Articles

Competition between Li^+ and Mg^{2+} for the Phosphate Groups in the Human Erythrocyte Membrane and ATP: An NMR and Fluorescence Study†

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ABSTRACT: We investigated the mechanism of competition between Li^+ and Mg^{2+} in Li^+-loaded human red blood cells (RBCs) by making ^7Li and ^{31}P NMR and fluorescence measurements. We used ^7Li NMR relaxation times to probe Li^+ binding to the human RBC membrane and ATP; an increase in Mg^{2+} concentration caused an increase in both ^7Li T_1 and T_2 values in packed Li^+-loaded RBCs, in suspensions of Li^+-loaded RBC ghosts, in suspensions of Li^+-containing RBC membrane, and in aqueous solutions of ATP, indicating competition between Li^+ and Mg^{2+} for binding sites in the membrane and ATP. We found that increasing concentrations of either Li^+ or Mg^{2+} in the presence of human RBC membrane caused an increase in the ^{31}P NMR chemical shift anisotropy parameter, which describes the observed axially symmetric powder pattern, indicating metal ion binding to the phosphate groups in the membrane. Competition between Li^+ and Mg^{2+} for phosphate groups in ATP and in the RBC membrane was also observed by both fluorescence measurements and ^{31}P NMR spectroscopy at low temperature. The ratio of the stoichiometric binding constants of Mg^{2+} to Li^+ to the RBC membrane was approximately 20; the ratio of the conditional binding constants in the presence of a free intracellular ATP concentration of 0.2 mM was approximately 4, indicating that Li^+ competes for approximately 20% of the Mg^{2+}-binding sites in the RBC membrane. Our results indicate that, regardless of the spectroscopic method used, Li^+ competes with Mg^{2+} for phosphate groups in both ATP and the RBC membrane; the extent of metal ion competition for the phosphate head groups of the phospholipids in the RBC membrane is enhanced by the presence of ATP. Competition between Li^+ and Mg^{2+} for anionic phospholipids or Mg^{2+}-activated proteins present in cell membranes may constitute the basis of a general molecular mechanism for Li^+ action in human tissues.

Despite the pharmacological importance of Li^+ in the treatment of bipolar disorders and some viral infections (Bach, 1990), its mode of action is not well-understood. Guanine nucleotide-binding (G) proteins are activated by Mg^{2+} and act as switchboards in the cell membrane to carry signals between outside stimulants and second messenger systems, such as the adenylate cyclase and phosphatidylinositol turnover systems. Two types of G proteins, one with stimulatory and the other with inhibitory functions, provide a common site for both the antimanic and antidepressant therapeutic effects of Li^+ (Avissar et al., 1988). Because of the similar chemical properties of these two metal ions, competition between Li^+ and Mg^{2+} for Mg^{2+}-binding sites in biomolecules, and in particular for the constituents of cell membranes, may be a general molecular mechanism for Li^+ action in biological systems. We have shown by using ^{31}P and ^7Li NMR methods that, in aqueous solution, competition occurs between Li^+ and Mg^{2+} ions for the phosphate groups of guanosine di- and triphosphate, which are the substrates of G proteins (Rong et al., 1992), as well as for the phosphate groups of adenosine di- and triphosphate (Abraha et al., 1991). In an *in vitro* study, we also showed by using ^{31}P NMR methods that Li^+ displaced Mg^{2+} from the phosphate groups of ATP in human Li^+-loaded red blood cells (RBCs);[1] we speculated at the time that the displaced Mg^{2+} was bound to the RBC membrane (Ramasamy & Mota de Freitas, 1989).

The free intracellular Mg^{2+} concentrations, $[Mg^{2+}]_f$, in most cell types are generally an order of magnitude larger than the dissociation constant (K_d) of MgATP (London, 1991; Mota de Freitas & Dorus, 1993). Intracellular ATP is therefore nearly saturated with Mg^{2+}, and the ^{31}P NMR method based on the chemical shift separation between the α- and β-phosphate resonance of ATP ($\delta_{\alpha\beta}$) may not be sensitive to small changes in the free intracellular Mg^{2+} concentration. To

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[1] Abbreviations: AA, atomic absorption; BPG, 2,3-bisphosphoglycerate; CSA, chemical shift anisotropy; CPMG, Carr–Purcell–Meiboom–Gill T_2 pulse sequence; F, fluorescence intensity at a specific wavelength; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; R, ratio of fluorescence intensities at two separate wavelengths; RBC, red blood cell; S, contribution of free dye or metal-bound dye to the fluorescence intensity at a specific wavelength; SM, sphingomyelin; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; Tris, tris(hydroxymethyl)aminomethane.

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observe an appreciable change in the $\delta_{\alpha\beta}$ values, in our previous ^{31}P NMR studies on the competition between Li^+ and Mg^{2+} for phosphate groups in ATP and GTP, we had to use a large excess of Li^+ over Mg^{2+} (Abraha et al., 1991; Ramasamy & Mota de Freitas, 1989; Rong et al., 1992). Because of the good match between the K_d value for the complex between Mg^{2+} and the fluorescence indicator furaptra and physiologically relevant $[Mg^{2+}]_f$ values, and because of the higher sensitivity of fluorescence relative to NMR spectroscopy (London, 1991; Mota de Freitas & Dorus, 1993), we investigated and report here the results of our fluorescence and low-temperature ^{31}P NMR measurements of the competition between Li^+ and Mg^{2+} in aqueous solutions of ATP and of RBC membrane.

Lipids play an important role in the maintenance of cell structure. Interaction of metal ions with human RBC membranes and synthetic membranes made up of phospholipids, such as phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS), has been studied with ^7Li, ^{31}P, and ^2H NMR (Akutsu & Seelig, 1981; Alten & Seelig, 1981; Brown & Seelig, 1977; Hope & Cullis, 1980; Pettegrew et al., 1987a; Riddell & Arumugam, 1988; Rong et al., 1993; Roux & Bloom, 1990; Roux & Neuman, 1986; Yeagle, 1984), infrared, and fluorescence spectroscopies (Casal et al., 1987; Pettegrew et al., 1987b), differential scanning calorimetry, and X-ray diffraction (Hauser & Shiley, 1981). The binding of metal ions to membranes causes motional rigidity, conformational changes in the phospholipid head groups, which are reflected in changes in the values of the ^{31}P NMR relaxation rate, chemical shift, and chemical shift anisotropy (CSA), and distinct changes in the ^2H NMR quadrupole splittings (Akutsu & Seelig, 1981; Alten & Seelig, 1981; Brown & Seelig, 1977; Hope & Cullis, 1980; Roux & Bloom, 1990; Roux & Neuman, 1986; Yeagle, 1984).

The strength of the metal ion interaction, which can be measured from these NMR parameters, increases with increasing charge and metal ion concentration. A previous ^2H NMR study has shown that the extent of binding of Ca^{2+}, Mg^{2+}, Li^+, Na^+, and K^+ to deuterated bilayers of PC and PS decreased from Ca^{2+} to K^+ (Roux & Bloom, 1990). Results obtained with pure PS and mixed bilayers with PC and phosphatidylethanolamine (PE) at various NaCl or LiCl concentrations indicated that interactions with Na^+ and Li^+ have very different effects on the ^2H NMR quadrupole splittings of the phospholipid head groups (Roux & Neuman, 1986). The addition of Ca^{2+} to PS vesicles caused rigid lattice ^{31}P NMR spectra, which corresponded to a strong and specific head group immobilization by the Ca^{2+} ion (Hope & Cullis, 1980). At pH 7.4, the hydrated PC from egg yolk and PS from human erythrocytes adopted the bilayer phase, whereas lowering the pH below 4.0 resulted in a transition from the bilayer to the hexagonal configuration, which was an indication of a conformational change in the phospholipid (Hope & Cullis, 1980). By differential scanning calorimetry and X-ray diffraction, it was shown that Li^+ interacted with liquid-crystalline PS bilayers, forming a highly ordered crystalline complex that retained the bilayer structure (Hope & Cullis, 1981). The stability of the complex was indicated by the high crystal to liquid-crystal transition temperature of about 90 °C and the high transition enthalpy ΔH = 16.2 kcal/mol (Hope & Cullis, 1981). The interaction of PS with Li^+ was found to mimic those of Ca^{2+} and Mg^{2+} (Hope & Cullis, 1981). A small fraction of most cell membrane surfaces is occupied by negatively charged lipids, such as PI and PS. The interactions of metal ions with the anionic phospholipids PI and PS, which are minor constituents of the inner leaflets of cell membranes, are relatively strong compared to those with the uncharged phospholipids PC, PE, and sphingomyelin.

