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Ca^{2+}-CARDIOLIPIN INTERACTION IN A MODEL SYSTEM

SELECTIVITY AND APPARENT HIGH AFFINITY

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The interaction of cardiolipin with Ca^{2+} was assessed by measuring the cardiolipin-mediated extraction of $^{45}Ca^{2+}$ from an aqueous to an organic (methylene chloride) phase. Cardiolipin binds Ca^{2+} with high affinity [K_d(apparent) = 0.70 ± 0.17 μM (S.D.)]. Cation-cardiolipin interactions are selective. Interaction of cardiolipin with Ca^{2+} is insensitive to Na^+, but is inhibited by divalent cations with $Mn^{2+} > Zn^{2+} > Mg^{2+}$. In addition La^{3+} and Ruthenium red are particularly potent inhibitors of Ca^{2+} binding by cardiolipin. Cardiolipin-mediated extraction of Ca^{2+} into an aqueous phase is also inhibited by phosphatidylcholine. Inhibition of Ca^{2+}-cardiolipin interaction by phosphatidylcholine (a phospholipid known to stabilize the bilayer conformation) may implicate inverted, non-bilayer lipid structures in the binding.

Introduction

The transport of calcium across biological membranes is central to the function of numerous cellular systems. In some cases, such as the sarcoplasmic reticulum of skeletal muscle, the agent that mediates calcium movement has been isolated and identified. A 100 kDa ATPase protein is the functional transporter in sarcoplasmic reticulum (for a review, see Ref. 1). In mitochondria, although several distinct Ca^{2+} fluxes, including Ca^{2+} uptake in response to an inward negative membrane potential, have been defined [2] a specific calcium transport protein has yet to be unequivocally demonstrated to function in situ [3–5].

It has been suggested that phospholipids, which are major components of all biological membranes, might be capable of calcium transport [6]. Naturally occurring phosphatidylethanolamines adopt inverted nonbilayer structures. Both the hexagonal H_{II} phase, consisting of cylindrical arrays with the lipid headgroups facing inward, and inverted micelles contain an aqueous core suitable for sequestering calcium. Furthermore, the acidic phospholipids, phosphatidic acid and cardiolipin, can be induced by calcium to form inverted structures (for a review, see Ref. 7).

Attempts to add phospholipids to naturally occurring membranes have suggested that cardiolipin may stimulate calcium entry into platelets [8]. Phosphatidate has been reported both to mimic depolarization-induced calcium uptake by synaptosomes [9] and to facilitate Ca^{2+} uptake by hepatocytes [10]. The behavior of phospholipids in a model system has been studied extensively by

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PC, phosphatidylcholine.

Tyson et al. [11]. Using a Pressman cell [12], they demonstrated that cardiolipin and phosphatidic acid both facilitate the movement of calcium from one aqueous phase to another through a stirred organic phase. Extraction of calcium from an aqueous into an organic phase by cardiolipin has also been reported [13]. More recently, Serhan et al. [14] have proposed that phosphatidate may facilitate calcium influx into liposomes.

Because previous studies have, in general, been concerned with the ability of phospholipids to translocate Ca^{2+} across the plasma membrane, the Ca^{2+} concentrations utilized have been ≥ 1 mM. In the experiments of Tyson et al. [11], for example, lipid and Ca^{2+} concentrations were 0.42 and 10 mM, respectively. In contrast, intracellular Ca^{2+} concentrations are exceedingly low (0.1–10.00 μM) [15,16]. Thus, if a phospholipid were to mediate Ca^{2+} transport across an intracellular membrane, it would require very high affinity for the ion.

We report here an examination of the interaction of cardiolipin, a major component of the inner mitochondrial membrane, with Ca^{2+}. Using a model system, in this case, the ability of the phospholipid to extract Ca^{2+} from an aqueous to an organic phase, and Ca^{2+} and cardiolipin concentrations in the micromolar range, we have found that the apparent affinity of cardiolipin for Ca^{2+} is much higher than anticipated ($K_d < 1$ μM). Furthermore, competition studies using a variety of cations suggest that cardiolipin-cation interactions in this model system are selective.

