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A23187—Channel behaviour: Fluorescence study

G JYOTHI, A SUROLIA and K R K EASWARAN*
Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India

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Abstract. Pyranine entrapped soylipid liposomes have been used as a model system to study the proton transport across membrane in the presence of A23187, a carboxylic ionophore specific for electroneutral exchange of divalent cations. An apparent rate constant (k_{app}) for transport of protons has been determined from the rate of change of fluorescence intensity of pyranine by stopped flow rapid kinetics in the presence of proton gradient The variation of k_{app} has been studied as a function of ionophore concentration and the results have been compared with gramicidin—a well known channel former under the similar experimental conditions. The rates thus obtained showed that A23187 is not only a simple carrier but also shows channel behaviour at high concentration of ionophore.

Keywords. A23187—calcium ionophore; proton transport; stopped flow kinetics.

1. Introduction

The discovery of various fluorescent probes and ionophores have opened the way for an understanding of the complicated phenomena of transmembrane ion-transport and mechanism of transport kinetics in both model and biological systems (Eidelman and Cabantchik 1989; Pressman 1976). In biological systems electrochemical proton gradient plays an important role as the primary intermediate in the process of energy transduction (Mitchell 1979). The combined action of integral channel proteins and low permeability of bilayer to charged ions is responsible for the maintenance of this gradient (Deamer and Bramhall 1986). The free movement of protons across membrane barrier is severely restricted due to the development of a proton diffusion potential (Bramhall 1987). The kinetics of proton movement across membrane is an important aspect in the study of energy coupling (Krishnamoorthy and Hinkle 1984). Measurement of proton gradient decay was earlier reported for valinomycin and gramicidin in model membranes by entrapping pyranine—a convenient pH sensitive fluorophore in liposomes (Kano and Fendler 1978). In the present study pyranine has been used to monitor the H^+ flux mediated by A23187.

A23187 (calcimycin) occupies an important place among divalent cation carriers owing to its high specificity for calcium ion (Reed 1982; Kolber and Haynes 1981). It has been used extensively to understand the role of calcium ion in several cellular phenomena. A great number of biological events like leukotrienes and interferon production (Samuelsson 1983; Braude and Criss 1986) have been shown to be modified by this ionophore. The intrinsic fluorescence property of A23187 has been used to determine the mechanism of divalent cation transport across phospholipid bilayers (Kolber and Haynes 1981; Clement and Gould 1981). The

*Corresponding author.

rate of ion transport by this ionophore is sensitive to lipid composition and charge of the lipid head groups.

It has generally been believed that A23187 function as a typical carrier ionophore. However, flux of ions mediated by A23187 across biological membranes have been found to be significantly greater as compared to a typical carrier ionophore (Casewell and Pressman 1972). This together with earlier studies from our laboratory demonstrating that at higher concentration, A23187 molecules aggregate as a dimeric stack in phospholipid vesicles the dimensions of which are sufficient to span the bilayer thickness (Balasubramanian and Easwaran 1989). This prompted us to undertake the characterization of the proton translocation properties of A23187 as a function of concentration by fast reaction kinetics. Our studies showed that at the concentration at which A23187 has been shown to aggregate into a stack, its kinetic properties are similar to the well established channel forming ionophores, such as gramicidin.

2. Material and methods

A23187 free acid, soylipid, gramicidin and HEPES are from Sigma Chemical Co (USA) and used without further purification unless otherwise specified. 8-Hydroxy-1,3,6 pyrene trisulphonic acid is from Molecular Probes Inc. (USA).

A23187 concentration ranging from 20–130 was cosolubilized with acetone washed soylipid (asolectin) (Kagawa and Racker 1971) in chloroform and thin dried film was formed according to the standard procedures. The dried film was hydrated for sufficient time using the buffer consisting of 10 mM HEPES, 150 mM KCl, 1 mM pyranine with pH 7·5. Liposomes were formed by sonicating the suspension using probe type sonicator for 15–25 min until the suspension becomes translucent to light. The liposome suspension was applied to a Sephadex G-50 column and the column was eluted with the same buffer to remove external pyranine (Krishnamoorthy 1986). The eluted vesicles were diluted to a final phospholipids concentration of 4 mM. This would correspond to a vesicle concentration of about 1·3 μM, we assume an average vesicle to have ~3000 phospholipid molecules (Watts *et al* 1978). The homogeneity was further checked by negatively stained electron microscopy (Johnson *et al* 1971) which showed single bilayer vesicles with diameter approximately of 30 nm. The final solution of liposomes was filtered through 0·2 μm membrane filter to avoid light scattering.

