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PERMEABILITY OF A MODIFIED LIPID MEMBRANE TO $^{22}Na^+$

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SUMMARY

1. Phospholipid membranes, modified with alamethicin, have been used to measure their permeability to $^{22}Na^+$ by diffusion.

2. It was found that the permeability coefficient (k) at 37° varied within the range of 10^{-9} to 10^{-7} cm/sec with increasing values of Δc, the $^{22}Na^+$ concentration difference across the membrane. The variation is expected as a solvent drag effect.

3. The variation of k with Δc was of the form $k = a \cdot \Delta c^b$, where $a = 8.2 \cdot 10^{-4}$ and $b = -0.94$. A correlation coefficient of -0.94 was obtained for the relationship.

4. The amount of alamethicin partitioning into the membrane phase was insufficient to make the mobile ion carrier mechanism probable in this instance.

5. It is concluded that alamethicin altered the cationic conductance of the phospholipid membrane through the formation of hydrophilic, ion-transporting structures.

INTRODUCTION

Enhanced permeability to cations of model lipid membranes has been frequently demonstrated following the addition of macrocyclic antibiotics such as valinomycin, dinactin, and the enniations[2-4]. Physicochemical studies of the phenomenon indicate, in general, that the neutral molecules possess a nonpolar exterior, which enables them to readily partition into a lipid membrane, and conformations, which provide a polar core for non-hydrated cations to bind to[5-8]. The ability to quickly exchange free and complexed cations[9] is another reason for attributing a mobile ion carrying function to these antibiotics.

Not all macrocyclic antibiotics appear to act as mobile ion carriers, however. For example, in the case of gramicidin A and alamethicin, evidence for the formation of tubular structures bridging a lipid membrane has been advanced[20-22]. Such structures need not be static in shape or duration[10,11] and could be formed by a stochastic process involving conformational changes induced by electromotive, electrostrictive, and other forces. The action potential-type voltage response of an alamethicin-modified bimolecular lipid membrane to a constant current pulse[21] and the development of step changes in conductance of membranes in the presence of gramicidin A (ref. 12) are examples of this process.

On the basis of the primary structure of alamethicin the molecule contains a

free carboxyl group[13] which, when ionized, imparts to the molecule a negative electrophoretic mobility. As previously suggested[21], this property of alamethicin may play some part in its ability to produce the voltage-dependent cationic conductance changes in lipid bilayer membranes. It is of interest, therefore, to examine further the ability of alamethicin to increase cationic conductance of lipid bilayers under conditions where no electric potential is applied and any change in cationic permeability is measuerd by diffusion. In this report the results of $^{22}Na^+$ diffusion experiments on alamethicin-modified lipid membranes are described. When compared with the diffusion results obtained earlier for an unmodified phospholipid membrane[24] the present data indicate that alamethicin alters the intrinsic cationic conductance of phospholipid membranes.

EXPERIMENTAL PROCEDURE

The permeability cell used in the $^{22}Na^+$ diffusion experiments has been previously described[14]. It permitted the water in the compartment into which the $^{22}Na^+$ diffused to be continually replaced with fresh, distilled water by a perfusion technique. Thus, during an experiment, all the $^{22}Na^+$ diffusing through the phospholipid membrane was collected in the perfusate.

The 2 % (w/v) phospholipid extract in chloroform–methanol (2:1, v/v) has been previously described[15]. Its major components consisted of phosphatidylcholine, phosphatidylethanolamine, cerebrosides, and cholesterol. Sphingomyelin, phosphatidylserine and inositol were also present in minor amounts. To make the solution from which the membranes were made 16 % (by vol.) of *n*-tetradecane (Eastman Kodak P2221, m.p. 2–4°) was added to the lipid extract.

Prior to membrane application, the flow of the perfusate (distilled water with a Na^+ content of less than 1 μM) was started and allowed to fill both compartments of the permeability cell. Initially, therefore, the solute composition of the aqueous medium in the two compartments was identical. Application of the membrane solution to the aperture in the Teflon septum was then made with a fine sable hair brush. The membranes were allowed to thin fully to a bimolecular thickness. Alamethicin* was added to the non-perfused compartment in 10-μl amounts as an aqueous solution (10^{-7} g/ml). The concentration of alamethicin in the non-perfused compartment was therefore $0.6 \cdot 10^{-7}$ M.

