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# ELECTRICAL MEASUREMENT OF ELECTRONEUTRAL FLUXES OF DIVALENT CATIONS THROUGH CHARGED PLANAR PHOSPHOLIPID MEMBRANES

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**The voltage-sensitive channel-former monazomycin is used as a conductance probe to monitor changes in the *trans* electrostatic surface potentials of negatively-charged planar phospholipid bilayers. *Cis*-to-*trans* electroneutral fluxes of divalent cations mediated by ionophores A23187 and X537A are sensed via the effect of transported divalent cations on the *trans* surface potentials. Quantitative determinations of neutral $Ca^{2+}$ and $Mg^{2+}$ fluxes are made and related to ionophore function.**

The planar bilayer lipid membrane has become a valuable tool in the study of membrane transport phenomena. Its great utility for electrical measurements has led to numerous studies of the conductance mechanisms of ionophores, channel-formers, and incorporated biological materials. However, planar-bilayer studies of electroneutral fluxes have been relatively limited by the cumbersome non-electrical techniques required for such experiments. In this report we demonstrate how neutral fluxes of multivalent cations through negatively-charged planar lipid bilayers can be measured by standard electrical techniques. We show that the monovalent-cation conductance of bilayer membranes treated with the voltage-sensitive channel-former monazomycin can be used to monitor electrically-silent transmembrane $Ca^{2+}$ and $Mg^{2+}$ fluxes promoted by the ionophores A23187 and X537A. To our knowledge, electroneutral transport of $Mg^{2+}$ through planar lipid bilayers has not been demonstrated before.

The rationale of these experiments is based on the ability of monazomycin (or, equivalently, antibiotic LL-A491) to probe changes in electrostatic surface potential at the *trans* interface of a bilayer lipid membrane [1,2]. (*Cis* refers to the monazomycin-treated side of a bilayer, and *trans* refers to the opposite side.) The addition of $Ca^{2+}$ or other divalent cations to the *cis* compartment of a monazomycin-treated negatively-charged bilayer, in the presence of monovalent electrolyte, causes little change of the monazomycin-mediated conductance, since this conductance is highly selective for monovalent cations and is insensitive to shifts of the *cis* surface potential [1,2]. However, if a divalent-cation ionophore is also added to the membrane, the monazomycin-mediated conductance is rapidly inhibited. We present evidence below to show that this inhibition results from transport of divalent cations through the membrane, where they become temporarily trapped by the diffusion barrier of the *trans* aqueous unstirred layer. In the steady state, divalent cations accumulated at the *trans* interface reduce the magnitude of the *trans* surface potential by screening and binding; it is this positive shift of $\psi^{o}_{trans}$ that inhibits the monazomycin conductance.

For a bilayer that is highly charged, small concentrations of *trans* interfacial divalent cation ($C^{2+}$) can produce sizeable changes of $\psi^{o}_{trans}$, as is evident from the Grahame and Stern equations of diffuse double layer theory [3]. Thus, small fluxes

of $C^{2+}$ through the membrane can produce sizeable changes of monazomycin conductance. Moreover, it does not matter whether the fluxes are electrogenic or electroneutral, since it is only the appearance of $C^{2+}$ at the *trans* interface that matters.

Quantitative determination of transmembrane divalent-cation fluxes requires a calibration measurement, which is done as follows: A monazomycin-treated bilayer, with no divalent-cation ionophore present, is current-clamped in a high monazomycin-conductance state [2]. With a fixed concentration of $C^{2+}$ in the *cis* compartment (in this case 63 $\mu$M $CaCl_2$), voltage shifts ($\Delta V$) are measured as a function of $[C^{2+}]$ added to the *trans* compartment (see Fig. 5 of Ref. 2). A plot of $\Delta V$ vs. $[C^{2+}]_{trans}$ provides the requisite calibration. The flux measurement is now done under the same conditions, but with no addition of $[C^{2+}]_{trans}$. Divalent-cation ionophore and $[C^{2+}]_{cis}$ are added in either order, and the steady-state $\Delta V$ is recorded. The calibration curve gives an effective $[C^{2+}]_{trans}$ corresponding to $\Delta V$. Following the analysis of Heyer et al. [4,5], we take this value of $[C^{2+}]_{trans}$ to be that existing near the *trans* interface just outside the diffuse double layer. This approximation amounts to neglect of the diffusion barrier of the diffuse double layer (approx. 3 nm thick in our case) compared to that of the unstirred layer (approx. 100 $\mu$m thick [6]). The flux of $C^{2+}$ through the unstirred layer into the *trans* compartment is now calculated from