The measurements were carried out using a Union Giken RA 401 stopped-flow spectrophotometer. In the stopped flow apparatus one compartment was filled with liposomes with entrapped pyranine and another compartment with buffer of pH 6·5. A proton gradient of 0·35 pH units was established by mixing of contents in equal volumes from two compartments (pH_{int}= 7·5 and pHext= 7·15). The resultant proton gradient leads to proton flux which was in turn measured from the quenching of pyranine fluorescence at excitation wavelength of 468 nm, band pass 7 nm and emission was monitored in the presence of suitable cut off filter. The dead time of the instrument was 0·5 ms.

3. Results and discussion

Decay of fluorescence intensity of pyranine at different concentrations of A23187 in the presence of proton gradient of 0·35 units is shown in figure la where Y

axis indicates the normalized fluorescence intensity values. This plot clearly shows that increase of A23187 concentration increases the rate of proton flux or quenching of pyranine fluorescence. When the lipid to ionophore ratio approaches 30:1 (figure 1F), the rate of quenching is found to be very rapid with $t_{1/2}$ of approximately 15·6 ms.

The apparent rate constant values for proton flux are determined from the plot of In fluorescence intensity vs time (ms) as shown in figure lb. These values are found to be increasing with concentration of ionophore and the measured rate constant values for lipid/ionophore ratio of 200: 1 and 30: 1 are 8·2 and 115 respectively.

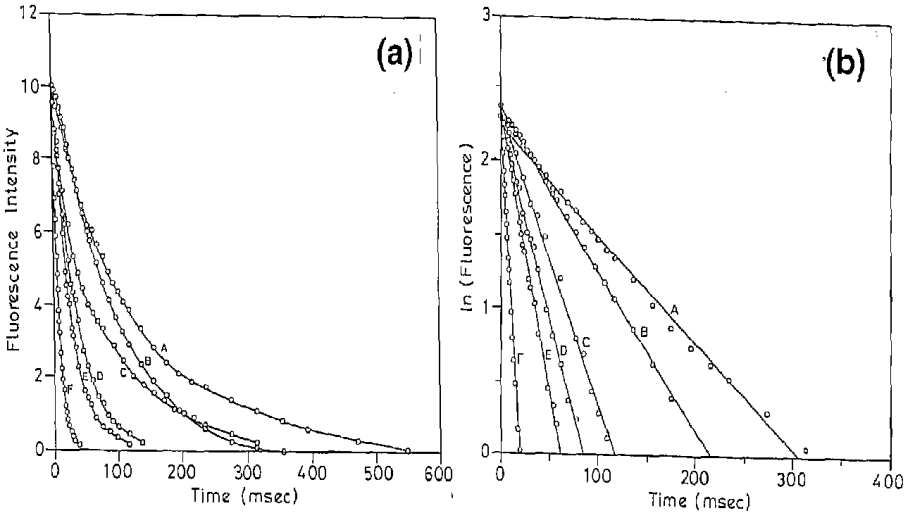

Figure 1. (**a**) Fluorescence decay curves of pyranine entrapped liposonles in the presence of proton gradient of 0.35 pH units at different concentrations of ionosphere, (**b**) In fluorescence intensity against time (ms) at different concentrations of ionophore.

Lipid to ionophore ratios are A, 200 : 1; B, 100 : 1; C, 66 : 1; D, 50 : : E, 40 : 1; and F, 30: 1. Concentration of soylipid is 4 mM.

A plot of initial rates *vs* ionophore concentration is shown in figure 2. The initial rates are measured from slopes of the fluorescence decay curves as shown in figure la and these values have been converted to the change in fluorescence intensity per min. The data thus obtained clearly show that rate of fluorescence change is linear up to the lipid/lonophore ratio of 50 : 1 beyond which it shows exponential behaviour (figure 2). The initial rates determined for A23187 at lipid ionophore ratio of 33 : 1 and 30: 1 are quite comparable with the values obtained with gramicidin at lipid : ionophore ratio of 400: 1 and 200 : 1.