Methods

Extraction of Ca^{2+} into an organic phase was measured by the method described by Jeng and Shamoo [5]. Lipid samples were placed in polypropylene test tubes and the solvents evaporated under nitrogen. The organic phase, 1.0 ml methylene chloride, was added to each tube and the tube vortexed briefly. A 0.5 ml aqueous phase containing 5 mM Hepes-Tris, pH 8.0, and $^{45}CaCl_2$, was then added. The tubes were stoppered and vortexed for 2 min. The phases were separated by centrifugation, and duplicate samples of the aqueous and organic phases were counted. (The organic phase was collected by puncturing the bottom of the tube.) Controls were run for all Ca^{2+} concentrations used. In the absence of lipids no Ca^{2+} could be detected in the organic phase. The data shown are representative of multiple replicate experiments.

Bovine brain phosphatidylcholine and the sodium salt of bovine heart cardiolipin were purchased from Sigma. Ruthenium red was recrystallized according to Luft [17]. Solutions were made fresh on the day of use and the concentration determined spectrophotometrically.

Phospholipid concentrations were determined by the method of Chen et al. [18].

Results and Discussion

The organic extraction technique measures the ability of a compound to interact with an ion, in this case Ca^{2+}, in such a manner that the resulting complex is soluble in the organic phase. A Scatchard plot for the interaction of cardiolipin with calcium, determined by organic extraction from aqueous phases containing 5–60 μM Ca^{2+}, is shown in Fig. 1. An apparent dissociation constant of 0.76 μM can be calculated from the plot. Five separate determinations, using cardiolipin con-

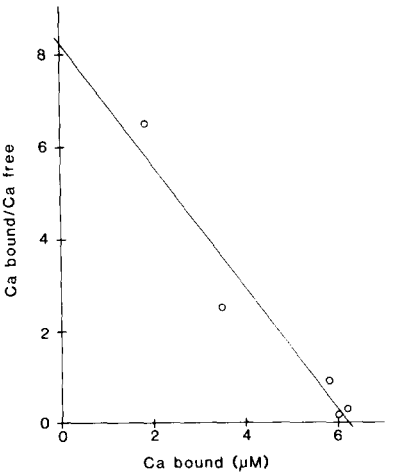

Fig. 1. A Scatchard plot for Ca^{2+} binding by cardiolipin measured by the organic extraction method. Each tube contained 9.6 nmol cardiolipin; Ca^{2+} concentrations were 5–60 μM in the aqueous phase. The line shown is a linear least-squares fit of the data. An apparent dissociation constant of 0.76 μM is computed from $-(1/\text{slope})$, and the x-intercept indicates the presence of 6.2 nmol of binding sites in the 1.0 ml organic phase.

centrations from 9.6 to 57.6 μM (assuming all the lipid to be present in the methylene chloride organic phase) yielded an apparent K_d of 0.70 $\pm$ 0.17 μM (S.D.). When toluene (dielectric constant = 2.4) was substituted for methylene chloride (dielectric constant 9.1), the apparent K_d increased to 3.4 $\pm$ 1.0 μM (three determinations). Clearly, at least in this assay system, the affinity of cardiolipin for calcium is high.

The dependence of Ca^{2+} extraction on cardiolipin concentration is demonstrated in Fig. 2 for a range of concentrations from 4.8 to 57.6 μM.

Tyson et al. [11] reported that, in the presence of excess Ca^{2+}, a mole ratio of Ca^{2+} to cardiolipin of 1 : 1 could be attained in the organic phase of their Pressman cell. If bulk extraction of Ca^{2+} in the absence of a lipophilic anion is observed, formation of such an uncharged complex is predicted. The same stoichiometry was determined by means of organic extraction (Fig. 3). Data are presented for two separate determinations carried

