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Comparison of Fluorescence, ^{31}P NMR, and ^{7}Li NMR Spectroscopic Methods for Investigating Li^+/Mg^{2+} Competition for Biomolecules

Louis Amari,* Brian Layden,* Qinfen Rong,* Carlos F. G. C. Geraldes,† and Duarte Mota de Freitas*,[1]

*Department of Chemistry, Loyola University Chicago, 6525 North Sheridan Road, Chicago, Illinois 60626; and †Department of Biochemistry and Center for Neurosciences, University of Coimbra, 3000 Coimbra, Portugal

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The biochemical action of lithium in the treatment of manic-depressive illness is still unknown. One hypothesis is that Li^+ competes for Mg^{2+}-binding sites in biomolecules. We report here our studies on metal ion competition by three distinct methods: fluorescence, ^{31}P NMR, and ^{7}Li NMR spectroscopy, using ATP as a model ligand. By fluorescence spectroscopy, we used the dye, furaptra, by measuring the increases in Mg^{2+} levels in an ATP solution as Li^+ levels were increased in the solution. This increase in Mg^{2+} levels was indicated by increases in the fluorescence intensity ratio (335/370) of furaptra. By ^{31}P NMR spectroscopy, this competition was demonstrated by changes in the ^{31}P NMR spectrum of ATP. The Li^+/Mg^{2+} competition was indicated by predictable changes in the separation between the α and β resonances of the phosphates of ATP. For ^{7}Li NMR spectroscopy, spin-lattice relaxation measurements were used, which provided free Li^+ concentrations that could be used for determining the free Mg^{2+} values in ATP solutions. The values of the free Mg^{2+} concentrations obtained by all three methods were in good agreement. The fluorescence and ^{7}Li NMR methods, however, proved to be more sensitive to low concentrations of Li^+ than the ^{31}P NMR method.

Key Words: **lithium; biomolecules; ATP; NMR; fluorescence.**

Lithium salts have been used over the past 50 years as one of the primary forms of treatment of manic-depressive illness. Thus far, the therapeutic action of the lithium ion within the cell is unknown. One hypothesis is that Li^+ competes for Mg^{2+}-binding sites. Because lithium and magnesium cations have similar ionic radii, and because of the diagonal relationship between these two elements in the periodic table, their chemical properties are similar. Therefore, competition between these two ions for binding to the same ligand-binding site is possible. Our previous studies demonstrated that Li^+ competes with Mg^{2+} for the substrates of guanine nucleotide-binding proteins (G-proteins)[2] (1) as well as for the phosphate groups of ATP and the human erythrocyte membrane (2–4). We have extensively studied Li^+/Mg^{2+} competition for biomolecules by use of three methods: (1) fluorescence spectroscopy with magnesium indicators, (2) ^{31}P NMR spectroscopy, and (3) ^{7}Li NMR spectroscopy. Prior to this study, we had not, however, validated these methods against each other, and, in the case of ^{7}Li NMR spectroscopy, we had not reported the procedure for calculating free Mg^{2+} concentrations from ^{7}Li NMR measurements.

Several fluorescent magnesium indicators are available (5, 6). Furaptra is the most widely used dye because it has the highest affinity for Mg^{2+} among all the different indicators. Upon Mg^{2+} binding, the spectrum is blue-shifted, from a λ_{max} value of 370 nm for the free form to a λ_{max} value of 335 nm for the Mg^{2+}-saturated

[1] To whom correspondence should be addressed. Fax: (773) 508-3086. E-mail: dfreita@luc.edu.

