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CALCIUM ION-FLUX ACROSS PHOSPHATIDYLCHOLINE MEMBRANES MEDIATED BY IONOPHORE A23187

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Summary

The antibiotic A23187 carries Ca^{2+} across Müller-Rudin membranes made from 1,2-dierucoyl-*sn*-glycero-3-phosphocholine and *n*-decane. The conductance of the membranes is not increased by the Ca^{2+}-transport. The flux depends linearly on Ca^{2+} concentration and ionophore concentration (above pH 6). It increases with increasing pH, approximately by a factor of 4—5 between pH 6 and pH 8. Maximal Ca^{2+}-fluxes of about 10^{-10} mol · cm^{-2} · s^{-1} were found. A counter transport of H^+ could not be detected.

The complex formation between A23187 and Ca^{2+} in egg phosphotidylcholine vesicles was studied spectroscopically. The results are consistent with the formation of a 2 : 1 complex. Optical absorption measurements on single phosphatidylcholine membranes were used to calculate the concentration of membrane-bound ionophore A23187.

Introduction

Ca^{2+} play an important role in several functions of living organisms like muscular contraction, synaptic transmission or visual excitation. In these processes Ca^{2+} is transported across membranes but the mechanism of the Ca^{2+} transport is not yet understood. Therefore, it is interesting to study molecules of known structure which are able to increase the permeability of membranes for divalent cations. One of these ionophores is the recently described A23187 [1,2]. Like the antibiotic X537A [3] it has one carboxyl group, but it is quite specific for divalent cations [4] whereas X537A also carries monovalent cations [5]. A23187 has been shown to act on many biological membranes (e.g. refs. 6—9).

Bimolecular lipid membranes of the Müller-Rudin type [10,11] have been

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successfully applied to study transport mechanisms of various ionophores. Therefore, we decided to investigate the transport of Ca^{2+} across bimolecular lipid membranes mediated by A23187. Whereas with most ionophores for monovalent cations like valinomycin or gramicidin electrical measurements are possible, it was already known that A23187 transports Ca^{2+} in a mainly electroneutral way [2,4,9]. This makes it necessary to measure Ca^{2+}-fluxes with radioactive $^{45}Ca^{2+}$ or with optical indicators.

Optical indicators are suitable for continuous measurements which is an experimental advantage. We have used Arsenazo III to follow continuously the Ca^{2+}-flux across bimolecular lipid membranes from phosphatidylcholine. This indicator is more sensitive and more stable than murexide which has been used in the early stage of this investigation. We are very grateful to Dr. M. Blaustein for suggesting Arsenazo III to us.

Materials and Methods

1,2-dierucoyl-*sn*-glycero-3-phosphocholine [di(22 : 1)-phosphatidylcholine], has been synthesized by K. Janko in our laboratory. For transport measurements this lipid was used throughout because it forms especially stable membranes. Egg phosphatidylcholine for vesicles was prepared in our laboratory by standard techniques. A23187 was a gift from Dr. R.L. Hamill from Eli-Lilly and Company, Ind., U.S.A., and was used without purification. HEPES buffer and Arsenazo III were products from Fluka, Buchs (Switzerland), whereas Tris buffer, 7-hydroxycumarin (Umbelliferon) and all inorganic salts (p.a.) were purchased from Merck, Darmstadt, G.F.R.

Black lipid membranes were formed by the standard technique [11] from solutions containing 1% (w/v) of di(22 : 1)-phosphatidylcholine in *n*-decane. The aqueous phase was 10^{-2} M buffer solution. A schematic top view of the cell used for all flux measurements is shown in Fig. 1 (inset).

By inserting a Teflon cup into a glass cuvette two compartments (I and II) were formed which could be stirred with magnetic stirrers. The compartments were connected by a hole (diameter 4 mm in all experiments) across which the membranes were formed. The geometry was chosen (small volume I about 1 ml, thickness of the cuvette 2 cm, large membrane) to give optimal sensitivity. The whole assembly was thermostated and placed in a Zeiss DMR 10 double beam spectral photometer. The sample beam passed through compartment I as shown in Fig. 1.

When Arsenazo III forms a Ca^{2+}-complex, the maximal change in its absorption spectrum occurs at 649 nm. Therefore, we used this wavelength to measure Ca^{2+} concentrations. Calibration measurements were performed in the same experimental setup as the flux experiments: after formation of a membrane, Arsenazo III was added to compartment I (to make the solution $5 \cdot 10^{-5}$ M). The extinction change at 649 nm was measured (at full scale deflection $E = 0.1$) after addition of known amounts $CaCl_2$ to compartment I. The calibration curve was a linear function of Ca^{2+}-concentration at least up to $1.5 \cdot 10^{-6}$ M. The minimal detectable concentration was $5 \cdot 10^{-8}$ M in our experimental setup. Since the spectral changes during Ca^{2+}-Arsenazo III complex formation

are pH dependent [12] a calibration curve was measured for each pH value used in the membrane experiments. A typical flux measurement was performed as follows. Both compartments were stirred continuously during the experiment. A23187 in ethanolic solution was added to compartments I and II to make the aqueous phase between $2 \cdot 10^{-8}$ M and $6 \cdot 10^{-6}$ M. After the membrane was formed, Arsenazo III was added to compartment I (final concentration $5 \cdot 10^{-5}$ M). When the membrane became completely black (usually after 15—30 min), Ca^{2+} was added to compartment II to make its concentration between 10^{-5} M and $5 \cdot 10^{-3}$ M. After Ca^{2+}-addition the extinction at 649 nm increased linearly for up to 1 h. Usually the slope was measured during 10—20 min. From this slope, together with the calibration curve the Ca^{2+}-concentration c flown into compartment I during the measuring period was obtained. With the volume V of compartment I and the membrane area A the Ca^{2+}-flux ϕ was calculated:

$$\phi = c \cdot V \cdot A^{-1} \text{ mol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$$

The smallest detectable flux ϕ was about 10^{-12} mol $\cdot$ cm^{-2} $\cdot$ s^{-1} and was limited by the sensitivity of the DMR10 spectrophotometer and by the stability of the extinction at 649 nm in the presence of lipid membranes within the measuring time of about 10 min. It was tested that Arsenazo III or its Ca^{2+}-complex do not cross the membrane under our experimental conditions.

