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Ionophore-Mediated Transmembrane Movement of Divalent Cations in Small Unilamellar Liposomes: An Evaluation of the Chlortetracycline Fluorescence Technique and Correlations with Black Lipid Membrane Studies

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Summary. Conceptual advances in the field of membrane transport have, in the main, utilized artificial membranes, both planar and vesicular. Systems of biological interest, *viz.*, cells and organelles, resemble vesicles in size and geometry. Methods are, therefore, required to extend the results obtained with planar membranes to liposome systems. In this report we present an analysis of a fluorescence technique, using the divalent cation probe chlortetracycline, in small, unilamellar vesicles, for the study of divalent cation fluxes. An ion carrier (X537A) and a pore former (alamethicin) have been studied. The rate of rise of fluorescence signal and the transmembrane ion gradient have been related to transmembrane current and potential, respectively. A second power dependence of ion conduction – including the electrically silent portion thereof – on X537A concentration, has been observed. An exponential dependence of "current" on "transmembrane potential" in the case of alamethicin is also confirmed. Possible errors in the technique are discussed.

Key words Membrane-transport · fluorescence · chlortetracycline · X537A · alamethicin · ionophore · liposome

Introduction

Much of the conceptual advance in the field of membrane transport has been made with artificial membranes – planar (bimolecular lipid membranes, or BLM) and vesicular (liposomes) (Mueller, Rudin, Tien & Wescott, 1962; Bangham, Standish & Watkins, 1965: Mueller & Rudin, 1967; Bean, Shepherd, Chan & Eichner, 1969; Bangham, Hill & Miller, 1974). The geometries of the two systems are complementary – the large surface-to-volume ratios of vesicles makes them ideally suited for measurements of net transmembrane solute transport while the BLM are preferred for studies of transmembrane potentials and transient ion conductances. Exchange kinetics in multilamellar liposomes are extremely complex (Johnson & Bangham, 1969). Unilamellar vesicles, wherein a single bilayer membrane encloses an aqueous compartment, provide an excellent model system. However, the size of small, unilamellar vesicles (SUV) creates problems in studying solute influxes at constant gradient as, for a 300-Å diameter vesicle, just 10 ions in the intravesicular space is of the order of 1 mM in the ion. Since the systems of interest – cells and organelles – have geometries and sizes better approximated by vesicles than by planar bilayers, methods for the extension of BLM-type studies to liposomes are required.

Of the available techniques for the measurement of intracellular potentials, the use of potential-sensitive dyes has been criticized on the basis of poor signal-to-noise ratios and specificity (Hladky & Rink, 1976), while problems of sealing and electrolyte leakage complicate the microprobe electrode technique. Further, microprobe electrodes cannot be introduced into SUV. There remain, then, the techniques of estimating intracellular/vesicular solute concentrations – with the advantage of detecting electrically silent transport. Problems of time resolution preclude the use of radioactive tracer techniques, leaving optical probes as the most attractive alternative. In this report we present a fluorescence technique, using the divalent cation probe, chlortetracycline (CTC) (Caswell & Warren, 1972; Mathew & Balaram, 1980), in small, unilamellar liposomes, for the study of the ionophorous activities of an ion carrier (X537A) (Caswell & Pressman, 1972) and a pore former (alamethicin) (Mueller, 1976) and relate the results so obtained with those of BLM experiments.

Materials and Methods

Egg phosphatidylcholine (EPC), X537A, CTC, Sephadex G-50, cholic acid and HEPES were from Sigma. Lanthanum chloride was obtained from Ventron. Alamethicin was synthesized as described earlier (Nagaraj & Balaram, 1981). $CaCl_2$ and $ZnCl_2$ were of analytical grade. SUV were generated by the removal of cholate from mixed micelles of EPC and cholate by gel filtration on Sephadex G-50 as described by Brunner, Skrabal and Hauser (1976). The vesicles so generated have been reported to be uni-