[2] Abbreviations used: G-proteins, guanine nucleotide-binding proteins; K_d, dissociation constant; RBC, red blood cells; R_{max}, fluorescence intensity ratio at 335 and 370 nm in presence of saturating amounts of Mg^{2+}; R'_{max}, intensity ratio in the presence of saturating amounts of Li^+; R_{min}, intensity ratio in the absence of metal ions; S_{max}, fluorescence intensity at 370 nm in the presence of saturating amounts of Mg^{2+}; S'_{max}, fluorescence intensity at 370 nm in the presence of saturating amounts of Li^+; S_{min}, fluorescence intensity at 370 nm in the absence of metal ions; T_1, spin-lattice relaxation measurement.

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indicator. The ratio of the fluorescence intensities at 335/370 is indicative of Mg^{2+} binding to the indicator and is used for determining free magnesium concentrations (5, 6). Furaptra and fluorescence spectroscopy can be used for investigation of the Li^+/Mg^{2+} competition for ATP by measurement of the change in the fluorescence intensity ratio of furaptra as the Li^+ levels vary.

One can use ^{31}P NMR spectroscopy for the investigation of Li^+/Mg^{2+} competition for nucleotides (1–3). The ^{31}P NMR method takes advantage of the fact that there is a decrease in the chemical shift separation between the α and β phosphates of the nucleotide as the mole fraction of phosphates complexed with Mg^{2+} increases which indicates an increase in free Mg^{2+} values (7). Thus, in a system in which Li^+ competes with Mg^{2+}, one would expect to see a change in the $\alpha\beta$ chemical shift separation as Li^+ displaces Mg^{2+}, causing the $\alpha\beta$ separation to shift from a value that resembles that observed in Mg^{2+}-saturated nucleotide to one that resembles Li^+-saturated nucleotide, as was shown previously by ^{31}P NMR spectroscopy for ATP in Li^+-loaded human RBCs (8).

The ^7Li NMR method uses observed lithium spin-lattice (T_1) relaxation measurements, which are weighted averages of free Li^+ and bound Li^+ values (9), and thus are indirectly indicative of free Mg^{2+} concentrations (4). In using this technique to measure Li^+/Mg^{2+} competition, one takes advantage of the fact that, as the free-Li^+ concentrations increase, the T_1 values increase. Therefore, the Mg^{2+} values can be determined indirectly from the observed Li^+ values in solution. The ^7Li isotope is highly abundant and is a high-receptivity NMR nucleus, which makes the use of ^7Li NMR a highly sensitive tool for monitoring Li^+/Mg^{2+} competition (10).

Our aim was to investigate Li^+ and Mg^{2+} binding to a model ligand, ATP, by fluorescence spectroscopy, ^{31}P NMR, and ^7Li NMR. Comparisons among the free Mg^{2+} concentrations calculated from all three methods will serve to validate and establish the relative merits and drawbacks of each method.

EXPERIMENTAL METHODS

Materials

All biochemicals and inorganic salts were purchased from Sigma Chemical Co. (St. Louis, MO). Furaptra was purchased from Molecular Probes (Eugene, OR).

Preparation of ATP Solutions

We maintained the ATP concentration constant at 5.0 mM to prevent base stacking of the nucleotides (11). ATP in its Na^+ form was used for all experiments. The amount of Na^+ binding to ATP is negligible (12) and was the same for each method. Because the binding of metal cations to nucleotides is dependent on pH, ionic strength, and temperature (13), the pH was adjusted to 7.4 and the ionic strength was maintained at 0.15 M by use of Tris–Cl buffer. The temperature used was 37°C for all experiments. All samples contained 2.5 mM $MgCl_2$. The samples were then titrated with LiCl from 0 to 100 mM. For the fluorescence experiments, 2 μM furaptra was added to each sample.

Fluorescence Spectrometer

Fluorescence experiments were conducted with a PTI QuantaMaster QM-1 fluorimeter. For the fluorescence experiments with furaptra, the excitation was scanned between 300 and 400 nm, with the emission set at 510 nm. The cuvette holder was water-jacketed to maintain the temperature at 37°C.

