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Carrier-Mediated Ion Transport Through Black Membranes of Lipid Mixtures and its Coupling to Ca^{++}-Induced Phase Separation

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Summary. Voltage jump-current relaxation experiments have been performed with valinomycin-doped membranes of mixtures of 1,2-dipentadecylmethylidene-glycero-3-phosphorylcholine (PC) and charged-phosphatidic acid (PA). Both relaxation processes predicted by a simple carrier model could be resolved which allowed the calculation of the rate constants of the Rb^+ transport. The dependence of the rate constants on the membrane composition indicates that (*i*) the lipids in the mixed membranes are homogeneously distributed and that (*ii*) no major difference exists between the composition of the membrane and that of the torus. The analysis of the stationary conductance data, however, shows that the valinomycin content of the mixed membranes depends strongly on their lipid composition. Addition of Ca^{++} ions to a 1:1 mixture induces a phase separation into PA domains of very low conductivity and PC-enriched regions of high conductivity. Half saturation is reached at $c_{Ca} = 5 \times 10^{-4}$ M. At 10^{-2} M Ca^{++} in the aqueous phase, the rate constants clearly indicate that all PA molecules are electrically "passivated" and only pure PC domains contribute to the membrane current. A detailed picture is thus derived of the coupling of a model transport system to the externally triggered membrane reorganization.

Key Words black lipid membranes · carrier-mediated ion transport · lipid mixtures · Ca^{++}-induced phase separation

Introduction

Ca^{++} ions play an essential role in many membrane-related biological functions like nerve excitation or cell-cell communication. Some of the observed modifications of ion permeabilities by Ca^{++} have been attributed to a change in the surface potential of the membrane (McLaughlin, 1977) while for others a direct action of the divalent ion on the transport system has been shown (Rose & Loewenstein, 1976). One of the striking effects of Ca^{++} on artificial membranes is the isothermal induction of phase separations in mixed membranes of neutral and charged lipids (Ito & Ohnishi, 1974; Ohnishi & Ito, 1974; Galla & Sackmann, 1975). However, it is not clear yet whether such Ca^{++}-induced phase separation phenomena are important also in biological membranes and how they influence or regulate physiological processes.

We have carried out investigations along this line by studying the characteristics of a model transport system, the ionophore valinomycin, incorporated into bimolecular lipid layers composed of a mixture of neutral phosphatidylcholine and charged phosphatidic acid. These black films are widely used as a model for biological membranes, namely for the investigation of ion transport mechanisms across a hydrophobic barrier (Läuger, 1972). We have chosen valinomycin for our study (1) because its action as a mobile carrier for alkali ions is well studied and (2) because it also has been tested as a molecular probe of structural properties of black lipid membranes (Stark, Benz, Pohl & Janko, 1972; Benz & Läuger, 1977). In particular, if it is possible to analyze kinetic experiments (voltage jump or charge pulse), substantial information about the physical state of the lipid matrix can be derived from the rate constants that determine the carrier transport. So far, only a few studies have been performed that deal with the question of how ionophores behave in lipid mixtures (Lesslauer, Richter & Läuger, 1967; Szabo, 1974; Benz & Cros, 1978; Apell, Bamberg & Läuger, 1979) and how they are influenced by externally triggered structural or organizational changes of the membrane (Krasne, Eisenman & Szabo, 1971; Boheim, Hanke & Eibl, 1980). It has long been discussed as to how phase transitions and especially phase separation phenomena may influence biological processes (Overath, Schairer & Stof-

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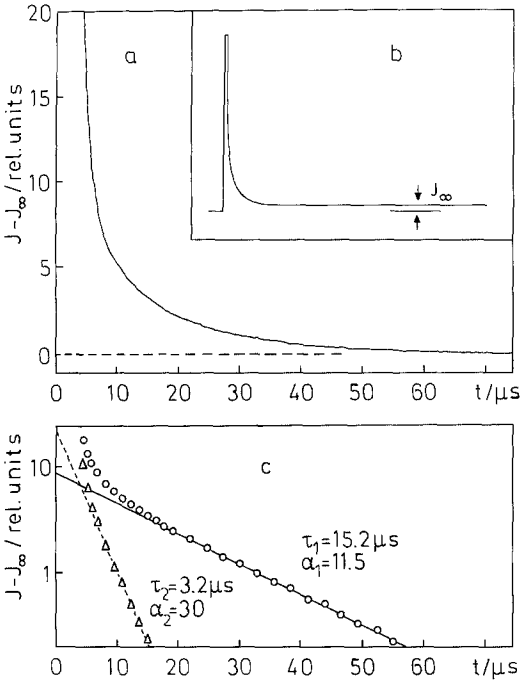

