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THE STOICHIOMETRY OF A23187- AND X537A-MEDIATED CALCIUM ION TRANSPORT ACROSS LIPID BILAYERS

LEA BLAU [a,*], RENEE B. STERN [a] and ROBERT BITTMAN [b]

[a] *Department of Chemistry, Yeshiva University, 245 Lexington Ave., New York, NY 10016 and* [b] *Department of Chemistry, Queens College of The City University of New York, Flushing, NY 11367 (U.S.A.)*

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Initial rates of ionophore-mediated Ca^{2+} transport across egg phosphatidylcholine bilayers of large unilamellar vesicles were measured using the absorbance change of arsenazo III at 650 nm as an indicator of Ca^{2+} translocation. A23187 induced the movement of Ca^{2+} in a 2:1 ionophore:Ca^{2+} complex, whereas its methyl ester (CH_3A23187) and X537A mediated Ca^{2+} movement in a 1:1 ionophore:Ca^{2+} complex. The relative potencies of these ionophores in transporting Ca^{2+} across lipid membranes were A23187 ≫ X537A > CH_3A23187.

The specificity of the naturally occurring ionophores A23187 and X537A to transport divalent cations has been studied extensively with particular focus on the cation-carrier interaction (see Ref. 1 for review). The stoichiometry of ionophore-metal ion complexes has been studied by many methods, and different values have been reported for the number of moles of ion per mole of a given ionophore. A 2:1 electroneutral complex of the anionic form of A23187 with Ca^{2+} was suggested from measurements of conductance and $^{45}Ca^{2+}$ movement across planar lipid membranes [2,3]. On the other hand, studies of the Ca^{2+} permeability followed by the use of radioactive Ca^{2+} through planar lipid bilayers of egg phosphatidylcholine (PC) and cholesterol showed that the rate of Ca^{2+} transport varied with A23187 concentration raised to the power of 1.0 to 1.7 [4]. This study also found a second-order dependence of X537A on the rate of Ca^{2+} transport, at 10 to 15 μM ionophore, indicative of a 2:1 X537A:Ca^{2+} complex [4]. A 2:1 complex having the composition $(X537A^-)_2:H^+$, Ca^{2+} was suggested from conductance measurements of planar egg PC bilayers [5]. Potentiometric studies in methanol indicated that both 1:1 and 2:1 A23187:Ca^{2+} complexes are formed, whereas only a 1:1 X537A:Ca^{2+} complex was detected [6]. Both 1:1 and 2:1 A23187:Ca^{2+} complexes were also detected at the surface of small unilamellar vesicles prepared from dimyristoyl-PC; however, it was shown by fluorescence studies that the translocation of Ca^{2+} by A23187 occurs as a 2:1 ionophore:Ca^{2+} complex [7]. A 2:1 ratio of ionophore:Ca^{2+} was found for the complex formation of A23187 [8] and X537A [9] in chloroform by NMR studies. Studies of $^{45}Ca^{2+}$ efflux from multilamellar vesicles showed a 2:1 A23187:Ca^{2+} complex formation at an ionophore concentration above 3 nM; however, the X537A-induced Ca^{2+} efflux occurred in a 1:1 ionophore:Ca^{2+} complex below 5 μM X537A and a 2:1 complex at 5 to 30 μM X537A [10]. Pohl and co-workers [11] studied the A23187-mediated efflux of divalent cations from sonicated egg PC

* To whom correspondence should be sent.

Abbreviations: CH_3A23187, methyl ester of A23187; PC, phosphatidylcholine.

vesicles at 6.3 to 15.7 nM ionophore and found a second-order dependence of the rates on the A23187 concentration.

The present study was undertaken to characterize the stoichiometry of the A23187 and X537A complexes with Ca^{2+} in large unilamellar vesicles (LUV). Using log-log plots of the initial rates of Ca^{2+} transport vs. ionophore or Ca^{2+} concentration, we show that the anionic form of A23187 forms a 2 : 1 ionophore : Ca^{2+} complex in lipid bilayer membranes, whereas X537A forms a 1 : 1 complex. We report similar rate measurements using a less active derivative of A23187 in which the carboxylate group is esterified (CH_3A23187). This derivative forms a 1 : 1 complex with Ca^{2+} in large unilamellar vesicles, suggesting that the stoichiometry of the transported complex in vesicles is dependent on the amphiphilic character of the ionophore and conformational changes that accompany deprotonation of A23187 [12].