Membranes thus modified were found to be noticeably more stable structurally. For instance, it was possible to maintain a modified membrane for varying periods of up to 110 days, whereas in our previous experiments the most durable unmodified counterpart lasted only 21 days. The improved structural stability of the modified membrane permitted permeability experiments in excess of 24 h. In a number of cases the alamethicin solution was added in 10-μl amounts to the perfused compartment as well, either once or every 2 or 4 h of the permeability experiment.

Two or more 30-min control samples of perfusate were then collected in separate, previously unused, 20 ml liquid scintillation bottles for background counting rate determinations before $^{22}Na^+$ (as $^{22}NaCl$; specific activity, 1 mC/mg) was added to the nonperfused compartment of the permeability cell and stirred. The amount of

* Alamethicin, a cyclopeptide antibiotic derived from Trichoderma viride, was kindly made available by Dr. E. L. Masson of the UpJohn Company of Canada.

$^{22}Na^+$ added to the non-perfused compartment was varied such that the estimated total Na^+ concentration difference across the membrane varied from 10^{-9} to 10^{-7} moles/cm^3. All experiments were run at 37° in a controlled-temperature room.

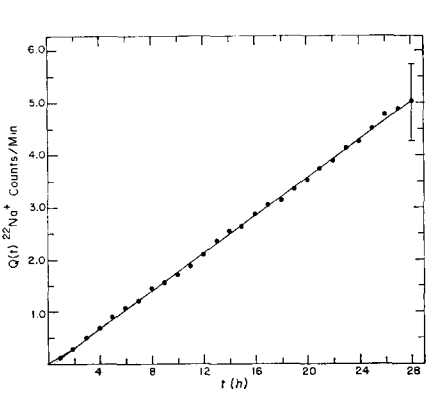
RESULTS

The $^{22}Na^+$ activity in the volume of perfusate collected each hour was measured by liquid scintillation counting as previously described[14]. Fig. 1 illustrates the results obtained in a 28-h experiment. $Q(t)$ is the time integral of the $^{22}Na^+$ counts and was calculated by summation of the net counts per hourly sample over the total number of samples included at time t. It is seen that after an initial delay, given by the diffusion lag time, $Q(t)$ increases linearly with time up to 28 h. The vertical bar associated with the point at 28 h represents the cumulative standard deviation.

From the steady state portion of the curve in Fig. 1 the amount of $^{22}Na^+$ permeating in a unidirectional manner through the membrane per unit time, dQ/dt, can be determined in counts/min per sec. The permeability coefficient k (cm/sec) may then be calculated from the equation

$$\frac{dQ}{dt} = k \cdot A \cdot \Delta c$$

where A is the area of membrane in cm^2 and the concentration difference $\Delta c = c - c_p \simeq c$, since c is the concentration of $^{22}Na^+$ in the non-perfused compartment (approx. 10^4–10^6 counts/min per cm^3) and c_p is the concentration of $^{22}Na^+$ in the perfused compartment (<0.1 counts/min per cm^3). It was found that the permeability coefficient depended on Δc as shown in Fig. 2. The variation in k with Δc was not significantly different in the situations where the phospholipid membrane was modified with alamethicin from one or both sides. The single line through the points is repre-



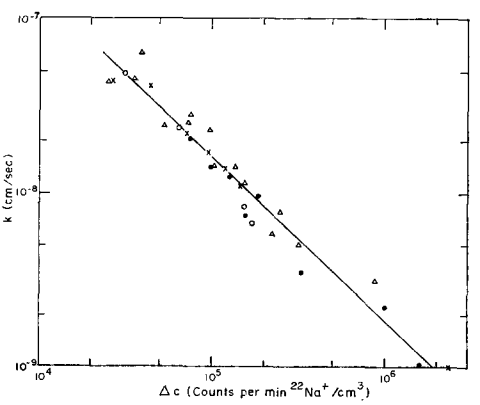


Fig. 1. Time integral of the $^{22}Na^+$ counts, $Q(t)$, *vs* diffusion time t. Membrane area, 0.03 cm^2. $\Delta c = 3.57 \cdot 10^4$ counts/min per cm^3. Permeability coefficient, $4.54 \cdot 10^{-8}$ cm/sec.

