Temperature Jump as a New Technique To Study the Kinetics of Fast Transport of Protons across Membranes

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ABSTRACT: Application of a temperature jump (2.5 °C) to a suspension of liposomes, having phosphate ($\Delta pK/\Delta T \sim 0.005$) as the internal buffer and tris(hydroxymethyl)aminomethane ($\Delta pK/\Delta T \sim 0.031$) as the external buffer, created a ΔpH ($pH_{in} - pH_{out}$) of positive sign in ca. 5 μs. Decay of this ΔpH was monitored by using the fluorescent pH indicator 8-hydroxy-1,3,6-pyrenetrisulfonic acid entrapped inside the liposome. This technique is useful to study transmembrane proton movement in the time range 5 μs–10 s at physiological pH values. The kinetics of proton transport aided by ion carriers such as nigericin, monensin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and valinomycin were studied by our method. The electrogenic nature of transport by CCCP and valinomycin and electroneutral ion transport by nigericin and monensin were shown. From the kinetics of proton transport aided by gramicidin, the time-averaged single-channel conductance of gramicidin channels was estimated to be $(2.1 \pm 0.5) \times 10^{-16}$ S for H^+ at pH 7.5.

The electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$ across membranes has been recognized as the obligatory intermediate in biological energy transduction [Mitchell (1961, 1979); see Skulachev and Hinkle (1981) for a review]. The maintenance of $\Delta\tilde{\mu}_{H^+}$ depends on the kinetics of its decay via various pathways. These pathways include (1) nonspecific leakage of ions through membranes and (2) specific movement of ions either through channels or aided by ion carriers. The kinetics of movement of protons across membranes is an important aspect in the study of energy coupling (Krishnamoorthy & Hinkle, 1984). The transport of protons by carriers and channels has been studied mainly by conductance measurements across planar lipid bilayers [e.g., Benz and McLaughlin (1983), Schindler and Nelson (1982), and O'Shaughnessy and Hladky (1983)]. Although being a powerful technique in the study of ion transport, conductance measurement has the following disadvantage in the study of proton currents. The ionic currents measured may not be specific to protons at physiological pH values due to a very low concentration of protons compared to other ions in the system. Further, electroneutral ion carriers (exchangers) such as nigericin and monensin cannot be studied by conductance measurements. Also, the conductance method is not easily applicable to vesicular systems and whole cells. In this paper we present a new method using temperature-jump (T-jump) technique (Eigen & DeMaeyer, 1966) to study the kinetics of fast movement of protons across lipid vesicle membranes. In this method, application of a T-jump[1] to a suspension of liposomes that had an external buffer having a high value of $\Delta pK/\Delta T$ created a ΔpH across the membrane. The decay of ΔpH was monitored by the fluorescent pH indicator pyranine (Kano & Fendler, 1978; Clement & Gould, 1981a) entrapped inside the liposome.

Materials and Methods

Pyranine-loaded soybean lipid vesicles were prepared by sonicating acetone-washed asolectin (Kagawa & Racker, 1971) at 50 mg/mL in a buffer containing 150 mM KCl, 20 mM KH_2PO_4, and 1 mM pyranine at pH 7.5. The sonication was carried out in a nitrogen atmosphere in a bath-type sonicator (Imeco Ultrasonics, Bombay). The extent of sonication was controlled by monitoring the optical density due to turbidity at 700 nm (0.35 at 1-cm-path length). Similar optical density values were obtained when the sonication was performed in a probe-type sonicator (Branson) at 70 W for about 30 min. Vesicles prepared in the bath-type sonicator were used in all our experiments. Removal of the external pyranine and change of the external buffer to Tris were carried out by passage through a Sephadex G-50 column eluted with a buffer containing 150 mM KCl and 20 mM Tris at pH 7.5. The phospholipid concentration of eluted liposomes was determined by inorganic phosphate assay (Ames, 1966). For some experiments, the vesicle suspension, after passage through Sephadex G-50, was subjected to a high-speed centrifugation (Barenholz et al., 1977) in order to remove any large size and/or multilamellar liposomes. Inorganic phosphate assays showed ~95% recovery in the top layer of the supernatant. Proton flux measurements (see Results) carried out with vesicles from the supernatant did not show any significant difference from uncentrifuged suspension, indicating the homogeneity of our sonicated preparations. The homogeneity was further checked by negatively stained electron microscopy (Johnson et al., 1971) which showed single-bilayer vesicles with diameters in the range 200–400 Å. Nearly 70% of the vesicles had diameters ~300 Å.