Nuclear Magnetic Resonance Spectrometer

NMR experiments were conducted with a Varian VXR-300 NMR spectrometer equipped with a multinuclear probe. ^{31}P NMR and ^7Li NMR measurements were made at 121.4 and 116.5 MHz, respectively. Samples were run in 10-mm NMR tubes spinning at 18 Hz. A 10-mm broad band probe was used. A variable-temperature unit was used for maintaining the probe temperature at 37°C. T_1 relaxation measurements were conducted by use of the inversion recovery pulse sequence.

Calculation of $[Mg^{2+}]_f$ Values from Fluorescence Spectroscopy Data

When we used furaptra, the free Mg^{2+} concentration, which was corrected for Li^+ binding to furaptra, was calculated from the following equation:

$$[Mg^{2+}]_f = K_d S_{min}(R - R_{min})/S_{max}(R_{max} - R) + K_d S'_{max}(R - R'_{max})[Li^+]_f/K'_d S_{max}(R_{max} - R), \quad [1]$$

where R is the fluorescence intensity ratio observed for the biological sample; R_{min}, R_{max}, and R'_{max} are the ratios of the fluorescence intensities at 335 and 370 nm in the absence of metal ions and in the 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 of metal ions and in the 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 K_d values of Mg^{2+} and Li^+ to furaptra were 1.5 mM at 37°C (6) and 237 ± 49 mM at 37°C (vide infra), respectively. Because Li^+ binds weakly to ATP (4), the free Li^+ concentration,

$[Li^+]_f$, was assumed to be equal to the total Li^+ concentration, $[Li^+]_T$.

Calculation of $[Mg^{2+}]_f$ Values from ^{31}P NMR Data

Because the mole fractions of unbound ATP (X_f), ATP bound to Li^+ (X'_b), and ATP bound to Mg^{2+} (X_b) are equal to 1, we calculated the free Mg^{2+} concentrations from the ^{31}P NMR data using the following equations:

$$X'_b = (K_1[Li^+]_f + (K_1)(K_2)[Li^+]_f^2)X_f \quad [2]$$

$$X_b = (\delta_{obs} - \delta_f)/(\delta_b - \delta_f) - X'_b(\delta_{b'} - \delta_f)/(\delta_b - \delta_f) \quad [3]$$

$$X_f = (\delta_b - \delta_{obs})/(\delta_b - \delta_f) - X'_b(\delta_b - \delta_{b'})/(\delta_b - \delta_f) \quad [4]$$

$$[Mg^{2+}]_{free} = X_b/K_bX_f, \quad [5]$$

where δ_f is the chemical shift difference between the α and β phosphates in the presence of zero Mg^{2+}; δ_b is the chemical shift difference between the α and β phosphates in the presence of saturating amounts of Mg^{2+}; $\delta_{b'}$ is the chemical shift difference between the α and β phosphates in the presence of saturating amounts of Li^+; and δ_{obs} is the chemical shift difference observed for a given sample. The δ_f, δ_b, and $\delta_{b'}$ values were measured to be 10.82, 8.43, and 9.80 ppm, respectively. Whereas Mg^{2+} binds ATP with a 1:1 stoichiometry, one or two Li^+ ions may bind to ATP (2). The value of K_b, the binding constant of Mg^{2+}-ATP, was 25,000 M^{-1} at 37°C. For Eq. [2], the values of K_1 and K_2, the binding constants of the first and second Li^+ ions to ATP, were 91 and 10.6 M^{-1} at 37°C, respectively (4). The K_1 and K_2 values used were determined from the data previously analyzed (4). The binding constants used were those that gave the best fit for our ^{31}P NMR and ^7Li NMR data. Additionally, the value of K_1 used is comparable to that previously reported (14). As before, $[Li^+]_f$ was assumed to be equal to $[Li^+]_T$ in Eq. [2].

