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^{7}Li NMR Relaxation Study of Li^+ Binding in Human Erythrocytes†

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ABSTRACT: We used ^{7}Li NMR spin–lattice (T_1) and spin–spin (T_2) relaxation time measurements to investigate the binding of Li^+ in human red blood cell (RBC) suspensions. In RBCs containing 1.4 mM Li^+, the intracellular ^{7}Li NMR T_2 relaxation value (0.30 ± 0.03 s) was much smaller than the corresponding T_1 value (6.0 ± 0.1 s), yielding a ratio of T_1 to T_2 of 20. For 1.5 mM LiCl solutions whose viscosities were adjusted to 5 cP with glycerol, the values of the T_1/T_2 ratios were as follows: 49 for unsealed RBC membrane (2.0 mg of protein/mL); 4.4 for spectrin (1.9 mg/mL); 1.5 for 5.4 mM 2,3-bisphosphoglycerate (BPG); 2.2 for 2.7 mM carbonmonoxyhemoglobin (COHb); 1.6 for 2.0 mM ATP; and 1.2 for a 50/50% (v/v) glycerol–water mixture. Intracellular viscosity and the electric field gradients experienced by Li^+ when traversing the spectrin–actin network therefore are not responsible for the large values of the T_1/T_2 ratios observed in Li^+-loaded RBCs. We conclude that the RBC membrane is the major Li^+ binding site in Li^+-loaded RBCs (K_b = 215 ± 36 M^{-1}) and that the binding of Li^+ to COHb, BPG, spectrin–actin, or ATP is weak. Partially relaxed ^{7}Li NMR spectra of Li^+-containing RBC membrane suspensions indicated the presence of two relaxation components, one broad and one narrow. At the same extravesicular Li^+ and protein concentrations, the T_1 values for right-side-out RBC vesicle suspensions were at least 2-fold larger than those for inside-out RBC vesicle suspensions; the inner layer of the RBC membrane, which has a larger percentage of anionic phospholipids than the outer layer, contributes mostly to Li^+ binding.

Membrane abnormalities are believed to be present in the cells of some psychiatric and hypertensive patients (Duhm, 1992). In bipolar disorders (formerly called manic depression), evidence supporting a cell membrane abnormality hypothesis emerged from studies of Li^+ transport in human RBC[1] (Meltzer, 1991; Mendels & Frazer, 1974). Some investigators have claimed that the efficacy of the treatment of bipolar patients receiving lithium carbonate can be monitored by the steady-state RBC/plasma lithium ratio; patients who responded to lithium treatment had a higher RBC/plasma ratio than normal individuals (Ostrow et al., 1978; Pandey et al., 1977). A component of erythrocyte lithium transport has also been identified and characterized that is significantly different in a subgroup of bipolar patients receiving lithium carbonate compared to normal individuals: the Na^+–Li^+ countertransport (or exchange) system (Ostrow et al., 1978; Pandey et al., 1977; Szentistvanyi & Janka, 1979). Although some researchers have claimed that the rates of Na^+–Li^+ exchange in RBC suspensions from bipolar patients receiving lithium carbonate are lower than those of normal individuals (Frazer et al., 1978; Greil et al., 1977; Mota de Freitas et al., 1990b; Ramsey et al., 1979), other investigators failed to find any significant difference with lithium-free patients (Mallinger et al., 1983; Richelson et al., 1986). In contrast, the rates of Na^+-Li^+ exchange in RBCs from treated and untreated hypertensive patients are significantly higher than those of normotensive individuals (Canessa et al., 1980; Ramasamy et al., 1990). A major autosomal gene locus encoding a polypeptide chain of a Na^+–Na^+ exchange protein, containing a high-affinity Li^+ binding site, has been identified (Dorus et al., 1983). Polymorphism, the presence of two or more alleles in the population, could account for the variation in the rates of RBC Na^+–Li^+ exchange within groups of bipolar and hypertensive patients, as well as for the overlap with the rates of normotensive individuals.

In recent studies the focus has been on membrane phospholipids, as they are known to modulate the activity of membrane proteins and possibly ion transport processes. One study has reported an increase in PC and PS for RBC and platelet membranes of schizophrenics and a decrease in PC and PS for bipolar patients (Sengupta et al., 1981); variations in phospholipid content have also been reported for the RBC membranes of hypertensive patients (Marche et al., 1985). PS is necessary to maintain Na^+–K^+ ATPase activity, while PI modulates Ca^{2+}–ATPase activity (Deutcke & Haest, 1987). Li^+ has also been shown to inhibit the enzyme inositol-1-monophosphatase, resulting in the alteration of the phosphoinositide signal transduction (Drummond et al., 1987; Worley et al., 1988). Several phospholipid vesicle studies substantiate the idea of specific Li^+–PS interactions (Casal et al., 1987; Riddell & Arumugam, 1988; Roux & Bloom, 1990). ^{7}Li NMR studies have demonstrated that the Li^+ ion interacts weakly with PC–PG liposomes (Fossel et al., 1985; Post & Wilkinson, 1991). However, not much attention has been given to metal ion interactions in RBCs, particularly the binding of intracellular Li^+ to RBC components.

Pettegrew and co-workers (Pettegrew et al., 1987a,b) have made elegant applications of ^{7}Li NMR and fluorescence

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[1] Abbreviations: NMR, nuclear magnetic resonance; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; RBC, red blood cell; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SM, sphingomyelin; SA, spectrin–actin network; ROV, right-side-out vesicles; IOV, inside-out vesicles; BPG, 2,3-bisphosphoglycerate; Hb, hemoglobin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; $Dy(PPP)_2^{7-}$, dysprosium triphosphate.

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methods to Li+-loaded RBCs. For Li+-loaded RBC, intracellular ^{7}Li T_1 values were much longer than T_2 values; a similar difference in Li+ relaxation times was observed with Li+–agar mixtures (Pettegrew et al., 1987a). Because a large difference in ^{7}Li NMR relaxation times was observed for both Li+-loaded RBCs and agar mixtures, Pettegrew et al. (1987a) speculated that diffusion of the Li+ ion across the heterogeneous electrostatic field gradients generated by the spectrin–actin (SA) network of the RBC membrane was responsible for this phenomenon. However, the ^{7}Li relaxation properties of the SA network were not tested directly as in this study; binding of Li+ to the cytoskeletal network or to other anionic RBC components could not be ruled out by the previous ^{7}Li NMR studies (Pettegrew et al., 1987a). Fluorescence anisotropy measurements (Pettegrew et al., 1987b) showed that the presence of Li+ ion may have increased the mobility of RBC membrane surface molecules and the surrounding water structure. In model membrane studies, Li+ has been found to bind to negatively charged phospholipids such as PS (Fossel et al., 1985; Riddell & Arumugam, 1988; Post & Wilkinson, 1991; Roux & Bloom, 1990). Interestingly, PS is found predominantly in the inner leaflet of the RBC membrane (Schwartz et al., 1984).

We report here the results of a systematic ^{7}Li NMR relaxation investigation of the interactions of the Li+ ion with the following anionic components of RBCs: the inner and outer leaflets of the RBC membrane, membrane phospholipids, spectrin, hemoglobin, 2,3-bisphosphoglycerate (BPG), and ATP.