T-jump experiments were performed in the homemade joule-heated instrument (Prabhananda, 1977). The temperature-jump cell had 1.1 mL of the liposome suspension (~3 mg of lipid/mL) thermostated at 25 °C. The excitation wavelength was 455 nm, and the emission was collected through a combination of a 500-nm cutoff filter and a 520-nm bandpass filter. An EMI 9558 QB photomultiplier was used to detect the fluorescence. Typically, temperature jumps of 2.5 °C were given. Following the temperature jump, the fluorescence signal was recorded on a storage oscilloscope and photographed. The photographs were then projected, and the initial rates were measured from the apparent "exponential" traces.

The internal buffering capacity of the liposomes was esti-

[1] Abbreviations: pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonic acid; T-jump, temperature jump; Tris, tris(hydroxymethyl)aminomethane; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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mated in the following way. The liposomes were titrated by using standard HCl in the presence and absence of nigericin in a recording pH meter to get the total and the external buffering capacities, respectively. The K^+/H^+ exchanger nigericin made the internal aqueous phase accessible to pH titration. The difference in these two buffering capacities was taken as the internal buffering capacity, which was 28 nmol of H^+ (pH unit)$^{-1}$ (mg of lipid)$^{-1}$ at pH 7.5 for soybean lipid vesicles.

The ionophores were added as ethanol solutions. When gramicidin (Sigma Chemical Co.) was added, the measurements were done after incubation at room temperature (20 °C) for 30 min to ensure the equilibrium distribution of gramicidin among vesicles. Control experiments showed that the above treatment is necessary and sufficient to reach the equilibrium distribution.

Results

Method of Measurement of Fast Transport of Protons. The basic system used in this work was pyranine (fluorescent pH indicator, $pK_a \sim 7.2$) entrapped in liposomes suspended in Tris buffer at pH 7.5. The internal buffer was potassium phosphate. The value of $\Delta pK/\Delta T$ of Tris is 0.031, whereas that of phosphate is 0.005. Hence a ΔpH ($pH_{in} - pH_{out}$) of positive sign is expected to be created on the application of a T-jump to this system. The magnitude of ΔpH can be estimated from the value of ΔT (2.5 °C for a discharge of 15 kV) to be 0.065.

The change in pyranine fluorescence observed subsequent to a T-jump in this system was characterized by a fast ($t_{1/2} < 5$ μs) decrease in fluorescence followed by a constant level of fluorescence (Figure 1A, trace a). Addition of nigericin (a H^+/K^+ antiporter) resulted in another and stronger phase of fluorescence decrease, the rate of which increased with increasing concentration of nigericin (traces b–d). The unresolved fast phase is likely to arise from the pH titration of residual pyranine in the external medium. The slower decrease in fluorescence, which presumably arises from the pH equilibration of the inner aqueous phase containing the fluorescent pH indicator, is the subject of this paper. The amplitude of the unresolved fast phase was less than 10% of the total signal (Figure 1A), contrary to the observation of Clement and Gould (1981a). Further, this fast phase was insensitive to the ionophore added. Figure 1B shows that when the two buffers were interchanged between the two phases (Tris inside and phosphate outside), the direction of the relaxation signal reversed. This confirms that the observed slower changes in fluorescence were indeed due to the decay of ΔpH and not due to any other interaction. The amplitude of the fluorescence change observed in the latter case (Figure 1B) was much smaller than that in Figure 1A, indicating a smaller magnitude of ΔpH created by the T-jump. It is likely that the internal phase is buffered dominantly by endogenous buffering groups in the lipid rather than by the buffer salt trapped inside. This would lead to a smaller ΔpH in the experiment shown in Figure 1B if the value of $\Delta pK/\Delta T$ of the dominant buffering groups in the lipid is lower than that of Tris buffer. The time constant associated with the creation of ΔpH was found to be close to the heating time (~ 5 μs) from the change in the fluorescence of pyranine in Tris buffer in a liposome-free system. The ΔT created by the T-jump decayed with a time constant ~ 15 s. The experiments described in this paper were performed with vesicles prepared from soybean phospholipids (Kagawa & Racker, 1971), which have been used extensively in reconstituting ion pumps. However, similar results were obtained with other lipids such as dimyristoylphosphatidylcholine (data not shown).