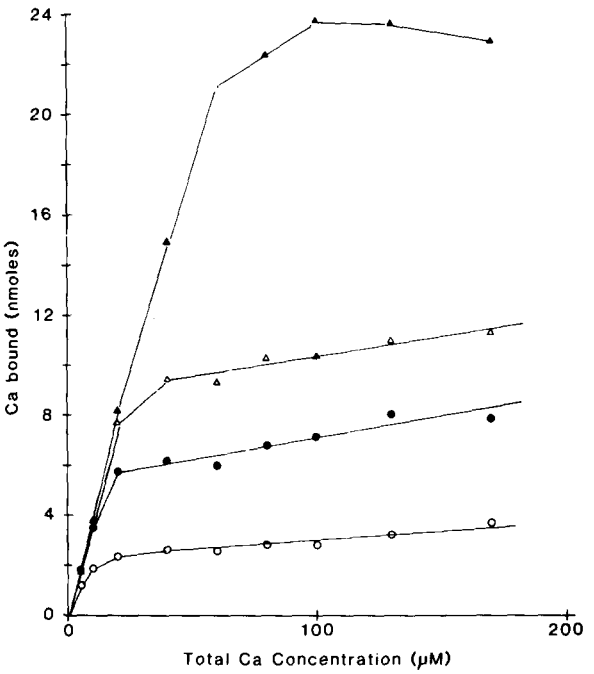

Fig. 2. Dependence of Ca^{2+} binding on cardiolipin concentration, determined by organic extraction. Tubes contained 4.8 (○), 9.6 (●), 19.2 (△), or 57.6 (▲) nmol cardiolipin. No detectable Ca^{2+} appeared in the organic phase when cardiolipin was omitted.

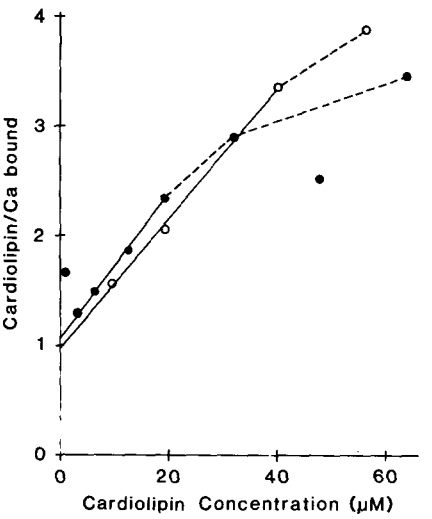

Fig. 3. Apparent stoichiometry of Ca^{2+} binding by cardiolipin. Results are shown for two Ca^{2+} concentrations: 40 μM (○) and 50 μM (●). The solid lines are least-squares fits of the data points they connect and extrapolate to stoichiometries of 0.97 and 1.06 cardiolipin per Ca^{2+} bound at infinite cardiolipin dilution, respectively. The broken curves connecting the points at higher cardiolipin concentrations were drawn by eye. They are consistent with observations at other Ca^{2+} concentrations (not shown). The single point (●) at approx. 2.4 2 nmol cardiolipin bound per Ca^{2+} in the 1.0 ml organic phase was not used in curve fitting.

out at 40 μM and 50 μM Ca^{2+}. The amount of Ca^{2+} bound is plotted as a function of the cardiolipin concentration. Extrapolation to infinite cardiolipin dilution yields cardiolipin to Ca^{2+} stoichiometries of 0.97 and 1.06, respectively. The accuracy of these stoichiometries will depend on the completeness of cardiolipin partitioning into the organic phase. Direct measurements of the phospholipid content of the two phases, made at 7–35 μM cardiolipin and 0–100 μM Ca^{2+}, demonstrated that less than 2% of the input cardiolipin was present in the aqueous phase. Under the conditions of Fig. 3, i.e., 50 μM Ca^{2+} and varying cardiolipin concentration, more than 90% of the cardiolipin was recovered in the organic phase (data not shown). Interfacial accumulation of cardiolipin is apparently a minor factor in this experimental system. (A stoichiometry of 1.55 cardiolipins per Ca^{2+} can be calculated from the intercept of the Scatchard plot in Fig. 1.)