Li^+ ions in Li^+-containing cell or membrane suspensions, as well as in Li^+-containing solutions of nucleotides, are in fast exchange on the ^7Li NMR time scale (Abraha et al., 1991; Rong et al., 1992, 1993). The observed ^7Li chemical shift, spin–lattice (T_1), and spin–spin (T_2) relaxation time values represent a weighted average of bound and free Li^+ ions. Because the $^7Li^+$ nucleus has a narrow chemical shift range (Mota de Freitas, 1993), ^7Li NMR chemical shifts are not sensitive to Li^+ binding to biomolecules. Li^+ binding to cell membranes was therefore previously studied with ^7Li T_1 and T_2 relaxation time measurements (Pettegrew et al., 1987a; Riddell & Arumugam, 1988; Rong et al., 1993); free nuclei have large T_1 and T_2 values, whereas those that are tightly bound have relatively smaller relaxation values. Unlike the partial visibility of the $^{23}Na^+$ NMR resonance, the $^7Li^+$ NMR resonance in Li^+-loaded RBC and membrane suspensions is fully visible (Mota de Freitas et al., 1990; Rong et al., 1993). Intracellular ^7Li T_1 values in Li^+-loaded RBCs were much larger than the corresponding T_2 values for a given intracellular Li^+ concentration (Pettegrew et al., 1987a; Rong et al., 1993); when the intracellular Li^+ concentration increased, the T_1 and T_2 values also increased, indicating a decrease in the fraction of bound intracellular Li^+ (Rong et al., 1993). A viscosity contribution was ruled out because, in a water/glycerol mixture whose viscosity was adjusted to that of intracellular RBC, Li^+ showed no significant difference between the ^7Li NMR T_1 and T_2 relaxation times (Pettegrew et al., 1987; Rong et al., 1993). It is well-established from NMR relaxation theory that the slow tumbling motions associated with metal ion binding contribute to the bound component of T_2 relaxation (Gadian, 1982). The large difference in ^7Li relaxation times was present in both intact Li^+-loaded RBCs and suspensions of Li^+-containing RBC membrane, but not in Li^+-containing solutions of purified spectrin, ATP, 2,3-bisphosphoglycerate (BPG), and hemoglobin (Rong et al., 1993). Whereas the large T_1/T_2 ratios observed in packed Li^+-loaded RBCs and in Li^+-containing suspensions of RBC membrane were highly dependent on Li^+ concentration, the small T_1/T_2 ratios observed in Li^+-containing solutions of spectrin, ATP, BPG, and hemoglobin were virtually independent of Li^+ concentration (Rong et al., 1993). These observations indicated that the Li^+ relaxation behavior observed in Li^+-loaded human RBCs was due to Li^+ binding to the RBC membrane and not to binding to intracellular RBC components or electric field gradients experienced by Li^+ when it transverses the spectrin–actin network, as previously postulated (Pettegrew et al., 1987a).

In this work, in addition to fluorescence and low-temperature ^{31}P NMR measurements of ATP- and/or membrane-containing solutions, we used ^{31}P NMR measurements to probe direct binding of Li^+ and Mg^{2+} ions to the phosphate head groups of phospholipids in the human RBC membrane, and we used ^7Li NMR T_1 and T_2 relaxation measurements to study the competition between Li^+ and Mg^{2+} ions for the phosphate groups in ATP and in the human RBC membrane.

EXPERIMENTAL PROCEDURES

Materials. Human RBCs were obtained from a blood bank (Life Source, Chicago, IL). The RBCs were used within a maximum of 3 days after blood drawing; the effect of storage on free intracellular Mg^{2+} concentrations (Bock et al., 1985) was therefore minimized. The Na^+ and Tris (tris(hydroxy-

methyl)aminomethane) salts of ATP, as well as sucrose, glucose, and choline chloride, were purchased from Sigma. LiCl (99.999%), $MgCl_2$ hexahydrate (99.999%), HCl, D_2O (99.9%), and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] were purchased from Aldrich. The ionophore A23187 was obtained from Boehringer Mannheim. The salt derivative of the Mg^{2+} fluorescence indicator furaptra was purchased from Molecular Probes. All reagents were used without further purification.

Preparation of Li^+- and Mg^{2+}-Loaded RBCs. Packed RBCs were washed three times for 5 min each by centrifugation at 6000*g* and 4 °C in an isotonic solution containing 100 mM choline chloride, 85 mM sucrose, 10 mM glucose, and 10 mM HEPES (pH 7.4). We removed the supernatant and the buffy coat by aspiration. LiCl loading of RBCs was achieved by incubation of the cells at 50% hematocrit in a medium containing 40 mM LiCl, 100 mM NaCl, 5 mM KCl, 10 mM glucose, and 20 mM HEPES (pH 7.2) at 37 °C for 12 h. An intracellular Li^+ concentration of 3 mM was achieved with this loading procedure. Mg^{2+} loading and depletion of RBCs were achieved by the incubation of Li^+-loaded RBCs in isotonic suspension medium containing either 20 mM $MgCl_2$ or 20 mM EDTA, respectively, in the presence of the ionophore A23187 (Ramasamy & Mota de Freitas, 1989). Under these loading conditions, no ATP hydrolysis, intracellular Li^+ and Mg^{2+} leakage, or intracellular pH changes were induced, as previously reported (Ramasamy & Mota de Freitas, 1989).

Preparation of Resealed and Unsealed RBC Membrane Suspensions. The procedure used for the preparation of human RBC membrane was adapted from the literature (Steck & Kant, 1974). Washed, packed RBCs (2 mL) were lysed at 4 °C in 40 mL of hypotonic lysing medium, 5 mM HEPES (pH 8.0). The suspension medium was centrifuged three times for 10 min each at 22000*g* and 4 °C in a Beckman J2-21 centrifuge with a J-20A rotor until the white membrane was observed. All membrane samples contained 5 mM HEPES (pH 7.4) and different concentrations of LiCl and $MgCl_2$. The membrane protein concentration was measured according to a published procedure (Bollag & Edelstein, 1991). Resealed RBC ghosts were obtained by incubation of the membrane preparation at 37 °C in a 5 mM sodium phosphate buffer (pH 8.0) (5P8) containing 1 mM $MgCl_2$ (Schwoch & Passow, 1973). Resealed Li^+- or Mg^{2+}-loaded RBC ghosts were obtained by incubation of the ghosts in 5P8 buffer containing Li^+ or Mg^{2+} at 4 times the desired intracellular concentration (Duhm et al., 1976). Mg^{2+} depletion of resealed Li^+-loaded RBC ghosts was achieved by incubation in an isotonic medium containing EDTA and the ionophore A23187 (Ramasamy & Mota de Freitas, 1989).

NMR, Fluorescence, and AA Measurements. ^7Li and ^{31}P NMR measurements were made at 116.5 and 121.4 MHz, respectively, on a Varian VXR-300 NMR spectrometer equipped with a multinuclear probe and a variable-temperature unit. Samples were run in 10-mm NMR tubes spinning at 18 Hz. Experiments were conducted at either 37 or 0 °C. We used 20% D_2O solutions to lock the frequencies of the ^7Li and ^{31}P NMR spectra. ^7Li T_1 and T_2 measurements were made by use of the inversion–recovery and Carr–Purcell–Meiboom–Gill (CPMG) pulse sequences, respectively (Gadian, 1982). Because there is no chemical shift difference between the free and bound forms of Li^+, the reported T_2 values are independent of the short pulse repetition rate used (0.25 s^{-1}) in the CPMG experiment (Laszlo, 1979). ^7Li NMR relaxation measurements were obtained with 2–8 transients for each increment between the two radio frequency pulses, and the number of increments used was 8–12. We made ^{31}P NMR measurements of membrane suspensions by using a single pulse sequence with ^1H decoupling and a receiver dead time of 12 μs (Smith & Ekiel, 1984). The preacquisition delay used minimized the spectral distortion resulting from probe and receiver ringdown (Smith & Ekiel, 1984). Because of the low signal-to-noise ratios of the ^{31}P NMR spectra of human RBC membrane suspensions at 37 °C, data were accumulated overnight. The effective chemical shift anisotropy (CSA) parameter was measured in powder spectra of membrane suspensions as the frequency separation in ppm between the main peak and the low-field shoulder, as previously described (Cullis & De Kruyff, 1976). ^{31}P NMR spectra of ATP solutions at low temperature were obtained with a single pulse sequence with approximately 1000 transients; we first calibrated the variable-temperature unit by using the chemical shift separation between the ^1H NMR resonances of methanol.