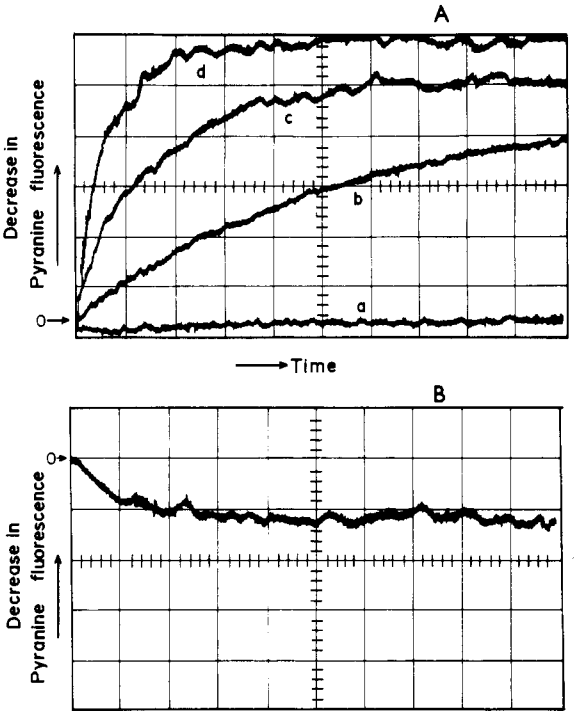

FIGURE 1: (A) Typical oscilloscope traces showing the decrease in fluorescence of the entrapped pyranine as a function of time following the T-jump in a suspension of soybean lipid vesicles (3 mg of lipid/mL). The internal medium was 150 mM KCl, 20 mM KH_2PO_4, and 1 mM pyranine at pH 7.5, and the external buffer was 150 mM KCl and 20 mM Tris at pH 7.5. The temperature was 25 °C, and ΔT was 2.5 °C. The time bases (ms/major division) and the concentrations of nigericin were (a) 100 ms and 0, (b) 100 ms and 0.09 μM, (c) 1 ms and 36 μM, and (d) 1 ms and 90 μM. The origin (i.e., the level of fluorescence before the T-jump) for each trace is shifted vertically to avoid conjestion. The symbol 0→ indicates the origin corresponding to trace c. (B) Oscilloscope trace showing the increase in pyranine fluorescence following the T-jump. The internal and external buffers were 20 mM Tris and 20 mM KH_2PO_4 (reverse compared to Figure 1A). The time base was 1 ms/major division, and the concentration of nigericin was 90 μM. Other conditions were the same as in Figure 1A.

The equivalent proton flux per unit ΔpH, J_{H^+/OH^-}, in units of μmol·min^{-1}·(mg of lipid)$^{-1}$·(ΔpH unit)$^{-1}$ was determined from the initial slopes of traces shown in Figure 1 and by assuming a linear relationship between J_{H^+/OH^-} and the driving force ΔpH (Mitchell & Moyle, 1967). The initial rates in terms of fluorescence intensity per minute were converted to internal pH per minute by using the calibrations of fluorescence intensity vs. pH. The internal buffering capacity of the vesicles (see Materials and Methods) was then used to get the J_{H^+/OH^-} in units of μmol·min^{-1}·(mg of lipid)$^{-1}$·(ΔpH unit)$^{-1}$.

Proton Transport Aided by Ion Carriers. Protonophores such as CCCP or SF 6847 did not catalyze the decay of ΔpH when present alone (data not shown), presumably due to the opposing diffusion potential created by H^+/OH^-. Inclusion of the K^+-uniporter valinomycin accelerated ΔpH decay by collapsing the diffusion potential. At high concentrations of valinomycin, J_{H^+/OH^-} was linearly proportional to the concentration of the protonophore CCCP (Figure 2A), showing that the rate of ΔpH decay was limited by transport of the proton itself. The flux J_{H^+/OH^-} in units of μmol·min^{-1}·(mg of lipid)$^{-1}$·(ΔpH)$^{-1}$ can be converted into electrical units (amperes) by using the Faraday constant. The surface area of the membrane was calculated by using the value 4×10^3 cm^2·(mg of lipid)$^{-1}$ (Mimms et al., 1981). The driving force, ΔpH, was converted into volts by using the relationship 1 ΔpH unit = 0.059 V. Typical conductance values calculated in this