$$\Phi = D\frac{[C^{2+}]^{o}_{trans}}{\delta} \qquad (1)$$

where $D$ is the divalent-cation aqueous diffusion coefficient, $\delta$ is the thickness of the *trans* unstirred layer, and $[C^{2+}]^{o}_{trans}$ is $[C^{2+}]$ near the *trans* interface as described above. The bulk concentration of $C^{2+}$ in the *trans* compartment remains negligibly small in the time-course of these experiments. Since, in the steady-state, $C^{2+}$ flux through the membrane must equal $C^{2+}$ flux through the unstirred layer, Eqn. 1 also yields the $C^{2+}$ transmembrane flux.

Bilayer lipid membranes were made from 1:1 mixtures of bacterial phosphatidylethanolamine (PE) and EDTA-washed bovine phosphatidylserine (PS), obtained as lyophilized powders from Avanti Biochemicals and dissolved as 1% (w/v) solutions in *n*-decane (Sigma). The teflon cell, membrane partition, electrodes, and current-clamp circuit have been described previously [2]. Both chambers were stirred with magnetic fleas at constant speeds of approx. 250 rpm. The aqueous solutions were 10 mM NaCl, buffered at pH 7.0 with 2 mM morpholinopropane sulfonic acid (Mops) (sodium salt). Merck ultrapure NaCl and deionized distilled water (Barnstead Nanopure, $> 10$ M$\Omega \cdot$ cm) were used in all experiments. Divalent-cation salts were reagent grade. Initial bare membrane conductances were typically $1 \cdot 10^{-8}$ S/cm$^2$. Antibiotic LL-A491 was obtained from Dr. E.L. Patterson, Lederle Laboratories, Pearl River, NY, and was stored refrigerated in distilled water. In this report we often refer to LL-A491 colloquially as 'monazomycin', since the two antibiotics appear to be functionally identical [2]. LL-A491 was added to the *cis* chamber to a final concentration of 0.2–0.5 $\mu$g/ml, which resulted in a typical steady-state conductance of approx. $4 \cdot 10^{-6}$ S/cm$^2$ with the membrane current-clamped at 65 nA/cm$^2$ ($V_{cis}$ positive). Electrolyte solutions were checked routinely for contaminating multivalent cations by monitoring the conductance of an LL-A491-treated bilayer while adding EDTA to the *trans* chamber. Ionophore A23187 was obtained from Dr. R.L. Hamill, Lilly Research Laboratories, Indianapolis, IN and ionophore X537A from Dr. W.E. Scott, Hoffmann-La Roche, Inc., Nutley, NJ. The ionophores were stored at $-20$°C in methanol or ethanol. We found it essential to rinse the membrane chamber between runs with hot ethanol in order to remove ionophore (especially A23187) which adheres strongly to teflon. All experiments were done at approx. 22°C.

In the figures, all bilayer lipid membranes have been pre-treated with *cis* additions of LL-A491 and are current-clamped at 0.5 nA. The *trans* compartment is virtual ground, so that positive values of $\Delta V$ represent a decrease in monazomycin conductance. It was shown in Ref. 2 that these $\Delta V$ shifts reflect changes of the *trans* surface potential, $\Delta\psi^{o}_{trans}$, which are closely equal to $\Delta V$.

In Fig. 1 we show the effect of repetitive additions of *cis* $Ca^{2+}$ at constant [A23187]. Initial addition of 0.5 $\mu$M A23187 to the *trans* chamber

causes a small (+2 mV) voltage shift; a similar effect occurs if A23187 is added *cis* (not shown). This decrease of monazomycin conductance is [A23187]-dependent and thus suggestive of A23187-monazomycin interactions in the bilayer. The effect becomes undetectable for [A23187] less than 0.5 $\mu$M, indicating that A23187-monazomycin interactions remain negligibly small in the experiments reported here. Repeated additions of *cis* $Ca^{2+}$ now produce positive $V$ shifts, indicating positive shifts of $\psi^{o}_{trans}$ caused by accumulation of transported $Ca^{2+}$ at the *trans* interface. To verify that these voltage shifts indeed result from the effects of transported $Ca^{2+}$, EDTA is added to the *trans* compartment, causing $V$ to fall to its original value. If instead EDTA is added to the *cis* chamber (not shown), a similar drop of $V$ occurs, presumably due to collapse of the transmembrane $Ca^{2+}$ gradient and cessation of $Ca^{2+}$ transport. Control experiments show that EDTA itself produces no $V$ shift under these conditions. Also, it does not matter whether A23187 is added *cis* or *trans* in these experiments. No significant electrical effects are observed here if either A23187 or monazomycin is omitted.

