Competition between Li^+ and Mg^{2+} in Neuroblastoma SH-SY5Y Cells: A Fluorescence and ^{31}P NMR Study

Louis Amari,* Brian Layden,* Joyce Nikolakopoulos,* Qinfen Rong,* Duarte Mota de Freitas,* Graça Baltazar,[#§] M. Margarida C. A. Castro,[#¶] and Carlos F. G. C. Geraldes[#¶]

*Department of Chemistry, Loyola University, Chicago, Illinois 60626 USA, and [#]Department of Biochemistry, [§]Department of Zoology, and [¶]Center for Neurosciences, University of Coimbra, Coimbra, Portugal

ABSTRACT Because Mg^{2+} and Li^+ ions have similar chemical properties, we have hypothesized that Li^+/Mg^{2+} competition for Mg^{2+} binding sites is the molecular basis for the therapeutic action of lithium in manic-depressive illness. By fluorescence spectroscopy with furaptra-loaded cells, the free intracellular Mg^{2+} concentration within the intact neuroblastoma cells was found to increase from 0.39 ± 0.04 mM to 0.60 ± 0.04 mM during a 40-min Li^+ incubation in which the total intracellular Li^+ concentration increased from 0 to 5.5 mM. Our fluorescence microscopy observations of Li^+-free and Li^+-loaded cells also indicate an increase in free Mg^{2+} concentration upon Li^+ incubation. By ^{31}P NMR, the free intracellular Mg^{2+} concentrations for Li^+-free cells was 0.35 ± 0.03 mM and 0.80 ± 0.04 mM for Li^+-loaded cells (final total intracellular Li^+ concentration of 16 mM). If a Li^+/Mg^{2+} competition mechanism is present in neuroblastoma cells, an increase in the total intracellular Li^+ concentration is expected to result in an increase in the free intracellular Mg^{2+} concentration, because Li^+ displaces Mg^{2+} from its binding sites within the nerve cell. The fluorescence spectroscopy, fluorescence microscopy, and ^{31}P NMR spectroscopy studies presented here have shown this to be the case.

INTRODUCTION

Lithium carbonate and lithium citrate have been used in the treatment of manic depressive illness (also called bipolar disorder) for a number of years. However, the molecular mechanism of Li^+ action remains unclear. For the majority of patients, lithium salts within the proper dosage range are successful in treating manic depression. Some patients, however, experience severe and toxic side effects, leading to failure of renal function, motor impairment, and even death. Understanding the mode of action of Li^+ will facilitate the development of better drugs that can perform the same function, but without the toxic side effects.

Several interrelated hypotheses have been proposed to explain the possible mechanism of Li^+ action. Among these are 1) a cell membrane abnormality (Metzler, 1991; Mota de Freitas et al., 1994a); 2) Li^+ inhibition of the enzymes inositol-1-monophosphatase and adenylate cyclase, causing depletion in brain inositol and cyclic AMP levels (Allison and Stewart, 1971; Berridge et al., 1989); and 3) competition between Li^+ and Mg^{2+} ions for Mg^{2+} binding sites in biomolecules, particularly guanine nucleotide-binding proteins (G proteins), which are involved in the signal transduction cascade (Avissar et al., 1988). Because Li^+ is ubiquitous within the cell, it is possible that the pharmacological action of Li^+ cannot be explained by a single mode of action.

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Address reprint requests to Dr. Duarte Mota de Freitas, Department of Chemistry, Loyola University Chicago, 6525 North Sheridan Road, Chicago, IL 60626. Tel.: 773-508-3091; Fax: 773-508-3086; E-mail: dfreita@luc.edu.

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Li^+ and Mg^{2+} ions have similar ionic potentials, because of their diagonal relationship in the periodic table of elements. As a result, Li^+ and Mg^{2+} exhibit similar chemical properties, and Li^+ may compete with Mg^{2+} for Mg^{2+} binding sites in biomolecules, especially O-containing and, on occasion, N-containing ligands (Frausto da Silva and Williams, 1976).

Using fluorescence spectroscopy and the Mg^{2+} indicator furaptra (Raju et al., 1989), we showed that there was competition between Li^+ and Mg^{2+} for the phosphate groups of adenosine triphosphate (ATP) and of the human red blood cell (RBC) membrane (Mota de Freitas et al., 1994b). We also showed by NMR spectroscopy that Li^+ competes with Mg^{2+} for the phosphate groups on guanosine triphosphate and guanosine diphosphate, which are the substrates for G proteins (Rong et al., 1992), and on the second messenger, inositol-1,4,5-triphosphate (IP_3) (Rong et al., 1994). Based on the conditional binding constants that we determined (Mota de Freitas et al., 1994b; Rong et al., 1994) and the physiological concentrations of Mg^{2+}, ATP, and IP_3 present in most cell types, we reasoned that the Li^+/Mg^{2+} competition mechanism may be operational in cells exposed to therapeutic levels of Li^+.

Neuroblastoma cells were chosen for our investigation because manic depression is a psychiatric disorder, and thus the primary site(s) of Li^+ action is presumably neuronal tissue. The human neuroblastoma SH-SY5Y cell line used in this study has a neuroblast-like morphology (Nakagawa-Yagi, 1994; Biedler et al., 1973). Previous studies conducted with this cell line have shown its suitability for the study of G-protein function in the brain, because the percentage composition of G proteins in SH-SY5Y cells is very similar to that in human brain cortex (Klinz et al., 1987; Carter and Medzihradsky, 1993), as well as the effects of

Li^+ treatment on phosphatidylinositol turnover (Carter and Medzihradsky, 1993; Stubbs and Agranoff, 1993). This cell line has also been used in an investigation of the ion regulatory properties of Li^+ and other metal ions such as Ca^{2+} (McDonald et al., 1994). Unlike primary cultures of neurons and glial cells, the SH-SY5Y cell line provides significantly higher cell densities, which are necessary for ^7Li NMR (Nikolakopoulos et al., 1996) and fluorescence measurements. Thus far, we have used the SH-SY5Y cell line in our laboratory to study the transport and binding properties of Li^+ by ^7Li NMR spectroscopy (Nikolakopoulos et al., 1996, 1998; Zachariah et al., 1996).