Egg PC, A23187 and cholesterol were purchased from Sigma Chemical Co. Cholesterol was recrystallized from methanol. Diazald was obtained from Aldrich Chemical Co. The ionophore X537A was donated by Dr. J. Berger of Hoffmann-LaRoche. To convert X537A to the protonated form a solution of X537A in methanol was acidified with 6 M hydrochloric acid and extracted with chloroform. The chloroform fraction was dried and the solvent was removed, yielding the free acid form of X537A [13]. Its purity was checked by spectrophotometric measurements and compared to known values [14]. Kinetic measurements were carried out with the protonated X537A. The Ca^{2+}-sensitive dye arsenazo III was purchased from Gallard-Schlessinger Chemical Corp. It was converted to the free acid by passing a concentrated aqueous solution through a column of AG50W-X H^+ form ion exchanger (Bio-Rad Laboratories). Concentrated hydrochloric acid was added to a final concentration of 6 M. Crystals of arsenazo III were separated at 4°C and collected by filtration. Excess water and hydrochloric acid were removed by dissolution of the crystals in acetone and evaporation of the solvent. Carboxyfluorescein was purchased from Eastman Organic Chemicals and was recrystallized from ethanol. The preparation of CH_3A23187 was carried out by using diazomethane generated from Diazald using a modification of the method of Lipsky and Landowne [15]. The purity of the product was checked by thin-layer chromatography on silica gel G plates (Analtech), eluted with cyclohexane/tetrahydrofuran/methanol (20 : 6 : 0.4, v/v/v). The major band was CH_3A23187 (molecular weight 537), as determined on a CEC21-110 mass spectrometer run at 70 eV. An impurity (molecular weight 551) of 2% was also present.

The vesicles were prepared by slowly injecting an egg PC solution in absolute ethanol (35 mM) into a stirred aqueous solution of 10 mM imidazole, (pH 7.0), containing 0.10 M $CaCl_2$ [16] or 7.6 mM arsenazo III in 0.15 M choline chloride solution [17]. The injection was carried out at a rate of 1 ml/h with a Brinkmann ip-4 peristaltic pump. The resulting vesicles were centrifuged with an SS34 rotor for 10 min at 10000 rpm to remove undispersed lipids. The ethanol and Ca^{2+} or arsenazo III in the external medium were removed by gel filtration. The vesicles were passed through a column (1.0 × 40 cm) of Sephadex G-50 (medium) and eluted with isotonic choline chloride solution containing 10 mM imidazole, pH 7.0. All aqueous solutions were prepared in 10 mM imidazole (pH 7.0). The eluted vesicles were free of untrapped Ca^{2+} or arsenazo III and were used within 24 h. No detectable spontaneous release of trapped Ca^{2+} or arsenazo III occurred during this period of time from vesicles stored at 4°C. The trapped volume of the vesicles was estimated by preparing the vesicles in 0.15 M carboxyfluorescein. The untrapped carboxyfluorescein was removed by passing the vesicles through a Sephadex G-50 column (1.0 × 40 cm). The vesicles were disrupted by mixing a 0.1-ml aliquot of vesicles with 0.1 ml of 1-propanol. The sample was further diluted with 10 mM imidazole, pH 7.0, to 1.0 ml, and the absorbance was measured at 490 nm. Similarly, the absorbance of the vesicles suspension was measured prior to column chromatography [16]. Phospholipid and cholesterol concentrations were determined by the methods of Taussky and Shorr [18] and Zlatkis and Zak [19], respectively. The volume of the trapped aqueous phase was 0.97 ± 0.12 l/mol lipid. The amount of Ca^{2+} captured in the vesicles was determined from the absorbance at 650 nm of vesicles disrupted

with 1-propanol and diluted with arsenazo III solution to a final arsenazo III concentration of 0.18 μM.