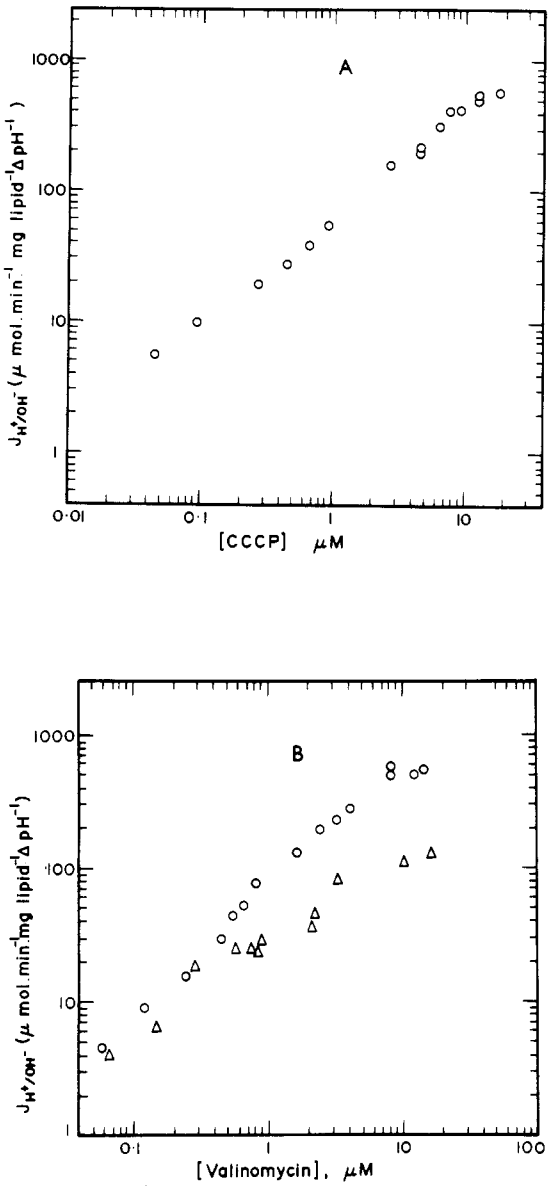

FIGURE 2: (A) Dependence of the equivalent proton flux per unit ΔpH, J_{H^+/OH^-}, on the concentration of the protonophore CCCP. The experimental conditions were the same as in Figure 1A. The vesicle suspension also had 16.4 μM valinomycin. The $t_{1/2}$ values of ΔpH-decay curves corresponding to J_{H^+/OH^-} values plotted in the figure were in the range of 2–300 ms. The value of J_{H^+/OH^-} in the absence of CCCP was ~1 μmol·min^{-1}·(mg of lipid)$^{-1}$·(ΔpH)$^{-1}$. (B) Dependence of J_{H^+/OH^-} on the concentration of valinomycin at 18.2 μM CCCP (○) and 1.82 μM CCCP (△). The value of J_{H^+/OH^-} in the absence of valinomycin was less than 0.1 μmol·min^{-1}·(mg of lipid)$^{-1}$·(ΔpH)$^{-1}$.

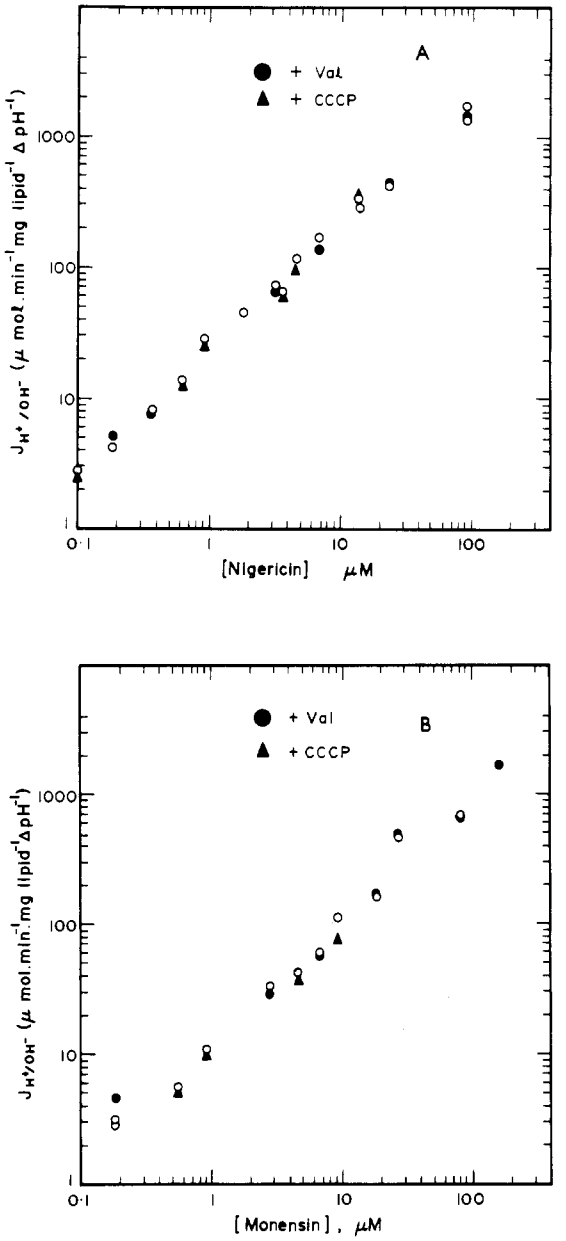

FIGURE 3: Dependence of J_{H^+/OH^-} on the concentration of nigericin (A) and monensin (B): (○) in the absence of valinomycin or CCCP; (●) in the presence of 2.4 μM valinomycin; (▲) in the presence of 27 μM CCCP. The $t_{1/2}$ values of ΔpH-decay curves corresponding to these data were in the range of 0.5–400 ms.