Serhan et al. [14] have reported the sequence $Mn^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$ for cation translocation by phosphatidic acid in multilamellar vesicles. Selectivity of cardiolipin-cation interaction under conditions of low lipid and ligand concentration was measured by testing the ability of a variety of cations (10–100 μM) to inhibit extraction of Ca^{2+}, from an aqueous phase 50 μM in Ca^{2+}, into an organic phase (Fig. 4). It can be seen that Na^+ is essentially without effect. Mn^{2+}, which is translocated into mitochondria by the Ca^{2+}-uptake system [19,20], inhibits Ca^{2+} extraction more strongly than Mg^{2+}, which is not translocated [19,20]. La^{3+} and Ruthenium red are particularly potent inhibitors of Ca^{2+} extraction. Fifty percent inhibition is observed at approx. 13 and 0.8 μM, respectively. Complete inhibition by ruthenium red appeared at < 3 μM or approximately one Ruthenium red per 13 cardiolipin. Under the conditions of this experiment, the controls bound 8.38 ± 0.46 nmol Ca^{2+}. Complete inhibition by Ruthenium red thus occurred at approximately one Ruthenium red per 5.6 Ca^{2+}. This stoichiometry suggests that each of the six positive charges on Ruthenium red can interfere with the binding of a calcium ion.

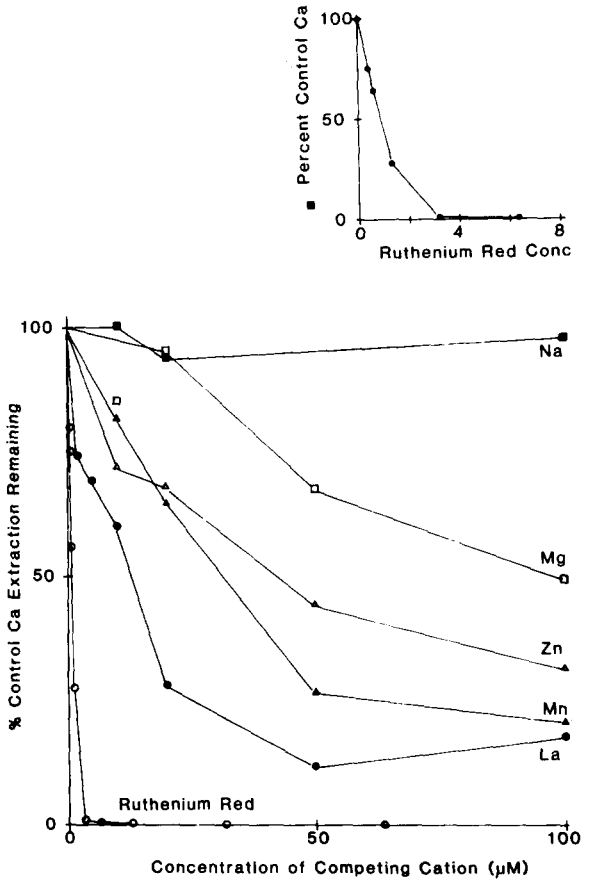

Fig. 4. Inhibition by cations of cardiolipin-mediated extraction of Ca^{2+} into an organic phase. Each tube contained 19.2 nmol cardiolipin. Ca^{2+} concentration in the aqueous phase was 50 μM. Controls bound 8.38 ± 0.46 nmol Ca^{2+}. Cations tested were, in order of increasing ability to inhibit, Na^+ (■), Mg^{2+} (□), Zn^{2+} (△), Mn^{2+} (▲), La^{3+} (●), and Ruthenium red (○). Inhibition by Ruthenium red is shown in expanded form in the insert.

Tyson and co-workers [11] failed to observe cation selectivity when cardiolipin-mediated cation translocation was measured in a Pressman cell. In fact, when cations were present in the donor phase at 10 mM, cardiolipin transported Rb^+ at a rate in excess of the rate measured for Ca^{2+}. Rb^+ was, however, unable to compete with Ca^{2+} for translocation. Rb^+ was also an ineffective competitor in the organic extraction system described here. In two experiments, Rb^+ and Mn^{2+} (100 μM) reduced Ca^{2+} extraction to an average of 85% and 30% of the control values, respectively, under the conditions of Fig. 4.