Efflux and influx of Ca^{2+} were monitored by following the change of absorbance at 650 nm of an arsenazo III solution (0.18 μM unless otherwise stated) after the addition of 10 μl of an ethanolic ionophore solution. The rate of Ca^{2+} efflux was monitored by adding a 50-μl aliquot of large unilamellar vesicles containing trapped Ca^{2+} to a cuvette containing 0.8 ml of arsenazo III solution in 0.15 M choline chloride and the ionophore. Influx of Ca^{2+} was followed by adding a 50-μl aliquot of large unilamellar vesicles containing trapped arsenazo III to a cuvette that contained the ionophore in 0.8 ml of isotonic $CaCl_2$ in choline chloride solution. The absorbance was measured in a Perkin-Elmer 559A spectrophotometer at 22° ± 1°C. No detectable leakage occurred in the presence of ethanol (1.1% by volume). Initial rates were calculated from the linear portion of a plot of absorbance vs. time (see inset to Fig. 1 for a typical example).

The initial rate of A23187-mediated Ca^{2+} efflux from large unilamellar vesicles containing trapped Ca^{2+} was monitored using arsenazo III in the external medium. Fig. 1 shows that incorporation of 26 mol% cholesterol into egg PC large unilamellar vesicles causes a large decrease (about 37%) in the rate of Ca^{2+} efflux at 22°C over a wide range of A23187 concentration. This is expected from the increase in lipid order in egg PC bilayers in the presence of cholesterol, which hinders the movement of the carrier and cation-carrier complex. The inset shows a plot of the absorbance at 650 nm vs. time following addition of 0.06 μM A23187; the initial rate (dA/dt) was calculated from the tangent at zero time to the absorbance vs. time curve. Plots of the logarithm of the initial rates of Ca^{2+} efflux from egg PC and egg PC/cholesterol

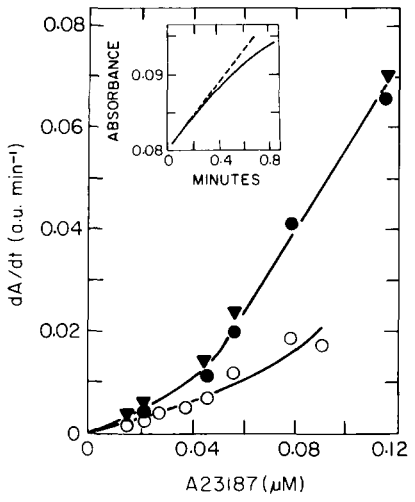

Fig. 1. The initial rates of Ca^{2+} release from large unilamellar vesicles prepared from egg PC as a function of the ionophore concentration. ●, 0.063 mM PC; ▼, 0.15 mM PC; ○, PC 0.075 mM, cholesterol 0.027 mM. Inset: Absorbance changes of the arsenazo III solution at 650 nm resulting from A23187-mediated Ca^{2+} efflux from large unilamellar vesicles as a function of time. The concentrations of PC and A23187 were 0.076 mM and 0.06 μM, respectively.

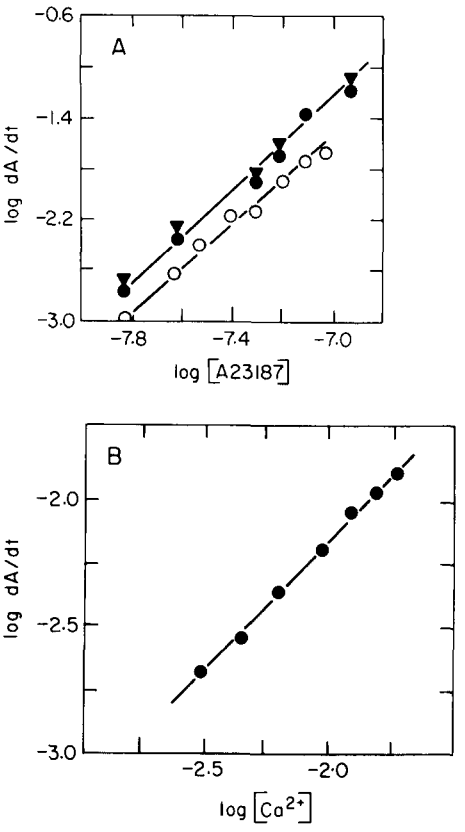

Fig. 2. Dependence of rates of Ca^{2+} efflux and influx on A23187 and Ca^{2+} concentrations. (A) A plot of the log of the initial rate of Ca^{2+} release from large unilamellar vesicles vs. the log of A23187 concentration. Symbols and lipid concentrations are as indicated in the caption to Fig. 1. (B) A plot of the log of the initial rate of Ca^{2+} influx into large unilamellar vesicles as a function of log of Ca^{2+} concentration. The PC concentration was 0.12 mM.

large unilamellar vesicles vs. the logarithm of the A23187 concentration were linear (Fig. 2A). The slopes of the straight lines and the standard deviations with egg PC large unilamellar vesicles are 1.82 ± 0.06 and 2.04 ± 0.08. With egg PC/cholesterol large unilamellar vesicles the slope is 1.83 ± 0.10. These results indicate that the rate of Ca^{2+} efflux has a second-order dependence of A23187 concentration.

