Ion Pairing between Cl^- or ClO_4^- and Alkali Metal Complexes of Ionophore Antibiotics in Organic Solvents: A Multinuclear NMR and FT-IR Study

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The extent of ion pairing in chloride and perchlorate salts was studied by measurement of the Cl^- and ClO_4^- resonances and the observation of the perchlorate stretching frequency by use of nuclear magnetic resonance (NMR) spectroscopy and Fourier transform infrared spectroscopy (FT-IR), respectively, for a variety of ionophores in various solutions and in large unilaminar vesicles (LUVs). The NMR line widths of chloride and perchlorate were larger in solutions containing the neutral ionophores valinomycin (Val) and nonactin (Non) than in solutions containing the negatively charged ionophores nigericin (Nig), lasalocid (Las), and monensin (Mon). The viscosity-corrected perchlorate NMR line widths in solutions containing Val and Las were significantly negatively correlated ($r^2 \geq 0.99$) with the dielectric constant of the solvent. Solvents with low dielectric constants favored ion pair formation. From methanolic solutions containing the Li^+, Na^+, K^+, and Cs^+ salts of Cl^- and ClO_4^-, it was determined that the cation with the highest selectivity for the ionophore affords the most ion pairing. A decrease in pH from 7 to 3 had no significant effect on the NMR line widths of chloride and perchlorate in methanolic solutions containing Val, whereas a similar decrease in pH in a methanolic solution containing Mon caused a 2-fold increase in the line widths. The FT-IR difference spectrum of $KClO_4$ in a methanolic solution containing Val showed splitting at the perchlorate stretching frequency. No band splitting was observed in the FT-IR difference spectrum of $KClO_4$ in methanolic solutions containing Las. The efflux of ^{35}Cl in LUVs containing the neutral ionophore Val followed first-order kinetics with an efflux constant of 1.70×10^{-3} min^{-1}, as determined by ^{35}Cl NMR spectroscopy. The induction of increased membrane permeability in LUVs by the ionophore was determined to be negligible for Val and Nig by fluorescence spectroscopy.

Introduction

Cl^- is one of the most abundant free anions in living cells. Quantitation of intracellular chloride activity and the mechanisms involved in regulation and transport of intracellular Cl^- in biological systems have been actively investigated.[1−3] Cl^- is transported across the cell membrane via three fundamentally different pathways: an electrically conductive transport pathway and two electroneutral pathways, Cl^- anion exchange and Cl^- cation cotransport. The contribution of each of these Cl^- transport pathways varies from system to system. The presence of defective Cl^- transport pathways has been observed in several disease states, including Duchenne muscular dystrophy,[4] congenital myotonia,[5] and cystic fibrosis.[6]

We have previously reported that ion carriers, such as ionophores, affect not only the cation distribution but also the anion distribution across cell membranes.[7] Cell membranes are relatively impermeable to small ions as a result of the large amount of energy needed to transfer an ion from the aqueous phase into the hydrocarbon, apolar, phase of the lipid bilayer.[8] This barrier is important in maintaining selectivity and regulation of ion permeability. With the aid of ionophores, however, the cell membrane permeability for metal cations is increased.[9]

The structures of the carrier ionophores used in this investigation are shown in Figure 1. Carrier ionophores function by binding the cation and transporting it through the membrane. This is accomplished by the extremely flexible ionophore backbone, which is composed of strategically placed oxygen atoms.[10] The selectivity sequences arise not only from the size ratio of the ionophore cavity to the radius of the cation but also from the asymmetric ionophore charge distribution.[11]

NMR spectroscopy, in addition to X-ray crystallography, circular dichroism, and FT-IR, has been used for determining not only the free-metal ionophore structures but also the ion-bound conformations.[10−12] Distinct conformations of metal-free ionophores exist in different solvents.[10] Solvent polarity controls the relative populations of the various conformations. Despite the information available on the structure and function of ionophores, considerable ambiguity exists on the effect of

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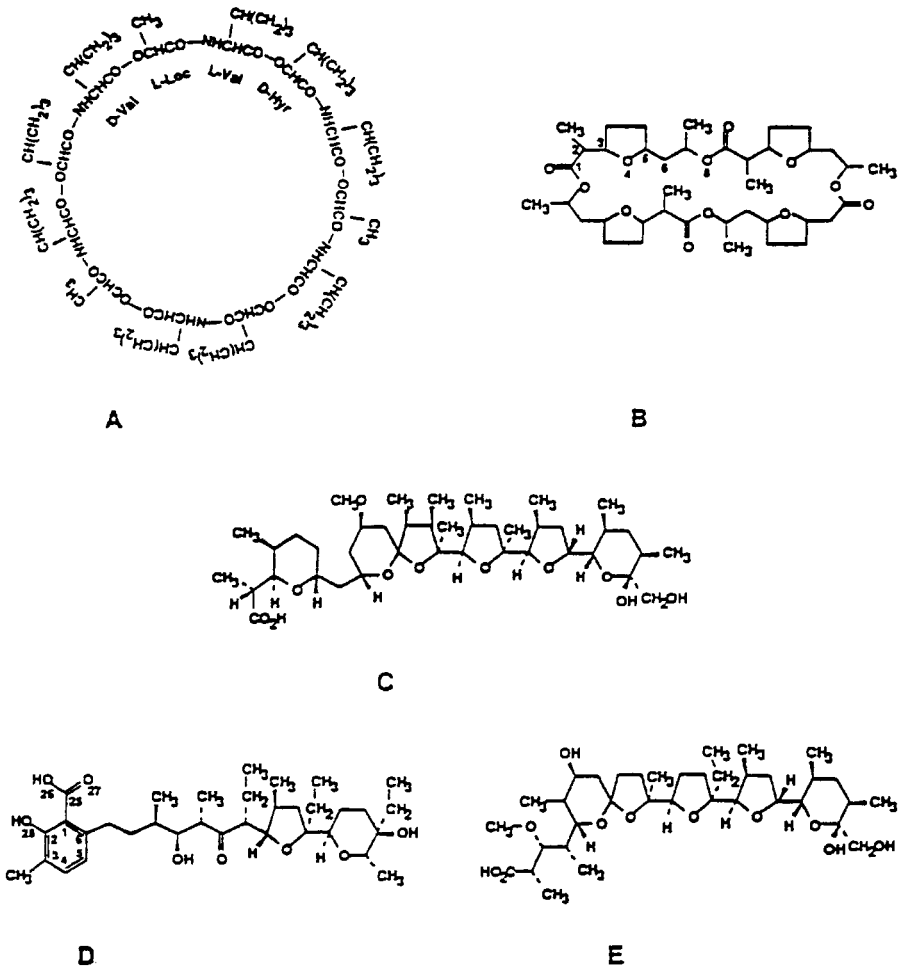

Figure 1. Structures of the ionophores used in this study: (A) valinomycin, (B) nonactin, (C) nigericin, (D) lasalocid (X-537A), and (E) monensin. A and B are neutral ionophores, whereas C, D, and E are carboxylic ionophores.

ionophores on the membrane potential of biological systems.[13,14] Transport of the positively charged neutral ionophore−cation complex out of the cell has been postulated as being accompanied either by Cl^- efflux through the anion transport band 3 protein or by proton exchange, thereby maintaining cell neutrality and not affecting the potential gradient.[13,14] Hladlky and Rink,[15] however, have clearly shown by fluorescence spectroscopy that Val does affect the membrane potential of RBCs; by using ^{19}F and ^{31}P NMR methods for measuring the transmembrane potential in RBC suspensions containing neutral ionophores,[7] we have confirmed these observations. Thus, the positively charged neutral ionophore−cation complex could form an ion pair with a Cl^- ion and provide an additional transport pathway for anions.