Calculation of $[Mg^{2+}]_f$ Values from ^7Li NMR Data

To calculate the free Mg^{2+} concentration from the ^7Li NMR data, we used the following equations:

$$x_f = (R_{obs} - R_b)/(R_f - R_b) \quad [6]$$

$$x_f = [Li^+]_f/[Li^+]_T \quad [7]$$

$$[ATP]_f = ([Li^+]_T/[Li^+]_f - 1)/(K_1 + 2K_1K_2[Li^+]_f) \quad [8]$$

$$[ATP]_T - [ATP]_f - K_1[Li^+]_f[ATP]_f - K_1K_2[Li^+]_f^2[ATP]_f = K_b[Mg^{2+}]_f[ATP]_f, \quad [9]$$

where x_f is the mole fraction of free Li^+; R_f is the reciprocal of the ^7Li relaxation in the presence of saturating amounts of Li^+; R_b is the reciprocal of the ^7Li relaxation in the presence of saturating amounts of substrate; and R_{obs} is the reciprocals of the ^7Li relaxation times at various Li^+ concentrations. The R_b value used (0.47 s^{-1}) was previously calculated from a model system in which the LiATP species predominates (4). The R_f value (0.08 ± 0.01 s^{-1}) was found experimentally at the end of each Li^+ titration by adding saturating amounts of Li^+ (500 mM). The K_b, K_1, and K_2 values were the same as for the ^{31}P NMR method. No assumptions were necessary when using Eqs. [6]–[9].

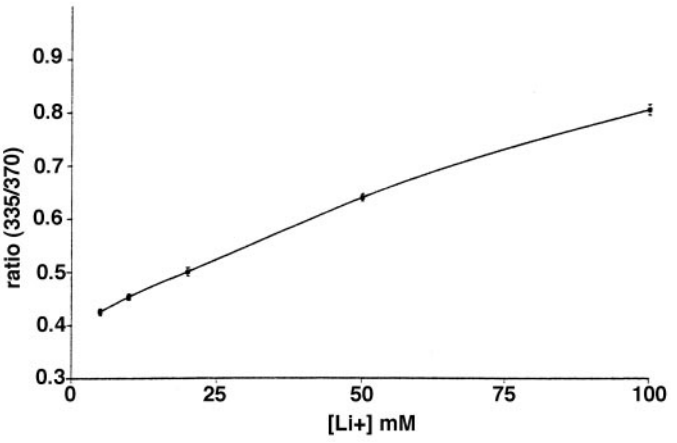

FIG. 1. Changes in fluorescence intensity ratios (335/370) for several Li^+ concentrations. The ATP and Mg^{2+} concentrations were 5.0 and 2.5 mM, respectively.

RESULTS

Competition between Li^+ and Mg^{2+} for the Phosphate Groups in ATP by Fluorescence Spectroscopy

The analysis of the fluorescence excitation spectra of furaptra in solutions containing 5.0 mM Na-ATP, 2.5 mM $MgCl_2$, 150 mM Tris–Cl, pH 7.4, at 37°C, and increasing concentrations of LiCl (0 to 100 mM) are shown by a plot of the fluorescence intensity ratios (335/370) vs Li^+ concentrations (Fig. 1). Upon addition of Li^+, the λ_{max} position was blue-shifted from 370 to 335 nm, which is indicated by an increase in the fluorescence intensity ratio (335/370).

Because Li^+ causes changes in the furaptra spectra similar to those of Mg^{2+}, it was necessary to determine the K_d of Li^+–furaptra. 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, two orders of magnitude larger than those of Mg^{2+}. The slope of the Hill plot provides the number of binding sites of Li^+ to furaptra, and we obtained the K_d value by dividing the slope by the y intercept. From the spectral intensity changes induced by the addition of increasing concentrations of Li^+, we calculated at 25°C a K_d value of 250 ± 70 mM (n = 8)

