Influence of N-dodecyl-N,N-dimethylamine N-oxide on the activity of sarcoplasmic reticulum Ca\(^{2+}\)-transporting ATPase reconstituted into diacylphosphatidylcholine vesicles: Effects of bilayer physical parameters

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Abstract

Sarcoplasmic reticulum Ca-transporting ATPase (EC 3.6.1.38) was isolated from rabbit white muscle, purified and reconstituted into vesicles of synthetic diacylphosphatidylcholines with monounsaturated acyl chains using the cholate dilution method. In fluid bilayers at 37 °C, the specific activity of ATPase displays a maximum (31.5 ± 0.8 IU/mg) for dioleoylphosphatidylcholine (diC\(_{18:1}\)PC) and decreases progressively for both shorter and longer acyl chain lengths. Besides the hydrophobic mismatch between protein and lipid bilayer, changes in the bilayer hydration and lateral interactions detected by small angle neutron scattering (SANS) can contribute to this acyl chain length dependence. When reconstituted into dierucylphosphatidylcholine (diC\(_{22:1}\)PC), the zwitterionic surfactant N-dodecyl-N,N-dimethylamine N-oxide (C\(_{12}NO\)) stimulates the ATPase activity from 14.2 ± 0.6 to 32.5 ± 0.8 IU/mg in the range of molar ratios C\(_{12}NO\) : diC\(_{22:1}\)PC = 0/C\(_{4}\) to 1/C\(_{2}\). In dilauroylphosphatidylcholines (diC\(_{12:0}\)PC) and diC\(_{18:1}\)PC, the effect of C\(_{12}NO\) is twofold—the ATPase activity is stimulated at low and inhibited at high C\(_{12}NO\) concentrations. In diC\(_{18:1}\)PC, it is observed an increase of activity induced by C\(_{12}NO\) in the range of molar ratios C\(_{12}NO\) : diC\(_{18:1}\)PC / C\(_{20}\) \(\leq 1.3\) in bilayers, where the bilayer thickness estimated by SANS decreases by 0.4 ± 0.1 nm. In this range, the \(^{31}\)P-NMR chemical shift anisotropy increases indicating an effect of C\(_{12}NO\) on the orientation of the phosphatidylcholine dipole N\(^+\) –P\(^-\) accompanied by a variation of the local membrane dipole potential. A decrease of the ATPase activity is observed in the range of molar ratios C\(_{12}NO\) : diC\(_{18:1}\)PC / C\(_{20}\) \(\geq 2.5\), where mixed tubular micelles are detected by SANS in C\(_{12}NO\) + diC\(_{18:1}\)PC mixtures. It is concluded that besides hydrophobic thickness changes, the changes in dipole potential and curvature frustration of the bilayer could contribute as well to C\(_{12}NO\) effects on Ca\(^{2+}\)-ATPase activity.

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1. Introduction

P-type ATPases are fundamental in establishing ion gradients by coupling the ATP hydrolysis to ion transport across biological membranes [1,2]. Among many P-type ATPases known today, Mg\(^{2+}\)-dependent Ca\(^{2+}\)-ATPase (ATP phosphohydrolase, EC 3.6.1.38, SERCA1) from skeletal muscle sarcoplasmic reticulum (SR) is structurally and functionally the best studied member [3]. SR Ca\(^{2+}\)-ATPase is one of the most studied intrinsic membrane proteins because it is a single-chain transmembrane protein [4] present at high concentration in the SR membrane with a well known function. It transports 2 moles of Ca\(^{2+}\) from the cytoplasm into the reticulum across the SR membrane with concomitant hydrolysis of 1 mol of ATP; two or three moles of H\(^+\) are counter-transported. The determination of crystal structures of the SR Ca\(^{2+}\)-ATPase with two bound Ca\(^{2+}\) ions in the transmembrane protein region [5], and in absence of Ca\(^{2+}\) ions and in presence of the inhibitor thapsigargin [6], provided an opportunity to interpret in structural terms Ca\(^{2+}\)-ATPase conformational changes accompanying the reaction cycle [7–10]. However, to fully elucidate the structure and function of Ca\(^{2+}\)-ATPase in the membrane,
and particularly the role of lipid–protein interactions that influence ATP hydrolysis and ion transport, it is necessary to reconstitute it into defined synthetic phospholipids. The most successful approach so far involves the use of various detergents for Ca\(^{2+}\)-ATPase solubilization and reconstitution. Using this approach, it has been found that the Ca\(^{2+}\)-ATPase activity depends on phase states, hydrocarbon chain lengths, structure and charges of polar head groups of annular lipids surrounding the protein [7,11–14]: a) the activity is practically zero in the solid-like (gel phase) bilayer, high in the fluid (liquid crystal) bilayer, but the particular value of fluidity in the fluid state has no effect; b) for high activity, a fluid bilayer from lipids with zwitterionic head groups is required—charged lipids support low activities; c) lower activity is observed in lipids under conditions when they form non-bilayer aggregates in isolation; d) the activity in (zwitterionic) diacylphosphatidylcholines is highest in the fluid bilayer of 1,2-dioleoylphosphatidylcholine, but lower in fluid bilayers with shorter or longer acyl chains. These results indicate that the ATPase activity is modulated by a delicate interplay of several physical factors—amongst which the most important seem to be hydrophobic thickness, hydrogen bonding potential, hydration, surface charge, dipole potential and curvature frustration of the bilayer.

