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^{7}Li and ^{23}Na NMR Studies of Transmembrane Cation Transport Mediated by Ionophore Lasalocid A

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Abstract: Ion transport across phospholipid vesicles was studied by ^{7}Li and ^{23}Na-NMR using an aqueous anionic paramagnetic shift reagent, dysprosium nitrilotriacetate $[Dy(NTA)_2]^{3-}$, mediated by ionophores, lasalocid A and A23187. The intra- and extracellular ^{7}Li and ^{23}Na-NMR signals were well separated (20 Hz) at mM concentration of the shift reagent. The observed data on the rate constant for lithium transport across DPPC vesicles at various concentrations of the ionophores indicated that lasalocid A is a more efficient carrier for lithium ion compared with the sodium ion transport by this ionophore, while A23187 was not specific to either of the ions (Li or Na). ©1998 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: ion transport; kinetics; lithium; NMR; shift reagent; vesicles

INTRODUCTION

In biological membranes the transmembrane ion transport is a very important process mediated by integral membrane protein [1–3]. Transport may take place by mechanisms involving either diffusive carrier (shuttle) or membrane spanning channel (pore) species. The discovery of small, linear and macro cyclic antibiotic transporter molecules (ionophores) [4–6] has contributed to our present understanding of the mechanism of cation transport across model and biological membranes. Studies on the structure and conformation of various ionophores and their cation complexes in solution and solid state and their interaction with model membranes, over the past few years by several groups, have clearly shown the importance of these studies in understanding the transmembrane cation transport at the molecular level [7].

The major cations transported across biological membranes have little access to direct spectroscopic evidence and are hard to monitor. Traditional methods of analysis use destructive methods to achieve the separation of intra- and extracellular compartments. To understand the kinetics of cation transport the NMR technique that uses the paramagnetic lanthanides [7,8] and cation-specific aqueous shift reagents [9–12] has been used successfully. The latter method had attained importance as one can observe the cation transported (^{7}Li, ^{23}Na, ^{39}K, ^{43}Ca etc.) directly using multinuclear NMR [10]. The development of aqueous shift reagents [9, 12,13] for cationic NMR has made it possible to use ^{7}Li, ^{23}Na, and ^{39}K-NMR to monitor their kinetics. The intracellular ion signals are usually inherently isochronous with extracellular

Abbreviations: CD, circular dichroism; DPPC, dipalmitoylphosphotidyl choline; $[Dy(NTA)_2]^{3-}$, dysprosium nitrilotriacetate; Dy_2O_3, dysprosium oxide; $N(CH_2COOH)_3$, nitrilotriacetic acid; TEA, $N(CH_2CH_2OH)_3$, triethanolamine; ULV, unilamellar vesicles.

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ion signals. The polyanionic paramagnetic aqueous shift reagents are negatively charged chelate lanthanides and allow the observation of the resolved resonances of intra- and extracellular ions without the requirement of physical separation. The detection of anisochronous resonances of ions on opposite sides of the membrane depends on the fact that the shift reagent does not cross the membrane and are therefore present only on the outside of the liposomes. The lipid vesicles are stable in the presence of shift reagent and thus the extracellular ions only experience the resonance shift. Intra- and extracellular Li^+ have also been discriminated in yeast cells by ^{7}Li-NMR spectroscopy [14]. Hill and Shulman also used ^{35}Cl-NMR spectroscopy [15] to monitor chloride levels in vesicles using Co^{2+} as a shift reagent to resolve internal and external signals.

The transport of alkali metal ions mediated by ionophores out of and into phospholipid vesicles and in living cells using high-resolution multinuclear NMR spectroscopy was studied by different groups [10, 16–20]. Lipids play an important role in the maintenance of cell structure. Li^+ ions in lithium-containing cell or membrane suspensions are in fast exchange on the NMR time scale [21, 22]. $^7Li^+$ chemical shifts are insensitive to Li^+ binding to biomolecules. Therefore the observed ^{7}Li chemical shift represents a weighted amount of bound and free ions.

Finding or designing a suitable Li^+ selective transporter system is very important because of its therapeutic effect in the treatment of manic depressive illness [24–26]. Despite the pharmacological importance of Li in the treatment of various disorders and some viral infections [26], its mode of action is not well understood. In this paper we report our studies on lithium and sodium transport across phospholipid vesicles using ^{7}Li- and ^{23}Na-NMR, induced by ionophores, lasalocid A and A23187. Use of triethanol ammonium–dysprosium nitrilotriacetate, an aqueous paramagnetic shift reagent made it possible to resolve and detect resonances of ions present inside and outside the lipid vesicles.