Several control measurements have been done with radioactive Ca^{2+}. ^{45}Ca was added to compartment II and small samples were taken out from compartment I at certain time intervalls. The ^{45}Ca concentration was determined in a scintillation counter by comparison with standard solutions of ^{45}Ca.

Conductance measurements and charge pulse relaxation measurements were done as described elsewhere [13].

In addition to the flux measurements, the concentration of the ionophore in the membrane was directly measured spectrophotometrically. The spectrophotometer was especially constructed for low absorption measurements of bilayer membranes and is described elsewhere [14]. A continuous record of the spectrum was not possible. The extinction at certain wavelengths was measured with and without membrane and from the difference the extinction of the membrane was calculated. The mean value of at least 5 membranes without ionophore was subtracted from the mean value of an equal number of membranes with ionophore to get the extinction. The standard error of measurements with A23187 doped membranes varied between 10 and 75% depending on the wavelength and the absolute value of extinction.

Vesicles were prepared with the method of Batzri and Korn [15] by injecting 200 μl $2.5 \cdot 10^{-2}$ M ethanolic solution of egg phosphatidylcholine into 7 ml 10^{-2} M HEPES solution. Spectra were taken with a Zeiss-DMR10 spectral photometer in 1 cm quartz cells. Identical vesicle suspensions were initially present in the sample and reference cell in order to eliminate from the measured absorption spectra the light scattering contribution caused by the vesicles. A23187 in ethanol was added to the sample, an equal amount of ethanol to the reference. Equal amounts $CaCl_2$ were added to both solutions.

Results

Ca^{2+}-flux across the membrane

Two typical experiments are illustrated by Fig. 1. In Expt. (a) (full line) A23187 was added symmetrically to the aqueous phases and after the membrane became optically black, Ca^{2+} was added to compartment II. Immediately the extinction at 649 nm increased linearly with time. When stirring was stopped for a few minutes at the points 1, the slope of the extinction curve decreased. When stirring was started again, at the points 2 the extinction sharply rose and continued to increase with the original slope. This experiment shows that stirring does not significantly influence the Ca^{2+} flux but only the homogenous distribution of Ca^{2+} in compartment I. If the stirrer was stopped only in compartment II the flux did not change significantly. This shows that the measured Ca^{2+}-flux is not controlled by diffusion in the aqueous phase. In Expt. (b) (dashed line) Ca^{2+} was added to compartment II before addition of the ionophore. No Ca^{2+}-flux was observed without A23187. After addition of the same amount A23187 as in Expt. (a) the same slope was reached but with a time-lag of about 10 min.

In Fig. 2 the Ca^{2+}-flux at constant Ca^{2+}-concentration is shown as a function of the A23187 concentration added symmetrically to the aqueous phase. Measurements were done at three pH values (5.5, 7, 8). The same buffer 10^{-2} M HEPES) was used for clearness in all three cases, although it does not buffer

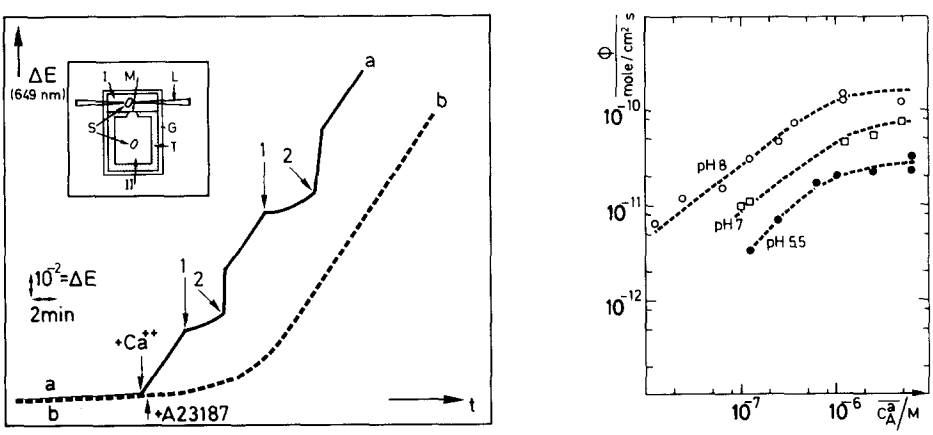
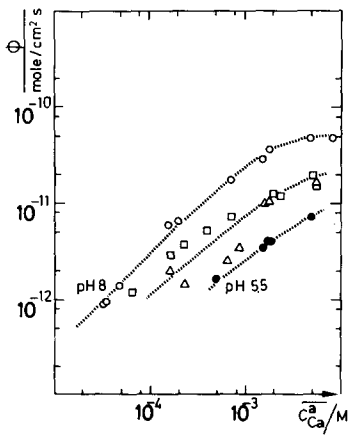


Fig. 1. Inset: topview of the cuvette in the sample compartment of the spectrophotometer DMR 10. L, light beam of sample compartment; G, glass cuvette (→ chamber I); T, Teflon cup (→ chamber II); M, membrane across 4 mm ϕ hole in the teflon cup; S, magnetic stirrers. Extinction change at 649 nm (absorption maximum of the Ca^{2+}-Arsenazo III-complex) versus time. Arsenazo III was $5 \cdot 10^{-5}$ M in chamber I. (a) ———, A23187 (10^{-6} M) symmetrically added first. At the indicated point $CaCl_2$ ($1.7 \cdot 10^{-3}$ M) was added to chamber II. HEPES 10^{-2} M, pH 7, T = 21°C at points 1 stirring was stopped at points 2 stirring was started again. (b) - - - - - -, $CaCl_2$ ($1.7 \cdot 10^{-3}$ M) was added first to chamber II. At the indicated point A23187 (10^{-6} M) was added symmetrically. KCl 10^{-2}, pH 7, T = 21°C.

Fig. 2. Ca^{2+}-flux through di(22 : 1)-phosphatidylcholine membranes versus A23187 concentration at constand Ca^{2+} concentration. The ionophore was symmetrically added from ethanolic stock solutions before membrane formation to give the indicated concentration. Fluxes were calculated from measured extinction increase at 649 nm using calibration curves as described under methods. c_{CaCl_2} = $1.7 \cdot 10^{-3}$ M, HEPES 10^{-2} M, T = 21°C.