The concept of ion paring pertains to two ions that are in the close vicinity of each other for a short time before their thermal motions tear them apart.[16] There are several proposed models for representing the possible ion associations that exist in solution.[16−18] Three types of ion pairs exist: solvated ions, solvent-separated ion pairs, and contact ion pairs, with the environment of the solvent and ions differing among these forms. NMR spectroscopy has proved to be a valuable technique for differentiating between the types of ion pairs existing in solution.[12,19−22] Quadrupolar nuclei, such as ^{35}Cl, have an electric field gradient that is very sensitive to the symmetric charge distribution around the observed nuclei.[17,23] Changes in both chemical shift (δ) and line width ($\Delta\nu_{1/2}$) parameters yield information on the electronic distribution around the nucleus of interest. Because ion pairing is a relatively weak interaction, changes in chemical shift values are rarely observed. In contrast, a decrease in relaxation time, leading to an increase in line broadening, as a result of ion association is easily observed. The NMR line width is the only parameter that distinguishes between two of the possible ion associations.

The intravesicular and extravesicular resonances of Cl^- can be resolved with the use of aqueous shift reagents (SRs). Cobalt(II) nitrate, $Co(NO_3)_2$, has been reported as a ^{35}Cl NMR SR in

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vesicles.[24,25] Vesicles have also been used for monitoring transport by ^{35}Cl NMR spectroscopy.[26] Recently, we reported that tris(glycinato)cobaltate(II), $[Co(Gly)_3]^-$, is a suitable and stable SR for the study of chloride transport in human erythrocyte suspensions by use of ^{35}Cl spectroscopy.[27] The use of $[Co(Gly)_3]^-$ in vesicles may aid in the study of Cl^- transport.

FT-IR provides a complementary approach to the study of ion pairing in solution. Formation of an ion pair can lead to changes in the point group symmetry of perchlorate from T_d to C_{3v} or C_{2v}, resulting in the appearance of new bands in the mid-infrared region.[23] The time scales of the NMR and IR measurements differ considerably. The time scale for 10 MHz NMR spectroscopy is of the order of 10^{-7} s, whereas that for vibrational spectroscopy is of the order of 10^{-12} s. The consequence of different observation times in the two techniques is that ion pair formation may be in the fast exchange domain in the NMR time scale, but in slow exchange in the IR time scale.

The electrostatic interaction of neutral ionophores with cations results in positively charged complexes that may ion pair with a counteranion such as Cl^- or ClO_4^-. Ion pair formation between Cl^- and ClO_4^- and alkali metal complexes of carboxylic ionophores is unlikely. In this paper, we present NMR and IR evidence that an ion pairing mechanism operates in solutions of neutral ionophores, and we demonstrate by experiments conducted with vesicles that ion pairing may provide an alternate transport pathway for anions in biological systems. Alkali metal complexes of valinomycin are known to ion pair with several paramagnetic anions, such as $CoBr_4^{2-}$ and $Co(SCN)_4^{2-}$.[28] However, prior to this study, no reports existed on ion pairing of ionophores with Cl^- or ClO_4^-.

Methods and Materials

Valinomycin (Val), nonactin (Non), nigericin (Nig), lasalocid (Las), monensin (Mon), and *n*-octyl-β-glucopyranoside were purchased from Sigma (St. Louis, MO). Chloride and perchlorate salts of lithium, sodium, and cesium were purchased from Aldrich (St. Louis, MO), as were HPLC-grade acetone, methanol, nitromethane, acetonitrile, and dimethyl sulfoxide (DMSO). Phosphatidylcholine was purchased from Avanti Polar Lipids (Alabaster, AL). High-purity calcein was purchased from Molecular Probes (Eugene, OR). All reagents were used as received except for some of the solvents, which were further purified as described below.

The organic solvents were purified as previously described.[29] In brief, acetone was dried with anhydrous calcium sulfate ($CaSO_4$) and then distilled. Anhydrous methanol was obtained by drying with calcium hydride (CaH_2), followed by distillation. Nitromethane was refluxed over CaH_2, distilled, and then dried with $CaSO_4$. Acetonitrile was dried with Linde 4 Å molecular sieves, followed by stirring with CaH_2 and fractional distillation over CaH_2. DMSO was dried with Linde 4 Å molecular sieves and then distilled under reduced pressure. The purified solvents were transferred under a nitrogen atmosphere.

Stock solutions of 5 mM KCl and 5 mM $KClO_4$ were prepared from their respective salts in various solvents**.** An aliquot of the KCl or $KClO_4$ stock solution was added dropwise to the ionophore in powdered form with vigorous stirring, resulting in a final ionophore concentration of 5 mM. To prevent distortion of the NMR line shape, it was necessary to ensure that complete solubility of the alkali salts and ionophores was achieved.

Phosphatidylcholine (PC) large unilaminar vesicles (LUVs) were prepared by detergent dialytic removal, as previously described.[30] In brief, PC (~25−30 mmol) and 15 equiv of *n*-octyl-β-glucopyranoside were dissolved in 1.5 mL of 100 mM NaCl during vortexing. The lipid was dissolved in the detergent solution and sonicated for 2 h. Detergent was removed by dialyzing of the sample twice against 2 L of 100 mM NaCl for 12 h at 37 °C. The NaCl solution was previously purged with N_2 for 12 h at 37 °C. At the time of analysis, the sample was passed through a 0.5 μm membrane under vacuum for homogenization of size and was passed into a septum-sealed NMR tube previously purged with N_2 gas. With the same method, different types of LUVs were prepared, such as 100 mM KCl LUVs in a 50 mM KCl and 50 mM KNO_3 suspension, and 50 mM KCl and 50 mM KNO_3 LUVs in a 100 mM KCl suspension.