MATERIALS AND METHODS

For these NMR studies the large unilamellar vesicles (ULVs) were prepared from dipalmitoylphosphatidylcholine (DPPC) obtained from Avanti Polar Lipids, Pelham, AL, by sonication of multilamellar vesicles (MLVs). The shift reagent used in these experiments was generated in situ and the NMR spectra were recorded on a Varian FT-80A spectrophotometer.

In a typical preparation of large unilamellar vesicles (ULVs) DPPC (18 mM) was dispersed in aqueous LiCl (450 mM) solution forming MLVs, which on ultra-sonication for 30 min form ULVs with lithium entrapped inside them. The supernatant unilamellar vesicular suspension, after centrifugation, was transferred into a 10 mm diameter NMR tube and ^{7}Li-NMR spectrum of the liposomes was recorded. An external Li standard was used from which the concentration of Li present was approximately calibrated as 150 mM. The NMR spectrum showed a single peak for the Li present inside and outside the liposomes. The ULVs and Li present both inside and outside the liposomes were dialysed two times (an average of 12 h each) against 2 l of 60 mM NaCl (Aldrich, Milwaukee, WI) to remove external Li^+. The ^{7}Li-NMR spectrum recorded after dialysis of ULVs also showed a single peak representing the lithium present outside and inside the vesicles. The concentration of Li was found to be ~80 mM using an external standard and also by comparison of the peak areas of Li before and after dialysis. 6 mM of dysprosium nitrilotriacetate $[Dy)(NTA)_2{}^{3-}]$, an aqueous paramagnetic shift reagent, was added to the vesicular suspension and the NMR spectrum recorded. The ^{7}Li-NMR of ULVs followed by the addition of the shift reagent showed two signals representing the lithium present outside and inside the unilamellar vesicles. The concentration of lithium entrapped inside the vesicles was found to be approximately 60 mM, while the external Li present outside after dialysis was around 18 mM. The decrease in the concentration of Li^+ ions in the liposomes was followed by ^{7}Li-NMR spectroscopy and the presence of Na^+ ions outside the liposomes was followed by ^{23}Na-NMR spectroscopy in an analogous manner.

The shift reagent triethanol-ammonium dysprosium nitrilotriacetate, $[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2COO)_3]_2$, was freshly prepared *in situ*, as given in the equation, from the heterogeneous reaction of dysprosium oxide (Dy_2O_3), nitrilotriacetic acid $[N(CH_2COOH_3]$ in triethanolamine $[N(CH_2CH_2OH)_3]$ [10, 11]; all three reagents were obtained from Aldrich, Milwaukee, WI. 1:4:6 equivalents of Dy_2O_3: $N(CH_2COOH)_3$: $N(CH_2CH_2OH)_3$ respectively were taken into a small flask and the reaction mixture stirred at room temperature for 25 min in a nitrogen atmosphere.

The weak equilibrium interaction probably involves an ion-pairing of alkali ions with dysprosium

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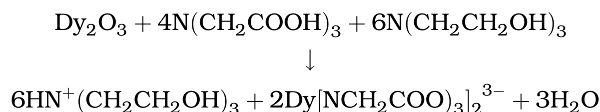
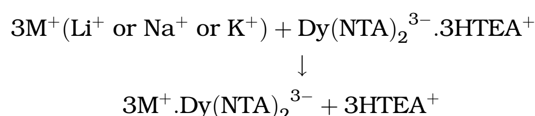


$$Dy_2O_3 + 4N(CH_2COOH)_3 + 6N(CH_2CH_2OH)_3 \downarrow 6HN^+(CH_2CH_2OH)_3 + 2Dy[NCH_2COO)_3]_2^{3-} + 3H_2O$$

nitrilotriacetate, $[Dy(NTA)_2^{-3}]$, replacing triethanolamine (TEA), which is observed on the NMR time scale:



$$3M^+(Li^+ \text{ or } Na^+ \text{ or } K^+) + Dy(NTA)_2^{3-}.3HTEA^+ \downarrow 3M^+.Dy(NTA)_2^{3-} + 3HTEA^+$$

NMR spectra were recorded at 31.09 MHz for ^7Li and at 21.04 MHz for ^{23}Na respectively on a Varian FT-80A spectrophotometer equipped with a multinuclear probe. In all these experiments D_2O (Aldrich, Milwaukee WI) was used as an external lock to the magnetic field and shimming was performed on a standard containing 0.5 M LiCl or NaCl in 100% D_2O, after which the suspension of the samples in 10 mm NMR tube were placed in the instrument. These alkali ions were found to be very sensitive to inadequate shimming.