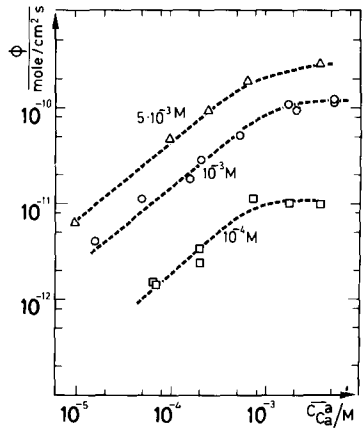


Fig. 3. Ca^{2+}-flux through di(22 : 1)-phosphatidylcholine membranes versus Ca^{2+} concentration at constant A23187 concentration. $C_{A23187} = 1.3 \cdot 10^{-7}$ M; $T = 21°C$; HEPES 10^{-2} M: ●, pH 5.5; △, pH 6; □, pH 7; ○, pH 8.

Fig. 4. Ca^{2+}-flux through di(22 : 1)-phosphatidylcholine membranes versus Ca^{2+} concentration at different A23187 concentrations added to the lipid phase. HEPES 10^{-2} M, pH 8; $T = 21°C$.

well at pH = 5.5 (pK 7,6). For special ionophore concentrations several flux measurements were performed, in order to prove reproducibility. We obtained for example a standard deviation of approximately 40% for 10^{-7} M ionophore at pH 8 (5 independant experiments). Similar standard deviations were assumed for the other concentrations. It is seen from Fig. 2 that: (1) the Ca^{2+}-flux increases linearly with concentration of A23187 (up to about 10^{-6} M); (2) above 10^{-6} A23187 the flux approaches saturation; (3) the flux increases with increasing pH.

In Fig. 3 the dependence of the Ca^{2+}-flux on the Ca^{2+}-concentration is shown at constant A23187-concentration but different pH values. The following results are found: (1) the flux increases linearly with the Ca^{2+}-concentration (at pH below 6 slightly less than linear). (2) at the higher pH values a saturation around 10^{-3} M Ca^{2+} is approached; (3) the flux increases approximately by a factor of 2, per pH unit.

A set of experiments was done with addition of A23187 to the membrane-forming solution. The results are given in Fig. 4 and can be summarized as follows: (1) the flux again increases linearly with low Ca^{2+} and ionophore concentrations. (2) Saturation is approached around 10^{-3} M Ca^{2+} in water and 10^{-3} M A23187 in the lipid phase. These measurements were done at pH 8.

Measurements with ^{45}Ca

To make sure that Arsenazo III does not influence the Ca^{2+}-flux, control measurements were made without the indicator but with $^{45}Ca^{2+}$. Again in the absence of A23187 no Ca^{2+}-flux was found, but after addition of the ionophore the radioactivity increased linearly with time in compartment I. The ^{45}Ca-flux was of the same order of magnitude as the value obtained spectroscopically under comparable conditions.

Conductance measurements

The membrane conductance was measured together with the Ca^{2+}-flux. Even with the highest Ca^{2+}-fluxes of 10^{-10} mol · cm^{-2} · s^{-1} and with ionophore-concentrations of 10^{-6} M, the conductance usually did not exceed 10^{-8} Ω^{-1} · cm^{-2}. Actually a small conductance decrease was usually observed after Ca^{2+}-addition. If Ca^{2+} would be transported as a positively charged complex, a flux of 10^{-10} mol · cm^{-2} · s^{-1} would carry an electric current $J = \phi \cdot A \cdot F$ of approx. 1 μA. The measured currents are 3—4 orders of magnitude smaller. This shows that the Ca^{2+}-transport is mainly electroneutral. As expected for an electroneutral transport, the Ca^{2+}-flux could not be influenced by potential differences of ±70 mV applied across the membrane.

Proton flux across the membrane

Since an exchange of H^+ for Ca^{2+} has been suggested during Ca^{2+} transport in biological membranes [7], we tried to measure proton fluxes directly by the pH indicator umbelliferon and with a glass-electrode.

A pH increase results in an extinction increase at 365 nm of the umbelliferon absorption (pK = 7.6). The experiments were done in 10^{-2} M KCl unbuffered with an approximate pH 6.7. This pH decreased less than 0.1 pH units during the measuring time. Control measurements confirmed that the Ca^{2+}-flux in unbuffered and buffered solutions were equal and nearly constant for 1 h. When umbelliferon was used instead of Arsenazo after addition of Ca^{2+} to chamber II no increase of the extinction at 365 nm in chamber I was observed. Addition of KOH amounts equivalent to the Ca^{2+}-flux into chamber I gave a large increase of the extinction at 365 nm. When both Arsenazo III and umbelliferon were present in chamber I again the extinction at 649 nm showed a normal Ca^{2+}-flux and the extinction at 365 nm did not change. When chamber II was made approximately 10^{-3} M in KOH, both the pH in chamber I and the Ca^{2+}-flux increased. But still the H^+- (or OH^-) flux was at least ten times smaller than the Ca^{2+}-flux. A similar experiment (without Arsenazo III but with Ca^{2+}, A23187 and umbelliferon symmetrically in chambers I and II) is shown in Fig. 5. Addition of 10^{-3} M KOH in chamber II lead to a pH increase in chamber I,

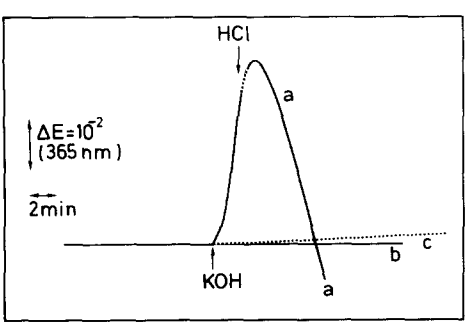


Fig. 5. pH-change in chamber I (as indicated by umbelliferon) induced by addition of 10^{-3} M KOH or HCl to chamber II. Solution in both chambers: 10^{-2} M KCl, pH 6.7; $C_{Umbelliferon} = 2.5 \cdot 10^{-5}$ M; $C_{A23187} = 1.3 \cdot 10^{-6}$ M (or zero). $C_{Ca^{2+}} = 1.3 \cdot 10^{-3}$ M (or zero). (a) with A23187, Ca^{2+} and KOH (or HCl). (b) without A23187, with Ca^{2+} and KOH; (c) with A23187 and KOH, without Ca^{2+}.