Total intracellular Mg^{2+} and Li^+ concentrations in RBCs and RBC ghosts were measured with a Perkin-Elmer 5000 atomic absorption (AA) spectrophotometer. Mg^{2+} fluorescence measurements were made in a PTI LS-100 fluorimeter. We measured fluorescence excitation spectra with the emission wavelength set at 510 nm (London, 1991).

Calculation of Binding Constants. The calculation of the binding constant, K_{Li}, of Li^+ to the human RBC membrane from ^7Li NMR T_1 measurements assumed a two-state (free, f, and bound, b, metal ions) model undergoing fast exchange and a total Li^+ concentration, $[Li^+]_t$, that is large with respect to the binding site concentration, [B] (Urry et al., 1989):

$$\Delta R^{-1} = (R_{obs} - R_f)^{-1} = K_{app}^{-1}\{[B](R_b - R_f)\}^{-1} + [Li^+]_t\{[B](R_b - R_f)\}^{-1} \quad (1)$$

where R_{obs}, R_f, and R_b are the reciprocals of T_{1obs}, T_{1f}, and T_{1b}, respectively. We have recently shown (Rong et al., 1993) that these assumptions made in the derivation of eq 1 are valid for Li^+-containing suspensions of human RBC membrane. K_{Mg}, the binding constant of Mg^{2+} to human RBC membrane, and K_{Li} were calculated from the apparent Li^+-binding constants, K_{app}, which were, in turn, determined from ^7Li T_1 values measured in the presence of increasing Mg^{2+} concentrations:

$$1/K_{app} = 1/K_{Li}(1 + K_{Mg}[Mg^{2+}]) \quad (2)$$

These equations assume one-to-one stoichiometry for Li^+ and Mg^{2+} binding to the RBC membrane. The metal ion-to-ligand ratios of the predominant species present in Li^+-containing solutions of ATP are 1:1 and 2:1 (Abraha et al., 1991); we obtained the binding constants and the limiting ^7Li relaxation rates, R_1, for the Li^+ complexes of ATP by using nonlinear least-squares fits of the ^7Li T_1 values. We used three different models, which assumed the formation of either the 1:1 or 2:1 species alone or a mixture of the 1:1 and 2:1 species (Abraha et al., 1991; Rong et al., 1992).

The dissociation constant of Li^+ to the dye was obtained from a Hill plot (Raju et al., 1989) of $\log[(F_{obs} - F_{min})/(F_{max} - F_{obs})]$ vs $\log [Li^+]$, where F_{obs} is the fluorescence intensity at 335 nm observed in the presence of various concentrations of LiCl, and F_{min} and F_{max} are the fluorescence intensities at 335 nm in the absence and presence of saturating amounts of Li^+, respectively. The stoichiometry of Li^+ binding to the dye was obtained from the slope, and the dissociation constant was calculated from the expression, antilog(y-intercept/slope) (Raju et al., 1989).

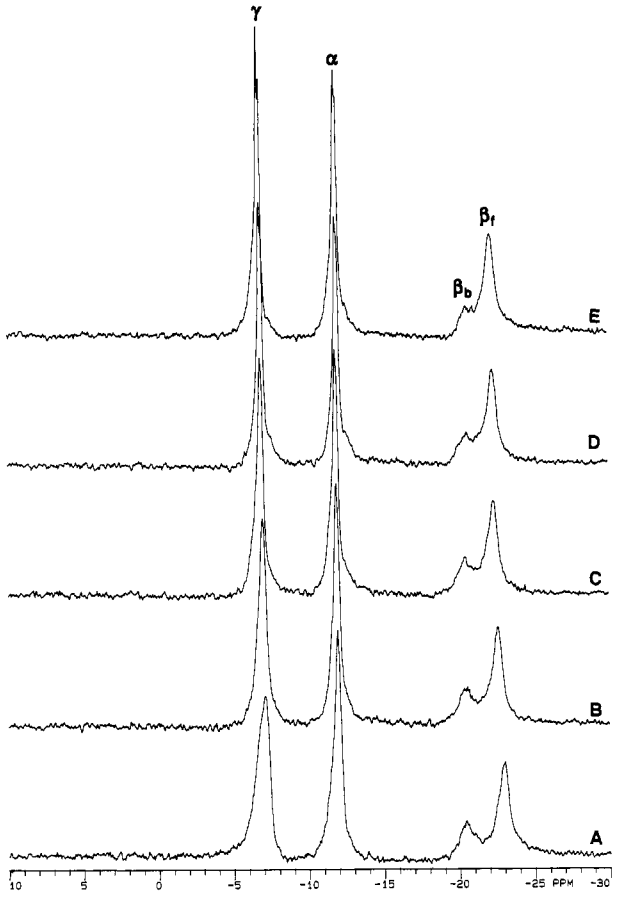

FIGURE 1: ^{31}P NMR spectra, at 0 °C and pH 7.5, of 3.0 mM NaATP in the presence of 1.0 mM $MgCl_2$ and (A) 0, (B) 20, (C) 40, (D) 60, and (E) 80 mM LiCl. The ionic strength of all samples was adjusted to 0.15 M by using Tris Cl. The assignments of the phosphate resonances are indicated in spectrum E; the subscripts f and b refer to Mg^{2+}-free and Mg^{2+}-bound forms of β-phosphate, respectively. Line broadening was 8 Hz.

Calculation of $[Mg^{2+}]_f$ *Values.* At low temperature (Figure 1), the ^{31}P NMR resonance of the β-phosphate of ATP is in slow exchange with Mg^{2+}, giving rise to two separate signals for the Mg^{2+}-free and Mg^{2+}-bound forms of ATP. In contrast, we observed, at low temperature, downfield shifts of the free β-phosphate resonance upon the addition of Li^+ to aqueous solutions of ATP, indicating that Li^+ was in fast exchange with the free species. We therefore calculated the values of $[Mg^{2+}]_f$ from the low-temperature ^{31}P NMR spectra of ATP by using the equation

$$[Mg^{2+}]_f = (\delta_{\alpha\beta}{}^f - \delta_{\alpha\beta}{}^b)/[K_\beta(\text{area ratio})(\delta_{\alpha\beta}{}^{obs} - \delta_{\alpha\beta}{}^b)] \quad (3)$$

where $\delta_{\alpha\beta}{}^f$ and $\delta_{\alpha\beta}{}^b$ are the chemical shift separations between the α-phosphate and β-phosphate resonances of Mg^{2+}-free ATP in the absence and presence of 150 mM LiCl, and $\delta_{\alpha\beta}{}^{obs}$ is the chemical shift separation observed in ATP solutions containing mixtures of LiCl and $MgCl_2$. The value of 1563 M^{-1} for the Mg^{2+}-binding constant to the β-phosphate of ATP, K_β, at 0 °C and pH 7.5 was estimated from values previously reported at 37 and 25 °C and pH 7.5 (Abraha et al., 1991; Bock et al., 1985; Prigodich & Haake, 1985). We obtained the area ratio by dividing the area of the Mg^{2+}-free β-phosphate resonance by that of the Mg^{2+}-bound β-phosphate signal. To obtain area ratios, we conducted deconvolution of the partially overlapped Mg^{2+}-free and Mg^{2+}-bound β-phosphate resonances by using the Marquardt peak-fitting menu of the NMR-286 software provided by Softpulse Software Co. (Guelph, Ontario, Canada).

Table 1: ^7Li NMR Relaxation Values of Li^+-Loaded RBCs, Li^+-Loaded RBC Ghosts, and Li^+-Treated RBC Membrane in the Presence of Increasing Concentrations of Mg^{2+}

sample	$[Mg^{2+}]_{in}$ (mM)[a]	$[Li^+]_{in}$ (mM)[a]	T_1 (s)[b]	T_2 (s)[b]
	Packed, Li^+-Loaded RBCs			
Mg^{2+}-depleted	0.0[c]	2.98 ± 0.02	4.9 ± 0.1	0.08 ± 0.01
normal Mg^{2+}	2.9 ± 0.1	2.99 ± 0.03	6.6 ± 0.1	0.15 ± 0.01
Mg^{2+}-saturated	9.8 ± 0.3	2.96 ± 0.03	7.8 ± 0.2	0.28 ± 0.02
	Packed, Li^+-Loaded RBC Ghosts			
Mg^{2+}-depleted	0.0[c]	2.97 ± 0.01	9.8 ± 0.3	1.1 ± 0.2
normal	2.9 ± 0.2	2.98 ± 0.02	11.1 ± 0.2	1.9 ± 0.1
Mg^{2+}-saturated	9.8 ± 0.2	2.96 ± 0.02	13.1 ± 0.5	2.4 ± 0.2
	Li^+-Treated RBC Membrane[d]			
	0.00	3.0	12.9 ± 1.0	0.16 ± 0.01
	0.25	3.0	16.2 ± 0.8	0.19 ± 0.01
	0.50	3.0	17.6 ± 0.2	0.21 ● 0.01
	1.0	3.0	18.6 ± 0.7	0.26 ± 0.01
	2.0	3.0	19.8 ± 1.1	0.30 ± 0.01

[a] The total intracellular Mg^{2+} and Li^+ concentrations in Li^+-loaded RBCs and Li^+-loaded RBC ghosts were measured by atomic absorption. [b] Each value, reported as mean ± standard deviation, is an average of three readings made on separately prepared samples, except for Li^+-treated RBC membrane where $n = 2$. [c] The Mg^{2+} levels measured were below the detection limit of 10 μM. [d] The membrane protein concentration was 1.8 ± 0.2 mg/mL. At Mg^{2+} concentrations of 1.0 mM or higher, the RBC membrane is resealed, whereas for lower concentrations fragmented membrane is present (Steck & Kant, 1974).