way were (4.1 ± 0.6) × 10^{-4} S cm^{-2} and (2.7 ± 0.3) × 10^{-3} S cm^{-2} at 1 μM and 10 μM CCCP, respectively. These values can be compared with 5.0 × 10^{-3} S cm^{-2} for 1 μM FCCP (Benz & McLaughlin, 1983) and 6.0 × 10^{-5} S cm^{-2} for 10 μM CCCP (O'Shaughnessy & Hladky, 1983). The difference in the estimates could arise from the following differences between our method and conductivity experiments: (1) the driving force in our experiment was ΔpH, in contrast to electrical potential, (2) differences in the type of lipid used, and (3) the presence of organic solvents in black lipid membranes used in conductivity measurements.

At lower concentrations of valinomycin, J_{H^+/OH^-} was dependent on the concentration of valinomycin (Figure 2B), showing the limiting of J_{H^+/OH^-} by the movement of the counterion, K^+. CCCP was present in excess in these experiments. This situation allows us to calculate the conductivity due to the movement of valinomycin–K^+ complex. The driving force, in this case, is the diffusion potential created by ΔpH/CCCP. The conductivity calculated from the data in Figure 2B was (3.4 ± 0.5) × 10^{-3} S cm^{-2} at 10 μM (aqueous concentration) valinomycin. This value may be compared with 7.5 × 10^{-3} S cm^{-2} measured in dioleoyllecithin planar membranes (Stark & Benz, 1971). Clement and Gould (1981b) used a similar system to measure the kinetics of valinomycin-mediated K^+ transport. Since they measured only the relative rates it is not possible to compare our data with theirs. Also, the absence of any protonophore in their system makes the comparison difficult.

Figure 3 shows the linear dependence of J_{H^+/OH^-} on the concentrations of H^+/K^+ antiporters nigericin and monensin. Separate additions of either a protonophore (CCCP) or the K^+ uniporter valinomycin to this system had no effect on J_{H^+/OH^-} (Figure 3, ● and ▲). This shows the strict electroneutral exchange behavior of nigericin and monensin. Com-

parison of parts A and B of Figure 3 shows that nigericin is more efficient than monensin especially at lower concentrations, in accordance with earlier reports (Pressman & Heeb, 1972; Henderson et al., 1969). The turnover rates calculated from Figure 3 were 68×10^3 and 24×10^3 protons translocated per minute per unit ΔpH for nigericin and monensin, respectively.

The ion carriers used in this study (Figures 2 and 3) were effective even at concentrations as low as 0.1 μM. This concentration would correspond to a ratio of one molecule of the carrier to about 10 vesicles [assuming an average of 3000 phospholipid molecules per vesicle (Watts et al., 1978)]. Further, the ΔpH-decay curves were monophasic at all concentrations of the ionophore used (Figure 1A). These observations show that these ionophores could exchange freely between vesicles in the time scales (ms) of our experiments.

Proton Transport through Gramicidin Channels. In contrast to ion carriers, the channel-forming ionophore, gramicidin, did not show exchange between vesicles during the time scale of our observation (ms) as expected. In this case, the amplitude of the fluorescence change increased with the concentration of gramicidin (Figure 4A), similar to the observations of Clement and Gould (1981c). The expected increase in the fraction of gramicidin-incorporated liposomes with an increase in gramicidin concentration in the suspension could explain this observation. The value of J_{H^+/OH^-} (which is normalized to the total amplitude of fluorescence change) remained nearly constant until about 2 μM gramicidin and then showed an increase with increasing concentrations of gramicidin (Figure 4B). Further, J_{H^+/OH^-} values did not increase in the presence of valinomycin (Figure 4B), showing that proton conductance through gramicidin channels was not limited by movement of counterions, similar to the conclusion by Clement and Gould (1981c). They measured the kinetics of development of ion flux through gramicidin channels and not the kinetics of ion movements.

