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THE TRANSLOCATION OF Ca^{2+} ACROSS PHOSPHOLIPID BILAYERS INDUCED BY A SYNTHETIC NEUTRAL Ca^{2+}-IONOPHORE

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Summary

The effect of a neutral synthetic Ca^{2+}-ligand, which induces selective Ca^{2+} transport in electrodialysis experiments in bulk membranes, on the Ca^{2+} permeability of phospholipid bilayers has been investigated. The ligand is able to promote the transport of Ca^{2+} across synthetic phospholipid bilayers and can therefore be classified as a Ca^{2+}-ionophore. Its activity is enhanced by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). The efficiency of the neutral carrier-mediated Ca^{2+} transport is rather low as compared with that of the charged Ca^{2+}-ionophore X537A.

The Ca^{2+} selectivity of the neutral ionophore is decreased by its incorporation in the low dielectric ambient of the phospholipid bilayer.

Introduction

In recent years, several ionophoric molecules able to render lipid membranes specifically permeable to Ca^{2+} have been described [1—4]. These molecules have in common a hydrophobic part which confers lipid solubility to them; and preferentially oxygen atoms which are arranged so that they can replace the water of solvation around the cation, and therefore represent the binding sites of the ligand.

The properties of two of these ionophores, the naturally occurring antibiotics A23187 and X537A, have been studied in detail [1,5]. They are charged molecules, and transport Ca^{2+} in the form of neutral complexes consisting of two ionophores sandwiched around a calcium ion [5,6]. This ratio,

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP, carbonyl cyanide *p*-triflоromethoxyphenylhydrazone; EGTA, ethylene-bis-(oxyethylenenitrilo)-*N,N'*-tetraacetic acid.

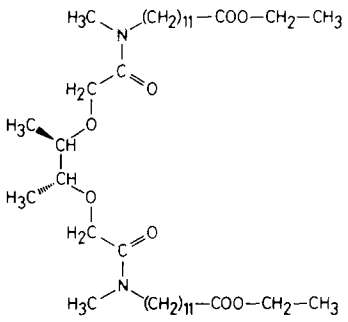

Fig. 1. Structure of the neutral synthetic Ca^{2+}-ligand.

however, can change when low concentrations of the ionophore are used [5], thus yielding a charged complex. Avenaciolide, a compound originally used as an inhibitor of the glutamate carrier in the inner mitochondrial membrane, has also been used as a Ca^{2+}-ionophore [4]. This molecule is neutral, has a wide range of cation specificity, and has a stoichiometry of its Ca^{2+} complex between 1 : 3 and 1 : 10 (cation to ligand).

To better understand the physicochemical properties of Ca^{2+}-ionophores, molecules have recently been synthesized in several laboratories which satisfy the theoretical requirements for Ca^{2+}-ionophoric activity [7]. Their ability to transport Ca^{2+} and other cations across hydrophobic phases has been investigated. We have recently reported on the properties of a variety of lipid-soluble electrically neutral molecules which selectively transport Ca^{2+} in bulk phases in electrodialysis experiments [8,9]. These molecules, in ambients of high dielectric constant such as certain liquid membranes, display a high selectivity for Ca^{2+} and have thus been used in Ca^{2+}-selective liquid-membrane electrodes [7,10]. In membranes having low dielectric constants, the Ca^{2+} selectivity decreases in favour of monovalent cations [8,11].

In this paper we report results on the ability of one of the synthetic neutral Ca^{2+}-ligands synthesized in our laboratory (Fig. 1) to promote the movement of Ca^{2+} across multilayer and single bilayer vesicular phospholipid membranes (liposomes), and across planar phospholipid bilayer membranes.

Materials and Methods

Planar lipid bilayer membranes were formed from 1% egg lecithin dissolved in *n*-decane. A small brush was used to apply the lipid to the hole in a plexiglass septum (2.8 mm in diameter), separating two chambers. Both chambers contained 1 mM $CaCl_2$ and about 10 μM ligand. A potential of 30 mV was applied across the lipid film during the experiments.