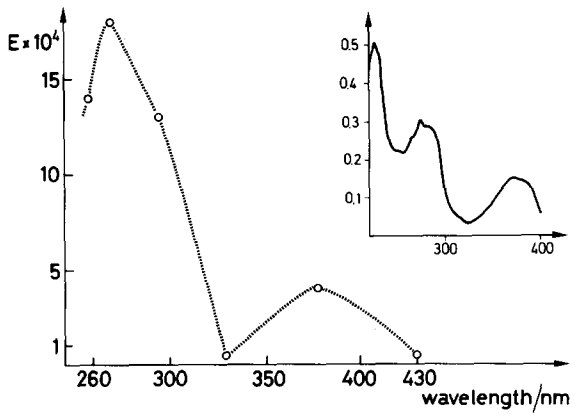


Fig. 6. Spectrum of A23187 in black lipid membranes. E is obtained as described in Materials and Methods by measurement of several membranes at each wavelength. Membranes were made from 1% di(22 : 1)-phosphatidylcholine in n-decane (w/v). A23187 was added 10^{-2} M to this solution. Inset: spectrum of $1.7 \cdot 10^{-5}$ M A23187 in n-decane.

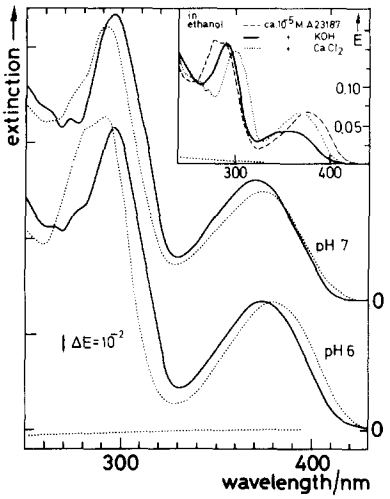
10^{-3} M HCl in chamber II to a pH decrease in chamber I. These effects were only found in the presence of both A23187 and Ca^{2+} (curve a). In the absence of A23187 (curve b) or Ca^{2+} (curve c) no extinction change at 365 nm was observed.

Spectroscopic measurements with single black films

Fig. 6 shows the spectrum of single membranes with 10^{-2} M ionophore in the lipid bulk phase. It is similar to the spectrum of the ionophore in n-decane (inset), although it is not clear whether the absorption peak in the membrane is closer to 270 nm (as in n-decane) or to 300 nm (as in egg phosphatidylcholine vesicles). The area concentration of the ionophore in the membrane may be calculated assuming the validity of Lambert-Beers law by $N_{tot} = E/10^3\,\epsilon$. Using ϵ (270 nm) $\approx 1.8 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ as determined in n-decane we obtain $N_{tot} = 10^{-10}\ mol \cdot cm^{-2}$.

Spectroscopic measurements with phosphatidylcoline vesicles

Absorption spectra measured in suspensions may be obscured by light scattering [16]. We tried successfully to eliminate these scattering artefacts by using as a reference a vesicle suspension identical to the sample except without A23187. Under these conditions the extinction measured away from the true ionophore absorbance (430—500 nm) was virtually zero at all pH values between 3 and 9. (Addition of $CaCl_2$ above pH 8 resulted in extinction values of $2 \cdot 10^{-2}$ between 430 and 500 nm in spectra like those of Fig. 7. This was probably due to aggregation of the vesicles.) A second error may arise from absorption flattening [17]. This effect does not influence our results to a measurable extent because we found a molar extinction coefficient at 280 nm $\epsilon = (1.70 \pm 0.15) \cdot 10^4\ M^{-1} \cdot cm^{-1}$ both in ethanol, n-decane solutions and in vesicle suspensions (pH 4). In the concentration range 10^{-6}—10^{-5} M A23187 Lambert-



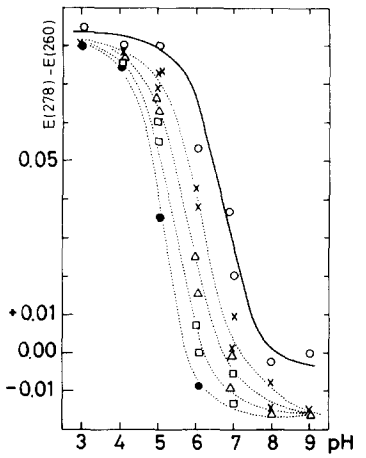


Fig. 7. Absorption spectra of A23187 in egg phosphatidylcholine suspensions or ethanolic solution (inset). Injection vesicles were prepared in 10^{-2} M HEPES of different pH as described in Materials and Methods and present in sample and reference cell. A23187 was added to the sample only, $CaCl_2$ to both cells. T = 21°C. ------, $\bar{C}_{\text{phosphatidylcholine}} = 7 \cdot 10^{-4}$ M $\bar{C}_{A23187} = 10^{-5}$ M, without Ca^{2+}; ———, $\bar{C}_{CaCl_2} = 1.5 \cdot 10^{-3}$ M was added. Inset: spectra of A23187 in ethanol. To 3 ml ethanol 10 µl 1 M KOH and 10 µl 0.1 M $CaCl_2$ in water were added.

Fig. 8. Extinction difference [E(278)—E(260)] of the ionophore in egg phosphatidylcholine vesicles in the absence and presence of $CaCl_2$. The values are taken from spectra with the conditions given for Fig. 7. ○, without Ca^{2+}; ×, 10^{-4} M $CaCl_2$; △, $4 \cdot 10^{-4}$ M $CaCl_2$; □, $1.6 \cdot 10^{-3}$ M $CaCl_2$; ●, $6.5 \cdot 10^{-3}$ M $CaCl_2$.