Fig. 1. Typical current relaxation of a PA membrane (doped with 5×10^{-4} M valinomycin in the membrane-forming solution) following a voltage jump of $U=50$ mV. $c_{RbCl}=0.1$ M, $c_{LiCl}=0.9$ M, $T=35$ °C, membrane area $=5\times10^{-3}$ cm^2. The current was measured as a voltage decay across an ohmic resistance of $R_A=100\,\Omega$. (*a*) Original curve as obtained on an *X-Y* plotter (DIN A3) after 512 averaged pulses. (*b*) Same experiment but with 4 times smaller time scale. The initial current spike is due to the loading of the membrane capacity ($\tau_{RC}=0.8$ μsec). (*c*) Semilogarithmic plot of the data from (*a*) and evaluation of the relaxation times and amplitudes according to Eq. (1) with I_∞ from (*b*). (○) original data, (△) after subtraction of the extrapolated slower process (——)

fel, 1970; Träuble, 1971; Lee, 1977; Sackmann, 1978). This paper for the first time presents data that allow one to derive a detailed picture of (1) how a membrane function can be coupled to the membrane structure and (2) how such a function can be regulated by changes in the lateral organization of the membrane.

Materials and Methods

The synthesis of the two lipids used in this study - 1,2-dipentadecylmethylidene-glycero-3-phosphorylcholine (PC) and 1,2-dipentadecylmethylidene-glycero-3-phosphatidic acid (PA) - is described elsewhere (Eibl & Nicksch, 1978). Membranes were formed (Mueller, Rudin, Tien & Wescott, 1962) from 1% (wt/vol) lipid solutions in *n*-decane (Fluka, purum). Valinomycin (Calbiochem) was added to the lipid phase to give a concentration of 5×10^{-4} M. The aqueous RbCl solutions (unbuffered, pH 5.8) contained also LiCl so that the overall concentration of monovalent ions was 1 M throughout the experiments. Studies of the Ca^{++} dependence were performed with electrolytes additionally containing $CaCl_2$ salt in various amounts.

The cell for the bilayer formation as well as the set-up for the voltage jump-current relaxation measurements are as described previously (Benz, Stark, Janko & Läuger, 1973; Pohl, Knoll, Gisin & Stark, 1976). A pulse generator with a rise-time of 4 nsec (Philips PM 5712) was used. The signal-to-noise ratio of the current relaxations was improved by signal averaging (Biomation waveform recorder, model 805 and Nicolet averager model 1070 with a high-speed buffer interface, model SD-78). All experiments were performed at 35 °C where both lipids in the decane-containing black membranes are fluid (Neher & Eibl, 1977; Blume & Eibl, 1981).

Analysis of the Current Relaxation

The current relaxation after a voltage jump of the valinomycin-mediated ion transport has been successfully interpreted by a carrier-model which is described in detail elsewhere (Läuger & Stark, 1970; Stark, Ketterer, Benz & Läuger, 1971; Benz et al., 1973; Laprade, Ciani, Eisenman & Szabo, 1974; Knoll & Stark, 1975). According to this model, the time course of the current I following a voltage jump U is given by

$$I(t)=I_\infty(1+\alpha_1 e^{-t/\tau_1}+\alpha_2 e^{-t/\tau_2}) \tag{1}$$

with I_∞ = stationary current at times $t\gg\tau_1$, τ_2, α_1, α_2 = relaxation amplitudes and τ_1, τ_2 = relaxation times. A plot of $\log[(I(t)-I_\infty)/I_\infty]$ versus t thus allows to separate the two relaxation processes. An example is given in Fig. 1. Parts *a*) and *b*) show the original plot after 512 averaged pulses (in part *b*) with a 4 times reduced time scale in order to determine I_∞) and part *c*) the corresponding semilog plot of these data. From the relaxation times and amplitudes derived in this way by a single experiment the rate constants which characterize the different steps in the carrier model can be evaluated (*see* Eqs. (2) through (9) in Knoll & Stark, 1975): k_D, the dissociation rate constant of the ion-carrier complex; k_S, the rate constant that describes the translocation of the free carrier; $k_R c_M^*$, the rate constant of the complex formation which can be determined only in combination with the interfacial ion concentration c_M^*; and the translocation of the complex $k_{MS}(0)$, which appears in the formalism as the sum $[k'_{MS}(U)+k''_{MS}(U)]$ (translocation from high to low potential and vice versa) and can be approximated (for small voltages) by

$$k_{MS}(0)=(k'_{MS}+k''_{MS})/2\cosh\frac{UF}{RT} \tag{2}$$

where F = Faraday constant, R = gas constant, and T = absolute temperature.