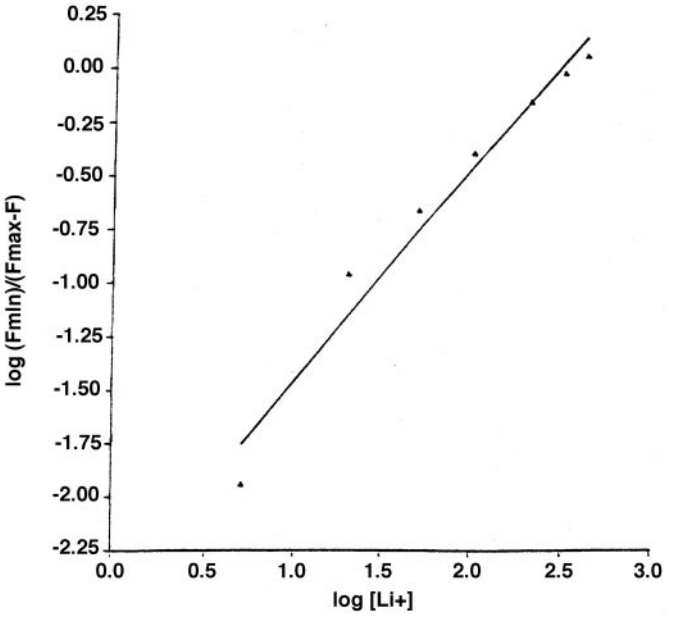

FIG. 2. Hill plot of Li^+ binding to furaptra. F is the fluorescence intensity at 370 nm, F_{min} is the fluorescence intensity at 370 nm in the absence of metal ions, and F_{max} is the fluorescence intensity at 370 nm in the presence of saturating amounts of Mg^{2+}.

and a stoichiometry of 1:1 for the Li^+–dye complex, using a Hill plot (Fig. 2). Additionally, we also determined the K_d value at 4°C to be 530 ± 135 mM (n = 5). For our experiments, we investigated Li^+/Mg^{2+} competition at 37°C. To determine the K_d value at 37°C (237 ± 49 mM, n = 2), we used the Van't Hoff equation and our two known dissociation constants. Additionally, it was necessary to determine whether there was an effect of ATP on the fluorescence spectrum of furaptra. The excitation spectrum of free dye did not change significantly in the presence of ATP, indicating that there was no specific dye–ATP interaction. In all samples, an increase in Li^+ concentration caused an increase in the values of $[Mg^{2+}]_f$ calculated by Eq. [1], demonstrating Li^+ displacement of Mg^{2+} from ATP (Table 1, column 2).

Competition Between Li^+ and Mg^{2+} for ATP by ^{31}P NMR Spectroscopy

Figure 3 shows the ^{31}P NMR spectra of solutions containing 5.0 mM NaATP and 2.5 mM $MgCl_2$, titrated with Li^+. As Li^+ is added, the $\alpha\beta$ chemical shift separation decreases. In the absence of Li^+, the β-phosphate peak is broad because Mg^{2+} binds primarily to the β-phosphate of ATP in slow exchange (13). The sharpening of the β-phosphate peak is indicative of an increase in the rate of metal ion exchange as the Li^+ concentration increased. The calculated free Mg^{2+} values are shown in Table 1, column 3, as determined from Eqs. [2]–[5].

Competition between Li^+ and Mg^{2+} for ATP by ^7Li NMR Spectroscopy

The addition of Li^+ caused significant increases in the observed ^7Li T_1 values (Fig. 4). The increases in free Li^+, which are indicated by increases in the observed T_1 values, were used for calculation of the free Mg^{2+} values (Table 1, column 4) from Eqs. [6]–[9].

Comparison of the Fluorescence, ^{31}P NMR, and ^7Li NMR Spectroscopy Methods

Table 1 shows the calculated $[Mg^{2+}]_f$ values for solutions containing 5.0 mM ATP and 2.5 mM Mg^{2+} titrated with Li^+, obtained via the fluorescence, ^{31}P NMR, and ^7Li NMR methods. In all cases, it was found that, as the concentration of Li^+ increased, the $[Mg^{2+}]_f$ concentration also increased.