We found that Li^+ binds weakly to the fluorescence indicator furaptra. We obtained free Mg^{2+} concentrations that were corrected for Li^+ binding from the ratio of the intensities at the wavelengths 335 and 370 nm in the fluorescence excitation spectrum (London, 1991):

$$[Mg^{2+}]_f = K_d S_{min}(R - R_{min})/S_{max}(R_{max} - R) + K_d S'_{max}(R - R'_{max})[Li^+]_f/K'_d S_{max}(R_{max} - R) \quad (4)$$

where R is the fluorescence intensity ratio observed for the biological sample; R_{min}, R_{max}, and R'_{max} are the fluorescence intensity ratios in the absence and presence of saturating amounts of Mg^{2+} or Li^+; S_{min}, S_{max}, and S'_{max} are the fluorescence intensities at 370 nm in the absence and presence of saturating amounts of Mg^{2+} or Li^+; and K_d and K'_d are the dissociation constants of the furaptra complexes of Mg^{2+} and Li^+, respectively. The values of $[Li^+]_f$ were calculated from the Li^+-binding constants for the LiATP and Li_2ATP species (*vide infra*).

RESULTS

NMR Studies. Table 1 shows that Mg^{2+} saturation of Li^+-loaded RBCs or Li^+-loaded RBC ghosts resulted in larger ^7Li T_1 and T_2 values. Conversely, Mg^{2+} depletion of Li^+-loaded RBCs or Li^+-loaded RBC ghosts resulted in shorter ^7Li relaxation values. Table 1 also shows ^7Li, T_1 and T_2 values for Li^+-treated RBC membrane in the presence of increasing Mg^{2+} concentrations. The T_2 errors in ghost samples are larger than those for the other two types of samples, presumably because of the larger sample variability associated with incomplete resealing of ghost preparations (Schwoch & Passow, 1973). Addition of a shift reagent (3 mM Dy-$(PPP)_2^{7-}$) to the preparations of packed Li^+-loaded RBCs or packed Li^+-loaded RBC ghosts did not yield extracellular $^7Li^+$ NMR resonances. The relaxation values reported in Table 1 for packed Li^+-loaded RBCs and packed Li^+-loaded RBC ghosts are therefore due to intracellular Li^+ and are not due to a mixture of intracellular Li^+ in exchange with an extracellular pool of Li^+ ions (Rong et al., 1993).

LiCl (at 3 mM) in water yields similar T_1 and T_2 values of approximately 20 s. In contrast, upon the addition of membrane, the T_1 value decreased to 12.9 ± 1.0 s, whereas the T_2 value decreased to 0.16 ± 0.01 s. This is due to the binding of Li^+ to the membrane and the high viscosity of the membrane solution (Rong et al., 1993). Li^+ ions tumble faster in water than in a viscous membrane-containing solution, thus the higher and equal T_1 and T_2 values in water. Upon titration of the membrane solution with increasing amounts of Mg^{2+}, the T_1 and T_2 values increased from 12.9 ± 1.0 and 0.16 ± 0.01 s in the absence of Mg^{2+} to 19.8 ± 1.1 and 0.30 ± 0.01 s, respectively, in the presence of 2.0 mM Mg^{2+}. As the Mg^{2+} concentration increased in the membrane suspension, the observed T_1 and T_2 values increased, approaching those of free Li^+; the observed T_2 values were, however, much smaller than those of free Li^+ because of the large T_1/T_2 ratio associated with Li^+ binding to the membrane (Rong et al., 1993). These observations indicate competition between Li^+ and Mg^{2+} for binding sites in the RBC membrane.

To determine whether Li^+/Mg^{2+} competition also occurs in the presence of physiologically relevant intracellular K^+ concentrations, we also measured the ^7Li T_1 and T_2 values for membrane suspensions (with a protein concentration of 1.9 ± 0.2 mg/mL) containing 3 mM LiCl and 150 mM KCl in the absence of $MgCl_2$ (T_1 = 18.9 ± 0.1 s; T_2 = 0.20 ± 0.02 s; n = 2) and in the presence of 0.50 mM $MgCl_2$ (T_1 = 19.8 ± 0.3 s; T_2 = 0.45 ± 0.06 s; n = 2). Regardless of the presence of a background of K^+ ions, an increase in Mg^{2+} concentration in membrane suspensions caused an increase in both ^7Li T_1 and T_2 values. The relaxation values observed in the presence of K^+ were, however, larger than those measured in its absence, indicating that Li^+ and Mg^{2+} binding to the RBC membrane decreases as the ionic strength of the suspension medium increases.

From the ^7Li T_1 values measured with RBC membrane suspensions (at a concentration of 3.0 ± 0.5 mg/mL) containing Li^+ concentrations in the range of 1.0–100 mM (data not shown), we calculated the Li^+-binding constant to the membrane by using eq 1 and found it to be (1.7 ± 0.1) × 10^2 M^{-1}. For each Li^+ concentration in the RBC membrane suspension (data not shown), we then varied the Mg^{2+} concentration between 0.1 and 1.0 mM; from the observed ^7Li T_1 values, we calculated, by using eq 1, the apparent Li^+-binding constants in the presence of Mg^{2+}. The Mg^{2+}-binding constant to the RBC membrane was calculated from eq 2 and was found to be (3.3 ± 0.1) × 10^3 M^{-1}.

We have previously shown that the addition of increasing concentrations of Mg^{2+} (0–2.5 mM) to aqueous samples containing 5.0 mM Li^+ and ATP (at 3, 5, or 7 mM) caused an increase in the ^7Li T_1 values because of competition between Li^+ and Mg^{2+} for phosphate groups in ATP (Abraha et al., 1991). We measured ^7Li T_1 values for 7.0 mM ATP solutions containing varying concentrations of Li^+ (1.0–1000 mM) and Mg^{2+} (0–1.0 mM). For a fixed Mg^{2+} concentration, an increase in the Li^+ concentration resulted in an increase in the ^7Li T_1 values because of an increase in the fraction of free Li^+; for a fixed Li^+ concentration, an increase in the Mg^{2+} concentration caused an increase in the ^7Li T_1 values because of metal ion competition for phosphate groups in ATP (data not shown). We fit the ^7Li T_1 relaxation data measured for 7.0 mM ATP solutions containing LiCl alone and $LiCl/MgCl_2$ mixtures to three different models (see Experimental Procedures) to generate the binding constants and the limiting R_1 values for Li^+ bound to ATP.

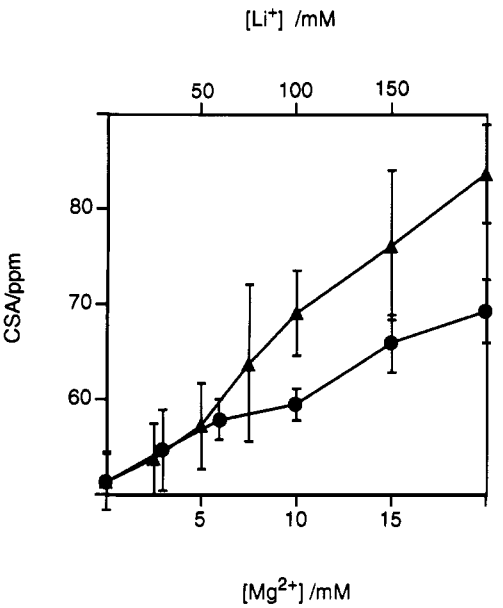

FIGURE 2: Dependence of CSA values of the phosphate groups of human RBC membrane on Li^+ (▲) and Mg^{2+} (●) concentrations. The top horizontal axis refers to Li^+ concentrations, whereas the bottom horizontal axis refers to Mg^{2+} concentrations. Each value is an average of two readings made on separately prepared samples. The membrane protein concentration for all samples was 7.5 ± 1.5 mg/mL.