It is known that the functional unit of the gramicidin channel is a dimer [e.g., Hladky and Haydon (1984) for a review]. Hence we expect the vesicles that show ΔpH decay to have at least one dimer of gramicidin per vesicle. The total number of gramicidin dimers in the system can be calculated from the concentration of gramicidin and the total surface area of the membrane (4×10^3 cm^2·(mg of lipid)$^{-1}$) (Mimms et al., 1981) by using the dimerization constant 6×10^{13} mol^{-1}·cm^2 (Veatch et al., 1975). The validity of this calculation rests on the assumption that gramicidin molecules can exchange between vesicles during the process of equilibration (30 min at 20 °C; see Methods). That the gramicidin molecules can exchange between vesicles was shown in the following experiment. Pyranine-loaded vesicles having high concentrations (14 μM aqueous concentration) of gramicidin were mixed with an equal volume of gramicidin-free vesicles. The amplitude of the fluorescence signal following the T-jump decreased by a factor of 2 on mixing, as expected. However, the fluorescence signal increased with time and reached 100% in about 20 min. This process ($t_{1/2} \sim 10$ min) is interpreted as equilibration of gramicidin among vesicles. The final concentration of phospholipid used in all our experiments was 2.7 mM. This would correspond to a vesicle concentration of about 0.9 μM if we assume an average vesicle to have ~3000 phospholipid molecules (Watts et al., 1978). The distribution of gramicidin dimers among the vesicles can then be calculated from the Poisson equation (Forster & Selinger, 1964; Khuanga et al., 1976)

$$P(x) = e^{-\mu}\mu^x/x!$$

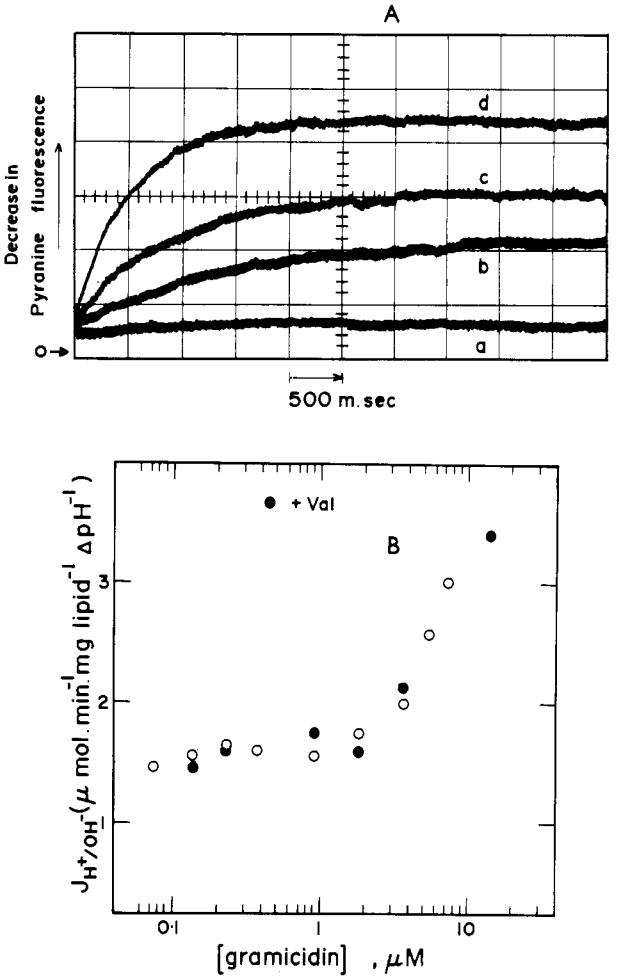

FIGURE 4: (A) Typical oscilloscope traces showing the increase in the amplitude of ΔpH-decay curves with increasing concentrations of gramicidin. The experimental conditions were the same as in Figure 1A except for the presence of gramicidin whose concentrations were 0 (a), 0.91 μM (b), 1.82 μM (c), and 7.3 μM (d). The symbol 0→ represents the origin corresponding to all the traces. (B) Plot of J_{H^+/OH^-} as a function of the concentration of gramicidin: (○) in the absence of valinomycin; (●) in the presence of 1.6 μM valinomycin. All the values of J_{H^+/OH^-} were normalized to the total amplitude corresponding to that of trace d in Figure 4A. The $t_{1/2}$ values were in the range of 0.4–1 s. Other experimental conditions were the same as in Figure 1A.

where $P(x)$ is the fractional population of vesicles with x number of gramicidin dimers per vesicle and μ is the ratio of the total number of gramicidin dimers and the vesicle. At 0.91 μM aqueous concentration of gramicidin (trace b in Figure 4A), the total concentration of the dimer can be calculated to be 0.33 μM. Hence, $\mu = 0.37$, and the values of $P(0)$, $P(1)$, $P(2)$, and $P(>2)$ are 0.69, 0.26, 0.05, and <0.01, respectively. Veatch et al. (1975) have shown that all the dimers are channels. Hence 69% of vesicles are expected to have no channel in them, and 26% will have with one channel incorporated. It is gratifying to note that trace b (Figure 4A) has nearly 30% of the total amplitude (trace d) of the fluorescence change, in agreement with the above calculation. Similarly, at 1.82 μM aqueous concentration of gramicidin ($\mu = 0.82$, trace c) the values of $P(0)$, $P(1)$, $P(2)$, and $P(>2)$ are 0.44, 0.36, 0.15, and 0.05, respectively. The observed 60% amplitude in trace c agrees once again with the 56% predicted by the above calculation. It should be mentioned that if we assume an average of 6000 phospholipid molecules per vesicle, the predicted amplitudes of traces b and c are 52% and 80%, respectively, in contrast to our observations. At $\mu < 1$ (aqueous