To specify the interplay of these different physical factors in the lipid-Ca\(^{2+}\)-ATPase protein interactions, the correlation between Ca\(^{2+}\)-ATPase activity and bilayer structural parameters in presence of various amphiphilic and hydrophobic compounds has been useful. Such studies using cholesterol and normal alkanes contributed to finding non-annular lipid binding sites in the Ca\(^{2+}\)-ATPase [7,12–16]. In the present study, we correlate effects of the zwitterionic amphiphile N\,-dodecyl-N,N-dimethylamine-N-oxide (C12NO) on the activity of purified Ca\(^{2+}\)-ATPase reconstituted into synthetic diacylphosphatidylcholines with its effects on the structural bilayer properties. We found in our previous studies that N-alkyl-N,N-dimethylamine-N-oxides (CnNOs) stimulate the activity of purified Ca\(^{2+}\)-ATPase at low concentration and inhibit it at high concentration [17,18]. In the delipidated monomeric form, Ca\(^{2+}\)-ATPase binds about 240 C12NO molecules that cover the hydrophobic surface of the protein in the form of a prolate monolayer ring [19]. It was also observed that C12NO predominantly interacts with the lipid component of Ca\(^{2+}\)-ATPase membranes [20]. C12NO is widely used as a mild biological detergent for solubilization, purification, reconstitution and crystallization of membrane proteins [21,22]. In the bilayer, CnNOs penetrate between phospholipids and affect the fluidity [23] and the thickness [24,25] of the bilayer. At high concentration, C12NO destabilizes the bilayers and converts them into non-bilayer phases [26] and mixed micelles [20,27,28]. In the present communication, we report results of the study of the effects of C12NO on the specific activity of purified Ca\(^{2+}\)-ATPase reconstituted into synthetic diacylphosphatidylcholines and correlate them with the effects of C12NO on the bilayer thickness, on the conformation of the phosphatidylcholine head group and on the bilayer stability.

2. Material and methods

2.1. Chemicals

Synthetic 1,2-dilauroylphosphatidylcholine (diC12:0PC), 1,2-dimyrystoleoylphosphatidylcholine (diC14:1PC), 1,2-dipalmitoleoylphosphatidylcholine (diC16:1PC), 1,2-dioleoylphosphatidylcholine (diC18:1PC), 1,2-diestearoylphosphatidylcholine (diC20:1PC), 1,2-dierucoylphosphatidylcholine (diC22:1PC) and 1,2-dinervonoylphosphatidylcholine (diC24:1PC) were purchased from Avanti Polar Lipids (Alabaster, USA). Egg yolk phosphatidylcholine (EYPC) was isolated from fresh hen eggs and purified by a column chromatography according to Singleton et al. [29] with modifications detailed in Ref. [28]. C12NO was prepared from N,N-dimethyl-1-dodecanamine by oxidation with hydrogen peroxide and purified as described by Devinsky et al. [30]. Cholic acid (Sigma, St. Louis, USA), Heps (Serva, Heidelberg, Germany), Tris (Serva, Heidelberg, Germany), sucrose (Slavus, Bratislava, Slovakia), histidine (Sigma, St. Louis, USA), dithiothreitol (DTT) (Sigma, St. Louis, USA), phenyl methyl-sulfonyl fluoride (PMSF) (Sigma, St. Louis, USA), Amberlite XAD-4 (Sigma, St. Louis, USA), EGTA (Sigma, St. Louis, USA), ATP (Sigma, St. Louis, USA), phosphoenolpyruvate (Boehringer, Mannheim, Germany), NADH (Sigma, St. Louis, USA) of the best available purity were used. Organic solvents of the p. a. purity obtained from Mikrochem (Bratislava, Slovakia) and water were redistilled before use. Heavy water (99.9% \(^{2}H\)) was from Isotec (Matheson, USA). Potassium cholate was prepared by the action of KOH on cholic acid and purified by crystallization and diethyl ether extraction. The other chemicals were from Lachema (Brno, Czech Republic).