Carrier Transport Through Mixed Membranes

In order to be able to resolve both relaxation processes predicted by the carrier model for all lipids and lipid mixtures investigated we had to choose 10^{-1} M RbCl in the aqueous phase. The ionic strength was kept constant at 1 M with LiCl. Figure 2 shows the relaxation times and amplitudes of the membrane current following a voltage jump of $U = 50$ mV for PA, PC and three mixtures of these lipids.

All four experimental parameters vary in a more or less monotonous way from pure PA to pure PC. From these data the four rate constants could be evaluated and are shown in Fig. 3. The two translocation processes, described by the rate constants k_S and $k'_{MS} + k''_{MS}$ are nearly unaffected by changing the headgroup of the lipid. The dissociation constant k_D increases slightly from PA to PC while $k_R c^*_M$ decreases by almost an order of magnitude. A pronounced nonlinear variation shows the membrane conductance λ_0 (shown in Fig. 4) which is related to the stationary current I_∞, at small voltage U by

$$\lambda_0 = \frac{I_\infty}{U \cdot A}$$

with A = membrane area.

Before discussing these results we have to try to answer two important questions: (1) Do we have a homogeneous lipid distribution in the membrane or do we see any phase separation or domain formation? (2) Are the two lipids in the black membrane in the same molar ratio as they are in the torus? We think that we can exclude at least a complete phase separation: Assume we have in a 1:1 mixture domains of pure PA and regions of pure PC. We would expect then that the relaxation of the current following a voltage jump is composed of two contributions originating from valinomycin molecules that are either in PC- or PA-environment. (This assumption, of course, requires that the domains are large enough so that boundary effects can be neglected.) Taking into account the relative current contributions of the two regions this superposition (shown in Fig. 5, dashed curve) would result in a current decay that does not correspond to the experimental data (full circles). Similar discrepancies

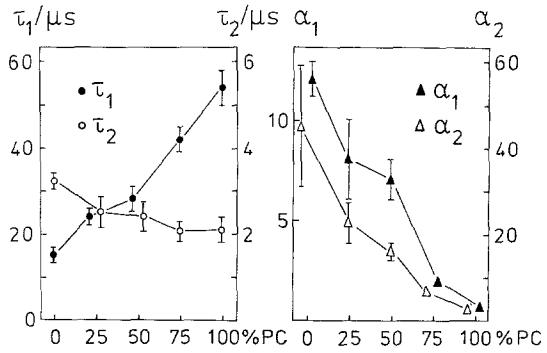

Fig. 2. Relaxation data as obtained from kinetic experiments with valinomycin in mixed PA/PC membranes. Plotted are the relaxation times (○, ●) and the relaxation amplitudes (△, ▲) as a function of the PC content. Each point represents the mean value of at least five membranes. Bars indicate the standard deviation. $T = 35\,°C$, $U = 50$ mV, $c_{Rb} = 0.1$ M, $c_{Li} = 0.9$ M

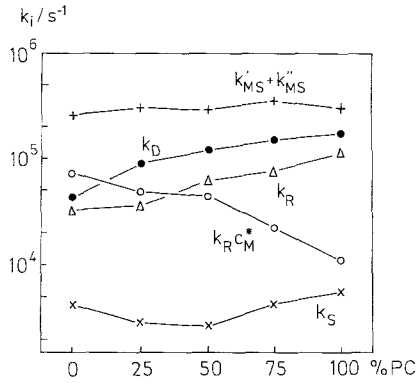

Fig. 3. Rate constants calculated from the relaxation data in Fig. 2 as a function of the PC content in the membrane