EXPERIMENTAL PROCEDURES

Materials. LiCl, NaCl, KCl, ammonium sulfate, choline chloride, glucose, sucrose, sodium phosphate, tetramethylammonium hydroxide, potassium cyanide, and potassium iron(III) hexacyanate were supplied by Aldrich. BPG, ATP (Tris form), HEPES, Tris base, Dextran T-70, DEAE–Sephadex A-50, glycerol, and sialidase were from Sigma. Packed RBCs were obtained from the Chicago Chapter of Life Source.

Preparation of Li+-Loaded RBCs and of Unsealed RBC Membrane. Packed RBCs were washed at least three times by centrifugation at 2000*g* for 6 min, with isotonic buffer containing 150 mM NaCl and 5 mM sodium phosphate (pH 7.4) at 4 °C. The plasma and buffy coat were removed by aspiration. Li+ loading of RBCs was achieved by incubation of the cells at 10% hematocrit and 37 °C in an isotonic medium containing 150 mM LiCl, 10 mM glucose, and 10 mM HEPES (pH 7.4) for 0–75 min. Under these loading conditions, the intracellular Li+ concentrations after 20 and 75 min were approximately 1.0 and 3.5 mM, respectively, as measured by atomic absorption. To remove extracellular Li+, the Li+-loaded cells were washed by centrifugation, at 7000*g* for 5 min at 4 °C, five times with an isotonic choline washing solution (CWS) containing 112.5 mM choline chloride, 85 mM sucrose, 10 mM glucose, and 10 mM HEPES (pH 7.4). Deoxygenated Li+-loaded RBCs (deoxyRBC) were prepared by gentle passage of moist nitrogen gas for 30 min through a suspension of washed Li+-loaded RBCs in an isotonic CWS at 25% hematocrit. Carbonmonoxygenated RBCs (CORBC) were prepared in a similar way by bubbling CO gas for 30 min through a Li+-loaded RBC suspension. DeoxyRBC and CORBC were then washed twice with CWS and repacked; their oxygenation states were verified by examination of their ^{31}P NMR spectra (Fabry & San George, 1983; Labotka, 1984).

Unsealed RBC membranes or ghosts were prepared by hypotonic lysis according to Steck and Kant (1974). Washed, packed RBCs were lysed in 20–40 vol of hypotonic 5 mM HEPES buffer (pH 8.0) (5H8). The membrane suspension was washed by centrifugation at 22000*g* and at 4 °C until the membrane was pale white. To avoid the possible interference of competing ions in our Li+ binding study, we modified the literature method (Steck & Kant, 1974) by replacing the sodium phosphate buffer (5P8) with a HEPES buffer whose pH was adjusted with tetramethylammonium hydroxide. Extraction of phospholipids from the RBC membrane was conducted according to published methods (Meneses & Glonek, 1988; Mota de Freitas et al., 1993). The purified phospholipids were suspended in a chloroform–methanol mixture (in a ratio of 5/2).

Preparation of Spectrin. Unsealed RBC membrane was prepared from 5P8 buffer as outlined above. The membrane sample was suspended twice in the extraction buffer, 0.3 mM sodium phosphate (pH 7.6), followed by centrifugation at 20000*g* for 30 min and incubation in 3 vol of extraction buffer at 37 °C for 20 min. The fragmented membranes were pelleted by centrifugation at 80000*g* for 1 h at 2 °C. Spectrin dimers, actins, and water-soluble proteins were present in the supernatant (Ungewickell & Gratzer, 1978). These products were characterized by SDS electrophoresis and the sample was found to contain 85–90% spectrin.

Preparation of Inside-Out (IOV) and Right-Side-Out (ROV) RBC Vesicles. Unsealed RBC membrane (1 mL), which was extracted in 5P8 buffer, was diluted to 40 mL with 0.5 mM sodium phosphate (pH 8.0) (0.5P8). After 0.5–1.5 h of incubation in ice, the membranes were pelleted at 28000*g* for 30 min, resuspended in 1 mL of 0.5P8 by vortex mixing, and passed through a No. 27 gauge needle three to five times to complete vesiculation. The vesicle suspension (2 mL) was overlayed on 3 mL of Dextran barrier (4.46 g of Dextran T-70 dissolved in 100 mL of 0.5P8, pH 8.3–8.5, d = 1.015 mg/mL). After centrifugation for 40 min at 29000*g*, the top band was collected and washed with 40 vol of 0.5P8 buffer at 29000*g* for 30 min. The preparation of ROVs was similar to that of IOVs, except for the addition of 0.1 mM $MgSO_4$ after incubation in ice (Macintyre, 1982; Steck & Kant, 1974). The two types of vesicle preparations were tested using sidedness assays of acetylcholine esterase and glyceraldehyde-3-phosphate dehydrogenase (Steck & Kant, 1974). The glyceraldehyde-3-phosphate dehydrogenase assay yielded a percentage of sidedness accessibility of 80 ± 8% for IOVs and 21 ± 3% for ROVs; in contrast, the acetylcholine esterase assay yielded a percentage of sidedness accessibility of 19 ± 4% for IOVs and 94 ± 2% for ROVs. The percentages of sidedness accessibility that we found for both IOV and ROV preparations are in agreement with those previously reported (Steck & Kant, 1974).

Preparation of Carbonmonoxy-, Deoxy-, and Methemoglobin. Washed, packed RBCs were suspended in 2 vol of cold distilled water, stirred gently for 30 min in a cold room, and restirred for another 30 min after the addition of $^1/_4$ vol of a neutral saturated ammonium sulfate solution whose pH was adjusted to pH 7.0 with NaOH. A precipitate formed, and the Hb solution was separated by centrifugation for 10 min at 18000*g*. Sulfate was removed by dialysis against 0.05 M Tris Cl and 0.001 M KCN buffer (pH 8.5); the buffer was changed at least three times every 4 h. Purification of hemoglobin was carried out by DEAE–Sephadex A-50 chromatography (Huisman & Dozy, 1965; Dozy et al., 1968). The column was prebalanced to pH 8.5 with the Tris–CN

buffer. Elution of the various Hb fractions was conducted using a pH gradient produced by Tris–CN buffers (pH 8.5–7.2) with a flow rate of 20 mL/h. To obtain COHb and deoxyHb, purified Hb was bubbled with CO or N_2 gas, respectively, for 1 h. We prepared metHb by treating the purified Hb solution (which was prepared as above except that the Tris buffer did not contain CN^-) with a slight excess of $Fe(CN)_6^{3-}$; the $Fe(CN)_6^{3-}$-treated metHb solution was purified by passage through a Sephadex G-25 column. By using optical spectroscopy, we characterized the three forms of Hb and determined their concentrations (Weissbluth, 1974; Winterbourn, 1985). The COHb preparation was more than 97% pure with less than 3% metHb; the metHb preparation contained approximately 87% metHb, 10% hemichrome, and 3% oxyHb. The deoxyHb preparation contained less than 3% metHb; by bubbling the Hb preparation with N_2 gas for 1 h most Hb was converted to the deoxy form.

Protein Concentration and Measurements of Viscosity and Osmolarity. The Coomassie Blue method was used for protein determination (Bradford, 1976). The viscosity of all solutions containing RBC components, measured with a Brookfield cone plate viscometer, was adjusted to 5 cP with glycerol. The osmolarity of all isotonic suspension media, measured with a Wescor vapor pressure osmometer, was adjusted to 300 mosM with glucose.