In Fig. 2 we show the effect of repetitive additions of A23187 at constant $[Ca^{2+}]_{cis}$. Each ionophore addition causes a positive $V$ shift. The flux corresponding to each $\Delta V$ can be calculated as described above from the calibration curve and Eqn. 1. A plot of log($Ca^{2+}$ flux) vs. log[A23187] for a single PS/PE bilayer is shown in Fig. 3, where a similar plot for X537A is also given. The initial slopes of these plots are 1.9 for A23187 and 2.3 for X537A. Although more data are required

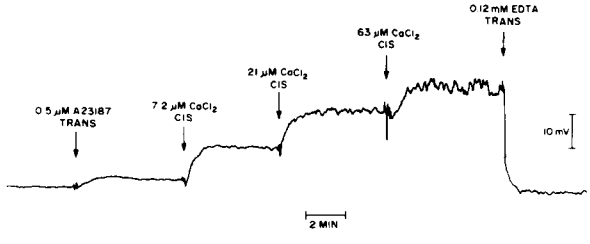


Fig. 1. Effect of additions of ionophore A23187, *cis* $Ca^{2+}$, and *trans* EDTA on transmembrane $V$ of a PS/PE (1:1) bilayer current-clamped at 0.5 nA and pretreated with 0.38 $\mu$g/ml LL-A491 in the *cis* compartment. Electrolyte is 10 mM NaCl, 2 mM (Na)Mops, pH 7.0. Total concentrations present after each addition are as indicated.

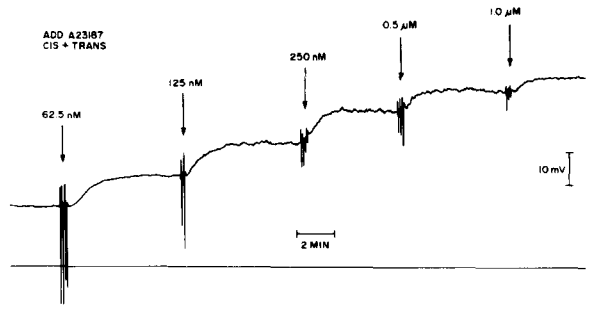


Fig. 2. Effect of repetitive symmetric additions of ionophore A23187 on transmembrane $V$ of current-clamped bilayer containing 63 $\mu$M $Ca^{2+}$ in the *cis* compartment. Total concentrations of A23187 present after each addition are as indicated. Conditions are the same as in Fig. 1.

for statistical reliability, both values are consistent with ionophore:$Ca^{2+}$ stoichiometries of 2:1 for the permeant species [7–14]. It is also evident in Fig. 3 that the potency of A23187 as a $Ca^{2+}$ ionophore exceeds that of X537A by nearly two orders of magnitude, in agreement with previously published findings [9,10]. The saturation behavior seen at the higher ionophore concentrations is consistent with approach to a limiting flux determined by the aqueous unstirred layers. The fluxes plotted in Fig. 3 are indeed electroneutral, since in the absence of monazomycin we found no

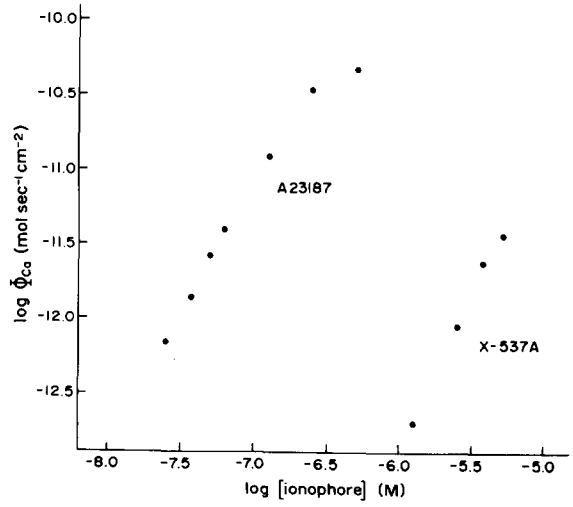


Fig. 3. Plot of $Ca^{2+}$ flux vs. aqueous ionophore concentration, as determined from experiments similar to that shown in Fig. 2. $[Ca^{2+}]_{cis} = 63$ $\mu$M. Fluxes are determined as described in the text from *trans* calibration curve and Eqn.1 with $D = 10^{-5}$ cm$^2$/s and $\delta = 100$ $\mu$m.

change of bare membrane conductance or e.m.f. at the highest ionophore concentrations pictured. For the A23187-mediated $Ca^{2+}$ flux, an electrical component of one charge per 8000 transported calcium ions would have been readily observable.