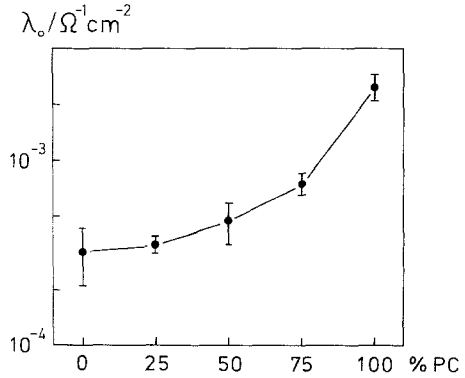

Fig. 4. Dependence of the stationary conductance λ_0 on the PC content in the membrane (PA/PC mixed membranes, $T = 35\,°C$, $c_{Rb} = 0.1$ M, $c_{Li} = 0.9$ M). Each point represents the mean value of at least five membranes ± standard deviation

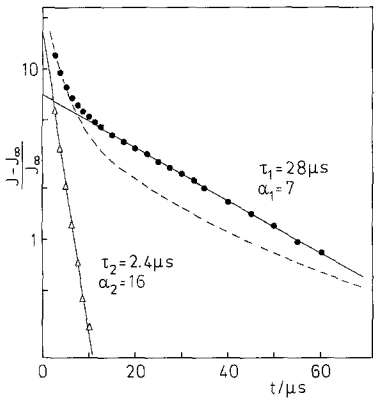

Fig. 5. Comparison between the experimentally determined current relaxation of a homogeneous 1:1 PA/PC mixture and the current decay of a hypothetical membrane composed of separated pure PA and pure PC domains. (●) semilogarithmic plot of the current relaxation following a voltage jump of $U=50$ mV for the 1:1 mixture ($c_{Rb}=0.1$ M, $c_{Li}=0.9$ M, $T=35$ °C); (△) current data after subtraction of the slower relaxation process. Relaxation times and amplitudes are evaluated according to Eq. (1). The dashed line is calculated assuming two independent membrane areas (equal in size) of PC and PA molecules according to

$$I(t)=0.5\,I_\infty^{PA}[1+\alpha_1^{PA}\,e^{-t/\tau_1^{PA}}+\alpha_2^{PA}\,e^{-t/\tau_2^{PA}}]$$
$$+0.5\,I_\infty^{PC}[1+\alpha_1^{PC}\,e^{-t/\tau_1^{PC}}+\alpha_2^{PC}\,e^{-t/\tau_2^{PC}}].$$

Stationary current, relaxation times and amplitudes for PA (I_∞^{PA}, τ_1^{PA}, τ_2^{PA}, α_1^{PA}, α_2^{PA}) and for PC (I_∞^{PC}, τ_1^{PC}, τ_2^{PC}, α_1^{PC}, α_2^{PC}) were taken from Figs. 2 and 4

between calculated and measured current relaxations can be seen for the other lipid mixtures (*not shown*). From these results we conclude that at least a complete phase separation does not occur in the membrane. Now, if one assumes that the components in the bilayer are homogeneously distributed, this then implies a gradual dilution of the surface charges of the PA molecules by adding PC molecules. According to (Läuger & Neumcke, 1973; McLaughlin, 1977)

$$\psi_S=2\frac{RT}{F}\ln\left\{\frac{\sigma}{\sigma_0}+\sqrt{\left(\frac{\sigma}{\sigma_0}\right)^2+1}\right\} \tag{4}$$

with $\sigma_0=\sqrt{8\varepsilon\varepsilon_0 RTc}$, $\varepsilon_0=8.85\times10^{-12}$ CV^{-1} m^{-1}, $\varepsilon=78.5$ the dielectric constant of water, $c=$the concentration of the 1:1 electrolyte, this means that by decreasing the surface charge density σ, one also decreases the surface potential ψ_S, and hence the enrichment of the positive ions at the (negative) lipid-water interface as predicted by the Boltzman relation

$$c_S=c_\infty\exp\{-\psi_S F/RT\}. \tag{5}$$

Assuming an area/lipid molecule of approx. 0.60 nm^2 we obtain for the pure PA bilayer (with $\sigma=-0.27$ Cm^{-2} and $c=1$ M) $\psi_S=-82$ mV. This should result in a 22-fold increase of the cation concentration at the interface. For the mixtures this enrichment is correspondingly lower which means that the relaxation experiments of the different membranes are all done in a different ionic environment. This should not affect the determination of the rate constants k_S, $k'_{MS}+k''_{MS}$, and k_D, as we could show in a separate experiment for PA and PC that these constants do not significantly change if one varies the bulk Rb concentration by almost two orders of magnitude (Schmidt et al., *in preparation*). The complexation step $k_R c_M^*$, however, depends greatly on the ion concentration at the interface. As a first approximation we can separate the ion enrichment factor $\exp\{-\psi_S F/RT\}$ for the different mixtures. The result is also plotted in Fig. 3. One can see that now all four rate constants vary in a more or less linear way with the molar ratio of the lipid components in the torus. This suggests that at least no major difference exists between the composition of the black membrane and the torus. (Results of Ca^{++}-induced phase separation experiments support this idea, *see* next section.)