Beers law was found to be valid. Absorption spectra of A23187 added to vesicles (molar ratio phosphatidylcholine: A23187 ≃ 70 : 1) were measured between pH 3 and 9. In Fig. 7 two examples are shown at pH 6 and 7 in the absence of Ca^{2+} (dashed line) and the presence of a saturating amount of Ca^{2+} (full line). No spectral changes were found below pH 4. All spectra and the inset show that increasing pH and Ca^{2+} concentration caused a decrease at 278 nm and a blue shift of the 380 nm peak. In the presence of Ca^{2+} the 280 nm peak was red shifted as described for ethanolic solutions [4] (see also inset). Absolute extinction values were difficult to reproduce. We suspect that aggregation-disaggregation phenomena of vesicles and/or ionophore which are influenced by unknown experimental parameters are the reason for this large data scatter (up to ±20%) of extinction values obtained with different vesicle preparations. We found that subtracting the extinction at 260 nm from the extinction at 278 nm in each spectrum gave reproducible differences ΔE for different samples (prepared under identical conditions). The reason is probably a partial elimination of scattering contributions caused by large particles in the vesicle solutions. Therefore, these extinction differences ΔE were used instead of absolute extinction values. ΔE is plotted against pH in Fig. 8 in the absence and presence of four constant Ca^{2+}-concentrations. From the curve without Ca^{2+} one can estimate a $pK \simeq 6.7 \pm 0.2$ of the ionophore in egg phosphatidylcholine vesicles. Extinction differences due to Ca^{2+} are small for pH < 4.5 and for pH > 7.5 and maximal between pH 5 and 7 (see Fig. 8).

Discussion

To get insight into the mechanism of Ca^{2+}-transport across lipid membranes mediated by A23187 from measured Ca^{2+}-fluxes a prerequisite is to show that the Ca^{2+}-flux is not controlled by diffusion in the aqueous phase. If the flux is limited by the unstirred layer adjacent to the membrane, the measurements would not allow conclusions on the flux through the membrane. The experiment reported in Fig. 1 clearly shows that diffusion in the aqueous phase did not limit the Ca^{2+}-flux. If this were so, the flux would depend on the stirring speed which was not found experimentally. Without stirring the flux-driving Ca^{2+}-gradient would be flattened and the flux would decrease. In the time intervall between points 1 and 2 (Fig. 1) less Ca^{2+} would flow than with stirring. This is not the case because when stirring is started again at point 2 the extinction increases to the point it would have reached with continuous stirring. The decrease in the extinction slope at point 1 is due to incomplete mixing in chamber I, but not to a decrease in the Ca^{2+}-flux. If the interpretation is correct the flux should not change when the stirrer is stopped in compartment II alone. This was in fact observed. The experiment has been done with a Ca^{2+}-flux of about 10^{-10} mol $\cdot$ cm^{-2} $\cdot$ s^{-1} (close to the highest obtainable flux) because in this case diffusion in the aqueous phase would be most limiting. A rough estimate of the flux across the unstirred layer from $\phi_d^{max} = D \cdot \Delta c_{Ca}/\delta$ would yield 10^{-9} mol $\cdot$ cm^{-2} $\cdot$ s^{-1} with $D = 10^{-5}$ cm^2 $\cdot$ s^{-1}, $\Delta c_{Ca} = 10^{-3}$ M and $\delta = 10^{-2}$ cm (D: diffusion coefficient of Ca^{2+}; Δc_{Ca}: concentration gradient; δ: thickness of the unstirred layer). The observed fluxes are 10% of ϕ_d^{max} or less, when the ionophore is added to the aqueous phase initially.

We believe that the complex formation between A^- and and Ca^{2+} occurs mainly at the membrane-water interface like in the valinomycin-K^+ system [18]. The Ca^{2+}-ionophore complex is mainly bound to the lipid membrane in vesicle experiments. It was found that at pH 9 about 94% of the ionophore (10^{-5} M) in water could be removed by centrifugation (10 min at 25 000 $\times g$, $T = 21°C$) after addition of 10^{-3} M $CaCl_2$, whereas in the presence of egg phosphatidylcholine vesicles ($\approx 10^{-3}$ M) centrifugation did not remove any ionophore. Assuming that all ionophore present formed the Ca^{2+} complex under the conditions used (pH $\geqslant$ 8) and assuming that the highest aqueous phase concentration of the Ca^{2+}-ionophore complex in the presence of vesicles is 6% of the total amount (as in the absence of vesicles), we can calculate a partition coefficient for the Ca^{2+}-ionophore complex $\gamma = n^m_{A_2Ca} \cdot V^a / n^a_{A_2Ca} \cdot V^m$ ($n^m_{A_2Ca}$ ($n^a_{A_2Ca}$) are the numbers of moles Ca^{2+}-ionophore complex in the membrane (aqueous) phases. V^a (V^m) are the volumes of the aqueous (membrane) phases. Inserting the numerical values obtained from centrifugation experiments, we calculate $\gamma \geqslant 2.1 \cdot 10^4$ in favour of the lipid membrane. Since the aqueous phase concentration may be smaller, this is taken as a lower limit of the partition coefficient. Analogous centrifugation experiments without Ca^{2+} at pH 9 and 3 allow to calculate a lower limit for the partition coefficients of A^- ($2.1 \cdot 10^3$) and AH ($4.2 \cdot 10^3$) in favour of the lipid phase. These findings make it unlikely that a complex formation in the aqueous phase contributes significantly to the Ca^{2+} flux but do not completely excluded that possibility. Fig. 1 (curves a, b) shows that a constant Ca^{2+}-flux is reached several seconds after addition of Ca^{2+} when the

membrane has been equilibrated with A23187 first, but it takes much longer (at least 50 times) to reach a constant flux when Ca^{2+} is added first and A23187 later. This may be explained by a slow incorporation of the ionophore into the membrane. One among other possible reasons for this delayed flux may be the slow diffusion of ionophore aggregates across the unstirred layer.

All flux measurements (for pH 8 and below saturation) can be expressed analytically. With addition of the ionophore A23187 to the aqueous phase, the following equation holds:

$$\phi(a) = a \cdot \overline{c^a_{Ca}} \cdot \overline{c^a_A} \tag{1}$$

with $a = 0.2 \pm 0.1$ [$M^{-1} \cdot cm \cdot s^{-1}$]. $\overline{c^a_{Ca}}$ and $\overline{c^a_A}$ are operationally defined as the amount [moles] of $CaCl_2$ or ionophore added to the cell divided by the volume of the aqueous phase.