Using fluorescence spectroscopy and microscopy, we studied the competition between Li^+ and Mg^{2+} by loading human neuroblastoma cells with the fluorescent Mg^{2+} indicator furaptra. We hypothesized that if the Li^+/Mg^{2+} competition mechanism were present in neuroblastoma cells, an increase in the total intracellular Li^+ concentration would result in an increase in the free intracellular Mg^{2+} concentration, because Li^+ would displace Mg^{2+} from its binding sites within the neuroblastoma cells.

^{31}P NMR spectroscopy can be used for studying Li^+/Mg^{2+} competition for nucleotides (Mota de Freitas et al., 1994b; Gupta and Gupta, 1987), as shown previously by us with Li^+-loaded human RBCs (Mota de Freitas et al., 1994b; Ramasamy and Mota de Freitas, 1989). The ^{31}P NMR method takes advantage of the fact that there is an increase in the chemical shift separation between the a and b phosphates of the nucleotide as Li^+ displaces Mg^{2+} from ATP. Thus, in a system in which Li^+ competes with Mg^{2+}, one would expect to see an increase in the $\alpha\beta$ chemical shift separation as Li^+ displaces Mg^{2+}, causing an increase in free Mg^{2+} values.

Most cell lines can be kept viable during the course of long NMR experiments only by continuously replenishing nutrients and oxygen. To perform perfused NMR experiments on the SH-SY5Y cell line, we had to immobilize the cells by using a technique that involved entrapment of the cells in agarose threads. The agarose threads containing the SH-SY5Y cells were perfused with fresh medium throughout the course of the experiment, which enabled the cells to maintain a high degree of viability (Nikolakopoulos et al., 1996, 1998; Egan, 1987; Swergold, 1992).

In this study, we used fluorescence and ^{31}P NMR spectroscopic methods as well as fluorescence microscopy to examine the competition between Li^+ and Mg^{2+} ions within intact human neuroblastoma SH-SY5Y cells. The studies described will take us a step farther in the understanding of the pharmacological action of Li^+ at the molecular level.

EXPERIMENTAL PROCEDURES

Materials

The SH-SY5Y human neuroblastoma cell line was provided by Dr. E. Stubbs, Jr. (Department of Neurology, Loyola University Medical Center); it was grown as a continuous cell line in our laboratory. All biochemicals and inorganic salts were purchased from Sigma Chemical Company (St. Louis, MO). Furaptra in both potassium salt and ester form was purchased from Molecular Probes (Eugene, OR). Low-gelling-temperature agarose was purchased from Fluka (Ronkonkoma, NY). Teflon capillary tubing was purchased from Read Plastics (Rockville, MD).

Preparation of neuroblastoma SH-SY5Y cells for fluorescence experiments

Human neuroblastoma SH-SY5Y cells were grown in monolayers in T-75 cm^2 polystyrene flasks in a humidified atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and harvested with Puck's D_1 solution (Stubbs and Agranoff, 1993). The cells (cell count of 4.0×10^7) were centrifuged and loaded at 37°C with modified phosphate-buffered saline (PBS) at pH 7.3: 5 mM KCl, 5 mM $Na_2HPO_4 \cdot 7H_2O$, 25 mM glucose, 120 mM sucrose, and 85 mM NaCl, containing a membrane-permeable ester derivative of the Mg^{2+} fluorophore, 10 μM furaptra, and an organic anion transport blocker, 2.5 mM probenecid (Di Virgilio et al., 1990), for 30 min. The neuroblastoma cells were then centrifuged and washed twice to remove any extracellular furaptra. Immediately before the addition of furaptra to the cell suspension, an aliquot of the cell suspension was taken for autofluorescence measurement.

After furaptra loading in PBS buffer containing 2.5 mM probenecid for 30 min, the washed cells were placed at 37°C in a solution containing 50 mM LiCl, 5 mM KCl, 5 mM $Na_2HPO_4 \cdot 7H_2O$, 25 mM glucose, 20 mM sucrose, 85 mM NaCl at pH 7.3, and 2.5 mM probenecid, for loading of the cells with Li^+. Aliquots of the nerve cell suspension were taken every 10 min for fluorescence measurements; to each aliquot, 1 mM $MnCl_2$ was added to quench the fluorescence of any leaked, extracellular furaptra (Raju et al., 1989). The cell viability was determined by the Trypan Blue dye exclusion method (Patterson, 1979).

We performed four control experiments to determine the optimal incubation procedure for the dye, as well as to determine whether probenecid was preventing dye leakage. Two control experiments (with and without 2.5 mM probenecid) were performed with a 30-min dye incubation (10 μM furaptra) in PBS buffer, followed by two washes for removal of excess dye and then fluorescence measurements every 20 min for 60 min with the cells in PBS buffer. The other two control experiments (with and without 2.5 mM probenecid) included a 30-min dye incubation (10 μM furaptra) in PBS buffer, followed by two washes to remove excess dye and another 30-min PBS incubation before measurements every 20 min for 60 min in PBS buffer.

The intracellular dissociation constant (K_d) and the fluorescence parameters for Mg^{2+} binding to furaptra were obtained from solutions of lysed neuroblastoma cells in which 2 μM furaptra in its salt form was added. Because it is difficult to measure directly the K_d and fluorescence parameters for Mg^{2+} inside the intact cell, our approach was to determine these parameters in a solution that mimics the intracellular milieu, which gives a good approximation of the intracellular values of these parameters (Murphy and London, 1995). The neuroblastoma cells were lysed by the addition of deionized water and were further lysed by sonication (2–3 min). The volume of the lysed cell suspension was adjusted by the addition of 0.5 M Tris-Cl (pH 7.3) buffer.