With this interpretation of the relaxation data, interesting conclusions can be drawn from the composition dependence of the conductance λ_0. If valinomycin is added to the lipid phase λ_0 is given by (Benz et al., 1973; Knoll & Stark, 1975):

$$\lambda_0=\frac{F^2 d c_s^b \gamma_s^{mb} k_{MS}(o) k_R c_M^*}{2RTk_D(1+2k_{MS}(o)/k_D+k_{MS}(o)\,k_R c_M^*/k_S k_D)} \tag{6}$$

where $c_s^b=$valinomycin concentration in the bulk lipid phase (torus), $\gamma_s^{mb}=$partition coefficient of the carrier S between the membrane and the bulk phase, $d=$membrane thickness. With the knowledge of the rate constants from relaxation experiments the only free parameter that determines the conductivity is the partition coefficient γ_s^{mb} which is given in Table 1 for the different membranes. The data show a highly nonlinear dependence on the composition which would be only slightly modified by taking into account the different membrane thicknesses. A possible explanation of this effect may be that the incorporation of valinomycin is highly sensitive to the packing density of the

Table 1. Partition coefficient γ_s^{mb} for the different PA/PC membranes as obtained from the experimental data in Figs. 3 and 4 (*see text* for details)

PA/PC	100/0	75/25	50/50	25/75	0/100
γ_s^{mb}	0.19	0.31	0.46	0.63	2.8

membrane. It is known from monolayer investigations that under otherwise similar conditions the area per PC molecule is always larger than that of a PA molecule and that the mean area/molecule for mixtures vary in a nonlinear way (Albrecht, 1979). The incorporation of a few PA molecules (with a small headgroup) into a PC monolayer increases the packing of the hydrocarbon tails considerably by compensating the too large headgroup of the PC molecules. Few PC molecules dissolved in PA monolayers, however, hardly change the mean area/CH_2 chain. This nonlinear dependence of the packing density on composition variations might be a reason for the observed behavior of the valinomycin content of the black membrane.

Ca^{++}-Induced Phase Separation

The next step was to investigate (1) how Ca^{++} ions influence the lateral organization of mixed PA/PC membranes and (2) how a model transport system like the carrier valinomycin responds to these changes. For that purpose, stationary and kinetic experiments with membranes of PC, PA and a 1:1 mixture of both components have been performed in the presence of different Ca^{++} concentrations ranging from 0 up to 10^{-1} M Ca^{++}. Figure 6 shows the influence of Ca^{++} on the conductance λ_0. Virtually no effect can be seen for PC membranes. PA membranes are stable only up to 3×10^{-4} M Ca^{++}. Nevertheless, a significant decrease in the conductivity is found. Quite a different behavior shows the equimolar mixture of PC and PA: λ_0 increases in an S-shaped way starting at about 10^{-4} M Ca^{++} and ending at about 10^{-2} M Ca^{++}. Further increase in the Ca^{++} concentration has no additional effect. The experimental conditions again had been chosen such that for all Ca^{++} concentrations both relaxation processes after a voltage jump could be resolved. Relaxation times and amplitudes derived from these measurements are shown in Fig. 7. Again a more or less S-shaped change of

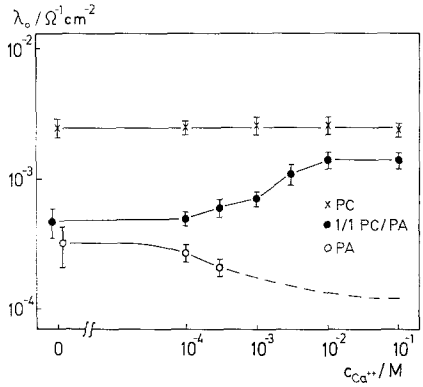

Fig. 6. Dependence of the stationary conductance λ_0 on the Ca^{++} ion concentration in the bulk aqueous phase. (×) PC membrane, (○) PA membrane, (●) 1:1 mixture of PC and PA. Data points represent mean values of at least five membranes. Error bars indicate standard deviations. $c_{Rb}=0.1$ M, $c_{Li}=0.9$ M, $T=35$ °C