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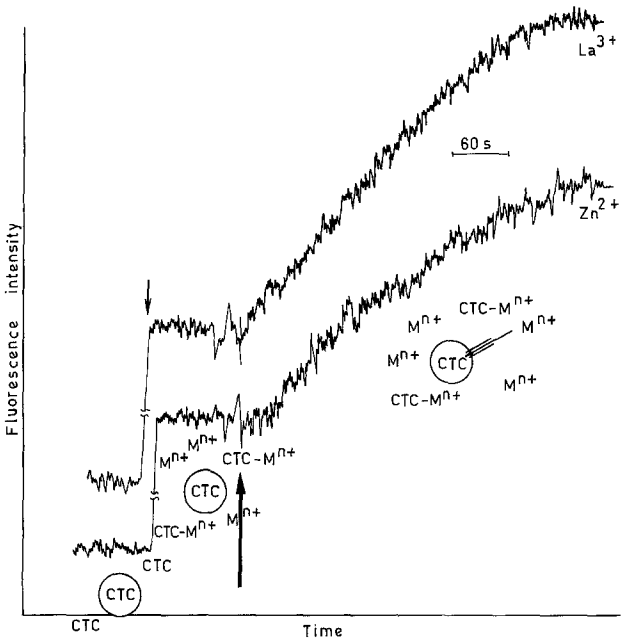

Fig. 1. Time-dependent increases in CTC-metal fluorescence on adding 5 μM alamethicin. Cation concentrations used: 500 μM La^{3+}, 1 mM Zn^{2+}. Addition of test cation is indicated with small arrow. Addition of ionophore is indicated with thick arrow. The state of the system following each addition is schematically represented against the traces

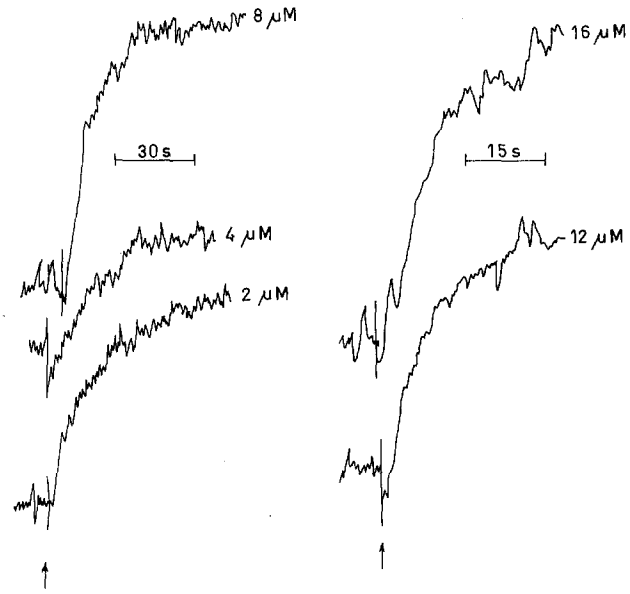

Fig. 2. Time-dependent increases in CTC−Ca^{2+} fluorescence on adding X537A. Concentrations of X537A are shown on figure. $[Ca^{2+}]_{ext}$ = 1 mM. Ionophore addition marked with an arrow

lamellar with a narrow size distribution of mean diameter 300 Å. Ion transport was followed using 25 μM CTC, 200 μg/ml vesicles in 5 mM HEPES, 100 mM NaCl, pH 7.0. After CTC had equilibrated across the membrane, the test cation was added at 1 mM, or as stated, following which ionophore was introduced. The fluorescence of the CTC-metal complexes was monitored on a Perkin-Elmer model MPF-44A fluorescence spectrometer operated in the ratio mode with 10 nm excitation and emission band pass; λ_{ex} = 390 nm, λ_{em} = 530 nm. 1-cm path-length cells were used and solutions stirred with a magnetic pellet to minimize settling. Vesicle integrity was checked by a step response after adding the test cation followed by a sharp rise on adding 20 M X537A.