Table 2: ^{31}P NMR Determined $[Mg^{2+}]_f$ Values for ATP Solutions in the Presence and Absence of RBC Membrane[a]

	without membrane			with membrane[b]		
$[Li^+]$ (mM)	area ratio[c]	$\delta_{\alpha\beta}$ (ppm)[d]	$[Mg^{2+}]_f$ (μM)[e]	area ratio[c]	$\delta_{\alpha\beta}$ (ppm)[d]	$[Mg^{2+}]_f$ (μM)[e]
0	1.5	10.9	380	1.9	11.0	280
20	1.5	10.8	490	2.2	10.7	360
40	1.5	10.5	800	2.6	10.5	410
60	1.8	10.4	890	2.8	10.4	710
80	2.5	10.3	960	3.1	10.3	770

[a] The reported values represent an average of measurements conducted in two separately prepared samples. All samples contained 3.0 mM NaATP, 1.0 mM $MgCl_2$, and 0.15 M Tris Cl (pH 7.5) at 0 °C. [b] The membrane protein concentration was 2.7 ± 0.1 mg/mL. [c] The errors in the determination of the area ratios were less than 5%. [d] The errors are less than 0.1 ppm. [e] The $[Mg^{2+}]_f$ values were calculated from eq 3, and the errors are less than 10%.

For ATP in the presence of LiCl alone and assuming an R_1 value of 0.05 s^{-1} for free Li^+, the Li^+-binding constants and the R_1 values for bound Li^+ were 12 M^{-1} and 1.5 s^{-1} (Σ^2 = 1.9 × 10^{-5}) for the model based on the LiATP species, 1.0 × 10^5 M^{-2} and 0.47 s^{-1} (Σ^2 = 4.9 × 10^{-3}) for the model based on the Li_2ATP species, and 3.2 × 10^2 M^{-1} and 0.21 s^{-1} for LiATP and 3.2 × 10^3 M^{-2} and 1.6 s^{-1} for Li_2ATP (Σ^2 = 1.7 × 10^{-5}) for the model based on a mixture of LiATP and Li_2-ATP. The Σ^2 values obtained for calculations with the ^7Li data in the presence of LiCl alone indicate that the best nonlinear least-squares fit to the ^7Li T_1 data was provided by the model based on a mixture of LiATP and Li_2ATP.

For ATP in the presence of mixtures containing 0.5 mM $MgCl_2$ and varying concentrations of LiCl, and assuming an R_1 value of 0.05 s^{-1} for free Li^+ and a binding constant of 2 × 10^4 M^{-1} for MgATP (Abraha et al., 1991), the Li^+-binding constants and the R_1 values for bound Li^+ were 7.3 M^{-1} and 1.8 s^{-1} (Σ^2 = 1.3 × 10^{-4}), respectively, for the model based on the LiATP species, 3.8 × 10^4 M^{-2} and 0.46 s^{-1} (Σ^2 = 3.0 × 10^{-3}) for the model based on the Li_2ATP species, and 8.7 × 10^2 M^{-1} and 0.14 s^{-1} for LiATP and 1.1 × 10^4 M^{-2} and 0.13

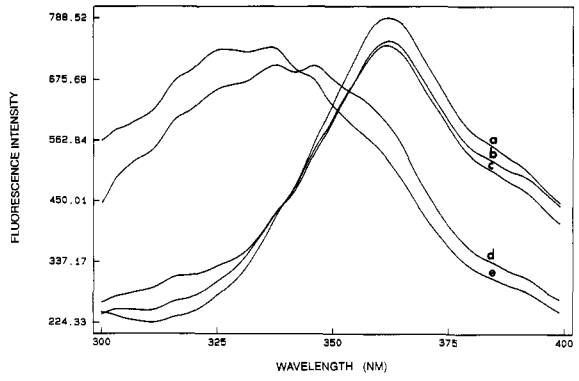

FIGURE 3: Fluorescence excitation spectra of 2 μM furaptra in the presence of (a) 0.1 mM EGTA, (b) 5.0 mM NaATP, (c) membrane (protein concentration was 2.0 ± 0.1 mg/mL), (d) 100 mM LiCl, and (e) 2.0 mM $MgCl_2$.

Table 3: Fluorescence-Determined $[Mg^{2+}]_f$ Values for ATP Solutions and RBC Membrane Suspensions[a]

	with ATP alone[b]		with membrane alone[c]		with both ATP and membrane[b,c]	
$[Li^+]$ (mM)	R^d	$[Mg^{2+}]_f$ (μM)[e]	R^d	$[Mg^{2+}]_f$ (μM)[e]	R^d	$[Mg^{2+}]_f$ (μM)[e]
0	0.85	70	1.09	220	0.65	30
5	0.91	90	1.14	420	0.66	50
10	0.95	100	1.20	480	0.69	80
20	1.04	130	1.29	600	0.73	110
50	1.19	210	1.43	800	0.90	220
100	1.31	280	1.44	610	1.02	200

[a] The reported values represent an average of measurements conducted in three separately prepared samples. All samples contained 2 μM furaptra, 1.0 mM $MgCl_2$, and 0.15 M Tris Cl (pH 7.4) at 25 °C. [b] The NaATP concentration was 2.0 mM. [c] The membrane protein concentration was 2.7 ± 0.1 mg/mL. [d] The errors in the determination of the R values (F_{335}/F_{370}) were less than 5%. [e] The $[Mg^{2+}]_f$ values were calculated from eq 4.

s^{-1} for Li_2ATP ($\Sigma^2 = 6.2 \times 10^{-5}$) for the model based on a mixture of LiATP and Li_2ATP. The Σ^2 values obtained for calculations with ^7Li data in the presence of $LiCl/MgCl_2$ mixtures also indicate that the best nonlinear least-squares fit to the ^7Li T_1 data was provided by the model based on a mixture of LiATP and Li_2ATP. The Li^+-binding constants appear to be larger in Li^+/Mg^{2+} mixtures than in the presence of Li^+ alone. The large error involved in the calculation of the binding constants suggests, however, that this difference may not be significant.

Figure 1 shows the ^{31}P NMR spectra of a Li^+ titration of an aqueous solution containing 3.0 mM NaATP and 1.0 mM $MgCl_2$ at 0 °C and pH 7.5 in the absence of human RBC membrane. Whereas the addition of up to 40 mM LiCl did not cause a significant change in the ratio of the areas of the Mg^{2+}-free and Mg^{2+}-bound β-phosphate resonances in the absence of human RBC membrane, the addition of 20 mM LiCl to an ATP-containing membrane suspension was sufficient to cause a significant increase in the area ratio (Table 2). However, we observed significant downfield shifts of the Mg^{2+}-free β-phosphate resonance upon the addition of 20 mM LiCl to ATP solutions in both the presence and absence of human RBC membrane. In both the presence and the absence of RBC membrane, the $[Mg^{2+}]_f$ values increased with an increase in the Li^+ concentration. In the absence of RBC membrane, the values of $[Mg^{2+}]_f$ increased for $[Li^+] \leq 40$ mM when no changes in the area ratios were apparent from the ^{31}P NMR spectra (Figure 1); these increases in free Mg^{2+} concentration originate from a decrease in free [ATP], as manifested by the downfield shifts observed in the Li^+ concentration range of 0–40 mM. For every Li^+ concentration studied, the $[Mg^{2+}]_f$ values were lower in the presence of membrane than in its absence (Table 2) because of additional Mg^{2+}-binding sites in the membrane.

Figure 2 shows changes in the effective CSA parameters obtained from the ^{31}P NMR powder spectra of human RBC membrane suspensions in the presence of increasing concentrations of Li^+ and Mg^{2+} ions; upon the addition of metal ions, the CSA value of 51 ± 3 ppm for membrane alone increased to 69 ± 3 and 84 ± 5 ppm in the presence of 20 mM Mg^{2+} and 200 mM Li^+, respectively. Because Mg^{2+} is divalent and Li^+ is a monovalent metal ion, smaller Mg^{2+} concentrations were sufficient to saturate the phosphate head groups of phospholipids in the RBC membrane; smaller concentrations of Mg^{2+} than of Li^+ were therefore used in these ^{31}P NMR experiments.