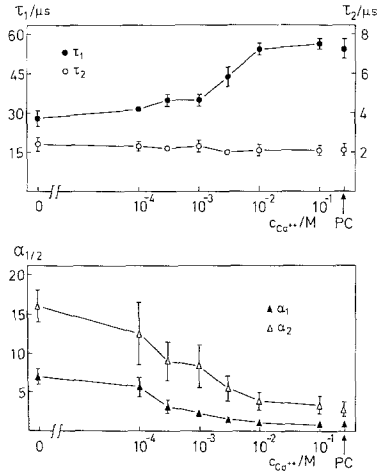

Fig. 7. Dependence of the relaxation times (○, ●) and amplitudes (△, ▲) on the Ca^{++} ion concentration in the bulk aqueous phase. The arrows point to the corresponding data for PC membranes which are independent of the presence of Ca^{++} ions up to 10^{-1} M. Data points are mean values ± standard deviation of at least five different membranes, $c_{Rb}=0.1$ M, $c_{Li}=0.9$ M, $T=35$ °C

the four parameters is found around 10^{-3} M Ca^{++} if the Ca^{++} concentration is increased.

For comparison also the experimental data for the pure PC membrane are added in Fig. 7. The presence of Ca^{++} up to 10^{-1} M has no influence on the carrier kinetic in a PC environment. The corresponding data for PA membranes (only up to $c_{Ca^{++}}=3 \times 10^{-4}$ M) are given in Table 2. Up to these concentrations only small changes of the relaxation data are found. Figure 8 shows the rate constants for the

Table 2. Relaxation times and amplitudes, and calculated rate constants of the valinomycin transport in pure PA membranes at different Ca^{++} ion concentrations[a]

$c_{Ca^{++}}$ (M)	τ_1 (μsec)	α_1	τ_2 (μsec)	α_2	$k_R c_M^*$ (sec^{-1})	k_D (sec^{-1})	$k'_{MS}+k''_{MS}$ (sec^{-1})	k_s (sec^{-1})
0	15	12.0	3.2	45	7.2×10^4	4.3×10^4	2.6×10^5	4.2×10^3
10^{-4}	16	12.0	2.8	40	7.0×10^4	5.9×10^4	2.8×10^5	3.6×10^3
3×10^{-4}	23	11.5	2.9	30	5.3×10^4	7.6×10^4	2.6×10^5	2.3×10^3

[a] $c_{Rb}=0.1$ M, $c_{Li}=0.9$ M, $T=35$ °C.

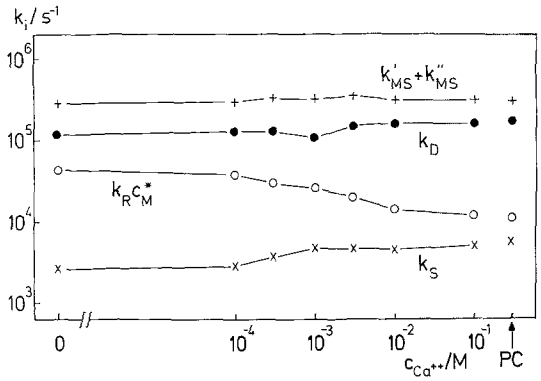

Fig. 8. Ca^{++} ion concentration dependence of the rate constants calculated from the relaxation data in Fig. 7

PA/PC mixture and for the pure PC as obtained from the experimental data in Fig. 7. $k'_{MS}+k''_{MS}$ and k_D are nearly unaffected by the presence of Ca^{++} ions, k_S shows a slight increase while $k_R c_M^*$ decreases again in a more or less S-shaped way.

The rate constants for the PA membranes (also shown in Table 2) exhibit only small changes up to $c_{Ca^{++}}=3\times10^{-4}$ M.

We first turn our attention to the very low and to the very high Ca^{++} concentration range. In the previous section we could show that in the absence of Ca^{++}[1] the difference in the ion transport characteristics of PA and PC membranes is mostly the result of a very different partition coefficient γ_s^{mb}. We also presented evidence that despite the low conductivity of the 1:1 PA/PC mixture the lipids in this membrane are probably in the same molar ratio as they are in the torus and that they are homogeneously distributed.