Fig. 2. Permeability coefficient k *vs* Δc, the $^{22}Na^+$ concentration difference across the membrane. Membrane area: ×, △, 0.03 cm^2; ●, ○, 0.06 cm^2. ×, ●, membrane modified with alamethicin added to non-perfused compartment only; △, ○, alamethicin added to both compartments for membrane modification.

sentative of either situation, and is of the form $k = a \cdot \Delta c^b$, where $a = 8.2 \times 10^{-4}$ and $b = -0.94$. A correlation coefficient of -0.94 was obtained for the line.

DISCUSSION

It is seen from Fig. 2 that under the experimental conditions described the permeability coefficient varied within the range of 10^{-9} to 10^{-7} cm/sec. These values are substantially less than those obtained at 25° in a composite system consisting of an albumin solution and a phospholipid sol[16]. Clearly the two structures must be quite different as expected.

The line through the data of Fig. 2 illustrates what has previously been stated[27], that the permeability coefficient may vary with Δc. The negative slope of the line suggests that a countercurrent flow of water slowed down the diffusing $^{22}Na^+$. The small values of Δc used in the experiments (10^4–10^6 counts/min per cm^3 are equivalent to $0.49 \cdot 10^{-9}$–$49 \cdot 10^{-9}$ moles/cm^3, respectively) indicate the sensitivity of interaction of the diffusing ions with the solvent in an alamethicin-modified membrane. Since "this interaction is not possible when solvent and test substance move along different pathways across the membrane"[17] it suggests the existence of hydrophilic, tubular, ion-transporting, structures in the membrane, a suggestion that is consistent with earlier observations[11]. Because there is an absence of any evidence for oscillations or fluctuations in the rate of $^{22}Na^+$ flow (Fig. 1) we cannot say whether the structures might be long lived or short lived (relative to the sampling time). Conductance measurements of the type referred to earlier[22,12] suggest that at least some are short lived.

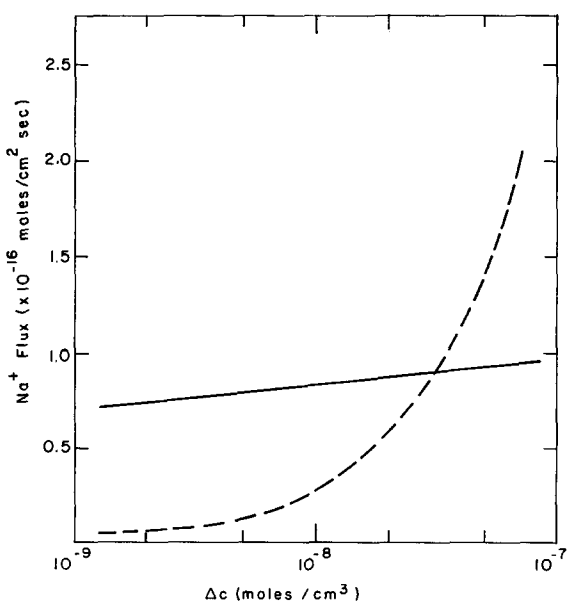


Fig. 3. Na^+ flux *vs* Δc, the Na^+ concentration difference across the membrane. ———, Na^+ flux through modified membrane (J_m). – – – –, Na^+ flux through unmodified membrane (J_u). $J_m = J_u$ at $\Delta c = 3.1 \cdot 10^{-8}$ moles/cm^3.

Given the variation in the permeability coefficient as shown in Fig. 2 the ion flux as a function of Δc may be determined[18]. In Fig. 3 the Na^+ flux through the alamethicin-modified membrane J_m (solid line) is shown as increasing logarithmically with Δc. The increase is quite moderate and suggests that the chemical potential, driving the diffusing ions through the membrane, is virtually counterbalanced by their interaction with water flowing osmotically in the countercurrent direction. Also shown in the figure is the Na^+ flux through an unmodified phospholipid membrane J_u (broken line) as calculated from data obtained earlier under otherwise identical conditions where $k \approx 0.27 \times 10^{-8}$ cm/sec and virtually invariant with Δc. It can be shown that the Na^+ flux through this unmodified membrane increases as a power function of the concentration difference. J_m is equal to J_u for $\Delta c = 3.1 \cdot 10^{-8}$ moles/cm^3.