Preparation of neuroblastoma cells for fluorescence microscopy experiments

The cells were grown overnight in chamber slides. One half-hour before use, the cells were loaded with 10 μM furaptra in PBS buffer. The PBS buffer was then removed, and the cells were rinsed three times with PBS to remove any extracellular furaptra. The chamber slides were then placed on the microscope, and the furaptra-loaded neuroblastoma cells were perfused with PBS containing 110 mM Li^+ at 37°C for 1 h to load the cells with Li^+. Because the cells were perfused, it was not necessary to use $MnCl_2$, for any extracellular furaptra would be washed away.

Preparation of Li^+-loaded and Li^+-free neuroblastoma cells in gel threads

The cells were harvested with Puck's D_1 solution. The Li^+-free neuroblastoma cells were immediately embedded in gel threads, whereas, for the loading of Li^+, the cells were suspended in a PBS solution containing 110 mM LiCl for 30 min, washed, and then embedded in gel threads. For embedding in the gel threads, the neuroblastoma cells were mixed with a solution of growth medium and low-gelling-temperature agarose to give a final agarose gel concentration of 0.7%. The gel-cell mixture was maintained at 37°C and extruded under mild pressure (12 p.s.i.) through a maximum of 1 m of teflon capillary tubing that was chilled in an ice bath (Nikolakopoulos et al., 1998; Foxall et al., 1984). The gel threads containing the cells were then collected directly in a 10-mm NMR tube containing growth medium. Perfusion with the growth medium was necessary to maintain cell viability during the course of the ^{31}P NMR experiments.

Fluorescence spectroscopy

Fluorescence experiments were conducted with a PTI QuantaMaster QM-1 fluorimeter. The excitation was scanned between 300 and 400 nm, with the emission set at 510 nm. The data were analyzed as a fluorescence intensity ratio of 335/370. All fluorescence spectroscopy experiments were performed in quadruplicate at room temperature.

Fluorescence microscopy

Fluorescence microscopy experiments were performed with a Nikon Diaphot fluorescence inverted microscope (Nikon Co., Tokyo, Japan). Images were acquired with a MagiCal System (Applied Imaging, Gateshead, England) and a Silicon Intensified Target (SIT) camera at a rate of 3 Hz. The light output of a 75-W xenon lamp was guided to a filter wheel that transmitted light alternately centered at 340 and 380 nm. The light beams exiting the filter wheel were directed onto the sample by means of a dichroic mirror and a 20× fluorite objective (Nikon Co., Tokyo, Japan). Light emitted above 510 nm was recorded by the SIT camera. The fluorescence intensity ratio used in these experiments was 340/380. The fluorescence ratio images were obtained by taking the ratios of the corresponding fluorescence images on a pixel-by-pixel basis. The ratio images were coded in pseudocolor (dark blue to red for the minimum to the maximum 340/380 ratios). The background fluorescence (obtained from a coverslip devoid of cells) was subtracted from all frames.

Nuclear magnetic resonance spectroscopy

^{31}P NMR experiments were made at 121.4 MHz with a Varian VXR-300 NMR spectrometer equipped with a multinuclear 10 mm broad-band probe. Samples were run in 10-mm NMR tubes, either spun at 16–18 Hz for the experiments without perfusion or without spinning for samples that were perfused. The probe temperature was maintained at 37.0 ± 0.5°C by use of a variable-temperature unit. The ^{31}P NMR spectra were obtained from ~1000 transients with a standard 90° pulse, a spectral width of 10,000 Hz, and 30,016 data points with proton decoupling. The signal-to-noise ratio was enhanced by exponential multiplication with a line broadening of 5–10 Hz (Nikolakopoulos et al., 1998). Furthermore, we found before that the phosphates of nucleotides other than ATP do not contribute significantly to the ^{31}P NMR spectrum of these cells (Nikolakopoulos et al., 1998).

Atomic absorption spectrophotometry

A Perkin-Elmer spectrophotometer (model 5000) equipped with a flame source was used for determination of Li^+ and Mg^{2+} concentrations.

Calculation of $[Mg^{2+}]_f$ values from fluorescence spectroscopy data

When furaptra was used, the free magnesium 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 ratio of the fluorescence intensities at 335 and 370 nm 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 (R_{min}) and in the presence of saturating amounts of Mg^{2+} or Li^+, respectively; S_{min}, S_{max}, and S'_{max} are the fluorescence intensities at 370 nm in the absence (S_{min}) and in the presence of saturating amounts of Mg^{2+} or Li^+, respectively; and K_d and K'_d are the dissociation constants of the furaptra complexes of Mg^{2+} and Li^+, respectively. Before any calculations, the autofluorescence of the unloaded cell suspension (without furaptra) was subtracted from the measured values.

The K_d of Mg^{2+}-furaptra was determined by titrating a neuroblastoma lysed cell suspension with Mg^{2+}. From a Hill plot, we calculated a K_d value of 1.9 ± 0.2 mM (n = 3) and a stoichiometry of 1:1 for the intracellular Mg^{2+}-dye complex. The K_d value was slightly higher within the neuroblastoma cells than in solution, the K_d in solution being 1.5 mM (Mota de Freitas et al., 1994b). The K_d value of Li^+ to furaptra at room temperature was previously found to be 250 mM (Mota de Freitas et al., 1994b). To obtain the maximum values, R_{max} and S_{max}, we added 50.0 mM Mg^{2+} to a neuroblastoma cell suspension, whereas for the determination of the minimum values, R_{min} and S_{min}, 50.0 μM EGTA was added. We used 2.5 M Li^+ to determine the value of R'_{max}. The values for R_{min}, R_{max}, and R'_{max} were found to be 0.35 ± 0.01 (n = 3), 3.40 ± 0.11 (n = 3), and 2.58 ± 0.02 (n = 3), respectively. Note that the second term of Eq. 1 is negative because R'_{max} is larger than R.