NMR Measurements. ^{7}Li NMR measurements were conducted at 116.5 MHz on a Varian VXR-300 NMR spectrometer, equipped with a multinuclear probe. The spectra were recorded using 10-mm NMR tubes. The probe temperature was kept constant at 37 °C. T_1 measurements of ^{7}Li NMR resonances were done by the inversion recovery method, while T_2 measurements were done by the Carl–Purcell–Meiboom–Gill method (CPMG) (Gadian, 1982). Spining was used for T_1 measurements of all samples, except for RBC suspensions for which cell settling was minimized by not spining. All T_2 measurements were conducted in nonspining samples. We calculated the T_1 and T_2 values by fitting the relaxation data to monoexponential functions using the software provided by the manufacturer of the NMR spectrometer; we obtained the slow and fast components of the T_1 and T_2 values from a line shape analysis of partially relaxed spectra (vide infra). The CPMG pulse sequence could accurately measure T_2 values ≥50 ms.

Calculation of Binding Constants. The Li^+ binding constant, K_b, to the RBC membrane was calculated from a James–Noggle plot (James & Noggle, 1969; Connors, 1987):

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

where R_{obs}, R_f, and R_b are the reciprocals of the observed (T_{1obs}), free (T_{1f}), and bound (T_{1b}) spin–lattice relaxation values, $[Li^+]_t$ is the total Li^+ concentration, and [B] is the binding site concentration. This equation is valid when $[Li^+]_t$ is large with respect to [B]. The equation also assumes 1:1 stoichiometry for the binding of Li^+ to binding sites in the RBC membrane.

RESULTS

Li^+-Loaded RBCs. Measurements of intracellular $^7Li^+$ T_1 and T_2 relaxation times for oxygenated Li^+-loaded RBCs are summarized in Table I. For all of the intracellular Li^+ concentrations studied, the T_1 values were much higher than the corresponding T_2 values. As the intracellular Li^+ concentration increased, so did T_1 and T_2 values because the fraction of free intracellular Li^+ also increased. These relaxation data are in agreement with those previously reported (Pettegrew et al., 1987a). The large difference between T_1 and T_2 values indicates that Li^+ interactions with a long correlation time (Gadian, 1982) must be present in Li^+-loaded RBCs.

Table I: ^{7}Li T_1 and T_2 Relaxation Values for Packed Li^+-Loaded RBCs and RBC Components[a]

sample	$[Li^+]$/mM	T_1/s	T_2/s	T_1/T_2
oxyRBCs[b]	1.0	5.6 ± 0.1	0.21 ± 0.04	24
	1.4	6.0 ± 0.1	0.30 ± 0.03	20
	2.3	6.3 ± 0.1	0.35 ± 0.01	18
	3.5	6.5 ± 0.2	0.46 ± 0.06	14
deoxyRBCs[b]	3.2	6.8 ± 0.2	0.41 ± 0.05	17
CORBCs[b]	3.2	6.0 ± 0.2	0.47 ± 0.06	13
2.0 mM ATP[c]	1.5	0.85 ± 0.03	0.53 ± 0.03	1.6
	3.0	0.98 ± 0.03	0.66 ± 0.00	1.5
	5.0	1.08 ± 0.01	0.69 ± 0.09	1.6
	8.0	1.22 ± 0.02	0.92 ± 0.00	1.3
2.7 mM COHb[c]	1.5	4.5 ± 0.5	2.0 ± 0.1	2.2
	3.0	5.3 ± 0.4	2.0 ± 0.3	2.7
	5.0	5.0 ± 0.6	2.0 ± 0.3	2.5
	8.0	4.9 ± 0.1	2.1 ± 0.1	2.3
2.7 mM deoxyHb[c]	1.5	4.7 ± 0.1	2.0 ± 0.2	2.4
	3.0	4.8 ± 0.1	2.1 ± 0.2	2.3
	5.0	4.6 ± 0.2	2.0 ± 0.1	2.3
	8.0	5.0 ± 0.2	2.1 ± 0.1	2.4
2.7 mM metHb[c]	1.5	4.2 ± 0.3	1.5 ± 0.1	2.8
	3.0	4.4 ± 0.1	1.7 ± 0.1	2.6
	5.0	4.3 ± 0.2	1.6 ± 0.1	2.7
	8.0	4.4 ± 0.4	1.7 ± 0.1	2.6
5.4 mM BPG[c]	1.0	1.17 ± 0.01	0.76 ± 0.05	1.5
	3.0	1.18 ± 0.02	0.73 ± 0.05	1.6
	5.0	1.17 ± 0.09	0.77 ± 0.04	1.5
	8.0	1.25 ± 0.03	0.94 ± 0.10	1.3
spectrin[c] (1.9 g of protein/mL)	1.5	3.0 ± 0.1	0.68 ± 0.10	4.4
	3.0	3.3 ± 0.2	0.77 ± 0.12	4.3
	5.0	3.7 ± 0.2	0.95 ± 0.20	3.9
membrane[c] (2.0 mg of protein/mL)	1.5	3.4 ± 0.1	0.07 ± 0.01	49
	3.0	4.2 ± 0.1	0.08 ± 0.01	52
	5.0	4.7 ± 0.2	0.13 ± 0.01	39
	8.0	5.1 ± 0.1	0.17 ± 0.02	30
phospholipid extract[d] (90 μg/mL)	1.5	0.56 ± 0.01	0.09 ± 0.01	6.2
	3.0	0.75 ± 0.01	0.12 ± 0.01	6.3
	5.0	0.91 ± 0.04	0.18 ± 0.02	5.1
	8.0	1.12 ± 0.02	0.34 ± 0.03	3.3

[a] Each value is expressed as the mean ± standard deviation of calculated values (see Experimental Procedures) obtained with two separately prepared samples, except for RBCs where four samples were studied. [b] The intracellular Li^+ concentrations were measured by atomic absorption after hypotonic lysis of Li^+-loaded RBCs. [c] Sample viscosity adjusted to approximately 5 cP with glycerol at 37 °C; the pH value was in the range 7.4–8.0. [d] Sample viscosity was approximately 0.5 cP at 20 °C.

The viscosity of the intracellular volume in RBCs is about 5 cP (Morse et al., 1979). Viscosity alone could be responsible for the observed difference in intracellular $^7Li^+$ T_1 and T_2. We therefore measured ^{7}Li T_1 and T_2 values for glycerol–water solutions of 1.5 mM LiCl. The viscosity of these samples was in the 0.7–5-cP range and was obtained with 0/100% to 50/50% (v/v) glycerol–water mixtures. Unlike RBCs containing 1.4 mM intracellular Li^+, in which there was a large difference between T_1 and T_2 values (Table I), for 1.5 mM LiCl in 50/50% glycerol–water (with a viscosity of 5 cP) there was only a slight difference between the T_1 (4.6 ± 0.1 s) and T_2 (4.0 ± 0.2 s) values. The T_1/T_2 ratio obtained for the viscosity-adjusted 1.5 mM LiCl solution was 1.2, which is considerably less than the T_1/T_2 ratio observed for RBCs loaded with 1.4 mM intracellular Li^+ (20; see Table I). The values that we obtained for Li^+-containing glycerol–water mixtures are in agreement with previously reported data (Pettegrew et al., 1987a). The large difference in intracellular

RBC $^7Li^+$ T_1 and T_2 values therefore is not due to viscosity effects.