As shown in Fig. 3, J_m is substantially more than J_u for low values of Δc, as expected if the concentration of the bare Na^+ in the membrane is substantially less than the concentration of the complexed Na^+ (ref. 7). For example, in the 28 h experiment (Fig. 1), where $\Delta c = 1.7 \cdot 10^{-9}$ moles/cm^3, $J_m = 0.73 \cdot 10^{-16}$ moles/cm^2·sec, while for an unmodified membrane J_u is about 12 times less at $0.06 \cdot 10^{-16}$ moles/cm^2·sec (ref. 14, Fig. 5B, p. 40). The net increase in the Na^+ flux due to modification with alamethicin is therefore equal to $0.67 \cdot 10^{-16}$ moles/cm^2·sec or $12.6 \cdot 10^{10}$ Na^+ in the 28 h period of the experiment. From the point of view of alamethicin as a possible unidirectional mobile ion carrier in the membrane this means that for a 1:1 stoichiometric Na^+–alamethicin complex $12.6 \cdot 10^{10}$ alamethicin monomer-type molecules are required to carry the net increase in Na^+ flux due to modification. Since this number is only 0.17 % of the total number of alamethicin molecules added to the non-perfused compartment it is conceivable in the event that all or some of the Na^+–alamethicin complexes could have sequestered into the perfused compartment after passing through the membrane. However, the fact that alamethicin partitions readily into a lipid phase[19] and no significant difference in the permeability coefficient was observed when the membrane was modified from both sides suggests that sequestration was probably not important.

To consider alamethicin as a mobile ion carrier it is reasonable to assume that the molecules moved through the membrane at a velocity equivalent to the permeability coefficient measured for Na^+. For the experiment shown in Fig. 1 the latter was equal to $4.54 \cdot 10^{-8}$ cm/sec and corresponds to a diffusion time of 6.6 sec (ref. 20) for a 60 Å thick membrane[21]. The calculated time is a measure of the period required for an alamethicin molecule to carry Na^+ by random walk from one interface to the other, assuming that any delay due to the formation and dissociation of the complex at the interfaces is negligible in comparison[9]. At a concentration of $0.6 \cdot 10^{-7}$ M the total number of alamethicin molecules added to the aqueous phase was $3.55 \cdot 10^{13}$ per ml. For a maximum concentration of alamethicin in phospholipid membranes of 40 times that in water, (experimental details to be described elsewhere) the number of alamethicin molecules in the membrane is $14 \cdot 10^{14}$ per ml and corresponds to an alamethicin:lipid molecular ratio of approx. $2 \cdot 10^{-5}$. For a uniform distribution of the molecules within the membrane the number per volume element, given by a membrane of area 0.03 cm^2 and $4.5 \cdot 10^{-8}$ cm deep (the thickness of an alamethicin molecule in the plane of the ring structure[19]), is equal to $1.9 \cdot 10^6$. The number of alamethicin molecules that may diffuse through the membrane from one interface to the other in 28 h is then equal to $2.9 \cdot 10^{10}$. This value is approx. 23 % of the number

of additional Na^+ transported across the membrane in 28 h following modification.

Unless an average stoichiometric ratio of 4–5 in the Na^+–alamethicin complex is assumed the result indicates that at maximum efficiency the amount of alamethicin present in the membrane was inadequate to carry the additional number of sodium ions across the membrane. Although the question of the number of sodium ions complexing with an alamethicin molecule appears not to have been answered it seems unlikely from the physical structure and known chemical properties of alamethicin that it would carry an average number of four to five sodium ions through the membrane at a time. In the face of this difficulty the mobile ion carrier is not favoured in this instance. It is therefore most probable that alamethicin altered the cationic conductance of the phospholipid membrane through the formation of hydrophilic ion-transporting structures.

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