2.2. Enzymes

Pyruvate kinase (EC 2.7.1.40) from rabbit muscle (Boehringer, Mannheim, Germany) and lactate dehydrogenase (EC 1.1.1.27) from pig heart (Boehringer, Mannheim, Germany) were used as obtained. Ca\(^{2+}\)-ATPase was isolated from the spinal region white muscle of a female rabbit (about 2.5 kg) and purified according to the methods outlined by Warren et al. [31,32] with modifications described by Karlovská et al. [18]. The protein concentration was determined by measuring the absorbance at 280 nm [33].

2.3. Ca\(^{2+}\)-ATPase reconstitution

The purified Ca\(^{2+}\)-ATPase reconstitution into phosphatidylcholine vesicles was performed using the cholate dilution method of Johannsson et al. [34]. Briefly, C12NO and phosphatidylcholine were mixed at the needed molar ratio in chloroform/methanol in a glass tube; the solvent was evaporated under a stream of gaseous nitrogen and its traces removed by an oil vacuum pump. The dry C12NO + phosphatidylcholine mixture was solubilized by adding 40 μl of the micellar solution of cholate in buffer (0.025 mol/l cholate, 0.01 mol/l Heps/Tris, 0.44 mol/l sucrose, 0.005 mol/l MgATP, pH 8.0).
The content was sealed under gaseous nitrogen, vortex-mixed, sonicated, eventually freeze-thawed until obtaining a transparent solution of mixed micelles. The purified ATPase (0.125 mg) was then added in the volume of 2 – 3 μl; the amount added was controlled by gravimetry. The content was vortex-mixed and incubated depending on the length of acyl chain as follows: diC12 : 1PC—15 min at room temperature and at 8 – 10 °C for a further 45 min, diC14 : 1PC — diC18 : 1PC—30 min at room temperature and at 8 – 10 °C for a further 30 min, diC20 : 1PC – diC24 : 1PC—1 h at room temperature. After this incubation, the samples were diluted by adding 0.4 ml of buffer (0.02 mol/l Hepes/Tris, 0.1 mol/l KCl, 0.005 mol/l MgSO4, pH 7.2 ± 0.1). The reconstitution procedure used is described in more detail by Filipek et al. [35].

2.4. Ca2+-ATPase activity

The ATPase activity was determined at 37 °C using the coupled assay system as described earlier [18,35]. The Ca2+-ATPase was diluted in the assay mixture and after incubation at 37 °C for 15 min, the reaction was started by the addition of a CaCl2 solution to reach the final volume 2.46 ml. The final composition of the assay system was 10.06 μg of Ca2+-ATPase per sample (2.46 ml), 0.079 mmol/l phosphatidylcholine, 40 mmol/l Hepes, 0.1 mol/l KCl, 5.1 mmol/l MgSO4, 2.1 mmol/l ATP, 0.53 mmol/l phosphoenolpyruvate, 1.1 mmol/l EGTA, 0.152 mmol/l NADH, 7.5 IU/ml of PK, 18 IU/ml of LDH, pH 7.2. The reaction was followed by measuring the decrease of NADH absorbance at 340 nm, at 37 °C. The specific activity A (in international units IU per mg of enzyme) was calculated according to: \[ A = \frac{\Delta A_{340}}{V_{\text{ass}}/6.22 m_{\text{prot}}} \]

where \( \Delta A_{340} \) is the change in NADH absorbance at 340 nm per min, \( V_{\text{ass}} \) is the final assay volume in ml (2.46 ml), and \( m_{\text{prot}} \) is the weight of Ca2+-ATPase in mg in the assay volume. The values of \( A \) given below are the mean values from triplicate experiments. The conditions for optimal pH, temperature, calcium and magnesium concentration and enzyme stability were determined for the assay system in preliminary experiments. Absorbance measurements were made using the diode-array HP8452A spectrophotometer (Hewlett Packard, Palo Alto, USA) and 1 cm quartz or plastic cuvettes.