PC vesicles for determination of membrane permeability were made by high-pressure extrusion as previously described.[31−33] In brief, PC was evaporated over a constant flow of nitrogen and resuspended in a Tris buffer (10 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 60 mM calcein, to a final PC concentration of 10 mg/mL. Samples were then subjected to 5 freeze−thaw cycles with liquid nitrogen to maximize the encapsulation volume and extruded 10 times through a high-pressure extrusion apparatus (Lipex Biomembranes, Vancouver, British Columbia) containing two 100 nm stacked polycarbonate filters (Whatman, Clifton, NJ) under 400 psi of nitrogen. The vesicle/dye mixture was then transferred to a Sephadex G-20 superfine gel size exclusion column, and fractions were collected. We then tested the fractions via fluorescence to determine into which fraction the vesicles eluted. Val and Nig were reconstituted from their lyophilized powder forms by use of EtOH and subsequently added to the appropriate aliquot of vesicle suspensions. EtOH was added to vesicle suspensions without ionophore as a control. Fluorescence spectra were acquired on a Photon Technologies Quanta-Master QM-1 fluorimeter equipped with a xenon-arc lamp, at an excitation wavelength of 494 nm. Maximum emission intensity was observed at 511 nm. Solutions were titrated with 0.03% (w/v) Triton X-100 stock solution for determination of the maximum fluorescence intensity.

The viscosities of the ionophore solutions were measured with a Brookfield cone plate viscometer, equipped for low-viscosity samples, by use of an 8° CP-40 cone, at 12 rpm. All FT-IR measurements were recorded on an Alpha Centauri FT-IR spectrometer (Northwestern University, Chicago, IL).

^7Li, ^{13}C, ^{23}Na, ^{35}Cl, and ^{133}Cs measurements were conducted at 116.4, 75.4, 79.4, 29.5, and 39.4 MHz, respectively, on a Varian VXR-300 NMR spectrometer equipped with a 10 mm multinuclear probe and a variable-temperature unit. A standard single pulse sequence was employed for obtaining the NMR spectra of all samples. Samples containing vesicle suspensions were run nonspinning. All NMR experiments were conducted at room temperature at the same gain and absolute intensity settings. Field-frequency locking on D_2O present in the external reference aqueous solution was used for all experiments. The line widths reported are corrected for line broadening.

^7Li NMR spectra were obtained at a flip angle of 60° (14 μs), an acquisition time of 0.97 s, a delay time of 35 s, a spectral width of 4500 Hz, and 32 transients. All ^7Li spectra were referenced to 150 mM LiCl. ^{13}C NMR spectra were obtained at a flip angle of 45° (8.7 μs), an acquisition time of 0.3 s, a 2 s delay, a spectral width of 13514 Hz, and 6000 transients. All ^{13}C spectra were referenced to TMS. ^{23}Na NMR spectra were obtained at a flip angle of 70° (20.0 μs), an acquisition time of 0.5 s, a spectral width of 10405 Hz, and 6000 transients. All ^{23}Na NMR spectra were referenced to NaCl−D_2O. ^{35}Cl NMR spectra of solutions containing Cl^- were obtained at a flip angle

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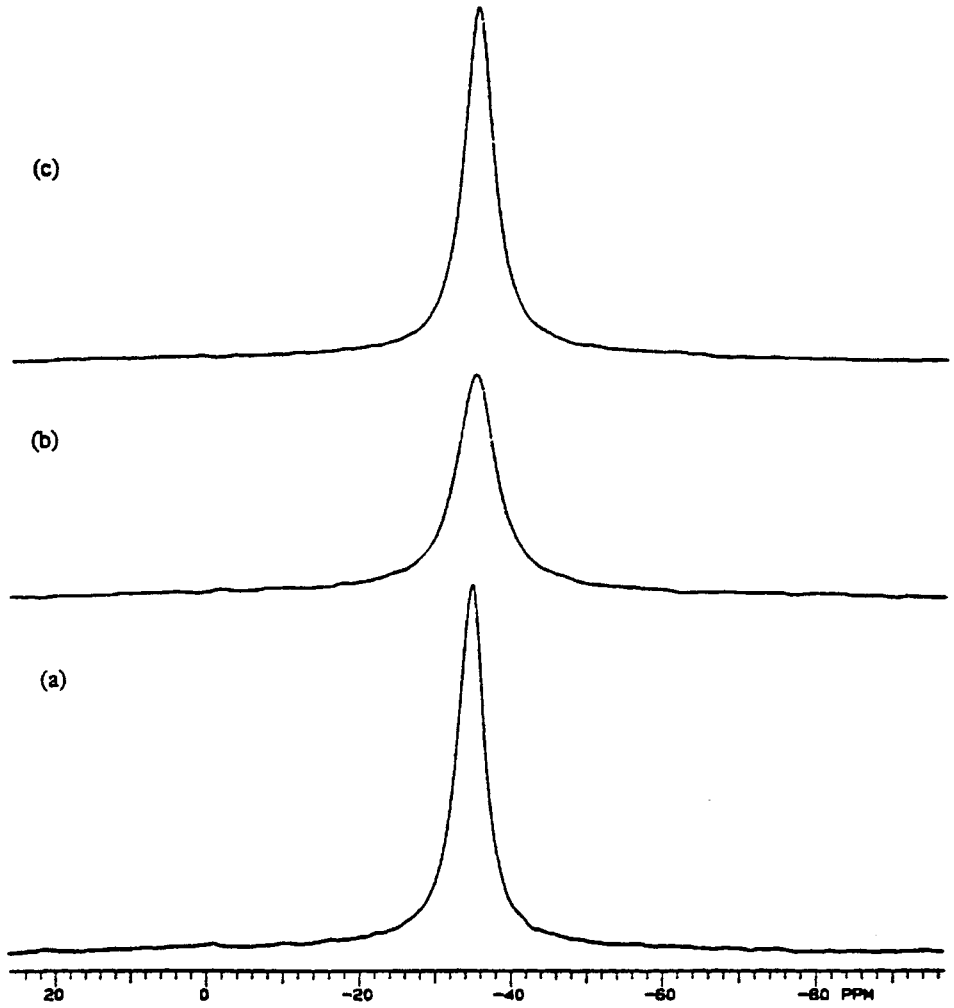

Figure 2. ^{35}Cl NMR spectra of 5 mM KCl in methanol (a) alone, and in the presence of (b) 5 mM Val and (c) 5 mM Mon.

of 45° (33.0 μs), an acquisition time of 0.1 s, a spectral width of 8413 Hz, and 100000 transients. ^{35}Cl NMR spectra of solutions containing ClO_4^- were obtained at a flip angle of 45° (33.0 μs), an acquisition time of 0.5 s, a spectral width of 5066 Hz, and 200000 transients. ^{35}Cl NMR spectra of Cl^- and ClO_4^- solutions were referenced to KCl and $KClO_4$, in D_2O, respectively. ^{35}Cl NMR spectra of LUV suspensions were made at a flip angle of 90° (78 μs), an acquisition time of 0.5 s, a spectral width of 1000 Hz, and 1024 transients. ^{133}Cs NMR spectra were obtained at a flip angle of 45° (16 μs), an acquisition time of 1.0 s, a spectral width of 8000 Hz, a 25 s delay, and 1024 transients.