The initial rate of A23187-mediated Ca^{2+} influx into large unilamellar vesicles was measured using vesicles containing trapped arsenazo III. The rate of Ca^{2+} transport increased with Ca^{2+} concentration. The log-log plot of the initial rate of Ca^{2+} influx into large unilamellar vesicles exposed to external Ca^{2+} in the range of 3–18 mM resulted in a straight line with a slope of 1.05 ± 0.06 (Fig. 2B). This indicates that the rate of Ca^{2+} influx is first order with respect to Ca^{2+} concentration.

Fig. 3 shows log-log plots of the initial rates of Ca^{2+} efflux from large unilamellar vesicles mediated by the ionophores $CH_3A23187$ and X537A. The slopes of the straight lines and their standard deviations are 1.08 ± 0.06 with $CH_3A23187$ and 1.02 ± 0.07 with X537A. Thus the rate of Ca^{2+} transport mediated by these ionophores is first order with respect to the concentration of the ionophore. The straight lines obtained in Fig. 3 were extrapolated to 0.096 μM ionophore concentration and compared with data presented in Fig. 2A for A23187. The initial rates of Ca^{2+} efflux at 0.096 μM ionophore were 49, 0.39 and 0.22 a.u./min for A23187, X537A and $CH_3A23187$, respectively. Thus the order of potency is A23187 $\gg$ X537A > $CH_3A23187$.

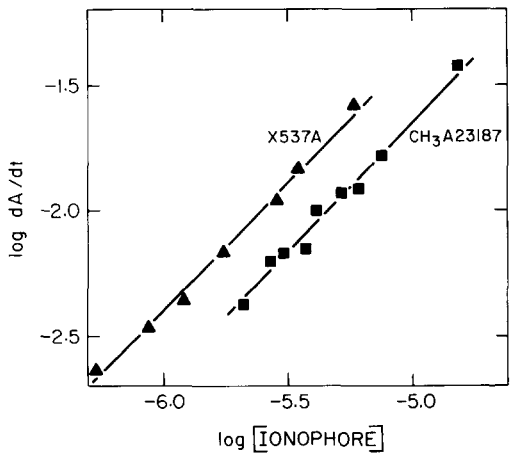

Fig. 3. Effect of log of ionophore concentration on the log of the initial rate of Ca^{2+} efflux from large unilamellar vesicles. ▲, X537A; ■, $CH_3A23187$. The PC concentration was 0.076 mM for X537A-mediated efflux and 0.060 for $CH_3A23187$-mediated efflux.

The rates of X537A-mediated Ca^{2+} influx into large unilamellar vesicles containing trapped arsenazo III were measured as a function of external Ca^{2+} concentration. When the X537A concentration was 14 μM, we found initial rate (ΔA_{650}/min) values of $5.0 \cdot 10^{-3}$, $10 \cdot 10^{-3}$ and $25 \cdot 10^{-3}$ with external Ca^{2+} concentrations of 10, 20 and 43 mM, respectively. These data support a first-order dependence of the initial rate on Ca^{2+} concentration.

In this study, we present data on Ca^{2+} movement across lipid membranes mediated by carboxylic ionophores using arsenazo III in the internal or external medium of the large unilamellar vesicles. The double logarithmic plot of initial rates of Ca^{2+} efflux vs. the ionophore concentration (Figs. 2 and 3) at a wide range of concentrations yielded straight lines which are analyzed and interpreted in terms of the order of the transport with respect to the components of the translocated complex. These lines are extrapolated to identical ionophore concentration, allowing us to estimate the relative potencies of the compounds investigated in this study. Our finding that esterification of the carboxyl group of A23187 results in a dramatic decrease in potency agrees with earlier observations of reduction of the stability of the ionophore : Mn^{2+} complex in ethanol when A23187 was substituted by $CH_3A23187$ [20]. It thus appears that the effectiveness of A23187 as a Ca^{2+} carrier is dependent on its ability to form a neutral complex with Ca^{2+}.

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