2.5. Small-angle neutron scattering

The dry diCn : 1PC powder was dispersed in heavy water to reach 1 wt.% concentration and closed in a plastic tube. The dispersion was sonicated in a bath sonicator and homogenized by hand shaking and vortex mixing. From the homogenized dispersions, extruded unilamellar vesicles were prepared: The diCn : 1PC dispersions were extruded through two stacked polycarbonate filters (Nucleopore, Plesanton, USA) with pores of diameter 50 nm mounted in the LiposoFast Basic extruder (Avestin, Canada) fitted with two gas-tight Hamilton syringes (Hamilton, Reno, USA). Each sample was subjected to 25 passes through the filters at room temperature. An odd number of passes were performed to avoid contamination of the sample by large and oligolamellar vesicles, which might not have passed through the filters. The final phospholipid concentration was not checked, but it was ≤ 1 wt.% in all samples because some amount of lipid could remain in the extruder. The samples were filled into 1 mm quartz cells (Hellma, Müllheim, Germany), closed and stored at room temperature. As the reference sample, the same cell containing heavy water without vesicles was used. The maximum period between the sample preparation and its measurement was 5 h. The neutron scattering experiments were performed on the PAXE spectrometer located at the end of the G5 cold neutron guide of the Orphée reactor (Laboratoire Léon Brillouin, CEA Saclay, France). The experiments were performed with sample to detector distances of 1700 and 5000 mm and the neutron wavelength of 0.6 nm. The sample temperature was set and controlled electronically at 30.0 ± 0.1 °C. The acquisition time for one sample was 40 min. The normalized SANS intensity \( I(q) \) as a function of the scattering vector value \( q = 4 \pi \sin \theta / \lambda \), where \( 2 \theta \) is the scattering angle, was obtained as described in detail by Kučerka et al. [36].

For the evaluation of \( I(q) \) data, we used the recently developed strip-function model of the coherent neutron scattering length density distribution \( \rho(z) \) taken perpendicularly to the bilayer surface [37]. In this model, the bilayer in unilamellar vesicles is divided into concentric strips with radii from the inner bilayer radius \( R_0 \) to the outer radius \( R_6 \). The methyl, methine and part of methylene groups of diCn : 1PC acyl chains are located in the region spanning two strips from \( R_2 \) to \( R_4 \), the dividing surface at \( R_3 \) is located at the centre of the bilayer. In the region from \( R_2 \) to \( R_4 \), the value of \( \rho(z) \) is constant. The strips from \( R_0 \) to \( R_2 \) and from \( R_4 \) to \( R_6 \) contain “dry” polar diCn : 1PC head groups (including choline, phosphate, glycerol and acyl chain carboxyls), some limited number of water molecules per one diCn : 1PC molecule \( N_w \), and the rest of diCn : 1PC acyl chain methylene groups. The contribution of the head groups to \( \rho(z) \) is triangular, increasing from \( R_0(R_0) \) to \( R_3(R_3) \) and decreasing then to \( R_4(R_4) \). The contribution of water molecules to \( \rho(z) \) decreases linearly from \( R_3(R_3) \) to \( R_2(R_2) \). Finally, the contribution of diCn : 1PC acyl chain methylene groups not located in the strips between \( R_2 \) and \( R_4 \) decreases linearly from \( R_2 \) to \( R_1 \) and from \( R_4 \) to \( R_6 \). The fragmental molecular volumes of constituents in \( R_0 \) to \( R_2 \) and in \( R_4 \) to \( R_6 \) strips are additive and the decrease in volume due to one constituent is compensated by the increase due to another one. The steric bilayer thickness is equal to \( d_s = R_6 - R_0 \). This model mimics the coherent neutron scattering length density distribution \( \rho_{\text{sim}}(z) \) obtained from the molecular dynamics (MD) simulations of fluid phosphatidylcholine bilayers more closely than other models frequently used in SANS data evaluation [37]. The experimental \( I(q) \) versus \( q \) data were fitted by the function minimization and error analysis program Minuit (CERN Program Library entry D506), using the vesicle structure factor derived from the strip model described above, convoluted by the Gamma function distribution of vesicle radii and by the PAXE spectrometer resolution function. Besides the experimental \( I(q) \) data, the input values were the fragmental volumes of different parts of the bilayer \( (\Delta \overline{\text{H}_2\text{O}} 0.030104 \text{ nm}^3) \), dry polar head group 0.319 nm³, acyl methine group 0.0218
nm³, acyl methylene group 0.0283 nm³, acyl methyl group 0.0522 nm³) taken from the literature [38–40] and the values of their coherent scattering amplitudes calculated by using the known scattering amplitudes of nuclei [41]. During the minimization, the distances of $R_2 - R_0 = R_6 - R_4$ were constrained to the value 1.2 nm obtained from MD simulations [37]. The result of fitting is a pair of $d_S$ and $n_W$ values; the area $A_L$ of one diCn:1PC molecule at the bilayer—aqueous phase interface is calculated from $d_S$ and $n_W$ using the known fragmental volumes of different parts of the bilayer.