We measured the ^7Li T_1 and T_2 values for packed deoxyRBC and packed CORBC which were loaded with 3.2 ± 0.2 mM Li^+. The T_1 and T_2 values for Li^+-loaded deoxyRBC were 6.8 ± 0.2 and 0.41 ± 0.05 s (T_1/T_2 = 17, n = 4), whereas the NMR relaxation parameters for Li^+-loaded CORBC were T_1 = 6.0 ± 0.2 s and T_2 = 0.47 ± 0.06 s (T_1/T_2 = 13, n = 4). The deoxy form of Hb present in N_2-treated RBCs is paramagnetic. In contrast, the CO form of Hb present in CO-treated RBCs is diamagnetic. At similar intracellular Li^+ concentrations, the values of the T_1/T_2 ratios found in packed Li^+-loaded CORBCs are significantly smaller (paired student's t-test, $p < 0.05$) than the values obtained in packed Li^+-loaded deoxy RBCs and oxygenated RBCs (Table I), suggesting that the paramagnetic relaxation induced by deoxyHb is small. The small paramagnetic susceptibility effect was present in deoxyRBCs prepared from two different blood batches, indicating that the variations in T_1 and T_2 values in different oxygenation states do not represent biological variability. Oxygenated Li^+-loaded RBCs may contain trace amounts of paramagnetic deoxyHb and metHb; not surprisingly, the values of T_1, T_2, and T_1/T_2 for oxygenated RBCs are intermediate between those observed for CORBCs and those for deoxyRBCs. Because the large difference in intracellular ^7Li T_1 and T_2 values was present in packed Li^+-loaded RBCs regardless of the state of oxygenation, we conclude that ^7Li NMR relaxation in RBCs is not controlled by paramagnetic relaxation induced by high-spin Fe^{2+} or by Fe^{3+} present in deoxyHb or metHb, respectively.

External Li^+ Binding Sites. Sialic acid residues are negatively charged and are located on the outer leaflet of the RBC membrane; they could be potential binding sites for the Li^+ ion. Li^+-free RBCs were incubated with 0.1 μM sialidase for 45 min at 37 °C to cleave off the sialic acid residues. The RBCs were washed at least three times with isotonic choline buffer containing 1.5 mM LiCl. To test for the possible interaction of Li^+ with sialic acid residues, the extracellular ^7Li NMR relaxation times were measured for sialidase-treated and untreated Li^+-free RBC suspensions. The relaxation parameters were unchanged by sialidase treatment (T_1 = 17.0 ± 0.2 s and T_2 = 16.5 ± 0.3 s, n = 3) and were comparable to those obtained in 1.5 mM LiCl solutions, indicating that Li^+ does not interact with sialic acid sites.

Internal Li^+ Binding Sites. We prepared ATP solutions containing 1.5–8.0 mM Li^+, whose viscosities were adjusted to 5 cP with glycerol. The ^7Li T_1 and T_2 values for the Li^+-containing ATP solutions are shown in Table I. The T_1/T_2 ratio ranged from 1.3 to 1.6, indicating that at the Li^+ concentrations typically present in Li^+-loaded RBCs Li^+ binds weakly to ATP.

Studies on Li^+ binding to Hb have been reported previously (Bull et al., 1973; Pettegrew et al., 1987a) and have shown very weak Li^+–Hb interactions. We have confirmed these observations by measuring ^7Li T_1 and T_2 relaxation times for freshly prepared 2.7 mM COHb, deoxyHb, and metHb solutions containing 1.5–8.0 mM Li^+ (Table I). ^7Li T_1 and T_2 relaxation times were also measured for spectrin (1.9 mg/mL) and 5.4 mM BPG; the values of the T_1/T_2 ratios also indicate weak Li^+ binding to these RBC components (Table I). In solutions of ATP, BPG, and spectrin, an increase in Li^+ concentration resulted in small increases in both the T_1 and T_2 values; this effect was also observed for Li^+-loaded RBCs (Table I) and is due to an increase in the mole fraction of free Li^+. The absolute values of T_1 and T_2 as well as the values of the T_1/T_2 ratios observed in Hb and spectrin solutions are significantly larger than those observed in ATP and BPG solutions; the larger difference between the T_1 and T_2 values observed in Hb and spectrin solutions is associated with the longer correlation times for Li^+ bound to the high molecular weight proteins Hb and spectrin (Pettegrew et al., 1987a).

The large difference in intracellular ^7Li T_1 and T_2 relaxation values characteristic of Li^+-loaded RBCs is also present in unsealed RBC membrane suspensions whose viscosities were adjusted to 5 cP with glycerol (Table I), indicating that binding of Li^+ to the RBC membrane is responsible for the unique relaxation behavior of Li^+-loaded RBCs. The NMR parameters for a 1.5 mM Li^+-containing RBC membrane suspension (2.0 mg/mL), whose viscosity was not adjusted with glycerol (1.7 cP at 37 °C), were T_1 = 9.1 ± 0.1 s and T_2 = 0.13 ± 0.02 s (T_1/T_2 = 70, n = 2). The globular Hb protein is present in RBCs but absent in RBC membrane suspensions; the larger values of the T_1/T_2 ratios observed in RBC membrane suspensions relative to those found with intact RBCs are presumably due to differences in viscosity between the two samples and additional Li^+ binding sites in packed RBCs.

We also conducted relaxation measurements on a 1.5 mM Li^+-containing phospholipid extract from the RBC membrane suspended in a methanol–chloroform mixture (see Experimental Procedures); we observed a significant difference between T_1 and T_2 values (T_1 = 0.56 ± 0.01 s, T_2 = 0.09 ± 0.01 s, T_1/T_2 = 6.2, n = 2) for solubilized membrane phospholipids (Table I). The absolute values of T_1 and T_2, as well as those of the T_1/T_2 ratios observed in suspensions of phospholipids extracted from the RBC membrane, were significantly smaller than those observed in unsealed RBC membrane suspensions because of differences in phospholipid concentration, viscosity, and solvent composition. The smaller values of T_1 and T_2 and the smaller difference between T_1 and T_2 values observed in suspensions of phospholipids are related, in part, to the shorter correlation time for Li^+ bound to the low molecular weight phospholipids. In addition, Li^+ binding to randomly oriented phospholipids obtained from solubilized RBC membrane is weaker than for unsealed RBC membrane fragments; the oriented head groups of the phospholipids at the surface of the membrane bilayer provide a large concentration of negative charges to which Li^+ can bind and thus afford larger T_1/T_2 ratios. The T_1/T_2 ratio decreased from 6.2 to 1.6 when the LiCl concentration increased from 1.5 to 20 mM in suspensions of phospholipids. In contrast to the behavior of ATP, BPG, and spectrin, the large dependence of the observed ^7Li relaxation values on Li^+ concentration suggests that phospholipids are the membrane components responsible for Li^+ binding.