Multilamellar liposomes were prepared in the following way: a chloroform solution containing 33 mg of egg lecithin : phosphatidylserine (10 : 1), was evaporated to dryness in a round bottom flask. The lipids were suspended in 1 ml of buffer I (20 mM HEPES, 11 mM Tris, 1 mM $Ca(OH)_2$, final pH 7.5) containing 1 mM murexide, by mixing vigorously for 1 min on a Vortex mixer and for some minutes on a magnetic stirrer. The separation of the vesicles from

the outside solution was obtained by gel filtration on a Sephadex G-25 column, using buffer I for elution. The effect of the Ca^{2+}-ionophores on the Ca^{2+} permeability of the vesicles was followed by measuring the changes in the absorbance of the Ca^{2+}-murexide complex at 540/507 nm using an Aminco 2W dual wavelength spectrometer. The assay mixture was buffer I plus 2 mM EGTA.

Single bilayer vesicles were prepared by sonication of the multilamellar vesicles suspended in buffer I (without murexide and with 1 mM $^{45}Ca^{2+}$), using a Branson sonifier for 30 min (output 30 W) at 0°C. After centrifugation at 100 000 × *g* for 45 min, the vesicles were separated from the free $^{45}Ca^{2+}$ on a Sephadex G-25 column using buffer I for elution. After diluting with buffer I the concentration of the lipid was about 3 mM. 1 ml of the $^{45}Ca^{2+}$-loaded vesicles was placed in Visking dialysis bags opened on one side and dialyzed against 1 l of buffer I containing 2 mM EGTA. When the ionophores (or the uncouplers) were added, the vesicles were preincubated with them for 2 min before being added to the dialysis bag. The release of $^{45}Ca^{2+}$ from the vesicles was followed by withdrawing 20-μl aliquots from the dialysis bags at the times specified in the legends of the figures. Radioactive counting was performed in a scintillation counter, using Instagel (Packard Instrument Comp. Inc., Downers Grove, Ill.) as the scintillation fluid.

The loading of the single bilayer liposomes with [^{3}H]sucrose and $^{24}Na^+$ was carried out as described for $^{45}Ca^{2+}$ loading, using 10^{-5} [^{3}H]sucrose (50 μCi ^{3}H) and 1.3 mM $^{24}Na_2CO_3$ (400 μCi ^{24}Na). Phosphate measurements were carried out with the method of Chen et al. [12].

Egg lecithin and phosphatidylserine were purchased from Lipid Products, Nutfield, U.K. The ionophore A23187 was a generous gift from Eli Lilly Research Lab. Indianapolis and X537A a generous gift from F. Hoffmann-La Roche and Co. AG, Basel. The neutral Ca^{2+}-ligand was synthesised as described previously [11].

Results

In the experiment shown in Fig. 2 the ability of the neutral Ca^{2+}-ligand to promote the movement of Ca^{2+} across phospholipid bilayers has been studied

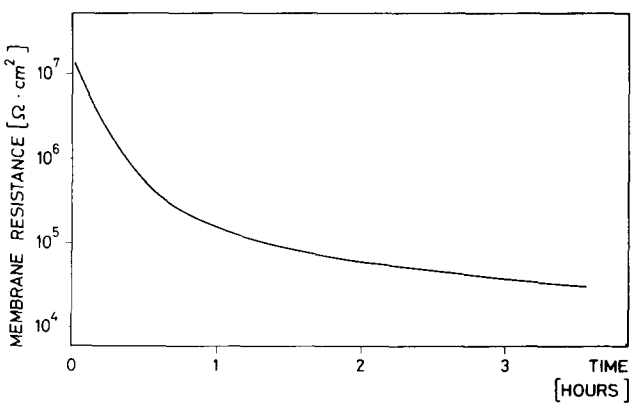

Fig. 2. Electrical resistance of planar phospholipid bilayer membranes in the presence of the neutral Ca^{2+}-ligand. Conditions: lecithin/decane membrane; aqueous phase: 10^{-3} M $CaCl_2$, $1.2 \cdot 10^{-5}$ M ligand.