Cardiolipin is reported to form the hexagonal H_{II} phase in the presence of Ca^{2+}, and an isotropic ^{31}P-NMR signal, possibly indicative of inverted micelles, can be detected using Ca^{2+}-cardiolipin mixtures [21,22]. Furthermore, ^{14}N-NMR studies [23] suggest that lipids in water-organic solvent systems are organized into inverted micelles in the organic phase. The cardiolipin-Ca^{2+} complexes found in the methylene chloride phase of these organic extraction experiments may thus be inverted micelles with Ca^{2+} sequestered in an aqueous interior. A preliminary test of this hypothesis was carried out by examining the effect of phosphatidylcholine (PC) on Ca^{2+} extraction by cardiolipin. PC is reported to stabilize bilayers and to inhibit formation of inverted structures [24]. From the observation that PC is required for the head groups of cardiolipin to be antigenic [25], it can be inferred that PC disrupts inverted structures formed by cardiolipin.

The effect of PC concentration on Ca^{2+} extraction by 9.6 nmol cardiolipin is shown in Fig. 5.

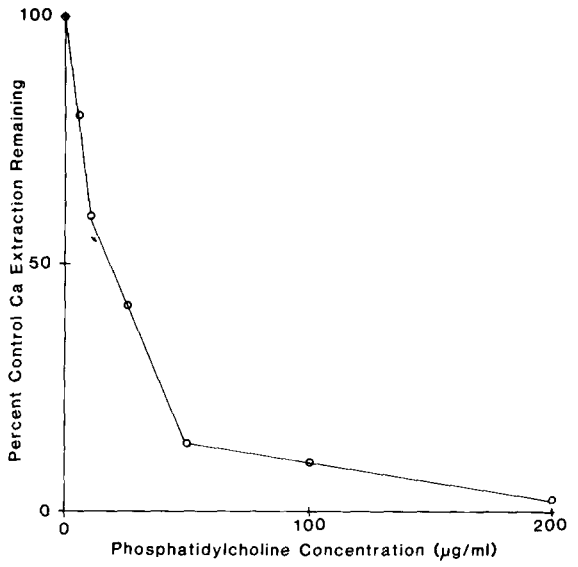

Fig. 5. Inhibition of cardiolipin-mediated Ca^{2+} extraction by phosphatidylcholine. Each tube contained 9.6 nmol cardiolipin. Ca^{2+} concentration in the aqueous phase was 50 μM. The controls bound 4.72 ± 0.36 nmol Ca^{2+}.

Measurable inhibition was observed at a PC/cardiolipin mole ratio of 1, and 50% inhibition occurred at PC/cardiolipin = 2.5 (using 750 as the molecular weight of PC). The apparent affinity of cardiolipin for Ca^{2+} was unaltered by PC. In experiments using the protocol of Fig. 5, the effect of PC concentration on cardiolipin-mediated Ca^{2+} extraction was determined for cardiolipin concentrations from 9.6 to 58 nmol per tube. Inhibition was not a simple function of either PC concentration or PC/cardiolipin ratio. No discontinuities in the curves were observed at the critical micelle concentration for PC in solvents of low dielectric constant (200 μg/ml for benzene [26]). The trivial explanation for the inhibitory effect of PC, namely that it tended to exclude cardiolipin from the organic phase, was eliminated by direct TLC analysis of the organic phase. PC concentrations sufficient to inhibit Ca^{2+} extraction > 90% did not visibly alter the cardiolipin content of the organic phase. Note that extraction of Ca^{2+} into the organic phase by PC also is of minimal concern. At 50 μM Ca^{2+}, even the highest PC concentration tested bound < 0.2 nmol Ca^{2+} whereas the cardiolipin control value for Fig. 5 was $4.72 \pm$ 0.36 nmol Ca^{2+} bound. Extraction of Ca^{2+} by PC itself may, however, explain the residual Ca^{2+} binding observed at high PC concentrations.