2.6. $^{31}$P-NMR spectroscopy

Changes in the phosphatidylcholine head group conformation were followed using the proton decoupled $^{31}$P-NMR spectroscopy. C12NO and EYPC were mixed at the needed molar ratio in chloroform/methanol in a glass tube; the solvent was evaporated under a stream of gaseous nitrogen and its traces removed by an oil vacuum pump. The dry EYPC+C12NO mixtures were transferred to other glass tubes and evacuated again. Redistilled water was added to these dry mixtures at the weight ratio $H_2O$:EYPC=1:1; the amount of water added was controlled by gravimetry. Finally, the tubes were flame sealed, and the content was homogenized by repeated freezing and thawing and by forth-and-back centrifugation. Before the measurement, the samples were equilibrated at room temperature in a dark place. $^{31}$P-NMR spectra were recorded on a VXR 300 NMR spectrometer (Varian, USA) at 121.4 MHz using the deuterium lock, HF pulse width 45–55° and the interpulse relaxation delay 0.7–1.0 s. The spectra were recorded using the strong proton inverse gated proton decoupling. The sample temperature was maintained at 25 °C using the gas-flow system of NMR spectrometer. Exponential multiplication of free induction decays corresponding to 50 Hz line broadening was applied prior to their Fourier transformation. The effective $^{31}$P-NMR chemical shift anisotropy, $-\Delta\sigma_{\text{eff}}$, was evaluated as the distance between extremes of the first derivative spectra and corrected for the Lorentzian linewidth broadening using the spectra simulated by computer. The details of the method were described by Uhríková [42].

3. Results and discussion

3.1. Effects of the phosphatidylcholine acyl chain length

We have found that the specific activity of Ca$^{2+}$-ATPase reconstituted in the fluid phosphatidylcholine bilayers is sensitive to the length of the acyl chain with a maximum at diC18:1PC while phosphatidylcholines with shorter or longer acyl chains support progressively decreasing activities when approaching the chain length extremes (Fig. 1). The maximum of activity at diC18:1PC was observed earlier by Lee [12], Lee et al. [16], Caffrey and Feigenson [43] and by Cornea and Thomas [44]. In the classical paper of Johansson et al. [34], the maximum was observed at diC20:1PC. This small discrepancy could be caused by differences in incubation time and temperature during the crucial step in reconstitution where the cholate-phosphatidylcholine mixed micelles interact with the ATPase: most probably 15 min and 0 °C in Ref. [34] resulted in a partial lipid substitution comparing to more extensive substitution at higher temperatures and longer incubation times in Refs. [12,16,44] and in the present work (detailed above in Material and methods). The chain length dependence of activity is frequently explained by the hydrophobic mismatch hypothesis: it is supposed that the thickness of the hydrophobic region of the bilayer must match the length of the hydrophobic part of Ca$^{2+}$-ATPase to support the maximum activity; increasing or decreasing of this thickness should cause conformation changes [12,45] and/or lateral aggregation [44] of Ca$^{2+}$-ATPase resulting in a reduced phosphohydrolase activity and Ca$^{2+}$ transport.

To relate the changes of the specific activity of Ca$^{2+}$-ATPase with the physical parameters of the bilayer, we estimated the steric thickness $d_S$, the surface area $A_L$ per lipid at the bilayer—aqueous phase interface and the number $n_W$ of water molecules per lipid located in the bilayer polar region of unilamellar diCn:1PC vesicles (Fig. 2). These parameters have never been measured so extensively, the only relevant work being that of Lewis and Engelmann [46] published more than 20 years ago. They estimated the phosphate–phosphate separation across the bilayer from the position of the first peak in the Patterson function after inversion of the small-angle X-ray scattering (SAXS) of sonicated unilamellar diC18:1PC, diC22:1PC and diC24:1PC vesicles at 20, 24 and 36 °C, respectively. However, their values are underestimated due to systematic truncation errors, i.e. inversion of SAXS data in a limited range of momentum transfer, as noted by Nagle and Tristram-Nagle [47]. Furthermore, the SAXS experiments were done at a constant reduced temperature above the gel–fluid transition temperature, while the protein interactions with phosphatidylcholine bilayers as a function of $n$ are studied experimentally at the same absolute temperature. As expected, we have found in our SANS experiments that the bilayer thickness increases with the increase of the length $n$ of the acyl chain: a simple linear fit
The dashed lines are drawn to guide eye. Full symbols: measurements at 30 °C, open symbols: extrapolations to 37 °C.