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

To calculate the free Mg^{2+} concentrations from the ^{31}P NMR data, we used the following equations, which take into account Li^+ binding to ATP:

$$[Mg^{2+}]_f = X_b/K_b X_f \quad (2)$$

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

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

$$K_b[Mg^{2+}]_T X_f = X_b + K_b[ATP]_T X_b X_f \quad (5)$$

where δ_f is the chemical shift difference between the a and b phosphates in the absence of Mg^{2+} (10.82 ppm); δ_b is the chemical shift difference between the a and b phosphates in the presence of saturating amounts of Mg^{2+} (8.43 ppm); δ'_b is the chemical shift difference between the a and b phosphates in the presence of saturating amounts of Li^+ (9.80 ppm); δ_{obs} is the chemical shift difference observed for a given sample; X_b is the mole fraction of ATP bound to Mg^{2+}; X_f is the mole fraction of unbound ATP; and X'_b is the mole fraction of ATP bound to Li^+. The δ_f, δ_b, and $\delta_{b'}$ values were obtained in cell-free solutions of ATP (Abraha et al., 1991; Iotti et al., 1996). The value of K_b, the binding constant of Mg^{2+} to ATP at 37°C, was 25,000 M^{-1} at pH 7.3. Because Li^+ loading does not significantly alter intracellular pH (Nikolakopoulos et al., 1998), the same K_b was used for the Li^+-free and Li^+-loaded cells. The total Mg^{2+} concentration within the neuroblastoma cells, $[Mg^{2+}]_T$, was determined by atomic absorption spectrophotometry (2.13 ± 0.7 mM, n = 7). The total ATP concentration, $[ATP]_T$, was determined by ^{31}P NMR to be 1.875 ± 0.147 mM (n = 3).

Equation 5 was not used to calculate $[Mg^{2+}]_f$ in Li^+-free cells. Only Eqs. 2–4 were used; the correction (X_b') to Eqs. 3 and 4 is equal to zero. To calculate $[Mg^{2+}]_f$ in Li^+-loaded cells, Eqs. 2–5 were used. It was necessary to calculate X_b' to solve Eqs. 2–4. By combining Eqs. 3–5 and through the use of a quadratic equation, X_b' can be determined.

RESULTS

Competition between Li^+ and Mg^{2+} in intact human neuroblastoma SH-SY5Y cells by fluorescence spectroscopy and furaptra

Table 1 shows the fluorescence intensity ratios (335/370) taken at 0, 20, 40, and 60 min and the percentage of intensity decrease at 370 nm with 0 min arbitrarily assigned as 100% dye concentration. The ratios were approximately the same regardless of incubation time and the addition of 2.5 mM probenecid (~0.5 throughout each control experiment). However, the intensity decreases were large (almost 50.0% intensity loss) when probenecid was not added in the experiment, but were small (less than 5.0%) when probenecid was added. Therefore, the optimal incubation time and method for the human neuroblastoma SH-SY5Y cells were the 30-min dye loading with 2.5 mM probenecid, immediately followed by the fluorescence measurements.

Fig. 1 *A* shows the fluorescence excitation spectra (taken at 0 and 40 min) of Li^+-free human neuroblastoma SH-SY5Y cells after the optimal loading period. There was no appreciable change in the *R* values (335/370 ratio) over the 60-min period (data in Table 1, 30 min dye with probenecid condition). For Fig. 1 *B*, the cells were incubated with the dye also according to the optimal loading procedure and then resuspended in a 50 mM Li^+-containing medium for 40 min. Fig. 1 *B* shows the fluorescence spectra over the 40 min of Li^+ loading, with measurements every 10 min. In contrast to the spectrum for Li^+-free nerve cells (Fig. 1 *A*), as Li^+ is loaded into the cells, the spectrum is blue-shifted (Fig. 1 *B*), indicating an increase in the free Mg^{2+} concentration.

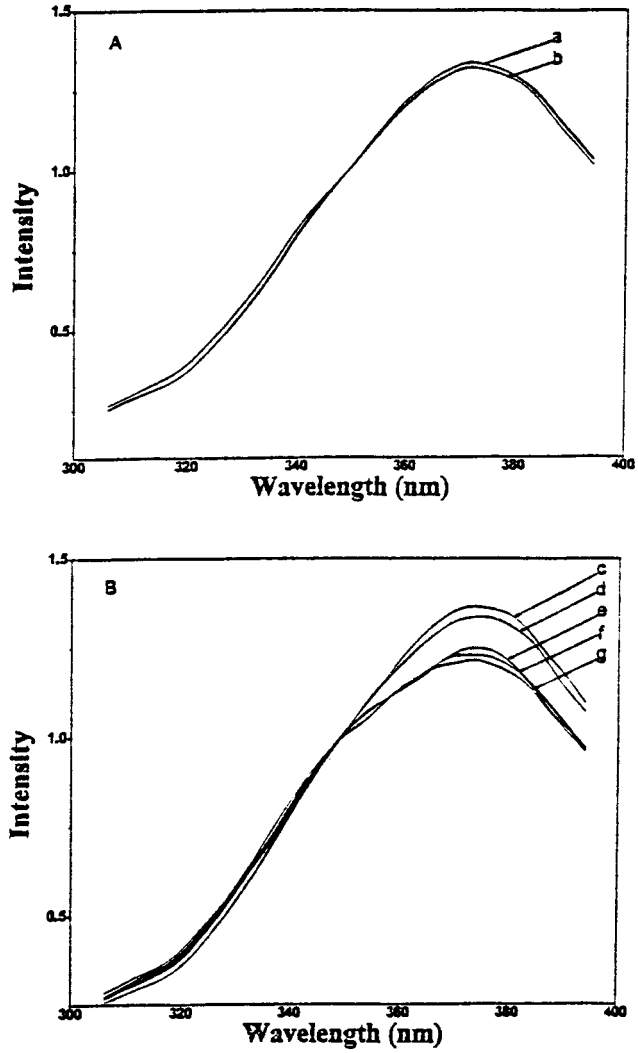

FIGURE 1 Fluorescence excitation spectra of human neuroblastoma Li^+-free (*A*) and Li^+-loaded (*B*) SH-SY5Y cells in PBS buffer containing 2.5 mM probenecid over a period of 40 min (measurements at 0, 40 min; spectra a and b, respectively) for the Li^+-free cells and over a period of 40 min (measurements at 0, 10, 20, 30, 40 min; spectra c–g, respectively) for the Li^+-loaded cells.