Fluorescence Studies. We observed a blue shift in the fluorescence excitation spectrum of 2 μM furaptra upon the addition of 2.0 mM $MgCl_2$ (Figure 3, spectra a and e), as previously reported (London, 1991). We also observed a blue shift in the fluorescence excitation spectrum of 2 μM furaptra upon the addition of 100 mM LiCl (Figure 3, spectrum d). The concentrations of Li^+ necessary to induce equivalent shifts in the λ_{max} position and intensity changes in the spectrum of the Mg^{2+}-free dye were, however, 2 orders of magnitude larger than those of Mg^{2+}. From the spectral intensity changes induced by the addition of increasing concentrations of Li^+, we calculated, from a Hill plot (see Experimental Procedures), a K_d value of 250 ± 70 mM (n = 8) and a stoichiometry of 1:1 for the Li^+–dye complex. The excitation spectrum of free dye did not change appreciably in the presence of either ATP or membrane (Figure 3, spectra b and c), indicating that there were no specific dye–ATP or dye–membrane interactions; the small decreases in fluorescence intensity at 370 nm observed upon the addition of either ATP or membrane to the dye-containing solution are attributed to dilution effects.

The fluorescence excitation spectra of furaptra in solutions containing 5.0 mM NaATP, 2.5 mM $MgCl_2$, 150 mM Tris Cl (pH 7.4), and increasing concentrations of LiCl (0–100 mM) are shown in Figure 4A; upon the addition of Li^+, the λ_{max} position was blue-shifted from 370 to 335 nm (Figure 4A, spectra a–f). The fluorescence intensities observed at 335 nm for Li^+-containing ATP solutions (no Mg^{2+} present; Figure 4B, spectrum i) were considerably lower than those observed in Li^+/Mg^{2+} mixtures (Figure 4B, spectrum j). In dye solutions containing membrane and 1.0 mM $MgCl_2$ (Figure 5A, spectrum b), the addition of 50 mM LiCl caused a further increase in the fluorescence intensity at 335 nm (Figure 5A, spectrum c); the fluorescence intensity observed in Li^+/Mg^{2+} mixtures was significantly larger than that observed in Li^+-containing RBC membrane suspensions in the absence of Mg^{2+} (Figure 5A, spectrum d). The spectra of Mg^{2+}–dye solutions containing both membrane and ATP (Figure 5B, spectrum e) resemble that of free dye in the presence of membrane (Figure 5B, spectrum a) because of the higher affinity of Mg^{2+} for ATP than for the RBC membrane. Addition of 50 mM LiCl to a Mg^{2+}–dye solution containing both membrane and ATP (Figure 5B, spectrum f) caused a smaller increase in the fluorescence intensity at 335 nm than did the addition of 50 mM LiCl to a Mg^{2+}–dye solution containing membrane, but no ATP (Figure 5B, spectrum c), at the same wavelength.

By measuring the fluorescence ratios and using eq 4, we calculated the values of $[Mg^{2+}]_f$ in 1.0 mM $MgCl_2$ solutions at 25 °C and pH 7.4 containing 2.0 mM ATP alone, membrane

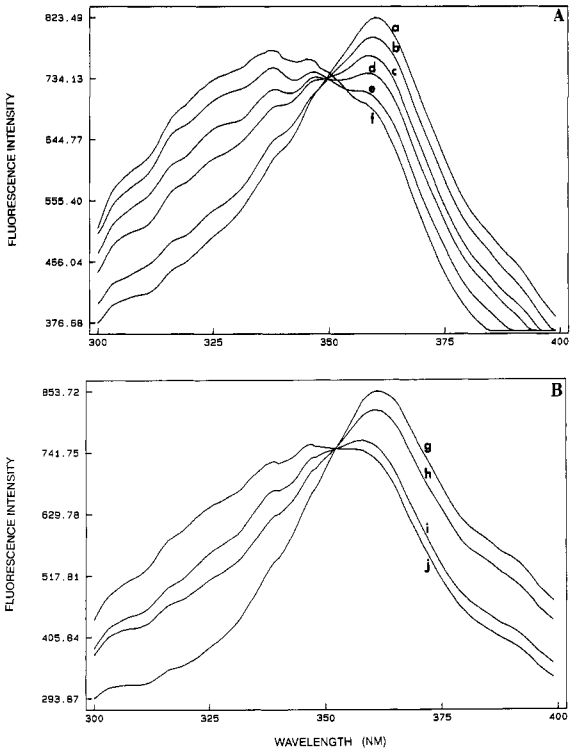

FIGURE 4: (A) Fluorescence excitation spectra of 2 μM furaptra in a solution containing 5.0 mM NaATP, 2.5 mM $MgCl_2$, 150 mM Tris Cl (pH 7.4) and (a) 0 mM LiCl, (b) 5 mM LiCl, (c) 10 mM LiCl, (d) 20 mM LiCl, (e) 50 mM LiCl, and (f) 100 mM LiCl. (B) Fluorescence excitation spectra of 2 μM furaptra in a solution containing 5.0 mM NaATP, 150 mM Tris Cl (pH 7.4) and (g) no metal ions, (h) 2.5 mM $MgCl_2$, (i) 100 mM LiCl, and (j) 2.5 mM $MgCl_2$ and 100 mM LiCl.

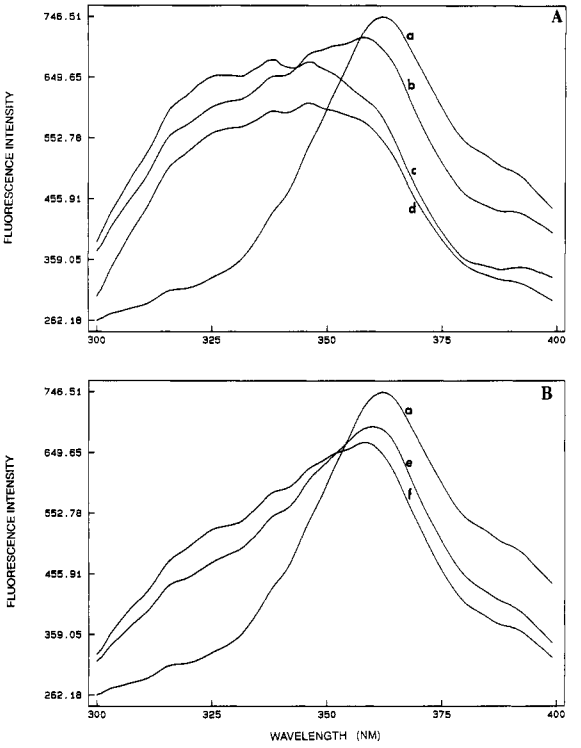

FIGURE 5: (A) Fluorescence excitation spectra of 2 μM furaptra in a membrane suspension (2.0 ± 0.1 mg/mL) in the absence of metal ions (a) and in the presence of (b) 1.0 mM $MgCl_2$, (c) 1.0 mM $MgCl_2$ and 50 mM LiCl, and (d) 50 mM LiCl. (B) Fluorescence excitation spectra of 2 μM furaptra in a membrane suspension (protein concentration was 2.0 ± 0.1 mg/mL) in the absence of metal ions (a) and in the presence of (e) 1.0 mM $MgCl_2$ and 2.0 mM NaATP and (f) 1.0 mM $MgCl_2$, 50 mM LiCl, and 2.0 mM NaATP. The solutions were buffered with 150 mM Tris Cl, pH 7.4.

alone, and both 2.0 mM ATP and membrane (Table 3). In all samples, an increase in the Li^+ concentration caused an increase in the values of $[Mg^{2+}]_f$, as expected. For a given Li^+ concentration, the $[Mg^{2+}]_f$ values obtained in the presence of ATP alone were much smaller than those calculated for membrane suspensions, because of the higher affinity of ATP for Mg^{2+} relative to membrane. The values of $[Mg^{2+}]_f$ calculated for solutions containing both ATP and membrane were smaller than those obtained for solutions containing ATP alone because of an increased number of Mg^{2+}-binding sites. Both fluorescence and ^{31}P NMR methods gave the same trends for $[Mg^{2+}]_f$ values when Li^+ and/or RBC membrane were present. For the same Li^+ concentration, the fluorescence-determined values of $[Mg^{2+}]_f$ were, however, smaller than those calculated from ^{31}P NMR measurements (Tables 2 and 3). This difference is presumably related to differences in temperature used in the fluorescence (25 °C) and NMR (0 °C) experiments and to the different sensitivities of the two techniques.

DISCUSSION

The binding sites shared by the three systems, Li^+-loaded RBCs, Li^+-loaded RBC ghosts, and unsealed RBC membrane, used in the ^7Li NMR relaxation experiments (Table 1) are located in the RBC membrane. In the three types of samples, a large difference between T_1 and T_2 values was present, and an increase in Mg^{2+} concentration led to a similar increase in ^7Li NMR relaxation values. We previously showed, by using ^7Li T_1 measurements of Li^+-containing suspensions of right-side-out and inside-out RBC vesicles, that the inner leaflet of the RBC membrane contributes mostly to Li^+ binding (Rong et al., 1993). We therefore conclude that Mg^{2+} displaced Li^+ from the inner leaflet of the RBC membrane in Li^+-loaded RBCs and Li^+-loaded RBC ghosts.