(weighted by uncertainties in \( d_S \)) gives \( d_S = (1.64 \pm 0.20) + (0.17 \pm 0.01) n \) in nm. Unexpectedly, the area \( A_L \) displays a maximum at \( n = 18 \). Similarly, the number \( n_W \) of water molecules located in the polar region of the bilayer depends on \( n \) (Fig. 2). Since the parameters in Fig. 2 were obtained at 30 °C and the \( \text{Ca}^{2+}\)-ATPase activities in Fig. 1 were measured at 37 °C, we have extrapolated \( A_L \) and \( d_S \) in Fig. 2 to 37 °C correcting for temperature effects. The area \( A_L \) was corrected using the lateral thermal expansivity \( \beta = 0.003 \text{ K}^{-1} \) as in [40], and the thickness of the bilayer using the transversal thermal expansivity \( \kappa = 0.001 \text{ K}^{-1} \) found recently for diC18:1PC [48].

The steric thickness of the bilayer increases with \( n \) as \( d_S = (1.66 \pm 0.20) + (0.17 \pm 0.01) n \) in nm at 37 °C, i.e. it remains almost unchanged. After temperature corrections, at 37 °C, the area \( A_L \) is slightly higher but the maximum is always for \( n = 18 \) (Fig. 2).

The maxima of \( A_L \) and \( n_W \) as a function of \( n \) seem surprising. Previous studies reported the decrease of \( A_L \) with \( n \) in bilayers prepared from diacylphosphatidylcholines with saturated acyl chains (diCn:0PC) a) in multilamellar vesicles in the solid-like gel state for \( n = 16–18 \) by small-angle X-ray diffraction (SAXD) [49], b) in multilamellar diCn:0PC vesicles in the fluid state for \( n = 12–18 \) by \( ^2 \text{H} \) NMR [50,51] and c) in unilamellar diCn:0PC vesicles in the fluid state for \( n = 12–18 \) by SANS [52].

The specific dependence of \( A_L \) (and \( n_W \)) on \( n \) in diCn:1PC bilayers in comparison to diCn:0PC bilayers can be due to the presence and position of the double bond in the diCn:1PC acyl chains (S. J. Marrink, personal communication): the position of the double bonds in diC14:1PC, diC16:1PC and diC18:1PC is 9-cis, in diC20:1PC 11-cis, in diC22:1PC 13-cis, and in diC24:1PC 15-cis. The value of the area \( A_L \) is the result of attractive and repulsive forces at the aqueous phase-bilayer interface. The main attractive components are the hydrophobic interaction, the van der Waals forces between acyl chains and the dipolar interactions between headgroups. The main repulsive components include steric interactions, hydration forces, and entropic contributions due to the ordering of acyl chains.

At constant temperature, the increase of \( n \) increases the van der Waals attraction what will reduce \( A_L \). However, the reduction of \( A_L \) yields a concomitant reduction of \( \text{gauche} \) conformers in chains, which decreases the chain disorder (i.e., the entropy) and this may depend on the position of the double bonds in the acyl chains. These two effects which act in opposite directions can cause the observed peculiar dependence of \( A_L \) (and \( n_W \)) in diCn:1PC bilayers. The value of \( A_L \) is thus a measure of lateral interactions in the bilayer. Irrespective of the actual mechanisms resulting in the chain length dependences of \( A_L \) and \( n_W \), the results summarized in Fig. 2 indicate that the bilayer hydration and lateral interactions can influence the activity of \( \text{Ca}^{2+}\)-ATPase reconstituted into diCn:1PC vesicles. Changes in lateral interactions yield changes of the bilayer lateral pressure profile which may affect the conformation of membrane proteins [53,54]. Beside the dominant “hydrophobic mismatch”, this could be another mechanism contributing to the dependence of the reconstituted \( \text{Ca}^{2+}\)-ATPase activity on the length of the acyl chain.