Results, Theory, and Discussion

CTC is weakly fluorescent, whereas its complexes with Ca^{2+}, Mg^{2+}, Zn^{2+} and La^{3+} have a much higher quantum yield. The probe can thus be used to detect the presence of these cations. Our technique utilizes the ability of CTC to permeate lipid membranes, which remain impermeant to cations in the absence of ionophore. Equilibration of CTC across the membrane foiled attempts to entrap the probe in the vesicles, followed by removal of external CTC by gel filtration. Extravesicular CTC leads to a large basal fluorescence in the presence of test cations and necessitates extensive background suppression. Figure 1 shows the effect of adding ionophore to this suspension. The slow rise in fluorescence emission intensity is attributed to the entry of cation into the vesicles and binding to intravesicular CTC, the complex being highly fluorescent. This is effectively a charge translocation process.

i.e., $F \alpha Q$ (1)

where F is the incremental fluorescence intensity over the base line and q the charge transported. It follows that

$$dF/dt = K \cdot dq/dt = KI \quad (2)$$

where I is the transmembrane current and K is a constant of proportionality. We are interested in studying ion translocation under conditions of specified gradient. With a vesicle diameter of 300 Å (Brunner et al., 1976) and volume of $\sim 1.5 \times 10^{-20}$ liter, the gradient is rapidly broken down, the influx of just 10 ions per vesicle equalizing the concentrations across the membrane. We shall, therefore consider only the initial slope of the $F-t$ curve

$$(dF/dt)_0 = KI_0. \quad (3)$$

We tested the dependence of ion translocating efficiency of X537A on ionophore concentration, under constant gradient conditions, for Ca^{2+}, Zn^{2+} and La^{3+} as shown in Fig. 2. Célis, Estrada-O and

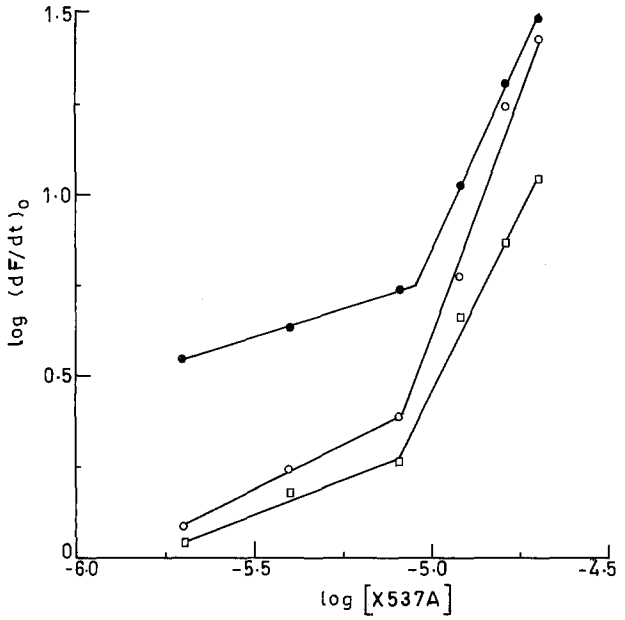

Fig. 3. Log-log plot of $(dF/dt)_0$ from Fig. 2 and similar experiments with Zn^{2+} and La^{3+} *vs.* X537A concentration. $[Ca^{2+}]$, 1 mM o—o; $[Zn^{2+}]$, 1 mM □—□; $[La^{3+}]$, 500 μM ●—●

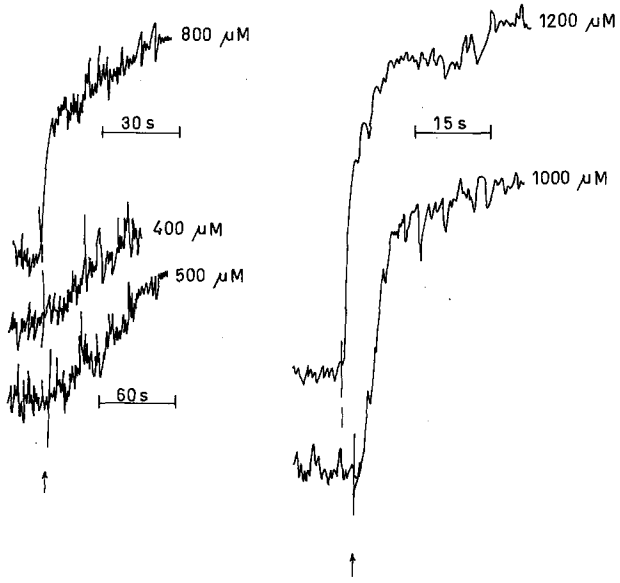

Fig. 4. Time-dependent increases in CTC–Ca^{2+} fluorescence on adding 5 μM alamethicin. $[Ca^{2+}]_{ext}$ concentrations are shown on figure. The upper time scale at left is for 800 μM $[Ca^{2+}]_{ext}$, the lower one for 400 and 500 μM. Time scale at right is for both 1000 and 1200 μM $[Ca^{2+}]_{ext}$. Ionophore addition is marked with an arrow