Results

Effect of Various Ionophores on the ^{35}Cl NMR Line Widths of Cl^- and ClO_4^- in Methanol. We used ^{35}Cl NMR spectroscopy to measure the Cl^- and ClO_4^- line widths for methanolic solutions in the presence and absence of K^+ complexes of the two subclasses of carrier ionophores. Figure 2 shows typical ^{35}Cl NMR spectra of KCl in the absence and in the presence of two different types of carrier ionophores. The Cl^- line width was much larger in the presence of valinomycin than in the presence of monensin, which, in turn, was larger than that observed in the absence of ionophores. The data shown in Table 1 indicate that samples containing the neutral ionophore, Val, exhibited much larger Cl^- or ClO_4^- line widths than did samples containing the carboxylic ionophores Nig, Mon, and Las; for each ionophore, the Cl^- line widths were much greater than the ClO_4^- line widths. To allow for variations in the viscosity of the solvents used in this study, we corrected the observed line widths by dividing them by the sample viscosity. The trends for $\Delta\nu_{1/2}/\eta$ reported in Table 1 also show more line broadening for Val relative to the carboxylic ionophores (Nig, Mon, and Las).

Table 1. ^{35}Cl NMR Line Widths for KCl and $KClO_4$ in the Presence and Absence of Ionophores in Methanol[a]

	$\Delta\nu_{1/2}$ (Hz)[b,c]		$\Delta\nu_{1/2}/\eta$ (Hz/cP)	
ionophore	KCl	$KClO_4$	KCl	$KClO_4$
nigericin	115 ± 9	9 ± 5	209 ± 16	14 ± 8
monensin	121 ± 7	11 ± 2	225 ± 13	20 ± 4
lasalocid	117 ± 4	12 ± 1	119 ± 7	24 ± 2
valinomycin	172 ± 9	16 ± 1	302 ± 16	28 ± 2
nonactin[d]				
without ionophore	102 ± 13	13 ± 1	181 ± 23	23 ± 2

[a] The ionophore and salt concentrations were both 5 mM. [b] Line broadening used in the Fourier transformation (25 Hz for KCl and 5 Hz for $KClO_4$) was subtracted from the observed values. There was no change in chemical shift from sample to sample. [c] Each value represents the average of measurements conducted in three separately prepared samples. [d] Nonactin was insoluble in methanol.

Effect of Solvent on the ^{35}Cl NMR Line Widths of ClO_4^- and Cl^-. Table 2 lists the ^{35}Cl NMR line widths of ClO_4^- in various solvents as well as the solvent parameters, donor number, and dielectric constant, for each solvent. The solvents were chosen on the basis of their range of dielectric constants and donor numbers. The ionophores and KCl were not soluble in every solvent (except for methanol and acetone−water mixtures; vide infra) used in this study. The ClO_4^- salts were, however, more soluble than the Cl^- salts, and we found that

Table 2. Effect of Solvent on the ^{35}Cl NMR Line Widths of $NaClO_4$[a]

	$\Delta\nu_{1/2}$, Hz ($\Delta\nu_{1/2}/\eta$, Hz/cP)[b]					
solvent (ϵ/DN)[c]	none	Val	Non	Las	Nig	Mon
acetone	10.5 ± 0.1	12.1 ± 0.1	13.0 ± 0.4	10.5 ± 0.1	10.3 ± 0.9	10.5 ± 0.5
(20.7/17.0)	(34.5 ± 0.5)	(39.6 ± 0.2)	(39.4 ± 1.2)	(34.6 ± 0.4)	(31.2 ± 2.7)	(35.0 ± 1.5)
methanol	9.7 ± 0.5	13.9 ± 0.4	*d*	10.5 ± 0.1	9.1 ± 4.8	11.2 ± 2
(32.7/25.7)	(17.8 ± 0.9)	(25.5 ± 0.8)		(19.3 ± 0.1)	(14.2 ± 7.4)	(19.6 ± 3.8)
nitromethane	10.3 ± 0.4	12.0 ± 0.3	12.1 ± 0.4	10.3 ± 0.4	*d*	*d*
(35.9/2.7)	(16.9 ± 0.7)	(19.7 ± 0.9)	(20.5 ± 0.3)	(16.8 ±0.6)		
acetonitrile	4.6 ± 0.2	6.5 ± 0.4	*d*	4.9 ± 0.1	*d*	*d*
(37.5/14.1)	(13.3 ± 0.6)	(19.1 ± 1.2)		(14.5 ± 0.2)		
DMSO	4.8 ± 0.1	4.9 ± 0.4	7.4 ± 1.7	3.8 ± 0.3	*d*	*d*
(46.7/29.7)	(2.4 ± 0.1)	(2.4 ± 0.2)	(3.6 ± 0.8)	(1.9 ± 0.1)		

[a] The ionophore and salt concentrations were both 5 mM. [b] The observed line widths at half-height are followed by the viscosity-corrected values in parentheses, and each value represents the average of measurements conducted in three separately prepared samples. Line broadening used in the Fourier transformation (5 Hz) was subtracted from the observed values. There was no change in chemical shift from sample to sample. [c] The dielectric constant, ϵ, and the donor number, DN, are given in parentheses under each solvent; the solvent parameters were obtained from ref 23. [d] Not measured.

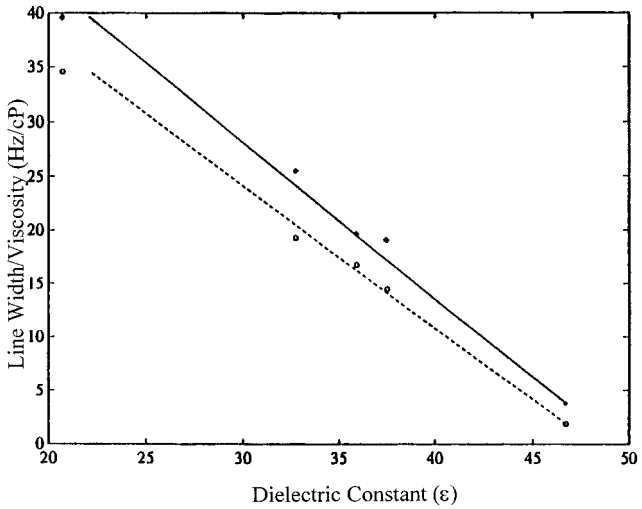

Figure 3. Plot of viscosity-corrected line widths of the ^{35}Cl NMR resonance of $NaClO_4$ versus dielectric constant, ϵ, for Val (◆) and Las (○) in acetone (ϵ = 20.7), methanol (ϵ = 32.7), nitromethane (ϵ = 35.9), acetonitrile (ϵ = 37.5), and DMSO (ϵ = 46.7).