TABLE 1 Fluorescence intensity ratios (335/370) and normalized intensity decreases in human neuroblastoma SH-SY5Y cells incubated with furaptra at two different incubation times with and without probenecid

Time of measurement (min)	30-min dye loading *R** (normalized intensity)#	30-min dye loading–30-min PBS *R** (normalized intensity)#
Without probenecid		
0	0.48 (100%)	0.55 (100%)
20	0.50 (78%)	0.51 (80%)
40	0.49 (62%)	0.51 (70%)
60	0.50 (49%)	0.52 (56%)
With probenecid		
0	0.48 (100%)	0.49 (100%)
20	0.50 (95%)	0.49 (98%)
40	0.51 (98%)	0.49 (95%)
60	0.50 (99%)	0.48 (95%)

* The ratios (335/370) were obtained during the 60-min measurement period (measurements at 0, 20, 40, and 60 min).
# The 370 nm intensity at 0 min was arbitrarily designated as 100%.

Table 2 shows the intracellular Li^+ concentrations for SH-SY5Y cells loaded with 50 mM Li^+ during a 40-min loading period. Aliquots of cells were taken every 10 min, and the Li^+ concentration was determined by atomic absorption spectrophotometry. These experiments gave $[Li^+]_T$, which we used as $[Li^+]_f$ in Eq. 1. This simplification, however, provides an overestimation of the $[Li^+]_f$, because some of the $[Li^+]_T$ is bound. Therefore, our free Mg^{2+} values are slight underestimates of their true values. Table 2 shows the calculated $[Mg^{2+}]_f$ values and ratios (335/370) obtained from Fig. 1 *B* and three other experiments ($n = 4$) in which we used separately prepared samples ($X \pm$ SD). The $[Mg^{2+}]_f$ increased from 0.39 ± 0.04 mM at 0 min to 0.60 ± 0.04 mM at 40 min.

TABLE 2 Fluorescence-determined $[Mg^{2+}]_f$ values of human neuroblastoma SH-SY5Y cells loaded with 50 mM Li^+ over 40 min*

Time (min)	$[Li]_T$ (mM)#	R	$[Mg^{2+}]_f$ (mM)
0	0	0.498 ± 0.015	0.39 ± 0.04
10	2.5 ± 0.2	0.543 ± 0.026	0.50 ± 0.08
20	3.8 ± 0.9	0.578 ± 0.027	0.59 ± 0.08
30	5.2 ± 0.8	0.584 ± 0.020	0.60 ± 0.07
40	5.5 ± 0.8	0.586 ± 0.014	0.60 ± 0.04

* The reported values represent an average of measurements conducted on four separately prepared samples.
# The intracellular Li^+ concentration was determined by atomic absorption spectrophotometry.

Cell viability during dye and Li^+ loading

We found that the viability determined by the Trypan Blue dye exclusion method (Patterson, 1979) to be between 80.0% and 90.0% throughout all of the experiments presented. The cell viability was determined before dye loading, after dye loading, and after Li^+ incubation. None of the experimental procedures that we employed decreased the cell viability significantly.

Binding of Mg^{2+}, Li^+, and Ca^{2+} to furaptra

We analyzed the changes in the fluorescence spectrum of furaptra (salt form) due to the three most relevant ions for this experiment, Mg^{2+}, Li^+, and Ca^{2+}, at typical intracellular concentrations. In Fig. 2, the addition of 0.5 μM Ca^{2+} (spectrum b) to 2.0 μM furaptra (salt form) shows only a minimal shift in the spectrum in comparison to the spectrum without any additional ions (spectrum a), whereas the addition of 15.0 mM LiCl (spectrum c) to 2.0 μM furaptra produces a greater shift. The greatest shift was observed when 1.0 mM Mg^{2+} was added to 2.0 μM furaptra (spectrum d).

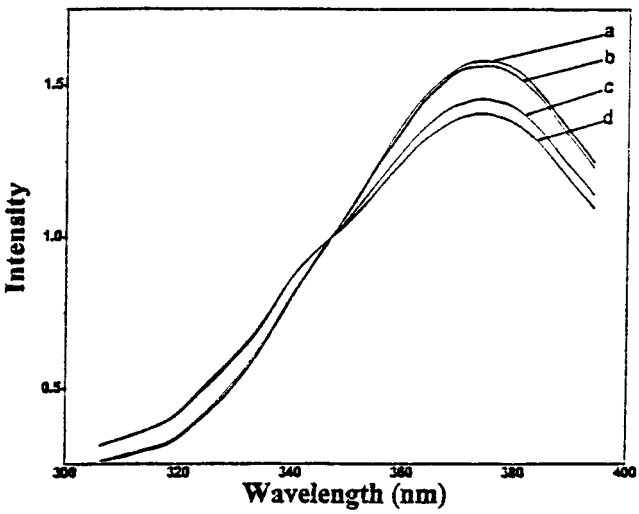

FIGURE 2 Fluorescence excitation spectra of 2 μM furaptra (salt form) in (*a*) the absence of metal ions and in the presence of (*b*) 0.5 μM Ca^{2+}, (*c*) 15.0 mM Li^+, and (*d*) 1.0 mM Mg^{2+}.

Competition between Li^+ and Mg^{2+} in intact SH-SY5Y cells by fluorescence microscopy

Fig. 3 *A* shows an image of Li^+-free neuroblastoma cells, and Fig. 3 *B* shows an image of Li^+-loaded neuroblastoma cells. To analyze the fluorescence microscopy data, we converted the image of the cells into a Windows-based Paint Shop Pro format. The number of pixels per cell image was then analyzed, and a ratio of the pixels for the wavelengths 340 and 380 nm was generated. Fig. 4 compares the time dependence of the ratios (340/380) for the Li^+-free and Li^+-loaded cells and shows that free intracellular Mg^{2+} levels are increasing for the Li^+-loaded cells while remaining constant for the Li^+-free cells.