With addition of the ionophore to the membrane forming solution (lipid bulk phase) we get:

$$\phi(b) = b \cdot \overline{c^a_{Ca}} \cdot \overline{c^b_A} \tag{2}$$

with $b = (1.4 \pm 0.4) \cdot 10^{-4}$ [$M^{-1} \cdot cm \cdot s^{-1}$] $\cdot \overline{c^a_{Ca}}$ as defined before and $\overline{c^b_A}$ [M] the amount of ionophore per volume of added lipid bulk phase.

From the measured Ca^{2+}-flux we can calculate the Ca^{2+}-permeability $P_{Ca} \simeq \phi/\Delta\overline{c^a_{Ca}}$. Using the linear regions in Figs. 3 and 4, we obtain the minimal permeability $P_{Ca} = 2.2 \cdot 10^{-6}$ cm $\cdot s^{-1}$ ($\overline{c^a_A} = 10^{-7}$ M, pH 5.5, 21°C) and the maximal permeability $P_{Ca} = 3.9 \cdot 10^{-4}$ cm $\cdot s^{-1}$ ($\overline{c^b_A} = 5 \cdot 10^{-3}$ M, pH 8, 21°C). These values are of the same order of magnitude as those given for phosphatidylcholine-cholesterol membranes in 0.1 M triethanolamine pH 7.5 in a recent paper [19].

From the maximal Ca^{2+}-flux ϕ_{max} found at a certain ionophore concentration one may calculate a turnover-number $t = \phi_{max}/N_{tot}$ (N_{tot}: concentration of ionophore in moles per cm^2 membrane area). N_{tot} was directly determined with a membrane-spectrometer as described in Results. Measurements could only be made in a very small concentration region ($\overline{c^b_A} = 3 \cdot 10^{-3}$ to 10^{-2} M).

We determined $N_{tot} \simeq 5 \cdot 10^{-11}$ mol cm^{-2} at $\overline{c^b_A} = 5 \cdot 10^{-3}$ M as described under Results. At the same $\overline{c^b_A}$ we found $\phi_{max} \simeq 2.7 \cdot 10^{-10}$ mol $\cdot cm^{-2} \cdot s^{-1}$. Therefore, $t = \phi_{max}/N_{tot} \simeq 5\ s^{-1}$. This turnover number is taken as a lower limit. As seen from Fig. 4 ϕ_{max} shows a tendency to saturate above $\overline{c^b_A} = 10^{-3}$ M. Therefore we expect that at $\overline{c^b_A} = 5 \cdot 10^{-3}$ some part of the ionophore present in the membrane does not contribute to the transport.

N_{tot} may also be determined by using the approximate partition coefficient γ for the Ca^{2+} ionophore complex obtained from vesicle experiments. When the total amount of ionophore added to the system (n^{tot}_A) is complexed by Ca^{2+} the following relation holds:

$$N_{tot} = \frac{\gamma}{V^a + \gamma V^m} \frac{n^{tot}_A}{2} d \text{ [mol cm}^{-2}\text{]} \tag{3}$$

The quantities in Eqn. 3 are defined and typical numerical values from our experiments given: $\gamma = c^m/c^a \geqslant 2.1 \cdot 10^4$. c^m, c^a: concentrations of the Ca^{2+}-ionophore complex in the membrane and water phase. $V^m = 6 \cdot 10^{-8}$ ml, $V^a = 4$ ml : volumes of the membrane and the aqueous phases. $n^{tot}_A = 4 \cdot 10^{-9}$ mol:

total amount of ionophore added to the system. $d = 5 \cdot 10^{-7}$ cm: thickness of the membrane. $N_{tot} = c^m \cdot d$: concentration of the ionophore in mol per cm^2 membrane area. $\phi_{max} = 10^{-10}$ mol $\cdot$ $cm^{-2} \cdot s^{-1}$: maximal Ca^{2+}-flux at this ionophore concentration. Inserting the numerical values in Eqn. 3 we obtain: $N_{tot} \geqslant 5.25 \cdot 10^{-12}$ mol $\cdot$ cm^{-2} and $t = \phi_{max}/N_{tot} \leqslant 19\ s^{-1}$. This seems in reasonable agreement with the value 5 s^{-1} calculated above. The question remains if the approximate γ determined from solvent-free vesicles can be used for decane-containing black membranes.

Another method to obtain partition coefficients and therefore N_{tot} would be to compare Ca^{2+}-fluxes at equal Ca^{2+} concentrations after adding ionophore either to the aqueous or the lipid bulk phase. This method would be analogous to the approach of Stark and Benz [18] in the case of Valinomycin-K^+. Its quantitative application depends on the detailed knowledge of the transport mechanism and was not used therefore at this stage of our investigations.

The discussion of the turnover number leads into the molecular interpretation of the transport mechanism, which is difficult to give at the moment. Two somewhat contradictory results must be explained. First, the fluxes are mainly electroneutral and second, they increase linearly or sublinearly with the ionophore concentration. Electroneutrality points to a 2 : 1 ionophore Ca^{2+} complex, the concentration dependence to a 1 : 1 complex.

We have tried to determine the stochiometry of the complex by spectroscopic studies with egg phosphatidylcholine vesicles. As already mentioned under Results, the spectral titrations was hampered by relatively large data scatter in spectra taken under identical conditions. All attempts to improve this situation by using different methods of vesicle preparation (injection or sonication method) or varying the lipid to ionophore ratio were unsuccessful. Nevertheless, the general features of the experiments were always reproducible. Therefore we have only attempted qualitative calculations for some special cases. It was not possible to fit all spectral titration curves quantitatively as discussed later. Assuming that Lambert-Beer's law is valid for all absorbing species i in a solution, the extinction at the wavelength λ is given by

$$E(\lambda) = \sum_i \epsilon_i^\lambda c_i d \tag{4}$$

We further assume that we can apply Eqn. 4 to the vesicle suspension containing A23187. In the absence of Ca^{2+} we obtain with $E/d \equiv m$