$NaClO_4$ was more soluble than $KClO_4$ in a wider range of solvents; this is why we chose $NaClO_4$ rather than chloride salts to investigate solvent effects.

We investigated the ^{35}Cl NMR line widths of $NaClO_4$ in acetone, methanol, nitromethane, acetonitrile, and DMSO, whereas the ^{35}Cl NMR line widths of KCl were measured in methanol and in a solvent mixture of 85% acetone−15% D_2O (data not shown). Several other solvents were tried, such as pyridine, chloroform, and THF. However, the insolubility of the K^+−ionophore complexes prevented ion pairing in these solvents. In every solvent studied, the ^{35}Cl NMR line widths of ClO_4^- in the presence of Val were significantly larger than those observed with carboxylic ionophores. In acetone, nitromethane, and DMSO, both Val and Non were soluble; in these solvents, line widths observed with neutral ionophores (Val and Non) were significantly larger than those observed with Las, suggesting that the line-broadening effects are general for this neutral ionophore subclass.

Figure 3 shows a plot of the viscosity-corrected ^{35}Cl NMR line widths of ClO_4^- in the presence of the ionophores Val and Las. An increase in the solvent dielectric constant resulted in a decrease in the viscosity-corrected line width for both Val- and Las-containing solutions with r^2 values of 0.996 and 0.991, respectively. We did not observe any significant correlation (r^2 ≈ 0.15) between viscosity-corrected line widths of ClO_4^- and the solvent donor number.

Table 3. Effect of Alkali Metal Cations on Ion Pairing Ability of Ionophores in Methanol

sample[a]	metal NMR line width /Hz	metal chemical shift[b]/ppm	^{35}Cl NMR line width[b]/Hz
LiCl	24.7	1.70	97
LiCl + Val	53	1.72	98
LiCl + Las	36	1.85	91
NaCl	20.5	−2.6	91
NaCl + Val	81.5	−2.9	114
NaCl + Las	104	−3.2	94
KCl	*c*	*c*	102
KCl + Val	*c*	*c*	172
KCl + Las	*c*	*c*	117
CsCl	6.3	−42.0	97
CsCl + Val	63.2	−12.4	117
CsCl + Las	9.5	−14.7	97

[a] Samples were prepared by dissolving each salt in methanol to a final concentration of 5 mM. Ionophores were added to the salt solutions to yield a final concentration of 5 mM. [b] Samples containing CsCl were referenced to 0.15 M CsCl−D_2O, those containing NaCl were referenced to 0.10 M NaCl−D_2O, and those containing LiCl were referenced to 0.15 M LiCl−D_2O. The ^{7}Li, ^{23}Na, ^{133}Cs, and ^{35}Cl NMR spectra were recorded twice, and the average is reported. Errors in chemical shifts are less than 1.0 ppm, and errors are less than 1.0 Hz for line width measurements. [c] Not measured.

Effect of Cation on Ion Pairing. Table 3 lists the ^{7}Li, ^{23}Na, and ^{133}Cs NMR chemical shifts and line widths for solutions containing LiCl, NaCl, or CsCl in the presence and absence of Val or Las; the corresponding ^{35}Cl NMR spectrum was also recorded for each. The largest broadening effect was observed for samples containing CsCl, with more than a 6-fold increase in the ^{133}Cs NMR line width for samples containing Val in place of Las. LiCl samples containing Val had ^{7}Li NMR line widths approximately 25% larger than those of samples containing Las. NaCl samples with Las, however, were found to have a larger ^{23}Na NMR line width than did those with Val. The ^{35}Cl NMR line widths for NaCl and CsCl were found to be larger for samples containing Val as opposed to Las. The extremely low selectivity for Li^+ by Val and Las was evident from the ^{35}Cl NMR line widths for LiCl in the presence of either Val or Las; the ^{35}Cl NMR line widths for LiCl solutions containing either Las or Val were approximately the same.

We made similar observations by ^{7}Li, ^{23}Na, and ^{35}Cl NMR for methanol-containing $LiClO_4$, $NaClO_4$, and $KClO_4$, in the presence and absence of Val and Las (data not shown).

Effect of pH on Ion Pairing. We investigated the effect of pH on the ^{35}Cl NMR line widths for samples containing Val or

Mon in methanol. The carboxylic group of Mon is protonated at low pH.[34] As the pH decreased from 7.5 to 2.9, the ^{35}Cl NMR line width of KCl in Mon-containing solutions increased from 121 to 214 Hz ($n = 3$). In contrast, a decrease in pH did not appreciably change the ^{35}Cl NMR line width of KCl in Val-containing solutions (172 Hz, pH 7.6; 164 Hz, pH 2.91; $n = 3$). Similarly, the ^{35}Cl NMR line width of $KClO_4$ in Mon-containing solutions increased from 11 to 20 Hz ($n = 3$) as the pH decreased from 7.2 to 2.9, whereas the line width of $KClO_4$ in Val-containing solutions remained essentially the same (16 Hz, pH 7.2; 18 Hz, pH 2.9; $n = 3$).

Effect of Various Ionophores on the FT-IR Difference Spectrum of ClO_4^- in Methanol. FT-IR spectra were obtained for perchlorate-containing ionophore samples in methanol; the wavelength region scanned ranged from 1800 to 500 cm^{-1}. There are four normal modes for vibration of the tetrahedral molecule ($KClO_4$); only ν_3 and ν_4 are infrared-active.[35] The ν_3 and ν_4 frequencies are in the range of 1050−1170 and 630 cm^{-1}, respectively. The 1130 cm^{-1} wavelength corresponds to the stretching frequency band ν_3; coordination of $KClO_4$ splits this band into two new bands at 1150 and 1120 cm^{-1}.[23] Figure 4 depicts the FT-IR difference spectra of perchlorate in the presence of Val (panel A) or in the presence of Las (panel B). When the spectral manipulations described in the caption below Figure 4 are used, the remaining absorbance is due to the change in the symmetry of the perchlorate ion. In Figure 4A, the singlet at 1130 cm^{-1} has split into two bands as a result of the change in symmetry due to the coordination of the ClO_4^- to the K^+ complex of Val. In Figure 4B, a singlet remains, indicating that the symmetry of the ClO_4^- in the Las-containing methanolic solution has not changed. The FT-IR difference spectra for methanolic solutions containing Nig and Mon were similar to those of Las (data not shown).

^{35}Cl NMR Study of Cl $^-$ Transport in LUVs in the Presence of [Co(Gly)$_3$] $^-$. We have characterized the effects of $[Co(Gly)_3]^-$ on the ^{35}Cl resonance of Cl^- in solution and have shown that, when added to suspensions of lipid vesicles, $[Co(Gly)_3]^-$ shifts the ^{35}Cl signal of the extravesicular Cl^-. Two peaks can be observed which show clear resolution. The spectra obtained with LUVs[24] and in the presence of 16 mM Co^{2+} are in agreement with those reported here. Cl^- transport experiments were conducted in the presence and absence of ionophore (0.005 mM Val and 0.005 mM Nig). The spectra are shown in Figure 5.