To determine which side of the RBC membrane contributes the most toward Li^+ binding, we measured ^7Li T_1 values for IOV and ROV suspensions containing Li^+ in the 2.0–9.0 mM range (Figure 1). The preparations of the two types of RBC vesicles were characterized by glyceraldehyde-3-phosphate dehydrogenase and acetylcholine esterase sidedness assays. Whereas glyceraldehyde-3-phosphate dehydrogenase is located in the inner leaflet of the RBC membrane, acetylcholine esterase is located in the outer leaflet. The percentages of sidedness accessibility that we found (see Experimental Procedures) agree with literature values (Steck & Kant, 1974). We also added a shift reagent, 3.0 mM $Dy(PPP)_2^{7-}$, to the RBC vesicle suspensions and observed only one ^7Li NMR resonance; the ^7Li T_1 data obtained in RBC vesicle suspensions therefore are due to extravesicular Li^+ and not due to an average of intra- and extravesicular Li^+. This follows from

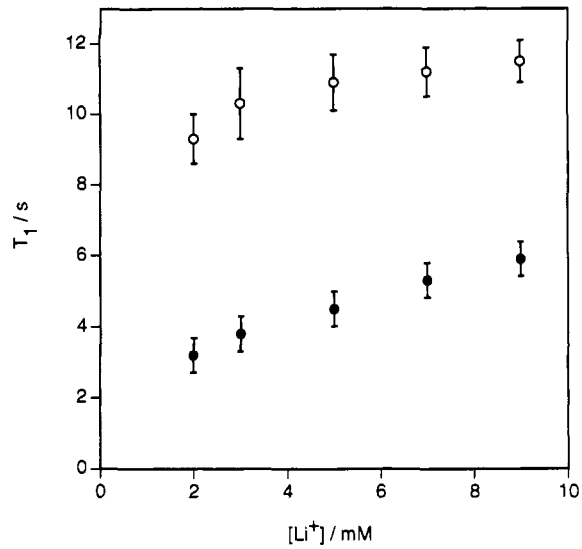

FIGURE 1: ^{7}Li T_1 values for Li$^+$-containing IOV (●) and ROV (○) suspensions. The data reported are averages of four separately prepared samples. The membrane protein concentrations in the IOV and ROV preparations were 3.5 ± 0.3 and 3.5 ± 0.7 mg/mL, respectively. At the same extravesicular Li$^+$ concentration, the T_1 values observed in ROV suspensions were at least 2-fold larger than those measured in IOV suspensions.

the known small size of RBC vesicles; the small amount of intravesicular Li$^+$ and the relatively poor sensitivity of ^{7}Li NMR spectroscopy preclude the detection of intravesicular Li$^+$.

For the same extravesicular Li$^+$ and membrane concentrations, the T_1 values observed in ROV suspensions are 2–3 times larger than those found in IOV suspensions (Figure 1). Because ROVs were generated from a Mg^{2+}-containing buffer (0.5P8-0.1Mg), Mg^{2+} may compete with Li$^+$ for binding sites on the surface of ROVs; this metal ion competition could provide an alternative explanation for the larger T_1 values observed in ROV suspensions. To rule out this latter possibility, we conducted a control experiment where we added 0.1 mM $MgSO_4$ to an IOV suspension containing 7.0 mM Li$^+$. Although the T_1 value increased by 15% (from 5.3 to 6.1 s), the T_1 value observed in Mg^{2+}-containing IOV suspensions (6.1 s, n = 2) is significantly shorter than that observed for ROV suspensions containing 7.0 mM Li$^+$ (11.2 s, n = 4). The large ^{7}Li T_1 values observed in ROV suspensions relative to IOV suspensions indicate weaker binding of Li$^+$ to the outer leaflet of the RBC membrane than to the inner leaflet; the difference in ^{7}Li T_1 values in ROV and IOV suspensions is not due to competition between Li$^+$ and Mg^{2+} for binding sites in the RBC membrane.

^{7}Li Relaxation Behavior and Binding to RBC Membrane. To understand the relaxation behavior of Li$^+$ in the presence of RBC membrane, we measured partially relaxed ^{7}Li NMR spectra of 20 mM LiCl in the presence of 6.0 ± 0.2 mg/mL unsealed RBC membrane (Figure 2). The spectra shown in Figure 2 are representative of spectra obtained for four separately prepared RBC membrane suspensions containing 20 mM LiCl. As the negative intensity decreased, we observed that the broad (or fast) component nulled and the $\Delta\nu_{1/8}/\Delta\nu_{1/2}$ value reached a minimum for a τ value of 5.6 ± 0.6 s (6.3 s for the sample shown in Figure 2); on division by ln 2 it gives a T_{1f} value of 8.0 ± 0.8 s (n = 4). For τ = 5.6 ± 0.6 s, only the narrow (or slow) component was observed; a value of 0.19 ± 0.02 s (n = 4) for T_{2s} was calculated by fitting the $\Delta\nu_{1/2}$ value into the equation, $T_{2s} = (\pi\Delta\nu_{1/2})^{-1}$. For a τ value of 6.6 ± 0.6 s (7.8 s for the sample shown in Figure 2), the

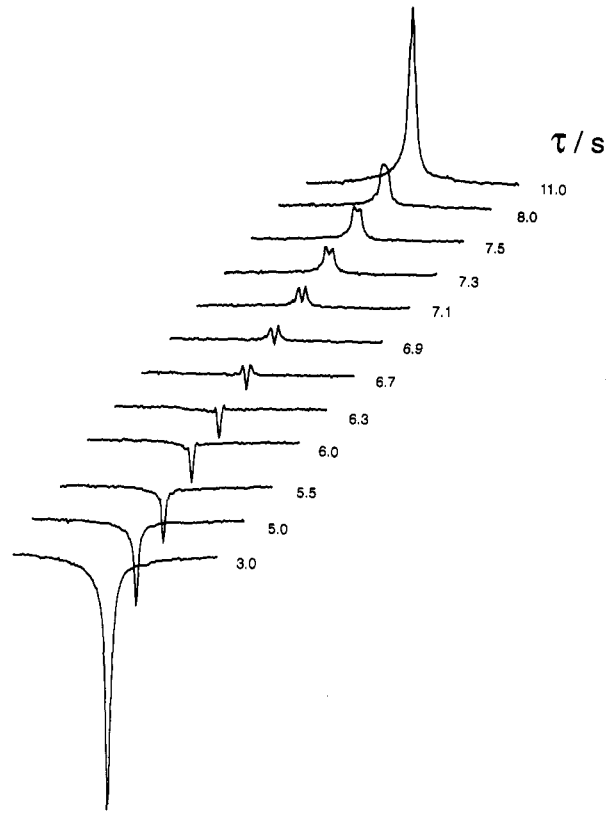

FIGURE 2: Partially relaxed ^{7}Li NMR spectra (at 20 °C) for 20 mM LiCl in the presence of unsealed RBC membrane (6.0 ± 0.2 mg/mL). The pulse sequence $(D\text{–}180°\text{–}\tau\text{–}90°)_n$ was used for recording the ^{7}Li NMR spectra. The interpulse delay (τ) values are indicated at the side of the spectra, and the preacquisition delay (D) values were 10 times the value of T_1. Each spectrum was obtained by averaging 30 transients (n). Only spectra close to the null point are shown. Biexponential behavior of the ^{7}Li resonance indicates the presence of broad and narrow relaxation components.