The quantitative validity of our flux measurements was verified by a $^{45}Ca$ tracer experiment, in which the radioactive and monazomycin-measured $Ca^{2+}$ fluxes were determined simultaneously with the same membrane. At $[A23187] = 1.2\ \mu M$ and $[Ca^{2+}]_{cis} = 62\ \mu M$, the tracer experiment yielded a $Ca^{2+}$ flux of $1.9 \cdot 10^{-11}$ mol/cm$^2$ per s, while the monazomycin technique gave $2.6 \cdot 10^{-11}$ mol/cm$^2$ per s, with $D = 10^{-5}$ cm$^2$/s and $\delta = 100\ \mu m$ in Eqn. 1. We consider these results to be in very good agreement. Although 100 $\mu$m is a typical unstirred-layer thickness for planar bilayer lipid membranes [6], the dependence of this parameter on chamber geometry and stirring speed renders its precise value in a given experiment somewhat uncertain. If the tracer flux above is used to determine the unstirred-layer thickness via Eqn. 1, we obtain $\delta = 140\ \mu m$ for this experiment. Use of this value for the flux calculations of Fig. 3 would lower all indicated fluxes by 0.14 log unit. A careful determination of $\delta$, however, requires further experiments such as measurement of radioactive butanol fluxes [15].

Fig. 4 shows a comparison of $Ca^{2+}$ and $Mg^{2+}$ transport by X537A. Bi-ionic potential and conductance measurements have shown that the electrogenic component of X537A-mediated cation transport through bilayer lipid membranes is much less selective for $Mg^{2+}$ than for $Ca^{2+}$ [8,16]. However, no such comparison has been made with planar bilayers for the large electroneutral component. Calibration of the *trans* voltage shifts for $Mg^{2+}$ indicates that in Fig. 4 the $Ca^{2+}$ flux exceeds the $Mg^{2+}$ flux by a factor of 3–10. More accurate determination of the $Ca^{2+}/Mg^{2+}$ specificity requires comparison of $Ca^{2+}$ and $Mg^{2+}$ fluxes over a broad range of ionophore concentrations. The specificity for transport of other multivalent cations can be determined in a similar manner.

An important consideration in these experiments is the effect of membrane surface potential on the observed $Ca^{2+}$-induced voltage shifts. Our mechanism predicts, for a given $Ca^{2+}$ flux, a sensitive dependence of $\Delta V$ on $\psi^o_{trans}$. Repetition of the experiment shown in Fig. 1 with an electrolyte of 0.1 M NaCl confirmed this prediction. Here $\Delta V$ following a *cis* addition of 63 $\mu$M $Ca^{2+}$ was reduced from 27 mV (cf. Fig. 1) to approx. 2 mV (not shown), as expected from Stern analysis [17]. Similarly, repetition of Fig. 1 with a PE membrane yielded no detectable $\Delta V$, also as expected. The sensitivity of our technique is therefore enhanced when $\psi^o_{trans}$ is highly negative. For a PS bilayer in 1 mM NaCl, we calculate that a $Ca^{2+}$ flux of $5 \cdot 10^{-16}$ mol/cm$^2$ per s is in principle detectable. Enhanced sensitivities would occur for tri- or higher-valent cations and for multivalent cations which bind more strongly to the membrane than does $Ca^{2+}$ to PS. Sensitivity could also be increased by use of a monovalent electrolyte whose cation binds less strongly to the membrane than does $Na^+$ (e.g., $K^+$, $Cs^+$, or tetramethylammonium$^+$ [18]). The sensitivity of this technique for measuring $Ca^{2+}$ fluxes through highly-charged planar bilayers equals or exceeds that obtainable in practice by ion-sensitive electrodes, dyes, or radioactive tracers [10,11].