A methanolic solution of lasalocid A or A23187 in the concentration range of 0.15 to 150μM, was injected to induce the transport of Li^+ and Na^+ across ULVs.

RESULTS AND DISCUSSION

^7Li-NMR spectrum of the sonicated dispersion of DPPC with LiCl entrapped inside was recorded at 31.09 MHz on a Varian FT-80A spectrophotometer equipped with a multinuclear probe. Figure 1 depict the ^7Li-NMR spectra of unilamellar vesicles of DPPC in which 60 mM LiCl is entrapped and 18 mM LiCl is present outside the large unilamellar vesicles. The single sharp resonance (Figure 1(a)) corresponds to Li^+ present both inside and outside the vesicles. The ^7Li-NMR spectrum of ULVs followed by the addition of 6 mM of $[Dy(NTA)_2^{3-}]$, an aqueous paramagnetic shift reagent, showed two signals (Figure 1(b)) representing the lithium present outside and inside the unilamellar vesicles. The small peak due to the external Li^+ present appeared 20 Hz upfield. The small upfield peak was shifted further upfield (data not shown) by increasing the concentration of $Dy(NTA)_2^{3-}$ and the magnitude of the splitting is consistent with the observed shift, which further confirms that $Dy(NTA)_2^{3-}$ shifts the lithium signal upfield. Thus, the assignment of the upfield peak as Li_{out} is clear. The shift reagent induced some broad-

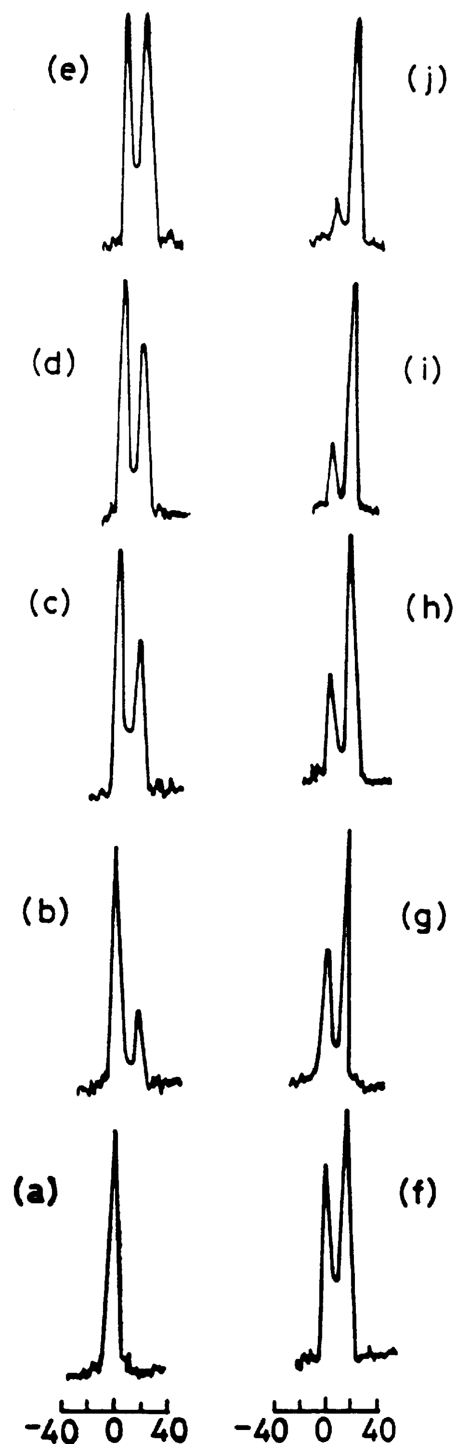


Figure 1 ^7Li-NMR (31.09 MHz) of a dispersion of DPPC ULV in aqueous LiCl solution: (a) spectra of LiCl (60 mM) inside the vesicles plus residual LiCl (18 mM) outside; (b) after addition of shift reagent $[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2COO)_3]_2$, (7.8 mM); (c–j) after the addition of 0.15 μM ionophore, lasalocid A, at 2.5, 5, 7, 14.2, 20.5, 37.5, 57.5 min and 24 h.