In the type of LUVs that contained 100 mM NaCl LUVs in a 50 mM KCl and 50 mM KNO_3 suspension, the intravesicular Cl^- signal peak decreased as time increased in the presence of Val. However, the intravesicular Cl^- signal peak area showed no significant change in the presence of Nig, or without ionophore. The Cl^- efflux in LUVs followed first-order kinetics. The transport rate constant was 1.70×10^{-3} min^{-1} in the presence of Val. In the other types of LUVs, such as 100 mM KCl LUVs in a 100 mM NaCl suspension, 100 mM NaCl LUVs in a 100 mM KCl suspension, and 50 mM KCl and 50 mM KNO_3 LUVs in a 100 mM NaCl suspension, all intravesicular signal peak areas showed no significant changes. The r^2 values were large ($r^2 > 0.98$) in all calculations, indicating the reliability of the kinetic data.

Determination of Membrane Permeability by Calcein Fluorescence. An alternative explanation for changes in the ^{35}Cl NMR spectra of vesicles incubated with Val (Figure 5B) is that addition of Val increases the Cl^- permeability of the vesicular membrane. In order to investigate the effect of the addition of ionophores on membrane permeability, we used calcein dye. Representative spectra for LUV samples incubated

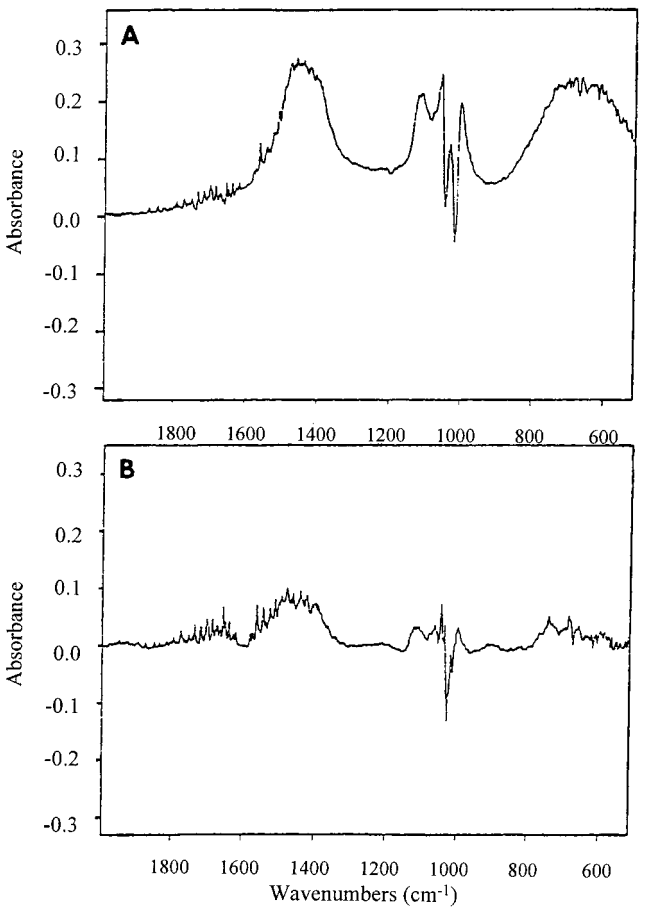

Figure 4. FT-IR difference spectra of methanol solutions containing (A) 5 mM $KClO_4$ and 5 mM Val or (B) 5 mM $KClO_4$ and 5 mM Las. The difference spectra were obtained by computer manipulation of the spectra obtained for the solvent alone, and for perchlorate and salt solutions in methanol. The algorithm used for the Val difference spectrum (A) was a + e − b − c for Val, whereas that used for the Las difference spectrum (B) was a + f − b − d; the symbols a through f represent the individual FT-IR spectra of methanol alone (a), $KClO_4$ in methanol (b), 5 mM Val in methanol (c), 5 mM Las in methanol (d), 5 mM $KClO_4$ with 5 mM Val in methanol (e), and 5 mM $KClO_4$ with 5 mM Las in methanol (f). All spectra were recorded in the absorbance mode.

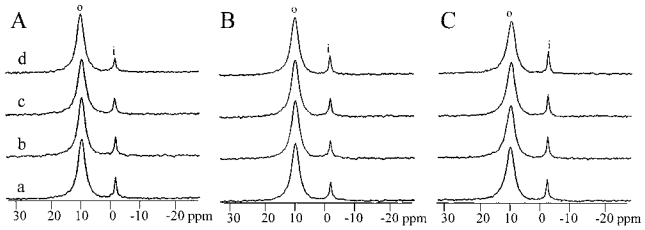

Figure 5. ^{35}Cl NMR spectra at the midpoints of data accumulation (a) 5.5 min, (b) 38.5 min, (c) 71.5 min, and (d) 104.5 min of 100 mM NaCl LUVs in a 50 mM KCl and 50 mM KNO_3 suspension at 37 °C. Lipid concentration was 15 mg/mL, in the presence of a mixture of 8 mM $[Co(Gly)_3]^-$ and (A) 0.005 mM Val, (B) 0.005 mM Nig, and (C) without ionophore. The spectra were the sum of 1024 scans and monitored Cl^- transport during a 1 h 45 min period. The symbols i and o denote the NMR resonances originating from the intra- and extravesicular compartments, respectively.

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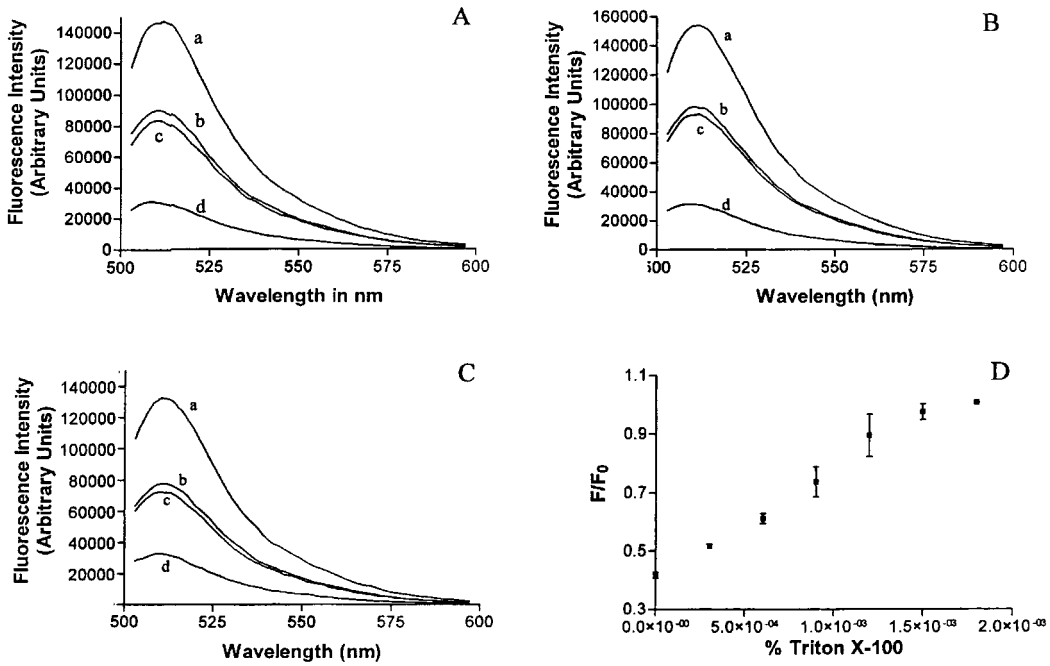