Conditional binding constants, as opposed to stoichiometric binding constants, are more useful in obtaining an understanding of competitive metal ion binding for biological ligands (Frausto da Silva & Williams, 1977). Most ligands that bind Li^+, including ATP, will also bind Mg^{2+} and Ca^{2+} with even higher affinity (Frausto da Silva & Williams, 1976). When ligands with high Mg^{2+} affinity (for example, the RBC membrane) are also present, the absolute values of the stoichiometric binding constants do not necessarily reflect the tendency of Li^+ to bind preferentially to ATP. On the basis of calculations of conditional binding constants, it was found that the organic ligands uramil diacetate and (*o*-carboxyphenyl)imino diacetate, which have a set of three oxygen and one nitrogen coordination sites, can compete for one-fourth or one-half of the Mg^{2+} bound to 3.2 mM ATP (Frausto da Silva & Williams, 1976). The free intracellular Li^+ and Mg^{2+} concentrations are on the same order of magnitude in the tissues of manic depressive patients undergoing lithium therapy; the free intracellular Ca^{2+} concentrations are, however, 4–5 orders of magnitude smaller than those of Li^+, implying that Ca^{2+} does not appreciably compete with Li^+ for binding to biological ligands.

The free intracellular Na^+ and K^+ concentrations are approximately 1–2 orders of magnitude larger than those of Li^+; any ligand with conditional binding constants that are 2 log units smaller for Na^+ and K^+ than for Li^+ will preferentially bind Li^+. Most ligands meet this criterion, and therefore competition between Li^+ and Mg^{2+} can conceivably

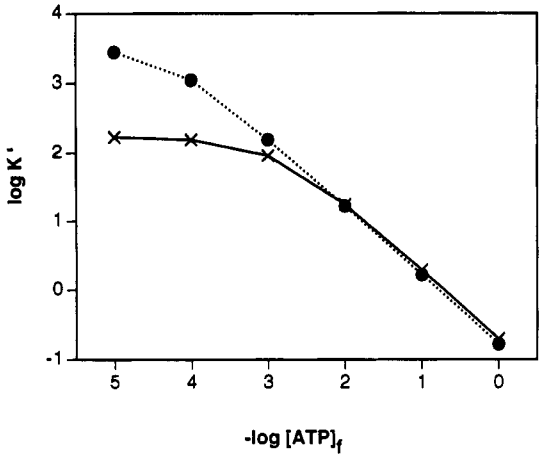

FIGURE 6: Logarithmic plots of the Mg^{2+} (●) and Li^+ (×) conditional binding constants to the RBC membrane as a function of free ATP concentration.

take place in the presence of physiologically relevant intracellular Na^+ and K^+ concentrations (Frausto da Silva & Williams, 1976). Li^+ loading of RBCs results in only small changes in intracellular Na^+ and K^+ levels (Canessa et al., 1980). Whereas packed Li^+-loaded RBCs contained high intracellular K^+ concentrations, low ionic strength buffers were used in the preparation of RBC ghosts (Steck & Kant, 1974). For unsealed RBC membrane preparations, both low and high ionic strength buffers were used (see Results). Despite the larger K^+ concentrations in Li^+-loaded RBCs and in some Li^+-containing suspensions of RBC membrane (see Results) relative to those present in suspensions of RBC ghosts and unsealed RBC membrane in low ionic strength buffer (Table 1), a large difference between ^7Li T_1 and T_2 values and a marked Mg^{2+} dependency of relaxation values were observed in all samples. We therefore conclude that physiologically relevant intracellular K^+ and Na^+ concentrations do not appreciably interfere with competition between Li^+ and Mg^{2+} for Mg^{2+}-binding sites in cell membranes. This conclusion is in agreement with those from previous ^2H and ^{31}P NMR observations, which showed that, whereas Li^+ and Mg^{2+} bind deep within the polar groups of the lipid bilayer, K^+ and Na^+ preferentially bind at the membrane surface (Roux & Bloom, 1990; Roux & Neuman, 1986).

On the basis of ^7Li T_1 data, the calculated stoichiometric binding constants of Mg^{2+} and Li^+ to the RBC membrane were approximately 3.3×10^3 (K_{Mg-M}) and 1.7×10^2 M^{-1} (K_{Li-M}), respectively. On the basis of ^{31}P chemical shift data (Abraha et al., 1991), the binding constant for the species MgATP was calculated to be approximately 2.0×10^4 M^{-1} (K_{MgATP}), whereas from ^7Li T_1 data the overall binding constants for the species LiATP and Li_2ATP were approximately 8.7×10^2 M^{-1} (K_{LiATP}) and 1.1×10^4 M^{-2} (β_{Li_2ATP}), respectively. In the presence of ATP, one can derive that the conditional binding constants for Mg^{2+} (K'_{Mg-M}) and Li^+ (K'_{Li-M}) binding to the RBC membrane are given by the following equations (Frausto da Silva & Williams, 1977):

$$K'_{Mg-M} = K_{Mg-M}/(1 + [ATP]_f K_{MgATP}) \quad (5)$$

$$K'_{Li-M} = K_{Li-M}/(1 + [ATP]_f K_{LiATP} + 2[ATP]_f[Li^+]_f \beta_{Li_2ATP}) \quad (6)$$

The conditional binding constants are dependent on the free concentrations of ATP ($[ATP]_f$) and Li^+ ($[Li^+]_f$). Figure 6 shows the calculated conditional binding constants, obtained from eq 5 and 6, as a function of free ATP concentration (in the range of 10^{-5}–1 M). Equation 6 requires an estimate of the free Li^+ concentration; to obtain an approximate concentration of free Li^+, we assumed that most Li^+ was bound to ATP in the form of Li_2ATP. For a typical total intracellular Li^+ concentration of 1.0 mM, the free Li^+ concentrations are on the order of 10^{-4} M; therefore, the term $2[ATP]_f[Li^+]_f\beta_{Li_2ATP}$ in eq 6 does not significantly affect the estimation of K'_{Li-M}. For $[ATP]_f$ values larger than 1.0 mM (Figure 6), Li^+ will bind to the RBC membrane as strongly as Mg^{2+}. Typical free intracellular ATP concentrations in RBCs are on the order of 0.2 mM (Ramasamy & Mota de Freitas, 1989). From Figure 6, we estimate, for typical free intracellular ATP concentrations ($-\log [ATP]_f = 3.69$), that the ratio of the conditional binding constants of Mg^{2+} and Li^+ to the RBC membrane is approximately 4. In contrast, in the absence of ATP, the ratio of the stoichiometric binding constants of Mg^{2+} and Li^+ to the RBC membrane is approximately 20. Using the calculated stoichiometric and conditional binding constants mentioned above and typical total concentrations of intracellular ATP and ions ($[ATP]_f$ = 2.0 mM, $[Li^+]_t$ = 1.0 mM, and $[Mg^{2+}]_f$ = 2.4 mM), as well as the estimated value of 0.8 mM membrane-binding sites (obtained from the $[Mg^{2+}]_f$ calculations in Table 3), we calculated the free metal ion concentrations in the absence of either ATP or membrane and in the presence of both. These calculations indicate that, for typical intracellular $[ATP]_f$ values, Li^+ can compete with approximately 14–21% of the Mg^{2+}-binding sites in the RBC membrane, depending on the relative values of the free intracellular Mg^{2+} and Li^+ concentrations; in the absence of ATP, however, Li^+ would compete for less than 5% of the Mg^{2+} membrane sites. Thus, in the presence of both ATP and RBC membrane, Li^+ can compete with some of the Mg^{2+} ions bound to ATP and the RBC membrane. Competition between Li^+ and Mg^{2+} for ATP (in 1:1 stoichiometries) favors Mg^{2+} binding (equilibrium constant of 4.3×10^{-2}); however, coupling of the reaction with ATP with binding of the displaced Mg^{2+} to the RBC membrane becomes energetically more favorable (equilibrium constant of 0.86). Therefore, the extent of the coupled reaction depends on the relative intracellular concentrations of Mg^{2+} and Li^+.