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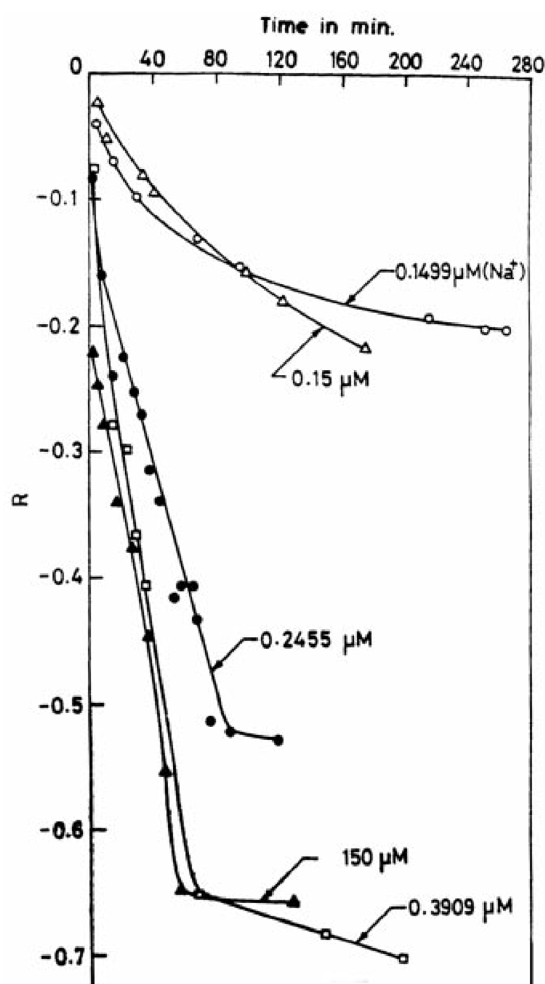

Figure 2 Plot of logarithm of the ratio of fractional area (inner/total) at any time to time zero (R) vs. time in minutes at different concentrations of ionophore.

ening of the peaks but its effect is negligible compared with the magnitude of the shift itself. To avoid any leakiness of the vesicles the spectrum was obtained within 2 h of the removal of LUV from NaCl dialysate.

A methanolic solution of the carboxylic ionophore, lasalocid A (0.15 μM), was injected into the sample after Figure 1(b) was recorded. The ionophore induces the rapid efflux of lithium down its concentration gradient. Figure 1(c)–1(j) shows the spectra recorded with the evolution of time. The outer peak increased at the expense of the inner peak.

Figure 2 shows the plot of the logarithm of the ratio of fractional area (inner/outer) at any time to time zero vs. time in minutes. The rate constants obtained (Table 1) show that the rate of transport of lithium increases as the concentration of ionophore increases. The transport of lithium across DPPC vesicles was studied at four different concentrations of the ionophore, lasalocid A. The efflux of lithium was found to increase as the concentration of the ionophore increases. The rate constants were converted to permeability coefficients (P) (Table 1), through the relationship $P\sim$ (rate constant) (vesicular inner aqueous volume) (vesicular surface area)−1 [8, 27]. The average values for the volume (6.54×10^{-15} ml) and area (3.5×10^{5} nm^{2}) were used to calculate the vesicular volume as given by Mimms *et al.* [27]. Our earlier results from CD and NMR studies [28] showed that lasalocid forms 1:1 (equimolar) and 2:1 (ion-sandwich) type complexes with Li ion, with the conformation depending on the cation concentration. The conformation of the lasalocid A–Li complex in our experiments probably exists as a 1:1 (equimolar) type complex with Li ion (Figure 3). The most reasonable explanation is the presence of very low relative concentration of the ionophore compared with the Li ions present in the system. Based on NMR data the conformational model for the complex showed that Li^{+} ion is preferentially bound to an end of the molecule to three oxygen atoms, while the salicylic acid part being relatively free. In the 2:1 complex, the Li ion is sandwiched between two lasalocid molecules, with each molecule providing three oxygen atoms to bind to Li ions [28].