### 3.2. Effects of \( \text{N-dodecyl-N,N-dimethylamine-N-oxide (C12NO)} \)

The effect of C12NO on the activity of \( \text{Ca}^{2+}\)-ATPase reconstituted into diC22:1PC is enormous—the activity increases up to \( A = 32.5 \pm 0.8 \text{ IU/mg} \) at the molar ratio C12NO:diC22:1PC=1.21 in sample (Fig. 3). This value is comparable to \( A = 31.5 \pm 0.7 \text{ IU/mg} \) observed in diC18:1PC, which is the lipid with the optimum length of the acyl chain, i.e. corresponding to the maximum activity (Fig. 1). Assuming that all the C12NO molecules are located in the bilayer, one calculates a value of the mean hydrocarbon chain length \( n = 18.23 \) carbon atoms in the bilayer at the molar ratio 0.8:0.4 C12NO:diC22:1PC (mol:mol).
C12NO:diC22:1PC=1.21. This value compares well with \( n = 18 \) in diC18:1PC which corresponds to the maximum activity. Therefore, the dominant contribution to mechanisms responsible for the increase of Ca\(^{2+}\)-ATPase activity in the mixed C12NO+diC22:1PC bilayer can be ascribed to the reduction of the hydrophobic mismatch between the protein and the bilayer.

The evolution of the changes of activity due to C12NO is different for the Ca\(^{2+}\)-ATPase reconstituted into diC12:0PC and diC18:1PC vesicles: the dependence of the activity on the molar ratio C12NO:PC goes through a maximum. This behaviour is seen in the dependences of normalized Ca\(^{2+}\)-ATPase specific activities \( A/A_0 \) on the molar ratio C12NO:PC in the sample (Fig. 4), where \( A \) and \( A_0 \) are the specific activities in presence or not of C12NO, respectively. Several other amphiphiles stimulate Ca\(^{2+}\)-ATPase activity at low concentration, e.g. oleic acid, methyl oleate, and oleyl alcohol [43,55], tertiary amine local anesthetics [56–59], pentobarbital [60], nonylphenol [61] and hexanol [62]. Ca\(^{2+}\)-ATPase is surrounded by a shell (annulus) of about 30–32 phospholipid molecules located at the bilayer–protein interface[13,63]. Besides these annular sites, the hydrophobic and amphiphilic molecules can bind to non-annular sites located between transbilayer helices or at protein–protein interfaces in Ca\(^{2+}\)-ATPase oligomers [13,15]. It was suggested that the stimulation of Ca\(^{2+}\)-ATPase activity could result from the binding of amphiphilic and hydrophobic molecules to these non-annular binding sites (see Froud et al. [55], Fernandez-Salguero et al. [60] and Lee [13] for references). While the increase of Ca\(^{2+}\)-ATPase activity depicted in Fig. 4 can be caused by the C12NO binding to the non-annular binding sites, the cause of the subsequent decrease of activity is not clear. Evidently, it cannot be the bilayer thickness in case of diC12:0PC. Because the length of the alkyl chain of C12NO is equal to that of diC12:0PC acyl chains, the insertion of C12NO into the diC12:0PC bilayer should not induce any significant change of the thickness, then the effect of hydrophobic mismatch on the activity must be excluded. The effect of the surface charge must be excluded too, because the phosphatidylycholines and C12NO are zwitterionic under experimental conditions (pH 7.2).

The effects of C12NO concentration on the behaviour of the Ca\(^{2+}\)-ATPase activity can be compared with a) those observed by Uhríková et al. [27] using SANS on the structure of bilayers of diC18:1PC vesicles and b) those observed in the present work by \(^{31}\)P-NMR spectroscopy on the conformation of the lipid head group in EYPC multilamellar vesicles. For this comparison, the molar ratio C12NO:PC in the lipid phase is needed because different experiments were performed at different diC18:1PC and C12NO concentrations in samples—the effect of the partition equilibrium on results must be thus eliminated. In the following, we calculate this molar ratio from the data obtained in our laboratory.

In the sample, the molecules of C12NO partition between the aqueous phase and the lipid phase; in equilibrium, this process is characterized by the molar partition coefficient \( K = \frac{c_{c12no,PC}}{c_{c12no,W}} \)

\[
K = \frac{c_{c12no,PC}}{c_{c12no,W}} = \frac{(n_{c12no,PC}/V_{PC})/(n_{c12no,W}/V_{W})}
\]

where \( c_{c12no,i} \) and \( n_{c12no,i} \) are molar concentrations and numbers of moles of C12NO, respectively, \( V_i \) are volumes, and indices \( i = W \) and \( i = PC \) denote the aqueous and lipid phases, respectively. Using simple algebra, one can calculate the molar ratio C12NO:PC in the lipid phase at any concentration of C12NO \( (c_{c12no}) \) and lipid \( (c_{PC}) \) in the sample using the known molar partition coefficient \( K \), the absolute specific volume of the lipid \( v_{PC} \) and the lipid molar weight \( M_{PC} \) as

\[
C12NO:PC = c_{c12no}/(c_{PC} + 1/v_{PC}M_{PC}K).
\]