From a Pearson correlation test, we determined that all three techniques were significantly correlated with each other. The ^7Li NMR and ^{31}P NMR spectroscopic techniques were the least positively correlated (r = 0.83, $P \leq 0.005$). The ^7Li NMR and fluorescence spectroscopic methods were more positively correlated (r = 0.91, $P \leq 0.0005$). The greatest positive correlation was found between the ^{31}P NMR and fluorescence spectroscopic methods (r = 0.98, $P \leq 0.0005$).

DISCUSSION

Competition between Mg^{2+} and Li^+ could occur for biologically relevant Mg^{2+} binding sites. Although Li^+ can displace Mg^{2+} bound to biomolecules, the activity of the biomolecules will not necessarily remain intact, and this is most likely due to the fact that Li^+ is monovalent, whereas Mg^{2+} is divalent, and the coordination of the two metal ions is not exactly the same.

TABLE 1

Calculated $[Mg^{2+}]_f$ Values (in μM) Obtained via Fluorescence, ^{31}P NMR, and ^7Li NMR Spectroscopy for Solutions Containing 5.0 mM NaATP and 2.5 mM Mg^{2+} (n = 3)

$[Li^+]$/mM	Fluorescence[a]	^{31}P NMR[b]	^7Li NMR[c]
5	157 ± 12	94 ± 7	196 ± 25
10	188 ± 11	130 ± 16	284 ± 59
20	235 ± 17	178 ± 20	528 ± 95
50	391 ± 16	376 ± 65	706 ± 106
100	561 ± 26	818 ± 230	769 ± 231

[a] The $[Mg^{2+}]_f$ values were calculated from Eq. [1].
[b] Values calculated from Eqs. [2]–[5].
[c] Values calculated from Eqs. [6]–[9].