using planar phospholipid membranes, made of egg phosphatidylcholine. Under the conditions employed the bilayer was stable for hours. The additions of the Ca^{2+}-ligand to the aqueous compartments separated by the bilayer did not induce changes in the electrical conductance of the membrane unless Ca^{2+} was also present in the chambers. In the presence of 10^{-3} M $CaCl_2$, and at a ligand concentration of approx. 10^{-5} M, and evident increase in the conductance of the bilayer was observed (Fig. 2). The conductance changes were rather slow, but the experiment nevertheless indicates that the ligand can act as a Ca^{2+}-ionophore also in phospholipid bilayers. This is in agreement with findings on planar phospholipid membranes recently reported by Amblard and Gavach [13] on a similar ligand.

The phenomenon was studied in greater detail using vesicular (liposomal) bilayer preparations. As shown in Fig. 3, the ability of the ligand to promote the release of isotopically labelled Ca^{2+} enclosed in the vesicles was not very pronounced, even when high concentrations of the compound were added. On the other hand, Fig. 3 shows that the charged Ca^{2+}-ionophore X537A was able to promote a fast release of the Ca^{2+} enclosed in the vesicles [5]. However, when neutral ionophores are used, a problem arises. The diffusion of the charged ionophore-Ca^{2+} complex out of the vesicle may create a membrane potential which would tend to limit the further translocation of the complex. To avoid this effect, the proton carrier FCCP was added to the medium. As expected (Fig. 3), the Ca^{2+}-ligand indeed promotes a faster release of Ca^{2+} from the vesicles when tested in the presence of FCCP. Alternative explanations for the effect of FCCP, such as the migration of ion pairs across the membrane, could also be considered. A plausible test of this alternative possibility would be the demonstration that K^+, added to Ca^{2+}-loaded liposomes in the presence of valinomycin (and the synthetic Ca^{2+}-ligand) promotes the efflux of Ca^{2+}. The significance of experiments of this type would however be greatly limited by the fact that the synthetic ligand, in the dielectric constant of the phospholipid bilayer, may also transport K^+. The dependence of the Ca^{2+} release process

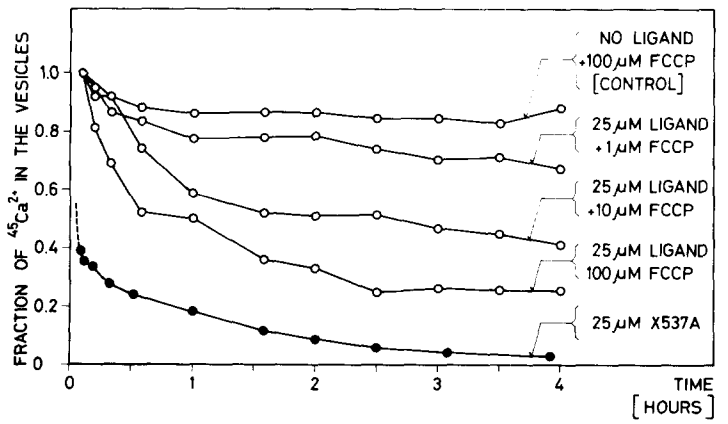

Fig. 3. Effect of the uncoupler FCCP on the ionophoric activity of the neutral Ca^{2+}-ligand. Experiment on Ca^{2+}-loaded lecithin liposomes. Medium: 30 mM Tris/HEPES buffer, 1 mM $Ca(OH)_2$, 2 mM EGTA. The liposomes were loaded with 30 mM Tris/HEPES buffer and 1 mM $^{45}Ca(OH)_2$. Phospholipid concentration: 2 mM organic phosphate. Ligand concentration: 25 μM. Other technical details in Methods.