At room temperature, the addition of large concentrations of Li^+ (100 mM) to aqueous solutions containing 5.0 mM Tris–ATP and 0.5 mM $MgCl_2$ caused an increase in the $\delta_{\alpha\beta}$ separation measured by ^{31}P NMR spectroscopy (Abraha et al., 1991); this observation is consistent with metal ion competition for ATP. Whereas the Mg^{2+}-free and Mg^{2+}-bound β-phosphate ^{31}P NMR resonances of ATP are in the fast-exchange domain at room temperature, they are in slow exchange at around 0 °C (Sontheimer et al., 1986). It was reported recently (Brown et al., 1993) that, at 0 °C, there was no evidence for competition between Li^+ and Mg^{2+} for ATP, on the basis of the relative changes in the areas of the ^{31}P NMR resonances of the β-phosphate goups of free ATP and Mg^{2+}-bound ATP in aqueous solutions of 5.0 mM NaATP and 2.5 mM $MgCl_2$, in the presence and absence of 2.5 mM LiCl. We also found that, for low concentrations of LiCl (2.5, 10, and 20 mM), there were no appreciable increases in the areas of the Mg^{2+}-free and Mg^{2+}-bound β-phosphate ^{31}P NMR resonances of 5.0 mM NaATP (data not shown). However, in either the presence or absence of RBC membrane, we observed significant increases in the $[Mg^{2+}]_f$ values for 3.0 mM NaATP solutions containing 1.0 mM $MgCl_2$ and $[Li^+]_t \geq 20$ mM (Table 2). Clearly, a large excess of Li^+ over Mg^{2+}

is required to make it possible to detect, by ^{31}P NMR spectroscopy, competition between Li^+ and Mg^{2+} for phosphate groups in ATP in the absence of RBC membrane (Figure 1).

^{31}P NMR spectroscopy is a useful and powerful method for the study of membrane structure because the P atom present in the phosphate head group of the phospholipids is located at the surface of the membrane (Smith & Ekiel, 1984). The ^{31}P nucleus is 100% naturally abundant, making this a sensitive method. Using the effective CSA parameter of the ^{31}P NMR spectrum, which is a nonhomogeneously broadened axially symmetric powder pattern, of a RBC membrane suspension, we investigated the degree of Li^+ and Mg^{2+} interaction with human RBC membrane (Figure 2). Because of the motional rigidity of the phosphate groups, the recorded broad spectrum comprised all of the resonances that are sensitive to the motions of the P atoms in the x, y, and z directions. The observed line shape arises primarily from nonhomogeneous broadening associated with CSA; a small portion of the line broadening observed originates from homogeneous relaxation and magnetic field inhomogeneities in the membrane samples. Because all samples measured contained similar membrane concentrations, natural line widths and inhomogeneities in the magnetic field contributed equally to the measured line-broadening values. We therefore attribute the increases observed upon metal ion addition in the line-broadening values of the ^{31}P NMR resonance in membrane suspensions (Figure 2) to increases in CSA values.

^{31}P NMR spectra of human RBC membranes at protein concentrations of 7.5 ± 1.5 mg/mL in the absence of Li^+ or Mg^{2+} yielded a CSA value of 51 ± 3 ppm (n = 3). The CSA value that we obtained for the human RBC membranes in the absence of metal ions is in good agreement with the CSA value previously reported for the same cell membrane (45 ± 2 ppm) (Yeagle, 1984), which was obtained with the Hahn spin–echo pulse sequence (Rance & Byrd, 1983). CSA is a measure of the degree to which the components of the chemical shift tensor are incompletely averaged; metal ion binding to phosphate head groups affects this averaging process in the ^{31}P NMR resonance (Akutsu & Seelig, 1981; Hope & Cullis, 1980; Yeagle, 1984). The observed CSA values were larger for Mg^{2+} than for Li^+ for a similar metal ion concentration (Figure 2). The CSA values of PC vesicles have also previously been found to increase when one goes from monovalent to trivalent cations, such as Na^+, Ca^{2+}, and La^{3+} (Akutsu & Seelig, 1981), which is in agreement with our line-broadening data obtained in the presence of Li^+ or Mg^{2+}. The CSA changes shown in Figure 2 are consistent with direct metal ion binding to the phosphate head groups of membrane phospholipids; they are not due to sample variation in membrane concentration, because the membrane protein concentration was kept approximately constant in all experiments. Our data show that metal ion binding to membranes caused increased motional rigidity of the phosphate head groups, which was manifested in nonhomogeneous broadening of the ^{31}P NMR resonance.

The mechanism of metal ion binding to the RBC membrane presumably involves competition between Li^+ and Mg^{2+} ions for the phosphate head groups of anionic phospholipids, in particular PS and PI, present in the inner leaflet of the human RBC membrane. Alternatively, competition between Li^+ and Mg^{2+} ions for the active site of Mg^{2+}-activated membrane-bound enzymes may occur. Direct metal ion binding to membrane-bound proteins, in particular G proteins, may result in changes in protein–lipid interactions. The structural changes in the membrane lipids triggered by different protein–lipid interactions may include conformational changes of the phosphate head groups of phospholipids and could also explain the observed increases in CSA values obtained upon the addition of metal ions to suspensions of RBC membrane (Figure 2). It is important to note that G proteins are present in the human RBC membrane (Codina et al., 1984) and that they are known to be activated by Mg^{2+} (Avissar et al., 1988).

The λ_{max} values for the fluorescence indicator furaptra in Mg^{2+}-depleted and Mg^{2+}-saturated media are 370 and 335 nm, respectively (Raju et al., 1989). Because Li^+ and Mg^{2+} have similar chemical properties, it is not surprising that Li^+ also binds to the Mg^{2+} fluorescence indicator (Figure 3). The K_d value for the Li^+–dye complex (250 ± 70 mM; n = 8) is, however, much larger than that of the Mg^{2+}–dye complex (1.5 mM) (Raju et al., 1989), indicating that Li^+ binds to furaptra more weakly than does Mg^{2+}. The difference in metal ion affinities for the fluorescence indicator is presumably related to the difference in charges of the Li^+ and Mg^{2+} ions. The calculated free Mg^{2+} concentrations in ATP and membrane solutions take into account the weak binding of Li^+ to the dye (Table 3).

Addition of Li^+ to a solution containing 5.0 mM NaATP and 2.5 mM $MgCl_2$ (Figure 4A) or membrane (2.0 mg/mL) and 1.0 mM $MgCl_2$ (Figure 5A) caused a blue shift in the λ_{max} position from 370 to 335 nm and an increase in the fluorescence intensity at 335 nm, which are in agreement with the calculated increases in free Mg^{2+} concentration (Table 3). In the absence of Li^+, there was a larger concentration of free Mg^{2+} in membrane suspensions (Figure 5A, spectrum b) than in ATP solutions (Figure 4A, spectrum a) because of the lower affinity of Mg^{2+} for the RBC membrane (K_b = 3300 M^{-1}) relative to ATP (K_b = 20 000 M^{-1}). Again, using the calculated stoichiometric and conditional binding constants and the total ion concentrations of Table 3 (with a total membrane site concentration of 0.8 mM), we calculated $[Mg^{2+}]_f$ values in good agreement with those obtained in Table 3 and showing the same trends in their dependence on Li^+, ATP, and membrane concentrations. Both the ^7Li T_1 values (Table 1) and the fluorescence data for solutions containing both ATP and RBC membrane are consistent with displacement of Mg^{2+} by Li^+ from MgATP, assisted by binding of the released Mg^{2+} to the RBC membrane. Whereas competition between Li^+ and Mg^{2+} for phosphate groups in ATP could be detected by ^{31}P NMR spectroscopy only at low temperatures for total Li^+ concentrations ≥20 mM (Figure 1), it was clearly observable either by fluorescence spectroscopy for total Li^+ concentrations ≥5.0 mM (Figure 4A, spectra a and b) or by ^7Li T_1 measurements for total Li^+ concentrations ≥1.0 mM (see Results). The ^{31}P NMR method was less sensitive than the fluorescence method for the detection of competition between Li^+ and Mg^{2+} ions for phosphate groups in ATP because almost all of the Mg^{2+} was bound to ATP (the K_d value for the MgATP complex is 0.05 mM), whereas larger Mg^{2+} concentrations are required for saturation of the fluorescence dye. [The K_d value for the Mg^{2+}–dye indicator is 1.5 mM (London, 1991).] For the Li^+ and Mg^{2+} concentrations studied, the ^7Li NMR relaxation method is as sensitive as the fluorescence method for investigating metal ion competition for phosphate groups in ATP because the limiting relaxation rates for free Li^+ and bound Li^+ are very different; small variations in the distribution between the free and bound states of Li^+ induced by Mg^{2+} competition result in significant changes in the observed ^7Li T_1 values.

In summary, we found that, regardless of the spectroscopic method used, competition between Li^+ and Mg^{2+} for Mg^{2+}-

binding sites in biological ligands is indeed feasible. It will be interesting to investigate whether a Li^+/Mg^{2+} competition mechanism for phosphate groups in GTP bound to purified G proteins (Avissar et al., 1988; Codina et al., 1984) also occurs at therapeutic Li^+ concentrations.

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