concentration of gramicidin less than 2 μM), the fraction of vesicles having more than one channel is expected to be much smaller than the fraction of vesicles with one channel. This would explain the invariance of J_{H^+/OH^-} up to $\sim$2 μM gramicidin and the increase at higher concentrations (Figure 4B). The time-averaged H^+ conductance of a single gramicidin channel can be estimated from the value of J_{H^+/OH^-} at $\mu < 1$. The flux of protons per gramicidin channel was calculated from the unnormalized value of J_{H^+/OH^-} (0.45 μmol·min^{-1}·(mg of lipid)$^{-1}$·(ΔpH)$^{-1}$ at 0.91 μM gramicidin, for example) and the total number of vesicles having at least one gramicidin dimer [$P(>0)$]. The conductance calculated in this way was (2.1 $\pm$ 0.5) $\times$ 10^{-16} S at pH 7.5 and a ΔpH equivalent to 3.8 mV. Divalent cations Ca^{2+} and Ba^{2+} have been shown to block the gramicidin channel (Bamberg & Laüger, 1977). Inclusion of 0.1 M of divalent cations reduced the H^+/OH^- fluxes in our system. The proton conductances calculated were 1.4 $\times$ 10^{-16}, 1.1 $\times$ 10^{-16}, and 1.1 $\times$ 10^{-16} S in the presence of Mg^{2+}, Ca^{2+}, and Ba^{2+}, respectively.

Addition of a protonophore such as CCCP or SF 6847 enhanced the rate of decay of ΔpH by gramicidin. In this system, presumably, H^+ was transported by the protonophore and the counterion K^+ by gramicidin channels. The K^+ conductance through the gramicidin channel can be estimated when the rate of decay of ΔpH is limited by K^+ movement through the channel and not by H^+ movement aided by the protonophore. This condition may be achieved by the use of very high concentrations of protonophore in vesicles containing gramicidin. However, the rates that increased with the increase in the concentration of SF 6847 did not show saturation even at the highest concentration (180 μM) of SF 6847 used. At 0.91 μM gramicidin and 180 μM SF 6847, the $t_{1/2}$ of ΔpH decay was $\sim$200 μs corresponding to $J_{H^+/OH^-} \sim 3$ mmol·min^{-1}·(mg of lipid)$^{-1}$·(ΔpH unit)$^{-1}$. This allows us to estimate the lower limit of the time-averaged single-channel conductance for K^+ as $\sim 1.1 \times 10^{-12}$ S.

Discussion

Creation of ΔpH across vesicle membranes by T-jump and subsequent detection of the decay of ΔpH by the use of entrapped fluorescent pH indicator pyranine has been shown as a useful technique to study the kinetics of proton transport. The rapid nature of ΔpH creation ($\sim$5 μs) and the slow decay of ΔT ($\sim$10 s) allow us to monitor proton movements in a wide time window of 5 μs–10 s. The superiority of our technique over conductance measurements across planar lipid bilayers lies in the specificity of the measurement to protons. The specificity of the latter technique to protons is questionable at physiological pH values where the concentration of protons is nearly 6 orders smaller than that of other ions in the system. At low pH values, lipid bilayers are unstable, leading to nonspecific ion channels (Kaufmann & Silman, 1983). Also, electroneutral ion carriers such as nigericin and monensin cannot be studied by conductance measurements. The obvious advantage of our method over the stopped-flow technique is the better time resolution. Further, the ability to perform repeated ΔpH creation–decay cycles in the same sample by our method reduces the amount of samples required.

