factor at pH ~6 (135 ms to 110 ms) when compared to that at pH ~7.5 (610 ms to 315 ms). As mentioned above, the significant intercept of the plot in Fig. 2 *b* also shows that in the rate-determining step of the "second mechanism," transport through gramicidin channels is not involved.

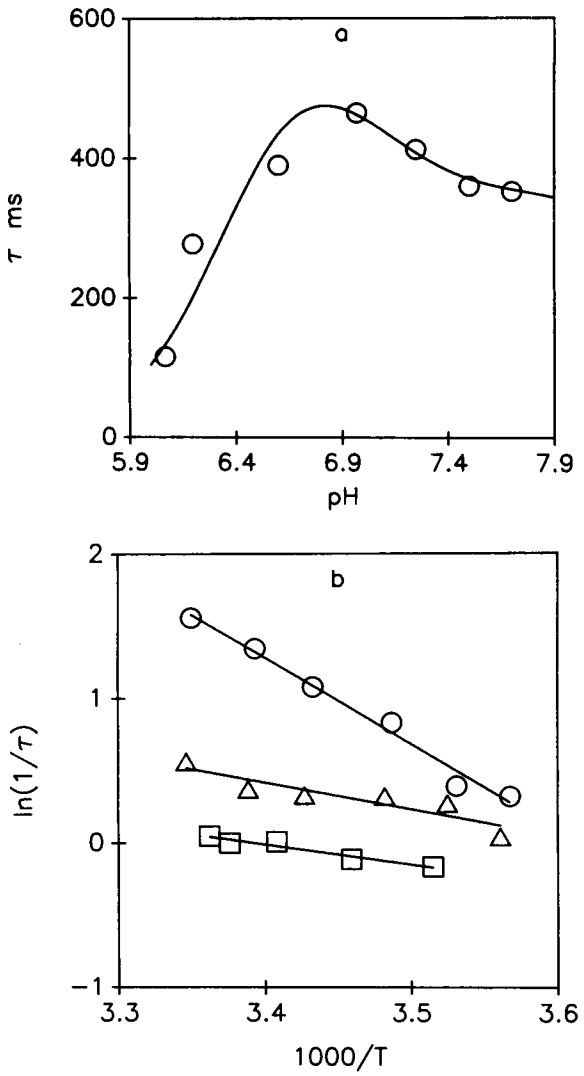

FIGURE 4 (*a*) pH dependence of ΔpH relaxation time τ in gramicidin-loaded vesicle solutions prepared from lipid mixtures containing 4.8 mM egg phosphatidylcholine and 0.3 mM phosphatidic acid. $[\text{gramicidin}]_0$ = 75 μM, inside vesicles 50 mM phosphate, outside vesicles 25 mM ACES and [KCl] = 300 mM. Equation 10 was used to calculate the solid lines as described in the text. (*b*) The dependence of τ (in seconds) on temperature (T in K) at pH 6.1 ○, pH 7.5 □, and pH 7.8 △ in SBPL vesicle solutions containing gramicidin ([lip] = 5.1 mM, $[\text{gramicidin}]_0$, = 75 μM, inside vesicles 50 mM phosphate, outside 25 mM ACES, [KCl] = 300 mM).

7. In the absence of gramicidin, the first term of Eq. 10 will be absent. Furthermore, the M^+ transport will be slower, making k_1 small and $k_2 \ll k_3$. Nevertheless, Eq. 10 shows that the pH-dependent factor $K/(K + [H^+])$, with pK ~7 (associated with the second term), should be detectable even in the absence of gramicidin in lipid vesicles that show the presence of the "second mechanism." The data in Fig. 4 *a*, obtained for a lipid mixture of 94% egg phosphatidylcholine and 6% phosphatidic acid loaded with gramicidin, show a decrease in τ with an increase in pH in the high-pH region similar to that from a dominant "second mechanism." The data in the absence of gramicidin, obtained in a similar mixture and given in Fig. 3 of the paper by Nichols and Deamer (1980), show a pH dependence close to that expected. However, a contribution at lower pH from H^+ transport through the water chains cannot be excluded in such data.

8. Equation 10 also predicts that the data obtained with two different [lip], keeping $[\text{gramicidin}]_0$ a constant, should show larger changes at pH ~6 than at pH ~7.5. This prediction was tested by a typical experiment with [KCl] = 300 mM, $[\text{gramicidin}]_0$ = 35 μM, 50 mM phosphate buffer inside vesicles, and 25 mM ACES outside vesicles. Our observations showed that on changing [lip] from 2.3 mM to 4.6 mM, τ changed from 87 ms to 192 ms at pH ~5.9 and from 510 ms to 580 ms at pH ~7.6. These changes are consistent with the expected behavior and are close to the values calculated using Eq. 10 and parameters given below.