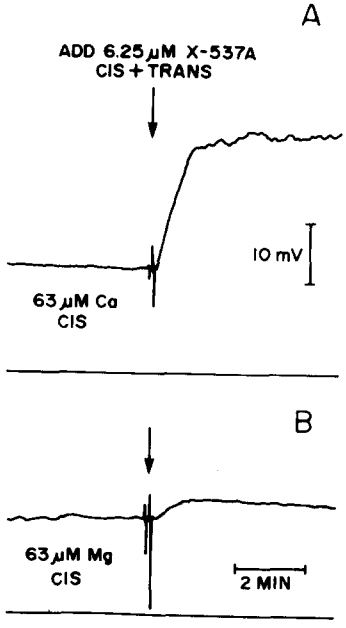


Fig. 4. Voltage shifts produced by symmetric addition of 6.25 $\mu$M ionophore X537A to current-clamped bilayer lipid membranes in the presence of (A) 63 $\mu$M *cis* $Ca^{2+}$ or (B) 63 $\mu$M *cis* $Mg^{2+}$. Voltage and time scales are identical for (A) and (B). Conditions are the same as in Fig. 1.

A further test of the credibility of our flux measurements is that of stirring effects. In the presence of 1 $\mu$M A23187 (and 63 $\mu$M *cis* $Ca^{2+}$), Fig. 3 indicates that the $Ca^{2+}$ flux is near saturation, hence limited by unstirred-layer diffusion. We found that elimination of *trans* stirring in this case caused a 6 mV increase of $\Delta V$, elimination of *cis* stirring caused a 7 mV decrease of $\Delta V$, and elimination of both *cis* and *trans* stirring resulted in little change of $\Delta V$. These results are fully consistent with the mechanism of flux detection proposed in this report and indicate approx. 100% increase of the *cis* and *trans* unstirred-layer thicknesses, respectively, when the *cis* and *trans* stirrers were turned off.

In this report we have demonstrated the feasibility of measuring neutral divalent-cation fluxes through charged planar bilayer lipid membranes by electrical monitoring of monazomycin conductance. We have also illustrated the significant effects that transported ions can have on membrane surface potentials, including inactivation of voltage-sensitive channels. The fact that ions are transported by electroneutral processes does not preclude their ability to interact electrostatically with the transmembrane surface. Our technique is well suited to study of interfacial reactions associated with transport, particularly with regard to the roles of surface potentials and asymmetric substrate (including $H^+$) availability. It is also possible that this method will permit sensitive detection of neutral divalent-cation transport through planar bilayers induced by incorporated biological materials.

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## References


1 Muller, R.U. and Finkelstein, A. (1972) J. Gen. Physiol. 60, 285–306
2 Cohen, J.A. and Moronne, M.M. (1978) Biochem. Biophys. Res. Commun. 83, 1275–1283
3 McLaughlin, S. (1977) Curr. Top. Membrane Transp. 9, 71–144
4 Heyer, E.J., Muller, R.U. and Finkelstein, A. (1976) J. Gen. Physiol. 67, 703–729
5 Heyer, E.J., Muller, R.U. and Finkelstein, A. (1976) J. Gen. Physiol. 67, 731–748
6 McLaughlin, S. (1975) Annu. Rev. Biophys. Bioeng. 4, 335–366
7 Case, G.D., Vanderkooi, J.M. and Scarpa, A. (1974) Arch. Biochem. Biophys. 162, 174–185
8 Célis, H., Estrada-O, S. and Montal, M. (1974) J. Membrane Biol. 18, 187–199
9 Hyono, A., Hendriks, T., Daemen, F.J.M. and Bonting, S.L. (1975) Biochim. Biophys. Acta 389, 34–46
10 Kafka, M.S. and Holz, R.W. (1976) Biochim. Biophys. Acta 426, 31–37
11 Wulf, J. and Pohl, W.G. (1977) Biochim. Biophys. Acta 465, 471–485
12 Pohl, W.G., Kreikenbohm, R. and Seuwen, K. (1980) Z. Naturforsch. 35c, 562–568
13 Haynes, D.H., Chiu, V.C.K. and Watson, B. (1980) Arch. Biochem. Biophys. 203, 73–89
14 Kolber, M.A. and Haynes, D.H. (1981) Biophys. J. 36, 369–391
15 Holz, R. and Finkelstein, A. (1970) J. Gen. Physiol. 56, 125–145
16 Schadt, M. and Haeusler, G. (1974) J. Membrane Biol. 18, 277–294
17 McLaughlin, S., Mulrine, N., Gresalfi, T., Vaio, G. and McLaughlin, A. (1981) J. Gen. Physiol. 77, 445–473
18 Eisenberg, M., Gresalfi, T., Riccio, T. and McLaughlin, S. (1979) Biochemistry 18, 5213–5223