The valinomycin kinetic in PC membranes is not influenced at all by Ca^{++}, even at high Ca^{++} concentrations. Unfortunately, pure PA membranes are not stable for Ca^{++} concentrations higher than 3×10^{-4} M (Laclette & Montal, 1977). That means that we cannot follow the influence of Ca^{++} on the valinomycin-mediated Rb^+-transport across these membranes up to those concentrations where for the mixed membranes a saturation of the Ca^{++} effect can be observed.

Nevertheless, the experimental data at 10^{-4} M and 3×10^{-4} M seems to indicate that Ca^{++} "passivates" the ion transport across PA membranes. This could be understood by the well-known effect of Ca^{++} to rigidify charged membranes (Träuble & Eibl, 1974): Addition of Ca^{++} ions in high concentrations to a phosphatidic acid dispersion shifts the phase transition temperature of these lipids by about 20 °K to higher temperatures. At lower Ca^{++} concentrations, only part of the membranes are transferred to the more rigid state (Galla & Sackmann, 1975). Similar effects can be observed by varying the pH in the aqueous phase in such a way that the degree of dissociation of the polar headgroups of the lipids change (Träuble & Eibl, 1974; Galla & Sackmann, 1975; Blume & Eibl, 1981). Increasing the H^+ concentration from pH 10 to pH 6 (which protonates one of the two negative charges of phosphatidic acid) again shifts the transition temperatures of these lipids by about 20 °K to higher temperatures. The influence of the pH-induced rigidity increase on the carrier transport has been reported for a phosphatidic acid with diphytanoyl chains (Knoll, 1976). Changing the pH of the aqueous phase from pH 8.5 to pH 5.8 decreases the stationary conductance λ_0 by a factor of 2.5 and slows down the valinomycin kinetic considerably: relaxation times decrease by a factor of 25, the rate constants decrease by more than an order of magnitude. Thus, from analogy, we conclude that the conductivity of a Ca^{++}-saturated PA membrane would be more than a factor of ten lower than

[1] The high concentration of RbCl and LiCl ($c_{Rb}+c_{Li}=1$ M) ensures that trace impurities of divalent cations do not influence the results even in the absence of EDTA. (*See also* Apell et al., 1979.)

the conductivity of a PC membrane at the same Ca^{++} level in the aqueous phase. This is indicated in Fig. 6 by the dashed line which extrapolates the measured values of λ_0 for PA to high Ca^{++} concentrations.

For the mixed membrane the rate constants of the carrier transport at high Ca^{++} concentrations ($c_{Ca^{++}} \geqq 10^{-2}$ M) very clearly demonstrate that under these conditions the current through the PA/PC membrane exhibits completely the characteristics of a current through a PC membrane; it is only about a factor of two smaller.

To explain this behavior we suggest a complete separation of PC and PA molecules induced by the binding of Ca^{++} ions to the negatively charged PA molecules. The domains of $PA * Ca^{++}$ complexes remain in the membrane beside the pure PC regions so that the overall composition of the membrane does not change. As discussed above, one can assume that the contribution of the PA domains to the overall current are negligible compared to the PC contribution. This means that the current through a completely demixed equimolar system should be only 1/2 of the current through a pure PC membrane, in good agreement with our data. This result also supports our statement that the membrane has the same composition as the torus. Although more complex mechanisms could also explain our results (like a Ca^{++}-dependent redistribution of the lipids between membrane and torus) we prefer the simplest explanation, i.e.: 50% of the membrane components of the 1:1 bulk lipid mixture can be passivated by Ca^{++}.

The question arises now which way the mixed membrane goes from the homogeneous mixture in the absence of Ca^{++} to the complete separation at high Ca^{++} concentrations. For the answer it is important to point out that the current relaxations at intermediate Ca^{++} concentrations always can be satisfactorily fitted by two exponentials. Therefore, we conclude that the 'active' membrane, i.e. that part of the membrane that contributes to the current, is always a homogeneous mixture. By comparison of the relaxation data obtained at different Ca^{++} concentrations (Figs. 7 and 8) with those measured with membranes of different composition (Figs. 2 and 3) it becomes evident that under the influence of increasing doses of Ca^{++} the electrically active part of the membrane changes its composition from a 1:1 PC/PA mixture gradually to pure PC.

From these observations we derive the following picture of the action of Ca^{++}: (1) The ions specifically bind to PA (a mere screening effect cannot explain the changes in all rate constants); (2) the bound molecules aggregate (to clusters of unknown size and unknown valinomycin content); (3) the conductivity of these domains is greatly reduced so that the current through that part of the membrane can be neglected; (4) the remaining part of the membrane gets more and more depleted of PA with a concomitant reduction in surface charge density σ (which causes the decrease of $k_R c_M^*$ via the decrease of the surface potential).