Parameters in Eq. 10 that predict the τ data in Figs. 2, 3, and 4

The solid lines in Figs. 2, 3 and 4 *a* have been calculated using Eq. 10 and $k_H^*(K_K/K_H) \approx 1.5 \times 10^6\ s^{-1}$ and $C_0k_1k_3/(k_2 + k_3) \approx 0.04\ M\ s^{-1}$ with variations within ±30%. (The variations in the parameters used to get good agreement with the observed data are presumably associated with differences in vesicle preparations and variations in the temperature.) C_0, which depends on the partitioning of the water to the membrane, can be very small, making the second term small. Because $K_K > K_H$, $k_H^* < 1.5 \times 10^6\ s^{-1}$. With such a limit for its value, k_H^* associated with the H^+ transport through the gramicidin channel can have a reasonable value, because it can be greater than the rate constant associated with the translocation of proton-bound or metal ion-bound nigericin (~$6 \times 10^3\ s^{-1}$) in SBPL vesicles (Prabhananda and Ugrankar, 1991).

Experiments that eliminate other explanations

Because gramicidin channels are known to enhance the transport rate of anions obtained by the deprotonation of acids (Ahmed and Krishnamoorthy, 1990) and because acids can transport H^+, it is attractive to suggest that the "second mechanism" is associated with anion transport. These could be due to Cl^- already present in vesicle solutions or free acid impurities in SBPL. The "second species" are unlikely to be Cl^- in view of the negligible increase in $1/\tau$ on increasing [KCl] at higher pH (see Fig. 3, *a* and *b*). This conclusion is further supported by our observation that replacing the salt KCl by KNO_3 does not affect the ΔpH decay rate significantly. (For example, in SBPL vesicle solutions at pH ~7.5 ([lip] = 5 mM, 50 mM phosphate buffer inside and 25 mM ACES outside vesicles and $[\text{gramicidin}]_0$ = 26 μM), the τ with [KCl] = 300 mM was ~0.95 s; with a mixture of [KCl] = 50 mM and $[KNO_3]$ = 250 mM τ was ~1.0 s.) The "second contribution" to the ΔpH decay is also not associated with free acid impurities in

SBPL vesicles. This could be inferred by preparing vesicles using lipid from a different source, which is unlikely to have impurities similar to that in SBPL. The data in Fig. 4 *a* obtained using 94% egg phosphatidylcholine and 6% phosphatidic acid lipid mixture also require us to assume the presence of "second species" at concentrations comparable to that in the vesicles prepared from SBPL.

Apart from Fig. 1 and the experiments of Ahmed and Krishnamoorthy (1990), the following argument also excludes changes in the membrane order on adding gramicidin as the source of the behavior attributed to the "second mechanism." Our observations (Prabhananda and Kombrabail, 1995) have shown that the changes in the membrane order causing increases in transport rate constants on adding membrane-permeant species is smaller at higher pH than at lower pH in the pH range 6–8. Therefore, such a change is unlikely to have caused changes in $1/\tau$ similar to that assigned to the "second mechanism" (which show an increase in $1/\tau$ with an increase in pH).

Discussion of the literature data using the above models

The ratio of the dissociation constants K_{Na}/K_K associated with the ion binding in the gramicidin channel reported in the literature by different authors shows substantial variation from ~1 to ~14 (see table 2 of Dani and Levitt, 1981). We can determine this ratio from τ data, also by using Eq. 4. In SBPL vesicle solutions containing a mixture of 50 mM KCl + 250 mM MCl ([lip] ~5 mM, $[\text{gramicidin}]_0$ ~75 μM, inside vesicles 50 mM phosphate, outside 25 mM ACES buffer, pH 6.1), τ ~190 ms and 133 ms were observed for $M^+ = K^+$ and Na^+, respectively. These data enable us to make an estimate of K_{Na}/K_K for the gramicidin channel-bound cations, assuming the "first mechanism" to be dominant at pH ~6.1. Our estimate (~1.5) is close to those given by Lauger (1973) and Eisenman et al. (1978). In a similar experiment at pH ~7.5, τ (~800 ms) did not change on changing M^+ from K^+ to Na^+, confirming our conclusion that the "first mechanism" is not dominant at this pH. We also note that the analysis of the τ data obtained with gramicidin at pH 7.5 reported in the literature (Krishnamoorthy, 1986) has to be revised, because it does not take into account the "second mechanism," which makes a >80% contribution to the ΔpH decay rate at this pH.

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