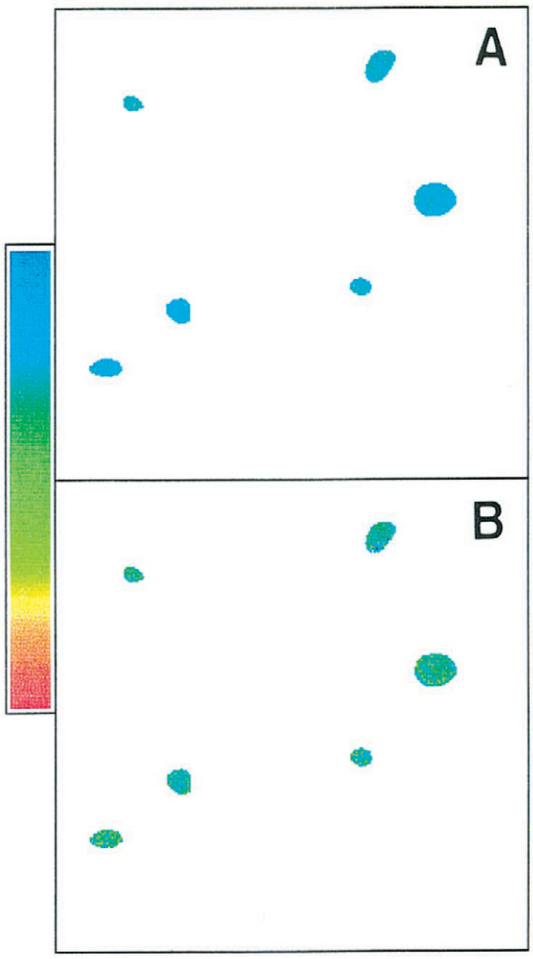

FIGURE 3 Fluorescence microscopy images of (*A*) Li^+-free and (*B*) Li^+-loaded SH-SY5Y cells. The images were coded in pseudocolor (*dark blue to red* for the minimum to the maximum 340/380 ratios).

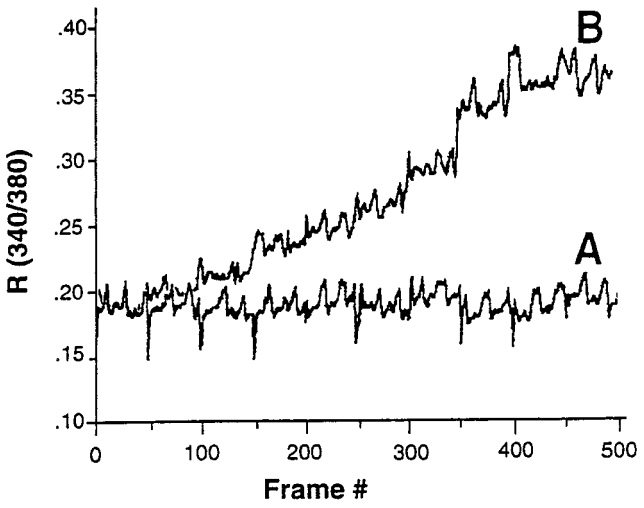

FIGURE 4 Time dependence of the ratios (340/380) from fluorescence microscopy experiments of (*A*) Li^+-free and (*B*) Li^+-loaded SH-SY5Y cells. The frames were collected every 0.33 s. Frame 0 indicates the start of the perfusion with either the Li^+-free or the Li^+-loaded solutions.

Competition between Li^+ and Mg^{2+} in intact SH-SY5Y cells by ^{31}P NMR spectroscopy

When comparing the ^{31}P NMR spectra (Nikolakopoulos et al., 1998) of Li^+-loaded and Li^+-free SH-SY5Y cells, we found that the $\alpha\beta$ chemical shift difference in Li^+-free cells was 8.67 $\pm$ 0.02 ppm ($n = 3$), whereas in Li^+-loaded cells this difference increased significantly ($p < 0.0005$) to 8.87 $\pm$ 0.02 ppm ($n = 3$). When we used Eqs. 2–5, the $[Mg^{2+}]_f$ values were 0.35 $\pm$ 0.03 mM for the Li^+-free cells and 0.80 $\pm$ 0.04 mM for the Li^+-loaded cells.

DISCUSSION

In studying Li^+/Mg^{2+} competition in this cell line, we first used fluorescence spectroscopy and the Mg^{2+} indicator furaptra (Raju et al., 1989; Murphy and London, 1995; Murphy, 1993). The furaptra dye, in its membrane-permeable ester form, was loaded into the cells by addition to cell suspensions. It permeated the cell membrane, and, once the dye was inside, the ester bonds were cleaved by intracellular esterases, resulting in dye in its salt form. Ester hydrolysis of the dye can take some time; therefore, we determined a proper incubation time to make certain that all of the ester bonds had been cleaved (Murphy and London, 1995). Furthermore, it was necessary to minimize the duration of the experiment to optimize the cell viability. Preliminary experiments (unreported data) demonstrated that a 30-min dye loading period was sufficient to load the cells with the dye. Therefore, we needed to analyze whether the dye was completely cleaved during this period or whether additional time was required to allow cleavage. Two incubation times were examined, a 30-min dye loading period with no additional time and a 30-min dye loading period with an additional 30-min incubation.

Some in vivo fluorescence dyes were shown to leak out of the cells during the course of the experiment. To minimize dye leakage in our experiment, we tested an organic anion transport inhibitor that has been used previously with the dye fura-2 in similar types of experiments (Di Virgilio et al., 1990). Because furaptra and fura-2 are structurally similar, we investigated whether probenecid would prevent leakage of furaptra from SH-SY5Y cells. In addition, it was important to assess whether probenecid would cause unwanted changes in the fluorescence ratios. Therefore, four control experiments were performed in which we compared two different incubation times (with and without 2.5 mM probenecid) to determine the correct incubation time and the effects of probenecid.