A plot of log (k_{app}/s^{-1}) against log ionophore concentration is displayed in figure 3, from which it is interesting to note that the curve shows distinct slopes. At low ionophore concentration the slope is found to be 0·8 and at higher ionophore concentration it is 3·80 (corresponding to A and B in the figure), thus indicating the aggregation of ionophore at higher concentration. A comparison of these results with those obtained for gramicidin showed that A23187 at higher concentration behaves very similar to the gramicidin channel. Though the actual ratio of lipid to ionophore in A23187 and gramicidin are not comparable, the apparent rate constants are comparable which suggests that A23187 not only functions as simple

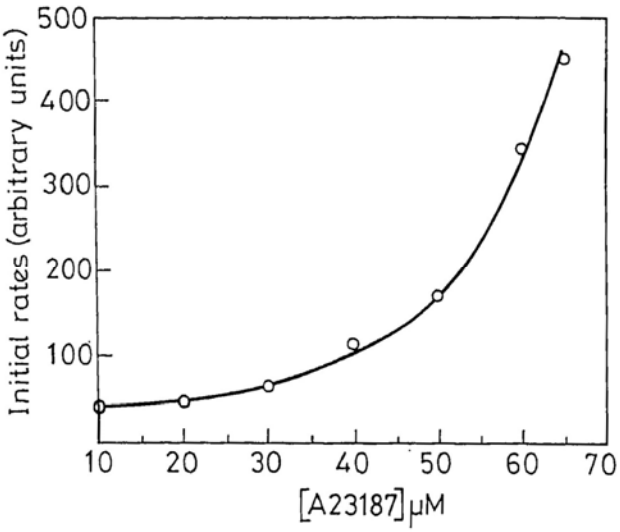

Figure 2. Initial rates of fluorescence decrease determined from fluorescence decay curves at different concentrations of ionophore.

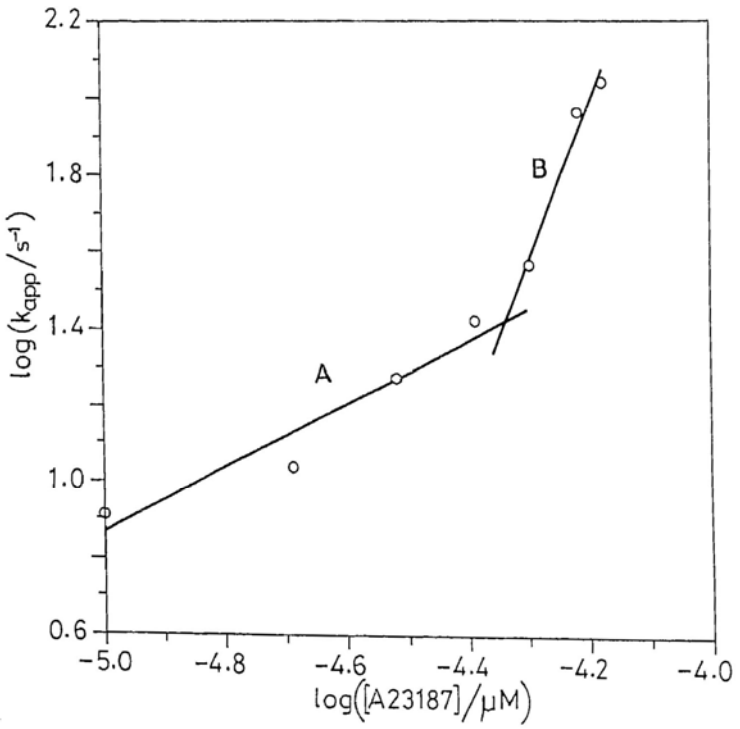

Figure 3. Plot of log (k_{app}/s^{-1}) against log ([A23187]/1 AM) showing two slopes. Slope of A, 0·8 and B, 3·8.

carrier but also shows concentration dependent channel behaviour. These results are further confirmed by fluorescence quenching and life time measurement studies (results not shown).

4. Conclusions

Stopped-flow kinetics studies on pyranine trapped soylipid liposomes containing calcium ionophore A23187 clearly establishes a concentration dependent aggregation for this ionophore. Earlier studies from this laboratory showed a time and concentration dependent aggregaton of A23187 in synthetic phospholipids such as DPPC and DMPC. The experiment discussed in this paper has been carried out with soylipid which is close to lipid composition of natural membrane systems.

If A23187 strictly acts as a carrier, then the relaxation rate should have shown a linear dependence on the concentration. However, a plot of log ionophore concentration *vs* log (k_{app}/S–1) for proton transport clearly shows that the ionophore behaves only as a carrier at low concentrations and as a channel at higher concentrations. In conclusion, the studies reported in this paper supports the channel forming ability of A23187 at concentrations where it stacks to form a aggregated structure in lipid vesicles (Balasubramanian and Easwaran 1989). This is also consistent with the recent data on conductance measurement in black lipid membranes with this ionophore (Balasubramanian *et al* 1992).

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