Montal (1974), in a similar experiment with BLM, obtained a sigmoidal curve in a log-log plot of membrane conductance *vs.* ionophore concentration. Since, at constant voltage, conductance is directly proportional to current, a similar curve should be obtained on plotting log $(dF/dt)_0$ against log (X537A concentration). This is, indeed, obtained in Fig. 3. The differential response of CTC to the different cations (Caswell & Hutchison, 1971; Mathew & Balaram, 1980) has been taken into account by dividing the initial slopes $(dF/dt)_0$ by the relative intensities of the complexes ($F_{CTC-M^{n+}}$). The latter portions of the curves in Fig. 3 are linear with slopes around 2 for all three ions under study, in agreement with the results of Célis et al. (1974) for Ca^{2+}. It is also in agreement with the results of Caswell and Pressman (1972), who proposed a 2:1 complex of X537A to metal, as the translocating unit. Note that the saturation of the curve reported by Célis et al. (1974) occurred only above 100 μM X537A.

These authors obtained a linear dependence of conductance on calcium gradient and concluded that the translocating unit contained one Ca^{2+} ion. Pressman (1973), however, reports that the amount of Ca^{2+} transported by X537A, as estimated by radioactive tracer techniques, is several orders of magnitude higher than that expected on the basis of electrical conductivity measurements – i.e., much of the transport is electrically silent. This is not entirely surprising as H^+ and Na^+ can both be transported by the ionophore, albeit less efficiently. Thus the net charge translocation detected represents only that fraction of the transport which is unaccompanied by the countertransport of H^+ and/or Na^+. Our technique, which measures transmembrane ion fluxes directly, yields the same result of a second power dependence on X537A concentration for transport, indicating that the translocating unit does contain two X537A molecules, possibly analogous to the asymmetric sandwich structure of the Ba^{2+} complex of the ionophore (Johnson, Herrin, Lin & Paul, 1970). The fact that charge translocation measurements arrive at the same conclusion could indicate that a constant proportion of the transport is electrogenic.

Changes in the transmembrane potential can be generated by varying the intra- and extra-vesicular ionic composition. It is expected that this potential should be approximately Nernstian (Sten-Knudsen, 1978), the concentrations of all permeant ions being considered. For alamethicin-modified membranes the distribution of all cationic species should be considered. Since the only asymmetrically distributed cation is Ca^{2+}, only the contribution of Ca^{2+} need be taken into account. An exponential dependence of membrane current on applied voltage has been shown in the case of alamethicin (Eisenberg, Hall & Mead, 1973). Thus, a log-log plot of

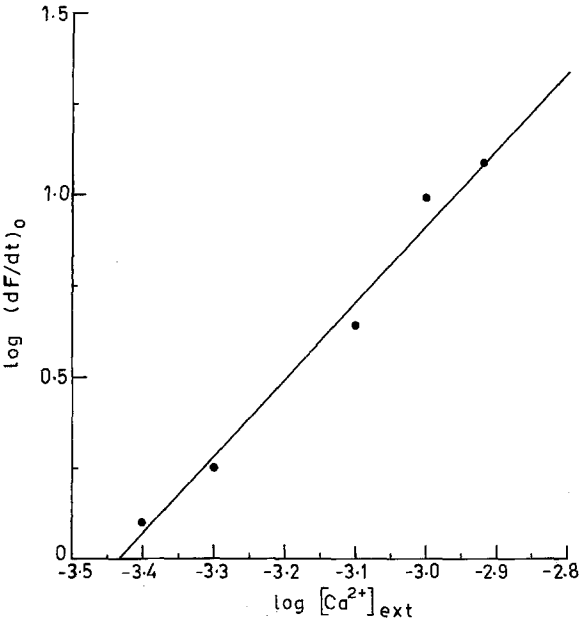

Fig. 5. Plot of $\log (dF/dt)_0$ from Fig. 4 *vs.* $\log [Ca^{2+}]_{ext}$

$(dF/dt)_0$ *vs.* external calcium concentration should be linear as the transmembrane potential will be given by