Table 1 demonstrates that the ratios (335/370) were consistently 0.50, regardless of incubation time or the addition of 2.5 mM probenecid. Therefore, we concluded that 30 min of dye loading was sufficient for the complete cleavage of the ester bonds on furaptra and that probenecid did not cause any changes in the ratios. The intensity decreases were approximately half of the starting fluorescence intensities when probenecid was not added to the cell suspension. Even though the ratios are independent of the overall dye concentration, we considered it important to maintain a high concentration of dye within the cells for the purpose of obtaining clearer spectra and thus more accurate estimates of $[Mg^{2+}]_f$. Probenecid was therefore used throughout the fluorescence spectroscopy experiments presented.

Fig. 1 *A* shows the fluorescence spectra of furaptra-loaded SH-SY5Y cells without the addition of Li^+ over a 40-min period immediately after the optimal loading procedure. There was no appreciable change in the fluorescence spectrum over this period; this indicates that Mg^{2+} levels remained constant. Fig. 1 *B* shows the fluorescence spectra of furaptra-loaded SH-SY5Y cells incubated with 50.0 mM Li^+ over a period of 40 min immediately after the optimal loading procedure. As Li^+ was loaded into the cells, the spectrum was blue-shifted, indicating an increase in the free Mg^{2+} concentration (Table 2), as well as Li^+ displacement of Mg^{2+} from Mg^{2+} binding sites within the neuroblastoma cells. Our observed initial free Mg^{2+} value of 0.39 mM is in close agreement with that observed for other neuronal cell types, which have free intracellular Mg^{2+} values ranging from 0.4 to 0.7 mM (Maguire, 1990; Murphy, 1993).

The Li^+ experiments were terminated at 40 min, because the free intracellular Mg^{2+} concentrations had stopped increasing at that time. Interestingly, the intracellular Li^+ levels had reached equilibrium at this point, demonstrating that this should be the time at which Mg^{2+}-free intracellular levels are highest if this competition is operational. This observation adds further validity to the hypothesis that Li^+/Mg^{2+} competition is occurring within the cell.

We performed additional experiments to examine the binding properties of Li^+ and Ca^{2+} ions because it was important to determine that the blue shift observed in Fig. 2 was due to Mg^{2+}. Studies have shown that Ca^{2+} significantly binds furaptra (Ca^{2+}-furaptra $K_d = 20.49$ μM) (Hur-

ley et al., 1992) and can cause, at high concentrations, a blue shift similar to that which Mg^{2+} produces with furaptra. Furthermore, Li^+ has caused similar blue shifts and has shown to bind furaptra weakly (K_d of Li^+-furaptra, 250 ± 70 mM) (Mota de Freitas et al., 1994b). Because both of these ions, Ca^{2+} and Li^+, are present in the neuroblastoma cells in these experiments and have been shown to bind furaptra (Mota de Freitas et al., 1994b; Hurley et al., 1992), the extent to which these two ions were contributing to the blue shift seen in Fig. 1 *B* was determined.

For both Ca^{2+} and Li^+, we used the maximum experimental free intracellular concentrations to determine how much these ions were contributing to the blue shift in the spectrum in Fig. 1 *B*. It is known from the literature (Connor and Henderson, 1996; Willars and Nahorski, 1994, 1995) on Ca^{2+} levels in SH-SY5Y cells that the maximum Ca^{2+} level is ~0.5 μM upon stimulation by agonists that increase Ca^{2+} levels, such as carbachol and bradykinin. It was possible that a small change in $[Ca^{2+}]$ could occur, because past studies have shown that platelets of Li^+-treated patients have higher free intracellular Ca^{2+} levels than do those of normal individuals (Tan et al., 1990). An increase in the Ca^{2+} level, if any, was not expected to be greater than 0.5 μM. Therefore, 0.5 μM Ca^{2+}, the maximum level, was added to 2 μM furaptra (*spectrum b* in Fig. 2). Because this maximum level caused no appreciable change in the spectrum (*spectrum a*, no ions; *spectrum b*, 0.5 μM Ca^{2+}), the effect of Ca^{2+} in this experiment was considered insignificant. Therefore, we concluded that a Ca^{2+} correction was unnecessary.

By atomic absorption spectrophotometry, we determined that the $[Li^+]_T$ within the neuroblastoma cells during an 110 mM Li^+ incubation at 60 min was ~15.0 mM. Therefore, 15.0 mM Li^+ was added to 2.0 μM furaptra (*spectrum c* in Fig. 2). This spectrum showed a considerable shift in comparison to the spectra without any additional ions (*spectrum a*). Therefore, a correction for Li^+ was added to Eq. 1; otherwise, our calculated Mg^{2+} concentrations would have been larger than their true values.

We also looked at Li^+/Mg^{2+} competition by using fluorescence microscopy. The cells were attached to a chamber slide, and the fluorescence excitation intensity was monitored at 340 and 380 nm. Figs. 3 *A* and 3 *B* are images of the neuroblastoma cells that were Li^+-free (*blue*) and Li^+-loaded (*green*), respectively. The Li^+-free cells appear blue and the Li^+-loaded cells are green, because the ratio of 340/380 is smaller for the Li^+-free and larger for the Li^+-loaded cells. In the case where the cells were perfused without Li^+, the ratios (340/380) remained constant throughout the experiment (Fig. 4 *A*), indicating constant Mg^{2+} levels. When the cells were perfused with Li^+, the ratios (340/380) increased (Fig. 4 *B*), which indicates that the free intracellular Mg^{2+} is increasing.

We also investigated Li^+/Mg^{2+} competition in intact human neuroblastoma cells by using ^{31}P NMR spectroscopy. The cells were embedded in agarose gel threads and perfused with medium over the course of the experiment. The increase in the $\alpha\beta$ chemical shift difference observed upon Li^+ loading was indicative of Li^+ displacement of Mg^{2+} bound to the phosphate groups of ATP, because it increased from a value that resembled Mg^{2+}-saturated ATP to one that resembled Li^+-saturated ATP (Mota de Freitas et al., 1994b; Ramasamy and Mota de Freitas, 1989).