Figure 6. Fluorescence spectra for 60 mM calcein encapsulated in PC vesicles with addition of Val in EtOH (A), Nig in EtOH (B), and EtOH alone (C). For all spectra, (a) maximum fluorescence intensity, (b) fluorescence intensity with ionophore or EtOH, (c) fluorescence of just vesicle suspension, and (d) fluorescence of Tris buffer are shown. Calibration of vesicles for maximum fluorescence intensity via addition of Triton X-100 is shown in panel D. Data in panel D are represented as average ± SEM, $n = 3$.

with ethanol solutions of Val and Nig and of ethanol alone are shown in Figure 6A−C. The mean fluorescence intensities for Val, Nig, and EtOH alone were 4.63 ± 1.04%, 3.92 ± 0.30%, and 3.32 ± 0.69%, respectively (mean ± SEM, referenced to maximum fluorescence intensity by Triton-X, $n = 3$ for all data points). The calibration curve used for obtaining maximum fluorescence intensity via addition of Triton-X is shown in Figure 6D. No significant difference in fluorescence intensity was observed among Val, Nig, or the solvent (ethanol) used to deliver the ionophore to the vesicle suspensions.

Discussion

In recent years, attention has been increasingly focused on using ionophores for studying and understanding the molecular basis of selective ion transport across biological membranes. Several biophysical studies have been undertaken in an effort to elucidate the molecular mechanism by which ionophores transport cations across membranes.[10−12,36] Most ionophore investigations have focused on cation−ionophore interactions and have neglected the role of anions. It was, however, discovered recently that the selectivity for extraction of alkali metal ion by neutral, synthetic ionophores is dependent on the nature of the anion.[37] In this study, we have focused on the capability of anions, in particular Cl^- and ClO_4^-, to form ion pairs in ionophore solutions.

The effect of ion pairing induced by ionophores in several solvent systems was examined for both Cl^- and ClO_4^- by use of ^{35}Cl NMR spectroscopy. A system in which ion pairing occurs is expected to have an increase in the ^{35}Cl NMR line width, whereas if no ion pairing occurs, the line width is expected to remain similar to that of the reference sample (without any ionophore present) after viscosity differences are taken into account.[23]

The neutral ionophores (Val and Non) form a positively charged complex once they interact with the alkali metal cation. We hypothesized that the Cl^- and ClO_4^- counteranions would be attracted to the alkali metal complexes of Val and Non and would form an ion pair. As shown in Tables 1 and 2, the ^{35}Cl NMR line widths of Cl^- and ClO_4^- did increase for the neutral ionophores in all solvents studied. The carboxylic ionophores (Las, Mon, and Nig) form an overall neutral complex when they interact with K^+ or Na^+. We hypothesized that, for carboxylic ionophores, the Cl^- and ClO_4^- counteranions would not be attracted to the neutral complex. We concluded from the ^{35}Cl NMR line width data that the neutral ionophores Val and Non, but not the carboxylic ionophores Las, Nig, and Mon, do interact, and form an ion pair, with Cl^- and ClO_4^- anions.

We used solvents varying in donor number and dielectric constant in order to mimic the range of polarities found in a biological membrane. As these parameters increase, the degree of ion pairing is expected to decrease. We found a significant correlation between the viscosity-corrected ^{35}Cl NMR line widths of $NaClO_4$ and the dielectric constant (Figure 3); no significant correlation between the viscosity-corrected ^{35}Cl NMR line widths of $NaClO_4$ and donor number was found (Figure 3). According to Marcus,[16] a combination of a low dielectric constant of the solvent, ϵ, which promotes the formation of ion pairs in general, and a low donor number of the solvent, DN, which causes only weak solvation of the cation, favors the formation of contact ion pairs. For sodium salts (Cl^- or ClO_4^-), the border between the contact ion pair and nonformation occurs when the value of the product of $DN\epsilon \approx 1000$; the $DN\epsilon$ values for acetone (352), methanol (840), nitromethane (97), and acetonitrile (529) are indicated in parentheses.[16] However, in the range of $DN\epsilon \sim 1000$, the ϵ may predominate in the ordering of the degree of ion pairing formation. The extent of the ionic association is qualitatively related to the ϵ of the solvent.[16] One can note that the extent of ion pairing decreases in the sequence acetone ($\epsilon = 20.7$), methanol ($\epsilon = 32.7$), nitromethane ($\epsilon = 35.9$), and acetonitrile ($\epsilon = 37.5$), which followed the trend of

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increased ionic association as the ϵ of the solvent decreased.[38] For ClO_4^- salts, one would predict that the largest ^{35}Cl NMR line widths would occur when acetone was the solvent, followed by methanol, and finally by DMSO (DNϵ = 1392). As shown in Table 2, the experimentally observed order of the ^{35}Cl NMR line widths of ClO_4^- coincides with the theoretically predicted ordering of solvents. This study is limited by the changes in ion−solvent interactions when going from one solvent to another. These changes depend on factors other than DN and ϵ. It would have been helpful to include a wider range of solvents; however, solubility problems precluded us from conducting a more extensive investigation of the effect of the solvent on the extent of ion pairing.

The effect of the cation on Cl^- and ClO_4^- ion pairing was also examined (Table 3). The selectivity of the ionophores for different cations is expected to vary from solvent to solvent. For the same solvent (methanol), we found that Cs^+ and Li^+ interacted more strongly with Val than with Las, but that Na^+ interacted more strongly with Las than with Val. With a radius of 1.69 Å, the Cs^+ ion fits snugly in the three-dimensional cagelike structure of Val.[11] Las, on the other hand, is the smallest of the carboxylic ionophores, with complexed ions sitting on, rather than within, the cage.[11] The smaller sizes of Na^+ (r = 0.95 Å) and Li^+ (r = 0.60 Å) account for the smaller changes in the ^{23}Na and ^7Li NMR line widths for samples containing Val, compared to salt solution alone. Ion pairing is therefore more readily observed when tight metal-ion binding is present, as with Na^+ and Cs^+, resulting in an increase in the ^{35}Cl NMR line width for samples containing Val as opposed to Las. The very low selectivity of both Val and Las for Li^+ yields very small changes in the ^{35}Cl NMR line widths. The neutral ionophore (Val) selectivity of alkali cations was found to decrease in the order K^+, Cs^+, Na^+, and Li^+; and the carboxylic ionophore (Las) selectivity of the alkali cations was found to decrease in the order Cs^+, K^+, Na^+, and Li^+.[11] The experimentally observed order of ^{35}Cl NMR line widths of ClO_4^- was consistent with the theoretical results.