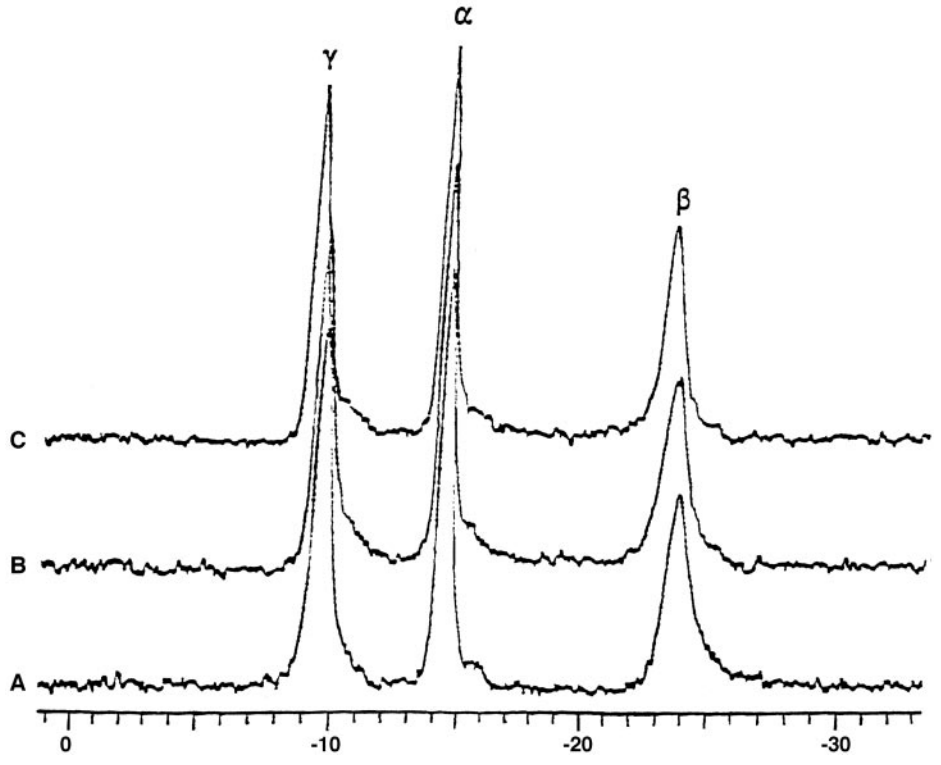

FIG. 3. ^{31}P NMR spectra of 5.0 mM ATP and 2.5 mM Mg^{2+} at 37°C alone (A), and in the presence of (B) 5.0 mM LiCl, and (C) 100.0 mM LiCl.

Our metal competition study was conducted with a model ligand, ATP, and by use of fluorescence, ^7Li NMR, or ^{31}P NMR spectroscopy. Upon addition of Li^+, the excitation fluorescence spectra were blue-shifted, indicating displacement of Mg^{2+} by Li^+. Because Li^+ and Mg^{2+} have similar chemical properties, it was not surprising that Li^+ also binds to the fluorescence Mg^{2+} indicator. At 37°C, the K_d value for the Li^+–dye com-

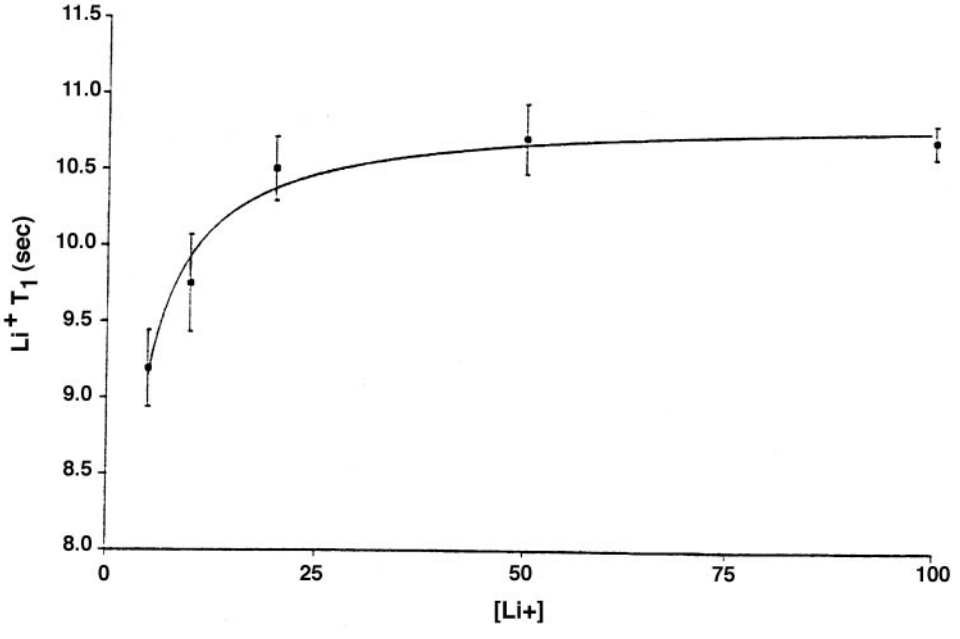

FIG. 4. Dependence of the observed Li^+ T_1 values at 37°C on the concentration of Li^+ (mM) in a 5.0 mM ATP solution with 2.5 mM Mg^{2+}.

plex (237 mM) was, however, much larger than that of the Mg^{2+}–dye complex (1.5 mM) (5), 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. For the free Mg^{2+} concentrations in ATP solutions calculated from the fluorescence intensity ratios, we took into account the weak binding of Li^+ to furaptra (Table 1, column 2) by adding a correction to Eq. [1].