$$m = \bar{\epsilon}_{AH}\bar{c}_{AH} + \bar{\epsilon}_{A^-} - \bar{c}_{A^-} \tag{4a}$$

when only two species AH and A^- are present in the vesicle suspension, $\bar{c}_{AH}$ or $\bar{c}_{A^-}$ are the concentrations of these species (operationally defined as the amount of each species divided by the volume of the vesicle suspension) and $\bar{\epsilon}_{AH}$, $\bar{\epsilon}_{A^-}$ the extinction coefficients obtained from $\bar{\epsilon} = m/\bar{c}$. In the absence of Ca^{2+} the vesicle-bound ionophore behaves like a monobasic acid (Fig. 8) and $E(278)$—$E(260)$ can be fitted with the Henderson-Hasselbach equation pH = pK + log c_{A^-}/c_{AH}. The obtained pK = 6.7 ± 0.2 corresponds to a $\bar{K}_s = 2 \cdot 10^{-7}$ M = $(\bar{c}_{A^-} c_{H^+})/\bar{c}_{AH}$ (assuming activity coefficients of unity).

In the presence of Ca^{2+} the titration curves are shifted toward the acid region. Increasing the Ca^{2+} concentration by a factor 65 corresponded to an

increase of the H^+ concentration by a factor of about 10. This can be understood with the following equations (assuming activity coefficients to be unity):

$$2\,AH + Ca^{2+} \rightleftharpoons A_2Ca + 2\,H^+ \qquad \bar{K} = \frac{\bar{c}_{A_2Ca}(c_{H^+})^2}{(\bar{c}_{AH})^2 c_{Ca^{2+}}} \tag{5}$$

$$\frac{\bar{c}_{A_2Ca}}{(\bar{c}_{AH})^2} = \bar{K}\frac{c_{Ca^{2+}}}{(c_{H^+})^2} \tag{5a}$$

c_{H^+}, $c_{Ca^{2+}}$: concentration of H^+, Ca^{2+} in the aqueous phase; $\bar{c}_{A_2Ca}$: concentration of the Ca^{2+}-complex in the vesicle suspension. The left-hand side of Eqn. 5a remains constant with increasing Ca^{2+}-concentration only when the H^+-concentration is increased at the same time by the square root of the Ca^{2+}-concentration (this means approximately that one moves from right to left over the titration curves of Fig. 8 at constant extinction difference). The experimental results seem to follow the prediction of Eqns. 5 and 5a sufficiently close to support the predominant formation of a 2 : 1 complex. Under these conditions the extinction modul would be given by:

$$m = \bar{\epsilon}_{AH}\bar{c}_{AH} + \bar{\epsilon}_{A^-} - \bar{c}_{A^-} + \bar{\epsilon}_{A_2Ca}\bar{c}_{A_2Ca} \tag{4b}$$

For high pH values (>8) c_{AH} is negligibly small. Eqns. 5 and 5a reduce to:

$$2\,A^- + Ca^{2+} \rightleftharpoons A_2Ca \qquad \bar{K}_2 = \frac{\bar{c}_{A_2Ca}}{(\bar{c}_{A^-})^2 c_{Ca^{2+}}} \tag{5b}$$

Defining the association degree

$$\beta = \frac{2\,\bar{c}_{A_2Ca}}{\bar{c}_{A^-}^{tot}} = \frac{m/\bar{c}_{A^-}^{tot} - \bar{\epsilon}_{A^-}}{\bar{\epsilon}_{A_2Ca/2} - \bar{\epsilon}_{A^-}}$$

we can write $\bar{K}_2$ in the following way:

$$\bar{K}_2 = \frac{\beta}{2(1-\beta)^2\,\bar{c}_{A^-}^{tot}(\bar{c}_{Ca^{2+}}^{tot} - \beta\,\bar{c}_{A^-}^{tot}/2)} \tag{5c}$$

$\bar{c}_{A^-}^{tot}$, $\bar{c}_{Ca^{2+}}^{tot}$: total concentrations of A^- (always 10^{-5} M) and Ca^{2+} (between $2.5 \cdot 10^{-5}$ M and $5 \cdot 10^{-3}$ M) added to the vesicles suspension.

From experimentally measured values of $\bar{\epsilon}$ and m we calculated $\bar{K}_2 = (3.7 \pm 0.4) \cdot 10^9$ [M^{-2}]. The relatively small standard deviation in a wide range of Ca^{2+} concentrations was taken as further evidence for the predominant 2 : 1 complex. We can further calculate $\bar{K} = \bar{K}_2 \cdot \bar{K}_S^2 = (1.5 \pm 0.9) \cdot 10^{-4}$. If the partition coefficient between the aqueous phase and the membrane phase are γ_{AH} and γ_{A_2Ca} for the ionophore and its Ca^{2+}-complex, respectively, and if the volume ratio of lipid to aqueous phase is X, we can obtain a modified formation constant K^m in the membrane:

$$K^m = \bar{K}\frac{\gamma_{A_2Ca}}{\gamma_{AH}^2} \cdot \frac{(1 + X \cdot \gamma_{AH})^2}{(1 + X \cdot \gamma_{A_2Ca})} \tag{6}$$

Inserting $\gamma_{A_2Ca} \simeq 2.1 \cdot 10^4$, $\gamma_{AH} \simeq 4.2 \cdot 10^3$ and $X = 7.3 \cdot 10^{-4}$ which are approximate values for our vesicle system we obtain $K^m \simeq (1.8 \pm 0.9) \cdot 10^{-7}$.

K^m differs from $\bar{K}$ mainly by taking into account the much smaller volume of the membrane lipid phase. Although this value of K^m is only an estimate, because the partition coefficients are not exactly known for our vesicle system it is interesting to note that it is similar to a formation constant $K = 3.7 \cdot 10^{-7}$ published recently [20] for the A23187-Ca^{2+} complex in a macroscopic two-phase-system.