The effect of pH on the ^{35}Cl NMR line widths of KCl or $KClO_4$ dissolved in methanol in the presence of Val or Mon was also studied. By decreasing the pH value to approximately 3, we also studied the effect of the protonation of the carboxylic group on Mon. The negative charge on Mon is neutralized at low pH, and therefore, at low pH, the interaction between monensin and K^+ results in the formation of a positively charged Mon−K^+ complex. The protonated Mon−K^+ complex has the same overall charge as the Val−K^+ complex, and both are now expected to have similar ion pairing abilities resulting in an increase in the ^{35}Cl NMR line width. As mentioned in the Results section, the ^{35}Cl NMR line widths of Cl^- and ClO_4^- in the presence of Mon increased when the pH was lowered. These observations indicate that the formation of an ionophore−cation with an overall charge of +1 yields an increase in the ^{35}Cl NMR line width, independent of the type of ionophore (neutral vs carboxylic).

The effect of ion pairing on the ^{13}C NMR spectrum of Val was also briefly examined (data not shown). Addition of KCl produced only small changes in the chemical shifts and line widths of the ^{13}C NMR resonances, which were attributed to cation binding.[19,22] This is not surprising if the cation−ionophore complex and Cl^- anion are pictured as two rotating spheres, such that no specific interactions with the ionophore backbone occur.

FT-IR studies on Val in the presence of $KClO_4$ have been performed.[39] The amide I region which appears at 1659 cm^{-1} has been assigned to the stretching vibration of amide C=O groups involved in intramolecular H-bonds for Val in nonpolar solvents. Complexation of cations to Val involves adjustments in the H-bonding network.[39] In solvents of high and medium polarity, the IR spectrum of Val and $KClO_4$ was observed to be more complex. We speculate that, in high-polarity solvents, most intramolecular H-bonds are being broken and intermolecular H-bonds are being formed between the solvent and the ionophore. Hence, the FT-IR spectra were too complex to allow us to draw any conclusions about any specific aspects of the cation−ionophore complex structure.

The change in symmetry of perchlorate from tetrahedral to C_{3v} upon ion pair formation with Val is clear in Figure 4A from the splitting of the ν_3 stretching band at 1130 cm^{-1} into two bands.[39] For Las, however, the band remains unsplit, indicating that the symmetry for perchlorate does not change (Figure 4B). From the FT-IR data, K^+−Val appears to form an ion pair with ClO_4^- in a monodentate fashion, whereas K^+−Las does not.

Shachar-Hill and Shulman[24] have characterized the effects of Co^{2+} on the ^{35}Cl resonance of Cl^- in suspension of lipid vesicles. They found that the ^{35}Cl chemical shift difference between intra- and extravesicular resonances was not changed over a 90 min period.[24] Recently, we found that, in the presence of Val and Co^{2+}, the intravesicular ^{35}Cl signal peak areas were decreased slightly as time increased. In the type of LUVs that contained 100 mM NaCl LUVs in a 50 mM KCl and 50 mM KNO_3 suspension, the intravesicular ^{35}Cl signal peak area decreased slightly in the presence of Nig or without ionophore. The Cl^- efflux was found to follow first-order kinetics. The rate constant had a 3-fold increase in the presence of Val compared with the presence of Nig, or without ionophore (data not shown). In the other types of LUVs (see Methods and Materials section), all intravesicular ^{35}Cl signal peak areas were decreased slightly with time in the presence of Val or Nig, or without ionophore (data not shown). The decrease may be due to Co^{2+}-induced passive chloride exchange, vesicles bursting, or vesicles fusing. However, when $[Co(Gly)_3]^-$ was applied as an SR, and the same types of LUVs were used for study of Cl^- transport in the presence or in the absence of ionophores, we found that only in the type of LUVs which contained 100 mM NaCl LUVs in a 50 mM KCl and 50 mM KNO_3 suspension, the Cl^- efflux also followed first-order kinetics with a rate constant of 1.70×10^{-3} min^{-1} in the presence of Val. Also, the intravesicular ^{35}Cl signal peak areas were not changed in the presence of Nig and in the absence of ionophore. This suggests that the neutral ionophore partially induces Cl^- efflux via ion pairing. Because Co^{2+} has a high positive charge, it may interact with negatively charged membrane phospholipids, resulting in passive Cl^- exchange. However, there is a small negative charge on $[Co(Gly)_3]^-$ which prevents any attractive interaction with membrane phospholipids and passive Cl^- exchange.

To determine the mechanism of ionophore-induced Cl^- transport, we used the other types of LUVs (see Methods and Materials section), and measured the Cl^- distribution by ^{35}Cl NMR spectroscopy. We did not find any significant changes in the intravesicular ^{35}Cl signal peak areas in the presence or absence of Val or Nig. The results indicate that LUVs are stable in the presence of $[Co(Gly)_3]^-$ and ionophore, and that a change in membrane potential occurs simultaneously with Cl^- transport.

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The Cl^- leakage of Co^{2+}-induced passive exchange in LUVs can be eliminated by use of $[Co(Gly)_3]^-$ as an SR. Riddell et al.[26] reported that Val had no appreciable effect on the rate of Cl^- transport across PC vesicle membranes. They measured the rate of Cl^- transport in vesicle suspensions and monitored the changes in the peak area of the intravesicular ^{35}Cl NMR resonance; the extravesicular ^{35}Cl signal was broadened by $MnCl_2$ present in the suspension medium.[26] Recently, Riddell and Zhou[25] reported that Cl^- can influx into PC vesicles at neutral pH with preparation of vesicles at 4 °C. We could not obtain the same results they described. In contrast, we found that Cl^- can efflux from the LUV in the presence of Val. This efflux does not appear to be a result of increased membrane permeability subsequent to the addition of the ionophore because an increase in permeability would have resulted in a dramatic increase in the calcein fluorescence, the result of a release from the dye in the vesicles, which was not observed (Figure 6).

Conclusions

From our ^{35}Cl NMR data, we conclude that positively charged neutral ionophore−cation complexes may form ion pairs with Cl^- and provide an alternate transport pathway for anions. The ^{35}Cl NMR method described here may be useful in providing a molecular understanding of abnormal Cl^- transport in cystic fibrosis and muscular dystrophy patients. Moreover, the Cl^-−ionophore complex may provide good model systems for characterization of the interactions between Cl^- and the cystic fibrosis transmembrane conductance regulator (CFTR) protein.

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