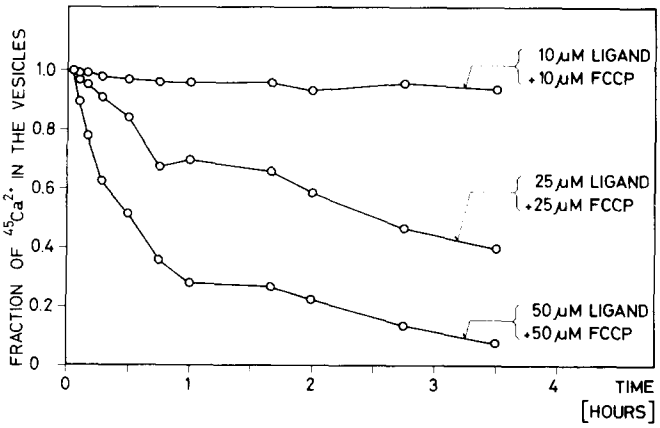

Fig. 4. Rate of Ca^{2+} release from liposomes at different ligand concentrations. Experimental conditions as in Fig. 3. Phospholipid concentration: 3.3 mM organic phosphate.

on the ligand concentration at which effects are evident is approximately one order of magnitude higher than that at which X537A is optimally active.

Similar studies have also been performed using multilamellar liposomes, and by monitoring Ca^{2+} with the specific metallochromic indicator murexide (ammonium purpurate). The results were similar to those obtained with $^{45}Ca^{2+}$ in monolamellar vesicles.

In the experiments shown in Figs. 2—4, rather high concentrations of the Ca^{2+}-ionophore and of FCCP had to be added to induce evident effects. It was therefore necessary to check whether the vesicles were structurally damaged during the process, and had thus become non-specifically permeable. Vesicles were therefore loaded with $^{45}Ca^{2+}$ and [^{3}H]sucrose, and then exposed to the Ca^{2+}-ligand (Fig. 5). Only insignificant amounts of labelled sucrose were lost during the ligand-induced Ca^{2+} release, indicating that the structural integrity of the lipid bilayer was not unspecifically affected by the ligand.

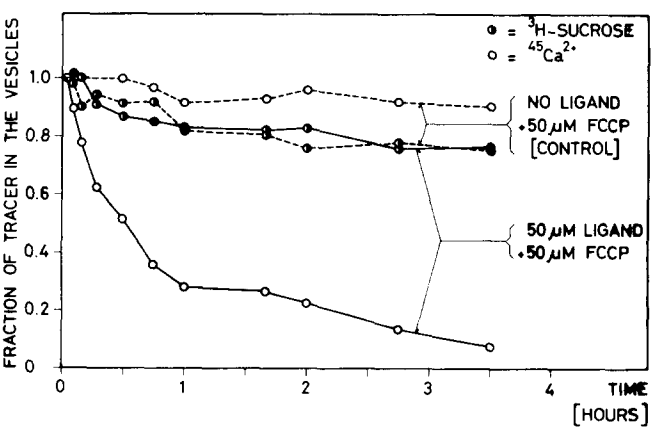

Fig. 5. Release of Ca^{2+} and of sucrose from Ca^{2+} and sucrose-loaded single bilayer lecithin liposomes. The medium was the same as in Fig. 3. The vesicles were loaded with 30 mM Tris/HEPES buffer, 1 mM $^{45}Ca(OH)_2$ and 0.01 mM [^{3}H]sucrose. Phospholipid concentration: 3.3 mM organic phosphate.