$$V = V_{salt} \cdot \ln [M^{n+}]/C_0' \tag{4}$$

where V_{salt} and C_0' are constants (Eisenberg et al., 1973) Fig. 4 shows the time-dependent fluorescence increase of CTC$-Ca^{2+}$ complexes in the presence of alamethicin, with varying concentrations of external calcium. Initial slopes of these curves are plotted against external calcium concentrations in a log-log plot in Fig. 5 and yields a straight line describable, in analogy with Eisenberg et al. (1973), as

$$\log (dF/dt)_0 = a + \log ([Ca^{2+}]_{ext}/b) \tag{5}$$

where a and b are constants. It is important to note that no such correlation is obtained with the carrier X537A where exponential $I-V$ curves, in the steady state, have not been reported in BLM studies either.

These results confirm the analogy of $(dF/dt)_0$ with transmembrane current and of $\log [M^{n+}]_{ext}$ with the transmembrane potential, thus allowing the construction of steady state $I-V$ curves with liposomes. The severe size restrictions imposed by SUV can be relieved by the generation of larger unilamellar vesicles. Unilamellar liposomes of up to 1000 Å can now be generated (Szoka & Papahadjapoulos, 1980) to better approximate organelle dimensions. The estimation of the stoichiometry of the translocating units is relatively simple in the case of BLM where transport across only one membrane at a time is being studied. In the liposome case, distribution of ionophore among vesicles has to be properly modelled. In the case of pre-formed aggregates entering the lipid phase, this is relatively simple, as for X537A. However, lipid phase aggregation to form oligometric aggregates, as has been proposed for alamethicin, is harder to model. It has been proposed that alamethicin enters the membrane as a monomer and subsequently undergoes reversible aggregation in the lipid phase (Mueller & Rudin, 1968). Boheim and Kolb (1978) have proposed that alamethicin pre-aggregates enter the membrane, under the influence of an applied electric field. However, Fringeli (1980) and Fringeli and Fringeli (1979), using Attenuated Total Reflection IR spectroscopy conclude that the ionophore enters the lipid phase and subsequently aggregates in this phase even in the absence of any field. Furthermore, noise in the system is currently too high to detect single-channel conduction even if the number of vesicles were to allow the controlled formation of a limited number of channels.

There exist three possible errors in this technique. Firstly, since the membrane is permeable to CTC an error could be introduced by the probe migrating across the membrane and binding external cation leading to a rise in fluorescence independent of ionophore. Secondly, CTC has been reported to act as a ionophore in pancreatic islet cells at concentrations above 100 μM (Sehlin & Taeljedahl, 1979). Ionophorous activity is not, however, expected at the concentrations used in our experiment. We observe a step response on adding the test cation to the CTC-loaded vesicle suspension (Fig. 1) with no slow rise in fluorescence with time, indicating that no further complexation is occurring after the initial rise attributed to the complexation of the extravesicular CTC. Further, a rise in fluorescence on adding ionophore (which does not, of itself, enhance CTC-metal fluorescence) indicates a nonuniform distribution of test cations and uncomplexed CTC across the membrane prior to ionophore addition. Thirdly, the observed rise in fluorescence could occur due either to cations entering the liposome or CTC leaving it, under the influence of added inophore. X537A is known to transport biogenic amines (Schadt & Haeusler, 1974) and CTC has a dimethylamino function. Transport of CTC by X537A is expected to be minimal as the ionophore complexes weakly with secondary and tertiary amines. The alamethicin pore is large enough to allow the passage of CTC or its complexes through it (Mueller, 1976). The fact, however, that good correlations have been obtained with a totally different technique, *viz.* BLM studies, indicates that such CTC fluxes are minimal during these experiments.

The technique thus serves to bridge the extant technologies of BLM and liposome work, allowing the study of transmembrane ion fluxes in liposome systems. An application of this technique to evaluate the structural requirements for pore formation by alamethicin and its synthetic fragments has been reported (Nagaraj, Mathew & Balaram, 1980).

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