$\Delta\nu_{1/8}/\Delta\nu_{1/2}$ value is close to 2.7, indicating that the narrow component reached its null point making only the broad component visible; the T_{1s} and T_{2f} values calculated from the values of τ and $\Delta\nu_{1/2}$ were 9.4 ± 0.9 and 0.06 ± 0.02 s (n = 4), respectively. From the T_{1f} and T_{1s} values and the integrated areas at the null points, we estimated that the experimental ratio of fast to slow relaxation components was 53/47. The equation relating T_2 to $\Delta\nu_{1/2}$ is valid only when field inhomogeneity effects are negligible. The T_2 and T_2^* values obtained by the CPMG pulse sequence or by line width measurements were 0.11 ± 0.01 and 0.06 ± 0.01 s, respectively. The estimated values for the spin–spin relaxation time of the slow and fast components are therefore T_{2s}^* and T_{2f}^*, which are slightly smaller than the true T_{2s} and T_{2f} values.

For a single Lorentzian line, the ratio of $\Delta\nu_{1/8}/\Delta\nu_{1/2}$ should be approximately 2.7 (Urry et al., 1989). At a pulse interval, τ, of 0.75 s (spectrum not shown), the $\Delta\nu_{1/8}/\Delta\nu_{1/2}$ value of 3.4 is larger than the theoretical value of 2.7; the inverted resonance observed for τ = 0.75 s is therefore composed of broad and narrow components. Similarly, the ^{7}Li NMR resonance observed for τ = 60 s (spectrum not shown) yielded a $\Delta\nu_{1/8}/\Delta\nu_{1/2}$ value of 3.5, also indicating biexponential relaxation for the ^{7}Li nucleus in the presence of RBC membrane. Whereas biexponential relaxation was observed for membrane suspensions containing 20 mM LiCl, only one relaxation component with Lorenztian line shape was observed in membrane suspensions (with the same protein concentration) containing 150 mM LiCl or in a glycerol–water mixture containing 150 mM LiCl (no membrane). Therefore, a large

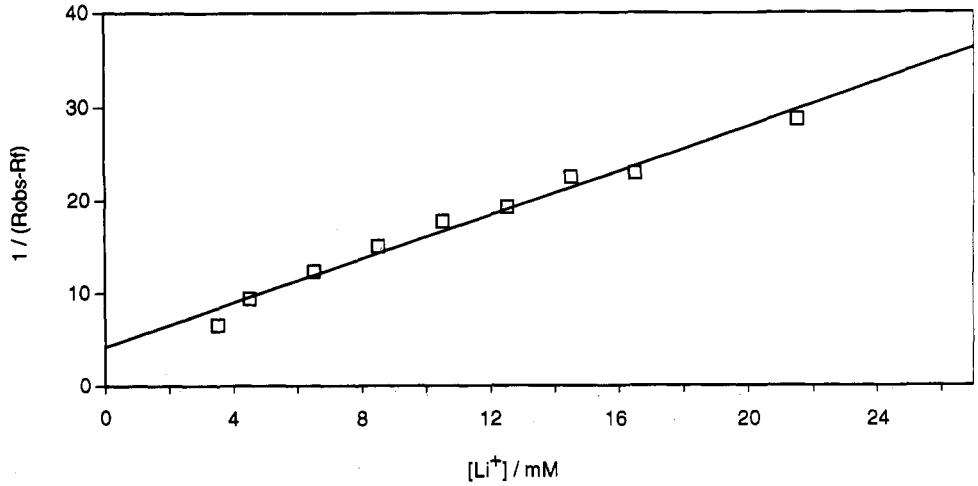

FIGURE 3: Plot of $1/\Delta R$ versus $[Li^+]$ for a Li^+-containing RBC membrane suspension. The membrane protein concentration was 3.0 ± 0.5 mg/mL. The T_1 values were obtained with the standard inversion recovery method. The value of K_b at 37 °C was 215 ± 36 M^{-1} ($r^2 > 0.95$, $n = 10$).

fraction of bound Li^+ must be present in the membrane suspension to observe biexponential relaxation.

We measured the areas of the ^7Li NMR resonances and the T_1 values in RBC membrane suspensions (at a protein concentration of 3.0 ± 0.5 mg/mL) containing Li^+ in the range of 3.5–22 mM (Figure 3). The areas of the ^7Li NMR resonances were directly proportional to the Li^+ concentrations present in the RBC membrane suspension, and in the presence of RBC membrane, the areas were at least 95% of the areas observed in the absence of membrane, indicating that there were no significant changes in the ^7Li NMR observable pool of Li^+ ions. From the observed ^7Li T_1 values, we calculated the binding constant of Li^+, K_b, and the concentration of Li^+ binding sites, [B], in the RBC membrane by using eq 1; the K_b value was 215 ± 36 M^{-1} ($r^2 \geq 0.95$, $n = 10$), and the value of [B] was in the range of 1–3 μM. The total concentration of Li^+ was therefore much larger than the concentration of Li^+ binding sites in the RBC membrane, confirming the assumption made in the derivation of eq 1. The K_b value that we obtained for Li^+ binding to the RBC membrane is in the same range as those measured by using ^{23}Na NMR relaxation times for Na^+ binding to the RBC membranes from hypertensive patients and normotensive individuals, 100–1000 M^{-1} (Ong & Cheung, 1986; Urry et al., 1980). Alkali metal relaxation measurements have been used previously to probe multiple binding sites, as in the case of gramicidin (Urry et al., 1989). The fit of our ^7Li T_1 data yielded only one K_b value; our relaxation measurements could not distinguish between subclasses of Li^+ binding sites in the human RBC membrane, presumably because the electric field gradients and the Li^+ affinities at the various binding sites within the membrane are not sufficiently different (Urry et al., 1980).

DISCUSSION

Slow motions contribute only to T_2, whereas fast motions such as those components of motions at the resonance frequency contribute to both T_1 and T_2 (Gadian, 1982). The observation of a large difference between $^7Li^+$ T_1 and T_2 values in Li^+-loaded RBCs (Table I) is indicative of a long correlation time for intracellular Li^+. When Li^+ ions are subject to substantial electric field gradients or are immobilized in the membrane, the ^7Li T_1/T_2 ratio increases; the larger the ratio, the stronger the interaction. We therefore used ^7Li T_1/T_2 ratio measurements to examine the internal and external Li^+ binding sites in RBC suspensions.

On the basis of the known RBC composition, one can predict what the Li^+ binding sites might be. Glycophorins account for 90% of the total sialic acid residues, and thus the outer cell surface has a negative charge (Steck, 1974). In principle, these residues can interact with or bind the positively charged Li^+ ion. No difference was found between the ^7Li T_1 and T_2 values of the extracellular Li^+ resonance for RBC suspensions treated and untreated with sialidase; the presumably weak interaction between Li^+ ions and sialic acid residues does not account for the large T_1/T_2 ratio observed in Li^+-loaded RBCs. It is possible that not all of the sialic acid residues were released by treatment with sialidase. However, if specific sialic acid–Li^+ interactions were present, one would expect an increase in ^7Li relaxation times after treatment with sialidase, which was not observed. The relaxation data do not support the presence of specific sialic acid–Li^+ interactions.