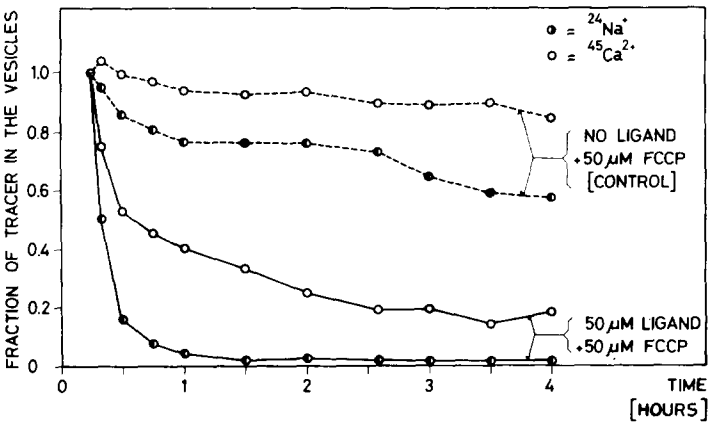

Fig. 6. Selectivity for Ca^{2+} in respect to Na^{+}. Release of Ca^{2+} and Na^{+} from single bilayer lecithin liposomes. The medium was the same as in Fig. 3. The vesicles were loaded with 30 mM Tris/HEPES buffer, 1 mM $^{45}Ca(OH)_2$ and 1.3 mM $^{24}Na_2CO_3$. Phospholipid concentration: 3.3 mM organic phosphate.

The neutral Ca^{2+}-ligand used in these experiments is rather specific for Ca^{2+} when applied to bulk membranes with a high dielectric constant [7,8]. However, in the lower dielectric constant of a phospholipid bilayer it may lose some of its selectivity in favour of Na^{+} (Fig. 5). In fact, the compound induces a Na^{+} release from liposomes which is even faster than that of Ca^{2+}. This is in variance with recent findings by Amblard and Gavach [13], who have reported that a similar ligand still prefers Ca^{2+} over Na^{+} in a glycerol-monooleate bilayer.

Discussion

The results presented demonstrate that the neutral Ca^{2+}-ligand is able to promote the transport of Ca^{2+} across artifical phospholipid bilayers and can thus be considered a Ca^{2+}-ionophore. The efficiency of the transport, however, seems to be rather low, compared to that of the charged Ca^{2+}-ionophore X537A (and presumably A23187); therefore, a high ligand concentration is required in order to obtain an evident increase in the permeability of the bilayer to Ca^{2+}. The high amount of ligand used, however, does not affect the structural integrity of the bilayer.

It must be pointed out here that similar results using a similar neutral Ca^{2+}-ligand and planar phospholipid membranes have been obtained by Eisenmann et al. (personal communication), and by Amblard and Gavach [13]. In the experiments with closed phospholipid vesicles the ability of the Ca^{2+}-ligand to translocate Ca^{2+} was stimulated by making the membrane permeable to protons. This was expected, since the diffusion of the charged complex would induce a membrane potential that would affect its further translocation. Similar effects are normally observed with neutral ionophores [14]; however, in the present case the FCCP concentration necessary for optimal rates of Ca^{2+} translocation was higher than normally required.

As previously shown [11] the ion selectivity of ligands is highly dependent on the dielectric constant of the environment. A decrease in the dielectric con-

stant of the membrane may lead to an increased preference of monovalent over divalent cations of the same size (Na^+/Ca^{2+}) [8,11,15]. In the low dielectric constant provided by the hydrocarbon chains of the phospholipids of the artificial membranes used, the neutral ionophore indeed loses its selectivity toward Ca^{2+} and becomes more selective for monovalent cations.

The neutral Ca^{2+}-ligand described in this paper is among the first to be shown to be active in phospholipid bilayer membranes. This is indeed the first instance in which release of Ca^{2+} from liposomes is induced by a neutral carrier. Recently, studies on mitochondria and on bulk phases have been carried out with avenaciolide, a naturally occurring neutral Ca^{2+} carrier, which also has a wide selectivity range [4,16]. The mechanism of action of avenaciolide, particularly the nature of the complex that traverses the membrane is still unclear, since a very high number of avenaciolide molecules seem to be required to promote translocation of Ca^{2+}. There is evidence available that predominantly 1 : 2 cation : ligand complexes of Ca^{2+} with the ionophore discussed here are formed in bulk membranes (see also ref. 17).

Studies are currently under way in this laboratory on the ability of the neutral Ca^{2+}-ligand described here to promote the transport of Ca^{2+} in natural membranes, having dielectric constants different from that of the artificial phospholipid membranes used in this study. Work is also in progress on structural modifications of the ligand to improve its selectivity towards Ca^{2+} in environments of low dielectric constants.

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