We tried to fit spectral titration curves (increasing Ca^{2+} concentration at constant ionophore concentration and pH) using $\bar{K}$, $\bar{K}_S$, $\bar{\epsilon}$ (for AH, A^- and A_2Ca: determined under conditions where only one of these three species was present) together with Eqn. 5 and the conditions of mass conservation. This fit was possible at pH $\geqslant$ 8 and showed that the formation of A_2Ca was completely saturated at about 30-fold excess of Ca^{2+} over A23187. The calculations also showed a dramatic decrease of the complex concentration at pH $<$ 5 even at a 500-fold excess of Ca^{2+} (compare Fig. 8). Between pH 5 and 8 a complete fit was not possible because the spectral data varied from experiment to experiment.

We now try to use the results found with vesicles to better understand the experiments with planar bilayer membranes under the assumption that both types of membranes are so similar that all reaction schemes and formation constants are the same in both systems. To simplify the scheme we use high pH conditions and write

$$\bar{c}_{A_2Ca} = \bar{K}_2 \cdot c_{Ca^{2+}} \cdot (\bar{c}_{A^-})^2 \tag{5c}$$

With $\bar{K}_2 = 3.7 \cdot 10^9$ M^{-2} and $c_{Ca^{2+}} = 1.7 \cdot 10^{-3}$ M we find that above $\bar{c}_{A^-} = 10^{-7}$ M all carrier is in the complexed form (Eqn. 5d) and therefor a linear dependence of the Ca^{2+}-flux from the ionophore concentration is expected.

$$\bar{c}_{A_2Ca} = \frac{\bar{c}_A^{tot}}{2} \tag{5d}$$

Only below these concentrations should a quadratic dependence of the flux on the ionophore concentration be found. Althoug the flux is close to the optical detection limit in this concentration range (Fig. 2) we should be able to detect a quadratic dependence on the ionophore concentration with our method. Such a quadratic dependence on the A23187 concentration (between 10^{-7} and 10^{-8} M) has in fact been when measuring Ca^{2+}-efflux from liposomes [21]. The experimental finding that the Ca^{2+}-flux depends linearly on the ionophore concentration below $c_A^a = 10^{-7}$ M is therefore not completely understood by comparison with the vesicle experimets. One reason for this observation may be that the reactions are not quantitatively identical in vesicle membranes and Müller-Rudin membranes. Some other possiblities may exist why the fluxes depend linearly on the ionophore concentration despite the formation of a 2 : 1 complex. One possibility would be that the species A^- could from larger aggregates in the presence of Ca^{2+} similar to phase separation in mixtures of neutral and negative lipids [22]. Such structures may possess hardly predictable transport properties, like a relay-mechanism for Ca^{2+}. Also the flux may be determined by the back transport of the ionophore. If A23187 acts like a classical carrier by binding Ca^{2+} on one side of the membrane, diffusing across the membrane and releasing Ca^{2+} on the other side, a back transport of the carrier is absolutely necessary,

because at the observed fluxes the high Ca^{2+} side would be depleted of carrier very soon and the flux would decrease or stop, which was not found. If Ca^{2+} is transported as the neutral 2 : 1 complex, the back transport of the ionophore should also proceed as a neutral species since no electrical effects were found as discussed earlier. The simplest way would be the protonation of the ionophore after Ca^{2+} release. This would result in the build-up of a relative large pH gradient, with the low Ca^{2+} side becoming more basic. The effect should be measurable, if one or two protons are transported per Ca^{2+}, but it was not found. A stoichometry of Ca^{2+} versus H^+ (or OH^-) transport cannot be given, but it seems that less than one H^+ is transported per Ca^{2+}. Nevertheless, the Ca^{2+}-fluxes are pH-dependant (see Figs. 2 and 3) which is explained by the competition of Ca^{2+} and H^+ for the ionophore as also shown by the vesicle experiments. Two further experiments show this pH influence on the Ca^{2+}-flux: formation of a proton gradient in the opposite direction as the Ca^{2+} gradient (addition of KOH to chamber II and buffering chamber I at pH 5.5 or 8) was found to increase the Ca^{2+}-flux. As described in Results (Fig. 5) the addition of KOH (or HCl) to the high Ca^{2+} side to make OH^- (or H^+) approximately 10^{-3} M induced a pH increase (decrease) on the low Ca^{2+} side but only in the presence of both Ca^{2+} and A23187. The saturation effects observed in Fig. 2 may be explained by the limited solubility of A23187 in water. The saturation effects seen in Figs. 3 and 4 may be due to a limitation of the backtransport of free carrier.

Recently it has been shown that besides the complex $(A_2M^{n+})^{+n-2}$ also $(A_2M^{n+}H)^{+n-1}$ and $(A_2M^{n+}H_2)^{+n}$ may exist [20]. The proton containing complexes would be positively charged which would be in contradiction to the electroneutral transport experimentally found in our system. Also by charge pulse experiments no unidirectional transport of a charged species could be detected (Wulf, J. and Benz, R., unpublished experiments). The detection limit of the method depends of course on the type of charge carrier. It is about 10^{-14} mol · cm^{-2} in the case of dipicrylamine ($k_i \approx 450\ s^{-1}$) and tetraphenylborate ($k_i \approx 7\ s^{-1}$) [13]. Making the assumption that a charged complex of A23187 behaves similar as these hydrophobic ions, less than $5 \cdot 10^{-12}$ or 10^{-13} mol · $cm^{-2} \cdot s^{-1}$ of a charged species would be transported in our case, whereas we find Ca^{2+}-fluxes up to $2 \cdot 10^{-10}$ mol · $cm^{-2} \cdot s^{-1}$.

It may be that a cotransport of anions makes the transport electroneutral. The scheme would be e.g. $(A_2CaH_2)^{+2}X_2 \rightleftharpoons A_2H_2 + Ca^{2+} + 2X^-$. Test experiments using $Ca(NO_3)_2$, $Ca(CH_3COO)_2$, $Ca(OH)_2$ and $CaSO_4$ in equal concentrations as $CaCl_2$ but in the absence of Cl^- gave Ca^{2+}-fluxes which were different within a factor of two compared with $CaCl_2$. Since this is within the experimental error of single measurements in our method, a large influence of the anion is excluded. Also increasing the Cl^- concentration 100 times by addition of KCl to chamber II did not change the Ca^{2+} flux. Furthermore, using Tris buffer instead of HEPES buffer did not influence the Ca^{2+}-flux. Apparently these results do not support an anion-cotransport, but the question has to be solved by further studies with radioactive anions.

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