RBCs are often thought of as packets of Hb. Intracellular Li^+ may also be interacting with hemoglobin. In the present study, Li^+ was also found to bind weakly to Hb (Table I). Our Hb data are in agreement with published results (Bull et al., 1973; Pettegrew et al., 1987a), which indicate weak Li^+–Hb interactions. We also found that the paramagnetic properties of deoxyHb or metHb, which might be present in partially oxygenated Li^+-loaded RBCs, are not responsible for the large value of the T_1/T_2 ratio. The small value of the T_1/T_2 ratio obtained for Li^+-containing glycerol–water mixtures indicates that the high intracellular viscosity, which is associated with large concentrations of Hb in RBCs, is not responsible for the large difference between ^7Li T_1 and T_2 values.

Pettegrew and co-workers (Pettegrew et al., 1987a) measured the ^7Li T_1 and T_2 relaxation values for RBCs incubated with 50 mM Li^+ (concentration expressed over total volume of cells and suspension medium) and found them to be approximately 5.1 and 0.15 s, respectively. They speculated that the large difference in relaxation times was due to the diffusion of Li^+ ions across the heterogeneous electrostatic field gradients generated by the SA network of the RBC membrane. However, no direct investigation of Li^+ diffusion through the SA network or of Li^+–SA binding was conducted by Pettegrew et al. (1987a); their speculation was based on measurements obtained with agar gels. In this study, we investigated directly the contributions of Li^+ diffusion through the SA network and those of Li^+–spectrin interactions by measuring ^7Li T_1 and T_2 values in spectrin solutions containing LiCl (1.5–5.0 mM) (see Table I). From the small values of

T_1/T_2 ratios observed in Li^+-containing spectrin solutions, we conclude that diffusion of the Li^+ ion through the SA network is not responsible for the large T_1/T_2 ratio present in Li^+-loaded RBCs. From the small dependence of the observed ^7Li relaxation values on Li^+ concentration and the small values of the T_1/T_2 ratios, we conclude that only weak Li^+ interactions with SA are present for the Li^+ levels typically present in RBCs. Ca^{2+}, and to a smaller extent Mg^{2+}, binds strongly to erythroid spectrin (Wallis et al., 1993); it is therefore unlikely that therapeutic concentrations of Li^+ would compete with physiological intracellular concentrations of Ca^{2+} and Mg^{2+}. The small values, and the small concentration dependence, of the T_1/T_2 ratios observed with Li^+ solutions containing physiological concentrations of BPG and ATP (Table I) also indicate weak Li^+–BPG and Li^+–ATP interactions. Human RBCs contain millimolar concentrations of Mg^{2+}, a metal ion with high affinity for ATP and BPG (Abraha et al., 1991; Smith & Martell, 1974); the extent of Li^+ binding to ATP and BPG in the presence of Mg^{2+} ions is expected to be even weaker.

The large values and the dependence on Li^+ concentration of the T_1/T_2 ratios observed with unsealed RBC membrane suspensions (Table I) indicate that the large difference between T_1 and T_2 values observed in Li^+-loaded RBCs is due to specific interactions between Li^+ and membrane binding sites. We also observed significant differences between T_1 and T_2 values for suspensions of phospholipids extracted from the RBC membrane (Table I), suggesting that phospholipids, but not proteins, provide the major Li^+ binding sites. Because stronger Li^+ binding was observed for extravesicular Li^+ in the presence of IOV than ROV (Figure 1), and no binding occurred for extracellular Li^+ in Li^+-free RBC suspensions, we conclude that the inner leaflet of the RBC membrane provides the major Li^+ binding sites in Li^+-loaded RBCs. The lipids of the erythrocyte membrane are asymmetrically distributed (Schwartz et al., 1984). The outer leaflet of mature human RBC contains approximately 40–50% PC, 40–50% sphingomyelin (SM), and 10–15% phosphatidylethanolamine (PE) of the total outer leaflet phospholipids, whereas the inner leaflet contains approximately 10–20% PC, 10% SM, 40–50% PE, 20–30% PS, and 1.4% PI of the total inner leaflet phospholipids (Cullis & Hope, 1985; Schwartz et al., 1984; Surgenor, 1974). Both anionic phospholipids, PS and PI, are found only in the inner leaflet. The intrinsic binding constants for interactions between some alkali and alkaline earth metal ions and PS have been reported (Newton et al., 1978): 0.8 M^{-1} for PS–Na^+, 4.0 M^{-1} for PS–Mg^{2+}, and 35 M^{-1} for PS–Ca^{2+}. Evidence for Li^+ interactions with PS-containing liposomes was previously obtained from ^7Li relaxation data (Riddell & Arumugam, 1988; Post & Wilkinson, 1991; Roux & Bloom, 1990). Therefore, it is likely that the anionic phospholipids PS and PI present in the inner leaflet of the RBC membrane contribute to Li^+ binding (Figure 3). The SA network is also present in the inner leaflet of the RBC membrane. For similar protein concentrations, however, the value of the T_1/T_2 ratio for spectrin solutions containing 1.5 mM Li^+ was 4.4, whereas that of RBC membrane suspensions was 49 (Table I). We therefore conclude that the SA network is not responsible for the enhanced Li^+ binding present in IOV suspensions (Figure 1).

Apparent affinity binding constants for Na^+ and Li^+ ions to the internal and external binding sites of the RBC Na^+–Li^+ exchange (countertransport) membrane protein were previously determined from Lineweaver–Burk plots (Hannaert & Garay, 1986; Sarkadi et al., 1978); on both membrane surfaces, a 15–18-fold preference of Li^+ over Na^+ was found. Interestingly, the absolute values for the binding affinities for Li^+ and Na^+ were 3-fold greater for the internal than for the external binding sites. The difference in ion affinities on the two RBC membrane surfaces may be due to excess internal negative charge associated with the presence of the anionic phospholipids PS and PI in the inner RBC membrane surface. Previous studies (Marche et al., 1985; Mota de Freitas et al., 1993; Sengupta et al., 1981) on the lipid composition of RBC membranes from bipolar and hypertensive patients have shown that the amounts of PS and PI are different from those present in RBCs from normotensive individuals.

Li^+ binding to RBC anionic phospholipids may be affecting the extent of lipid–protein interactions in the RBC membrane. Since the Na^+–Na^+ exchange protein, which mediates RBC Na^+–Li^+ countertransport, is a membrane protein, it is feasible that different extents of Li^+ binding to phospholipids, and in turn different extents of interactions between anionic phospholipids and the membrane-bound Na^+–Na^+ exchange protein, could be responsible for the variations in RBC Li^+ countertransport reported for bipolar (Frazer et al., 1978; Mota de Freitas et al., 1990b; Ramsey et al., 1979) and hypertensive (Canessa et al., 1981; Ramasamy et al., 1990) patients relative to normotensive individuals. We have recently found (Mota de Freitas et al., 1993) significant differences in Li^+ affinity and PS content between the RBC membranes from lithium-treated bipolar patients and those from matched normal individuals; changes in lipid–protein interactions in the RBC membrane of lithium-treated bipolar patients presumably result in stronger Li^+ binding to membrane phospholipids and in slower rates of RBC Na^+–Li^+ exchange. Although the Na^+–Na^+ exchange protein is known to bind Li^+ with high affinity (Hannaert & Garay, 1986; Sarkadi et al., 1978), its low abundance in the RBC membrane makes it unlikely that Li^+ binding to the Na^+–Na^+ exchange protein is solely responsible for the drastically short intracellular ^7Li T_2 values observed in Li^+-loaded RBC suspensions. The contribution of the Na^+–Na^+ exchange toward the observed ^7Li T_1/T_2 ratio in Li^+-loaded RBCs cannot be determined directly at the present time, because the RBC Na^+–Na^+ exchange membrane protein has not been isolated yet, and highly specific transport inhibitors are not available.