Analysis of these data by use of Eqs. 2–5 gave $[Mg^{2+}]_f$ values of 0.35 ± 0.03 mM for the Li^+-free cells and 0.80 ± 0.04 for the Li^+-loaded cells. We also performed three additional fluorescence spectroscopy experiments with 110 mM Li^+-loading solution so that we could compare our $[Mg^{2+}]_f$ values from the ^{31}P NMR experiments to our fluorescence spectroscopy method. These fluorescence experiments gave a free intracellular Mg^{2+} concentration of 1.03 ± 0.10 mM when the cells were completely loaded with Li^+.

The $[Mg^{2+}]_f$ values determined by ^{31}P NMR for the Li^+-free cells (0.35 ± 0.03 mM) were in good agreement with our fluorescence-determined Li^+-free values of 0.39 ± 0.04 mM (Table 2, 0 min), as well as previously determined $[Mg^{2+}]_f$ values for other neuronal cell types (Maguire, 1990; Murphy, 1993). Our ^{31}P NMR calculated $[Mg^{2+}]_f$ values for the Li^+-loaded cells (0.80 ± 0.04 mM) were 0.2 mM higher than our fluorescence-determined Li^+-loaded (50 mM Li^+-loading solution) values (0.60 ± 0.04 mM; Table 2, 40 min), and for our fluorescence experiments with 110 mM Li^+-loading solution our calculated $[Mg^{2+}]_f$ values were 0.4 mM higher than for our fluorescence experiments with 50 mM Li^+-loading solution. These differences in $[Mg^{2+}]_f$ values are understandable when one considers that for the ^{31}P NMR experiments and the fluorescence spectroscopy experiments with 110 mM Li^+-loading solution, a higher concentration of Li^+ was used to load the cells, whereas for the other fluorescence experiments, 50 mM Li^+ was used for loading. The reason for using a higher concentration of Li^+-loading solution is that more Li^+ will enter the cell (110 mM Li^+-loading solution results in a final $[Li^+]$ in the cell of 16 mM, whereas 50 mM Li^+-loading solution results in a final $[Li^+]$ in the cell of 5.5 mM). We would expect that with more Li^+ entering the cell that the competition for Mg^{2+} binding sites would be greater—hence the larger $[Mg^{2+}]_f$ value.

The ^{31}P NMR method was less sensitive than the fluorescence method for detection of competition between Li^+/Mg^{2+} within the SH-SY5Y cells, which is demonstrated by the small discrepancy in our 110 mM Li^+-loaded cell calculated $[Mg^{2+}]_f$ (0.80 ± 0.04 mM for the ^{31}P NMR method and 1.03 ± 0.10 mM for the fluorescence method). For ^{31}P NMR, ATP within the cells is almost saturated with Mg^{2+} (the K_d value for the MgATP complex is 0.05 mM), whereas, for the fluorescence methods, larger Mg^{2+} concentrations are needed for saturation of the fluorescent dye (the K_d value for the Mg^{2+}-dye indicator within the neuroblastoma SH-SY5Y cells is 1.9 ± 0.2 mM). Therefore, it was necessary to use a higher concentration of Li^+ (110 mM) for the ^{31}P NMR because this technique was less sensitive. The fluorescence method proved to be more sen-

sitive because the fluorescence indicator binds Mg^{2+} weakly, whereas the ^{31}P NMR indicator, ATP, strongly binds Mg^{2+}. Another problem with the ^{31}P NMR method is the difficulty of obtaining the true limiting shifts for the ATP within the cell (Iotti et al., 1996).

The fluorescence spectroscopic method that we used to examine Li^+/Mg^{2+} competition in intact SH-SY5Y neuroblastoma cells proved to be a sensitive technique, with a few limitations. One obstacle to the use of this cell line for fluorescence studies was that the cells are very delicate. For NMR experiments, the cells are embedded in gel threads or there is some other means of attachment, and the cells are perfused with medium (Nikolakopoulos et al., 1996, 1998; Zachariah et al., 1996). However, this was not practical with our fluorescence setup. To maintain a high level of cell viability, we always used fresh, oxygenated solutions. Under these conditions, the cell viability was maintained above 80% over the course of the experiment. When the solutions were not oxygenated, the viability at times would drop below 60%. For the fluorescence microscopy experiments, the cell viability was not a concern because the cells were grown on the coverslips and were continuously perfused with fresh medium.

To understand the physiological relevance of these experiments, we have to consider how much Li^+ accumulates in nerve cells exposed to therapeutic levels of Li^+ and compare these levels to how much Li^+ we allowed to accumulate in our cells. Previously, it has been shown that the tissue-to-plasma concentration ratio for Li^+ is 1.3 (Lam and Christensen, 1992) and that the plasma concentration of Li^+-treated patients is between 0.3 and 1.5 mM Li^+ (Bach, 1990). Therefore, the total intracellular Li^+ in tissue can reach 2 mM and in some tissue types (i.e., muscle cells), the tissue concentration can exceed these average levels (Komoroski et al., 1997). For our fluorescence spectroscopy experiments, the final intracellular [Li^+] levels were 5.5 mM (Table 2, 40 min). These levels are comparable to the therapeutic levels previously determined (Lam and Christensen, 1992), especially at the earlier times during the Li^+ incubation (10 and 20 min), at which the [Li^+] levels were 2.5 and 3.8 mM, respectively. For our ^{31}P NMR experiments, however, a higher intracellular Li^+ concentration, 16 mM (determined by atomic absorption spectrophotometry), was obtained. These levels are therefore less relevant to therapeutic Li^+ levels.

In summary, all three methods used for the study of Li^+/Mg^{2+} competition in intact neuroblastoma cells showed that there was competition between these two ions. Future studies by fluorescence microscopy are have been commenced in our effort to determine which organelles within the cell have the strongest affinity for Li^+. Fluorescence microscopy studies may take us a step closer to identification of the Li^+ binding sites within neuroblastoma cells and thus to an understanding of the pharmacological action of Li^+ at the cellular and molecular levels.

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