From the above presented data we can calculate an equilibrium constant for Ca^{++} binding at PA molecules if we assume that the PA clusters contain only a negligible amount of PC molecules. In the absence of Ca^{++} the ratio of PC molecules and PA molecules in the membrane is given by

$$x_o = [PC]/[PA]. \tag{7}$$

If, by binding of Ca^{++}, some of the PA molecules, $PA * Ca^{++}$, are passivated, then the remaining active membrane has changed the ratio of its components to

$$x = \frac{[PC]}{[PA] - [PA * Ca^{++}]}. \tag{8}$$

From a combination of Eqs. (7) and (8) we derive for that fraction of the PA molecules that has bound Ca^{++} the relation

$$\frac{[PA * Ca^{++}]}{[PA]} = 1 - \frac{x_o}{x}. \tag{9}$$

As mentioned already, we can get $x = x(c_{Ca^{++}})$ by comparing the relaxation data of different Ca^{++} concentrations with those of the different mixed membranes. From that we can calculate the fraction of bound PA as a function of $c_{Ca^{++}}$. The result is plotted in Fig. 9. If we analyze the concentration dependence of $[PA * Ca^{++}]/[PA]$ on the basis of a Langmuir adsorption mechanism we derive a half-saturation concentration of $c = 5 \times 10^{-4}$ M.

A different approach to determine the binding properties of divalent cations has been reported in the literature (McLaughlin, 1977): deviations of stationary conductance data from the predictions of the Gouy-Chapman theory for charged membranes have been used to derive binding constants for Ca^{++}. We want to

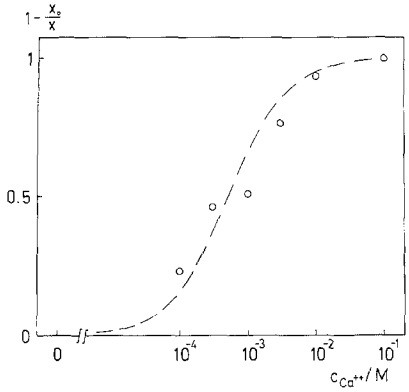

Fig. 9. Bound PA as a function of the Ca^{++} concentration in the aqueous phase. Half saturation is reached at 5×10^{-4} M. For details *see text*

point out that our evaluation of the half-saturation concentration of Ca^{++} binding to membranes of lipid mixtures does not depend on the validity of the Gouy-Chapman formalism. But the molecular process that we analyze includes a reorganization of the lipids in the membrane. A separation of Ca^{++} binding and lipid redistribution could be achieved by comparing our data for mixed membranes with those of pure PA membranes. For that purpose, however, measurements are necessary on Montal-Mueller type membranes which are reported to be stable also at high Ca^{++} levels (Laclette & Montal, 1977).

Conclusions

The presented results are obtained by voltage jump-current relaxation experiments analyzed on the basis of a simple carrier mechanism. Since it was possible to resolve experimentally both relaxation processes predicted by the model, the rate constants that characterize the different steps in the ion transport could be derived in a rather straightforward way. Thus it was possible to use the carrier as a molecular probe of the structure and lateral organization of the membrane in which it was incorporated.

The present data clearly demonstrate that Ca^{++} added to a homogeneously mixed PA/PC membrane induces not only a phase separation but also electrically passivates those membrane constituents to which it has bound. The conductance increase in the remaining active PC domains is predominantly the result of an increase of the valinomycin concentration in the membrane. This is only possible because in the Mueller-Rudin type black membrane the torus can act as a carrier reservoir. Even if the PA domains were completely depleted of valinomycin this could not account (starting with a 1:1 PA/PC mixture) for a fivefold carrier enrichment in the PC regions. No redistribution of valinomycin between membrane and torus, however, would be necessary if the starting mixture were a 4:1 mixture.

Our data give a rather detailed demonstration of one of several possible mechanisms for the coupling of membrane structure and function. Of course, a threefold change in the conductivity of a membrane by the variation of its lateral lipid organization is not what one would call a 'lipid switch of a protein function'. Our study shows, however, that it is possible to derive detailed models of the interaction of membrane structure and (transport) functions and how both can be modified by external parameters.

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