Table 1 The Rate Constants for Lithium/Sodium Transport across DPPC Vesicles at Different Concentrations of Ionophore–Lasalocid A

Concentration of ionophore (μM)	Ion transported	Rate constant (s^{-1})	Vesicular volume ($cm\,s^{-1}$)
0.15	Li	1.0×10^{-3}	1.87×10^{-9}
0.2455	Li	4.43×10^{-3}	8.27×10^{-9}
0.3909	Li	6.03×10^{-3}	11.25×10^{-9}
150	Li	7.61×10^{-3}	14.21×10^{-9}
0.149	Na	5.38×10^{-4}	1.0×10^{-9}

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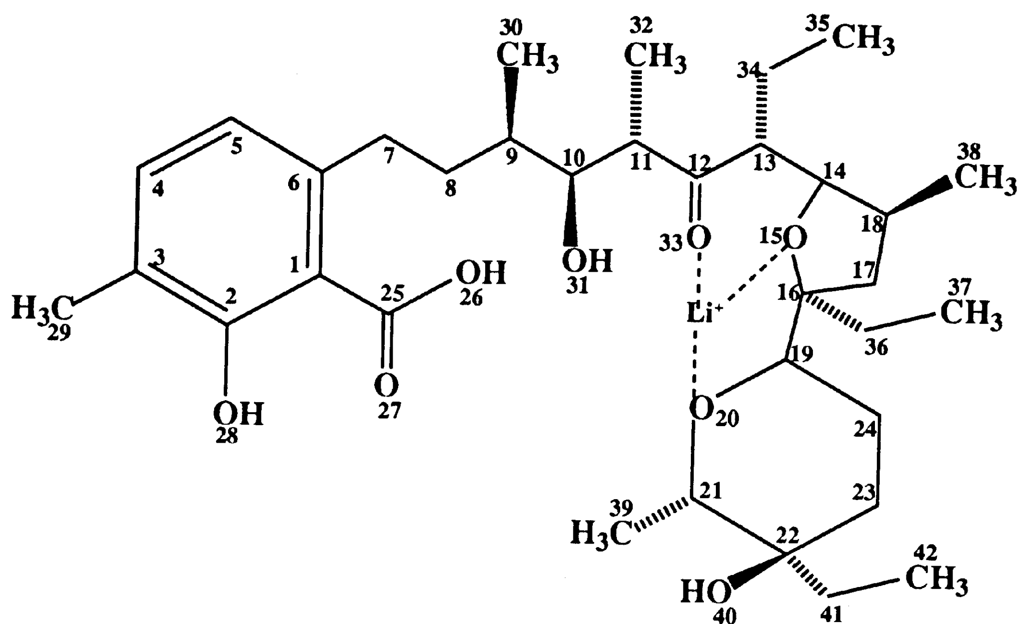

Figure 3 Lasalocid A–Li^+ complex.

Similar experiments were carried out to study the transport of Na^+ across phospholipid vesicles mediated by lasalocid A. Comparison of the rate of transport of Na^+ and Li^+ ions (Table 1) made at 0.15 μM concentration of the ionophore showed that under similar experimental conditions the rate constant for the transport of the Li^+ ion across DPPC vesicles by lasalocid A is an order of magnitude faster than for the Na^+ ion. Preliminary experiments with K^+ also confirmed the selectivity of lasalocid A towards Li^+ ion as compared to Na^+ and K^+.

The ionophore A23187 was used to study the kinetics of Li and Na ion transport across DPPC vesicles under similar experimental conditions as studied for lasalocid A. The rate of transport of Na^+ and Li^+ at different concentrations of the ionophre showed no specificity for one particular ion. These results suggest that ionophore lasalocid has specificity in binding to Li while A23187 is non-specific and carries both the alkali metal ions with approximately equal capability.

CONCLUSIONS

Our results on lithium and sodium transport across phospholipid vesicles using ^7Li and ^{23}Na-NMR and aqueous shift reagent dysprosium nitrilotriacetate show that both lasalocid A and A23187 transports these cations across DPPC vesicles but that lasalocid A is a more specific and efficient carrier for lithium. It is suggested that Li^+ selectivity of the ionophore lasalocid A has useful physiological relevance and encourage the study of this system at the molecular level as well as in the *in vivo* systems. Further studies are underway to investigate the ionic selectivity of this ionophore system not only with divalent cations (Mg^{2+} and Ca^{2+}) but also monovalent anions (Cl^- and I^-), and also to study the effect of Li^+ uptake into several tissues when Li^+ complexed to the ionophore is injected into rats in comparison with lithium as a salt.

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