We also performed experiments with ^{7}Li NMR relaxation measurements to study the competition mechanism and to compare the results obtained with those obtained by fluorescence and ^{31}P NMR spectroscopy. The $[Mg^{2+}]_f$ values obtained by the ^{7}Li NMR relaxation method are in good agreement with those obtained with the other methods (Table 1). We also used ^{31}P NMR spectroscopy to study the competition mechanism, and to compare the results obtained with those by fluorescence spectroscopy and ^{7}Li NMR relaxation measurements. The comparison of the three methods is shown in Table 1. The values obtained by ^{31}P NMR spectroscopy are similar to those with fluorescence spectroscopy and ^{7}Li NMR relaxation measurements.

^{31}P NMR spectroscopy is the least sensitive of the three techniques used to study Li^+/Mg^{2+} competition. The decreased sensitivity of the ^{31}P NMR spectroscopic method presumably arises because almost all Mg^{2+} was bound to ATP (the K_d value for the Mg–ATP 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+}–furaptra indicator is 1.5 mM) (15). Additionally, the $\alpha\beta$ separation of ATP, which is used for measuring free Mg^{2+} concentrations, produces only small decreases in ppm with increasing concentrations of metal ions, whereas furaptra provides a much larger change in the fluorescence intensity ratios. For the Li^+ and Mg^{2+} concentrations studied, the ^{7}Li NMR relaxation method is approximately as sensitive as the fluorescence method 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 ^{7}Li T_1 values. One problem with the ^{7}Li NMR relaxation method as well as with the ^{31}P NMR method is the need for knowing the association constants of the model ligand to obtain estimates of free Mg^{2+}. In contrast, for the fluorescence method knowledge of association constants is not required.

We performed these studies to validate the three methods. Pearson correlation coefficients showed that all three techniques were significantly correlated. Even though the ^{31}P NMR technique is the least sensitive, it has some advantages over the fluorescence method. The ^{31}P NMR method provides a technique for which an endogenous indicator, ATP, is available (7, 16). Because the ATP signal can easily be observed by ^{31}P NMR in most cell suspensions, this technique allows us to measure $[Mg^{2+}]_f$ without adding an exogenous indicator such as furaptra, as is necessary for the fluorescence method. The ^{31}P NMR technique provides obvious advantages for *in vivo* studies because the difficulties of loading an indicator and possible adverse effects of an indicator on the cells can be avoided. Additionally, the ^{7}Li NMR method provides us with a technique for studying this competition in which no outside indicators are necessary because the ^{7}Li nucleus has a high NMR receptivity (10). The ranking of the sensitivities of the methods (starting with the highest) is fluorescence, ^{7}Li NMR, and ^{31}P NMR. The results obtained compare favorably for all three methods, with each method having its own limitations and merits. For independent use of these methods, assumptions are necessary in two (^{31}P NMR and fluorescence method) of the three methods. For the free Mg^{2+} calculations using the ^{31}P NMR and fluorescence methods the equations assume that $[Li^+]_f$ is equal to $[Li^+]_T$. This assumption slightly overestimates the true $[Li^+]_f$ value. By using the ^{7}Li NMR method, however, $[Li^+]_f$ can be determined (Eq. [6]). For example, when $[Li^+]_T$ is 5.0 or 100 mM, $[Li^+]_f$ is equal to 4.6 and 96.3 mM, respectively. Therefore, this assumption, $[Li^+]_f = [Li^+]_T$, is reasonable when ATP is the model ligand. Furthermore, it is possible to combine these techniques to avoid any assumptions.

The biochemical action of Li^+ may be explained by its ability to compete for Mg^{2+} binding sites in biological systems involved in signal transduction, in particular G-proteins. Furthermore, it has been speculated that the therapeutic action of Li^+ in manic-depressive illness occurs by modulation of the activity of G-proteins by competing for the Mg^{2+} binding sites on these proteins (17). Currently, our laboratory is performing experiments with purified G-proteins to understand this competition further. The methods presented in this study provide us with techniques for characterizing this competition between Li^+/Mg^{2+} for different biomolecules.

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