Conclusions

We have demonstrated in a model system that interactions of cardiolipin with Ca^{2+} can occur at physiological intracellular Ca^{2+} concentrations and micromolar phospholipid concentrations. The apparent K_d for the interaction (< 1 μM) is comparable to that of the mitochondrial Ca^{2+}-uptake system determined from initial-rate studies [19] and to that of the Na^+/Ca^{2+} exchanger [2]. The interaction of cardiolipin with Ca^{2+} is further characterized by a differential sensitivity to other cations and pronounced sensitivity to Ruthenium red and lanthanum.

Calcium uptake by the mitochondrial uniporter is electrophoretic [19,20], yet the 1 : 1 stoichiometry of calcium-cardiolipin interaction suggests that the complex is electrically neutral. Similarly, studies of cardiolipin effects on artificial black lipid membranes formed from PC, PE and varying amounts of cardiolipin up to 25% showed no ionophoretic activity to calcium or potassium (Drzymala and Shamoo, unpublished observations). A primary role for cardiolipin in the function of the uniporter thus seems unlikely. Calcium also crosses the mitochondrial inner membrane by several mechanisms that are independent of membrane potential: Na^+/Ca^{2+} exchange, H^+/Ca^{2+} exchange, and Ca^{2+}-triggered Ca^{2+} release (for a review, see Ref. 2). The possibility thus exists that cardiolipin may be involved in the binding step of one of these transport functions.

We have considered the possibility that the cardiolipin utilized in these experiments might contain residual protein. 100 μg cardiolipin gave a Lowry-positive response equivalent to 1.5 μg bovine serum albumin. No amino acids could be detected when an equivalent sample was subjected to amino acid analysis. The observed capacity of the cardiolipin to bind Ca^{2+} with 1 : 1 stoichiometry, when Ca^{2+} is in excess, thus vastly exceeds the capacity of the contaminant protein.

The experiments reported here deal only with binding and sequestration of Ca^{2+} by cardiolipin. Serhan et al. [14,27] have incorporated cardiolipin

into multilamellar vesicles in more ratios up to 5%. Under these conditions, no Ca^{2+} entry into the liposomes was detected. This suggests that cardiolipin does not function as a classical ionophore. We have, however, looked at Ca^{2+} binding to unilamellar vesicles containing varying amounts of cardiolipin. Only when cardiolipin is present in a mole ratio in excess of 5% is significant Ca^{2+} binding observed (Brenza and Shamoo, unpublished observation). Cardiolipin accounts for 20% of the total mitochondrial phospholipid by weight [28] or approx. 10 mol%.

Extrapolation from experiments with model systems to biological membranes must be made with care. The formation of inverted structures, for example, may be observed with a synthetic lipid, but not with the naturally occurring forms of the lipid [29]. The behavior of any one particular lipid will be influenced by the other lipids present. The cytoplasmic membrane of *Escherichia coli* K12 strain CE1163 (which lacks phospholipase A in the outer membrane) shows evidence of an isotropic phase at 37°C [30], but an *E. coli* mutant strain (T2GP) deficient in the synthesis of cardiolipin and auxotrophic for fatty acids shows no signs of non-bilayer phases between 30 and 40°C [29]. Similarly, the protein constituents of biological membranes alter the behavior of the membrane lipids in ways that cannot be easily predicted. Thus, the lipids extracted from liver endoplasmic reticulum form only bilayers whereas the spectrum of the membranes themselves has a pronounced isotropic component [31]. In contrast, the isolated lipids of rod outer segments display spectra characteristic of the hexagonal H_{II} phase and an isotropic phase while the lipids of the intact photoreceptor membrane adopt a bilayer configuration [32]. Particularly relevant to the experiments discussed here, the spectra of both the inner mitochondrial membrane and its constitutent lipids indicate the presence of a Ca^{2+}-enhanced isotropic component, but the component is not detected in intact mitochondria [33].

Acknowledgements

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