The ^7Li nucleus has a nuclear spin, I, of $^3/_2$, and is therefore a quadrupolar nucleus. The quadrupole moment of ^7Li is, however, small (Mason, 1987). Nuclear Overhauser enhancement measurements and H_2O–D_2O exchange experiments showed that dipolar coupling to ^1H contributes approximately 20% toward the relaxation of intracellular Li^+ in RBC suspensions (Gullapalli et al., 1991; Pettegrew et al., 1987a). Contributions from spin rotation, chemical shift anisotropy, and scalar relaxation mechanisms have also been ruled out (Gullapalli et al., 1991). Our observations of similar T_1/T_2 ratios for RBCs bubbled with either N_2 or CO and of small T_1/T_2 ratios for both paramagnetic (deoxyHb and metHb) and diamagnetic (COHb) forms of Hb (Table I) indicate that paramagnetic relaxation is not an important relaxation mechanism for intracellular Li^+ in RBCs. Despite its small quadrupole moment, the major mechanism for relaxation of the ^7Li nucleus in Li^+-loaded RBCs is therefore quadrupolar relaxation.

Under the extreme narrowing condition ($\omega^2\tau^2 \ll 1$, where ω is the NMR observation frequency and τ is the correlation time), the T_1 value should be equal to the T_2 value (Mota de Freitas, 1993). Because the intracellular ^7Li T_1 values are significantly larger than the T_2 values in Li^+-loaded RBCs

(Table I), the extreme narrowing condition does not apply to the relaxation of intracellular Li^+. Outside the domain of motional extreme narrowing, and assuming that the relaxation and exchange times in the bound state are much shorter than those in the free state, the quadrupolar relaxation decay for a nucleus with $I = {}^3/_2$ is biexponential (Bull, 1972). In similarity to the two-state model for spin ${}^3/_2$ nuclides undergoing chemical exchange, a model that assumes asymmetric continuous distribution of correlation times for the fluctuating electric field gradients experienced by the spin ${}^3/_2$ nuclides in biological samples also predicts biexponential relaxation (Rooney & Springer, 1991). A Lorentzian line shape, which is observed in the extreme narrowing condition, is characterized by a $\Delta\nu_{1/8}/\Delta\nu_{1/2}$ ratio of 2.7 (Urry et al., 1989). Outside the domain of motional extreme narrowing, however, a non-Lorentzian line shape is observed that can be deconvoluted into a narrow Lorentzian curve, originating from the slow T_{2s} relaxation component and accounting for 40% of the total signal intensity, and a broad Lorentzian curve, owing to the fast T_{2f} relaxation component and accounting for the remaining 60% of the total signal intensity; similarly, the T_1 values under this condition can be decomposed into slow T_{1s} components that contribute 80% and fast T_{1f} components that account for the remaining 20% of the T_1 relaxation. The narrow or slow component of T_1 or T_2 relaxation is associated with the $+{}^1/_2$ to $-{}^1/_2$ transition, whereas the broad or fast component is due to the $+{}^3/_2$ to $+{}^1/_2$ and $-{}^1/_2$ to $-{}^3/_2$ transitions (Urry et al., 1989). The value of the $\Delta\nu_{1/8}/\Delta\nu_{1/2}$ ratio of the fully relaxed spectrum of 20 mM Li^+ in the presence of RBC membrane was 3.5, indicating the occurrence of two relaxation components. The experimental ratio (53/47) of fast to slow components that we estimated was in good agreement with the theoretical ratio (60/40).

Our partially relaxed ^7Li NMR spectra of RBC membrane suspensions containing 20 mM LiCl (Figure 2) provided evidence for biexponential relaxation for the ^7Li nucleus. When the mole fraction of free Li^+ was very large relative to that of bound Li^+, as was the case in RBC membrane suspensions containing 150 mM LiCl or in a glycerol–water mixture with the same Li^+ concentration, we were, however, unable to detect biexponential decay for the T_1 relaxation. The T_1 values observed in Li^+-containing RBC membrane suspensions represent weighted averages of free Li^+ in exchange with Li^+ bound to membrane sites. Because the relaxation of Li^+ in the absence of RBC membrane is monoexponential, the relaxation behavior of 150 mM LiCl in the presence of RBC membrane also appears to be monoexponential because of the large fraction of free Li^+. The T_{1f} and T_{1s} values for 20 mM Li^+ in the presence of RBC membrane were 8.0 ± 0.8 and 9.4 ± 0.9 s ($n = 4$), yielding a ratio of less than 2 for the spin–lattice relaxation rates. Low sensitivity may preclude the separation of the fast and slow relaxation components when they differ by a factor of less than 2 (Bull, 1972).

Gullapalli et al. (1991) did not observe biexponential T_1 or T_2 relaxation or a double-quantum ^7Li NMR resonance for Li^+-loaded RBCs. Whereas we searched for biexponential behavior by conducting an inversion recovery experiment, Gullapalli et al. (1991) performed a double-quantum experiment which is known to have a much lower inherent sensitivity (Kirk, 1990). Moreover, saturating intracellular Li^+ concentrations in RBCs are $\leq$10 mM; however, the Li^+ concentration present in our RBC membrane suspensions was 20 mM. Even if Li^+-saturated RBCs were used in Gullapalli's study (1991), a significantly higher Li^+ concentration was used in our study. The inability to detect a double-quantum NMR resonance for the ^7Li nucleus in Li^+-loaded RBCs may therefore be associated with low sensitivity (Kirk, 1990). We observed full visibility for the ^7Li nucleus in Li^+-containing RBC membrane suspensions (this study) and in Li^+-loaded RBCs (Mota de Freitas et al., 1990). The observation of partial visibility of the intracellular ^7Li NMR resonance and the inability to detect biexponential T_1 or T_2 relaxation in Li^+-loaded RBCs (Gullapalli et al., 1991) may also be associated with low NMR sensitivity under the experimental conditions used previously. Precedents for biexponential relaxation and partial visibility of the ^{23}Na nucleus in human RBC and rat liver have, however, been reported (Bansal et al., 1993; Gullapalli et al., 1991; Shinar et al., 1993). Apparent monoexponential ^{23}Na relaxation and a large difference between T_1 and T_2 were also reported for intracellular Na^+ in human packed RBCs (Pettegrew et al., 1984).

Changes in Li^+ binding sites, in particular anionic phospholipids, may be responsible for the abnormal Li^+ transport properties in RBCs from bipolar and hypertensive patients. Changes in the phospholipid composition (Hitzemann & Graver, 1982) or activities of enzymes involved in phospholipid metabolism and interconversion (Callahan, 1985) require investigation; such studies may contribute to an understanding of the etiology of bipolar illness and other neurological diseases, as well as essential hypertension. Our ^7Li NMR relaxation results indicate the promise of relaxation measurements to probe Li^+ interactions in disease states.

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