The molar partition coefficient \( K=630 \) has been recently measured for C12NO in the system consisting of unilamellar diC18:1PC vesicles in the aqueous phase at 37 °C (Karlovska and Balgavý, in preparation) using methods described in Ref. [64]. The absolute specific volume of diC18:1PC is \( v_{PC}=0.9985 \) ml/g at 30 °C [40] and \( M_{PC}=786.12 \) g/mol. By using these data and the thermal volume expansion coefficient \( \gamma = 0.0008 \) K\(^{-1}\) found experimentally for diC18:1PC [40], one obtains the molar ratio C12NO:PC in the lipid phase used in Ca\(^{2+}\)-ATPase experiments as well as in SANS experiments (Fig. 5). The partition coefficient \( K=507 \) was measured for C12NO in unilamellar EYPC vesicles in the aqueous phase at room temperature [64]. Since the volume of aqueous phase in EYPC samples for NMR spectroscopy was the same as the volume of EYPC lipid phase, the effect of C12NO partitioning is neglected and the values of C12NO: EYPC molar ratio in the lipid phase are taken to be the same as those in the samples (Fig. 5).

The SANS parameter \( r \) characterizes the geometry of C12NO+diC18:1PC aggregates: \( r=1 \) for bilayers, \( r=2 \) for rod-like micelles and \( r=3 \) for globular micelles (see Refs. [27,65] and references therein). The SANS parameter \( d_P \) characterizes the thickness of the bilayer; its value follows the distance between phosphate groups \( d_{PP} \) across the bilayer.
Ca\textsuperscript{2+}-ATPase activity. Seelig [68] calculated that the N\textsuperscript{−}–P\textsuperscript{−} dipole reorientation is accompanied by variations of the local membrane dipole potential of the order of 10\textsuperscript{5} V/cm and suggested that this could play a regulatory role in the membrane function. Cafiso [70] suggested that changes in membrane dipole potentials could affect the conformation of the protein in the membrane. It is possible that the reorientation of the N\textsuperscript{−}–P\textsuperscript{−} dipole of diC18:1PC induced by C12NO is another factor that compensates the influence of the decrease of the thickness of the bilayer on the activity of Ca\textsuperscript{2+}-ATPase.

The specific activity of Ca\textsuperscript{2+}-ATPase reconstituted into diC18:1PC bilayers decreases at molar ratios C12NO:PC > 2 where mixed rod-like (cylindrical) C12NO + diC18:1PC micelles (r > 1) are formed (Fig. 5). The geometry of the surfactant + phospholipid aggregates consisting of two components depends on the effective molecular packing parameter [71,72]. The theory predicts that, depending on the packing parameter δ, molecules form spherical micelles (δ < 0.33), normal cylindrical micelles (0.33 < δ < 0.5), curved bilayers (0.5 < δ < 1), flat bilayers (δ = 1) or inverted micelles (δ > 1) [71,72]. We conclude that the inhibition of Ca\textsuperscript{2+}-ATPase observed in diC18:1PC bilayers is most probably caused by a severe deformation of the bilayer resulting in the formation of normal tubular mixed C12NO + diC18:1PC micelles in isolation (δ < 0.5). A decrease of the activity of Ca\textsuperscript{2+}-ATPase is observed when reconstituted into phosphatidylethanolamines under conditions where the phosphatidylethanolamine in isolation forms an inverse hexagonal phase consisting of inverted cylindrical micelles [12,43]. This situation corresponds to an effective molecular packing parameter δ > 1. The inhibition of the activity of Ca\textsuperscript{2+}-ATPase is observed therefore for both positive and negative deviations from the optimal bilayer packing (δ = 1). These deviations do not necessarily result in the formation of non-bilayer lipid aggregates in contact with the protein; instead, there is a change of the lateral pressure profile resulting in a change of the protein conformation, thus of the function of the protein, as suggested by Cantor [53,54]. Similarly, the change of the thickness of the bilayer found in isolation does not imply any change in the thickness of the lipid annulus around the protein, because the bilayer can deform to match the protein. However, the result could be again a change of the bilayer lateral pressure profile.

In conclusion, we have observed that C12NO modulates the activity of Ca\textsuperscript{2+}-ATPase through several mechanisms, depending on the protein lipid environment. Besides the hydrophobic mismatch between the lipid bilayer and the protein, the conformation of the lipid head group and the deformation of the bilayer could contribute to the optimal protein function.

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