The usefulness of our method has been exemplified by some representative experiments on ionophore-aided proton transport (Figures 2–4). The strict electroneutral nature of ion transport by nigericin and monensin and the electrogenic transport by valinomycin and CCCP have been shown. The conductance value calculated for valinomycin was similar to that obtained by other workers. Our system would be very useful in testing the various models proposed for the transport of protons by uncouplers [Terada (1981) for a review]. We have estimated the time-averaged conductance of a single gramicidin channel to be (2.1 $\pm$ 0.5) $\times$ 10^{16} and $>1.1 \times 10^{-12}$ S for H^+ and K^+, respectively, at pH 7.5. Our estimates would correspond to the product of single-channel conductance and the fractional mean channel open time. Hladky and Haydon (1972) have measured the single-channel conductance of gramicidin to be 2.2 $\times$ 10^{-10} and 2.8 $\times$ 10^{-11} S for H^+ (at pH 2) and K^+, respectively. The difference ($\sim 10^6$) in the estimates of the proton conductance values is likely to be caused by (1) the difference in proton concentration ($\sim 10^6$) in the two experiments and (2) the fractional mean channel open time <1. Further, the driving force in our experiments is ΔpH rather than $\Delta\psi$ used in conductance measurements. The dependence of J_{H^+/OH^-} on the type of driving force has been observed in the case of unaided transport (Krishnamoorthy & Hinkle, 1984). Though our estimate is likely to be less accurate (due to possible heterogeneity in the vesicle–gramicidin complex) than conductivity measurements, it is precise enough to monitor small changes induced by a variety of perturbations to the channel and the membrane. The partial blocking of the gramicidin channel by divalent cations has been shown as a representative example in this regard. Studies on the proton conductance of the F_0 segment of F_0F_1-ATPase and other channels by our method are being pursued currently in our laboratory. Finally, this work has shown the usefulness, in ion-transport studies, of conventional T-jump equipment that, so far, has been used primarily to study chemical reactions.

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Registry No. CCCP, 555-60-2; H^+, 12408-02-5; nigericin, 28380-24-7; monensin, 17090-79-8; valinomycin, 2001-95-8.

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Use of Antibodies Specific to Defined Regions of Scorpion α-Toxin To Study Its Interaction with Its Receptor Site on the Sodium Channel

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ABSTRACT: Five antibody populations selected by immunoaffinity chromatography for their specificity toward various regions of toxin II of the scorpion *Androctonus australis* Hector were used to probe the interaction of this protein with its receptor site on the sodium channel. These studies indicate that two antigenic sites, one located around the disulfide bridge 12–63 and one encompassing residues 50–59, are involved in the molecular mechanisms of toxicity neutralization. Fab fragments specific to the region around disulfide bridge 12–63 inhibit binding of the ^{125}I-labeled toxin to its receptor site. Also, these two antigenic regions are inaccessible to their antibodies when the toxin is bound to its receptor site. In contrast, the two other antigenic sites encompassing the only α-helix region (residues 23–32) and a β-turn structure (residues 32–35) are accessible to their respective antibodies when the toxin is bound to its receptor. Together, these data support the recent proposal that a region made of residues that are conserved in the scorpion toxin family is involved in the binding of the toxin to the receptor.

The early studies on scorpion venoms were motivated by the problem of medical care of envenomation in several regions of the world (Vachon, 1952). The neurotoxic activity of the venom is due to the presence of small amounts (El Ayeb & Rochat, 1985) of basic proteins made up of 60–65 amino acid residues (Miranda et al., 1970), cross-linked by four disulfide bridges (Kopeyan et al., 1974). Although the immunogenicity of these compounds was recognized about 40 years ago (Grasset et al., 1946), the efficiency of the antiserum in therapeutic trials proved to be low (Balozet, 1971) when the whole venom was used as the immunogen. The venom of a definite species contains several α-neurotoxins that all bind to the same site on the sodium channel (Rochat et al., 1979; Catterall, 1984), even if they exhibit structural (Rochat et al., 1970) and antigenic polymorphism (Tessier et al., 1978; Delori et al., 1981; El Ayeb et al., 1983a). Moreover, the amount of each type of neurotoxin differs among individuals within a species (El Ayeb & Rochat, 1985), and finally, despite some structural homologies, toxins differ from species to species. Thus, this structural variability strongly complicates a rational design of polyvalent and efficient serotherapy.

Our laboratory has developed over the last few years several approaches to get a better understanding of the molecular features that are responsible for toxicity and antigenicity in the toxin family. A number of chemical modifications of the toxins have been performed to localize residues involved in the receptor binding or pharmacological properties of toxins. Lysines-56 for toxin I (Sampieri & Habersetzer-Rochat, 1978) and -58 for toxin II of *Androctonus australis* Hector (AaH I and AaH II)[1] and for toxin V of *Leiurus quinquestriatus quinquestriatus* (Lqq V), lysines-2 and -60 of Lqq V (Darbon

[1] Abbreviations: AaH II, toxin II of *Androctonus australis* Hector; Lqq V, toxin V of *Leiurus quinquestriatus quinquestriatus*; Bot III, toxin III of *Buthus occitanus tunetanus*; FAaH II, anti-AaH II Fab fragments; NRF BIII, anti-AaH II Fab fragments not retained on Bot III–Sepharose; RF BIII, anti-AaH II Fab fragments retained on Bot III–Sepharose; IgG, immunoglobulin G; PS I IgGs, PS II IgGs, PS III IgGs, and PS IV IgGs, anti-AaH II IgGs retained on the respective synthetic peptides; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin.

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