Neuropsychopharmacology (2004) 29, 759–769
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Chronic Lithium Treatment Attenuates Intracellular Calcium Mobilization

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Elevated basal intracellular calcium (Ca^{2+}) levels ($[Ca^{2+}]_B$) in B lymphoblast cell lines (BLCLs) from bipolar I disorder (BD-I) patients implicate altered Ca^{2+} homeostasis in this illness. Chronic lithium treatment affects key proteins modulating intracellular Ca^{2+} signaling. Thus, we sought to determine if chronic exposure to therapeutic lithium concentrations also modifies intracellular Ca^{2+} homeostasis in this surrogate cellular model of signal transduction disturbances in BD. BLCLs from BD-I ($N=26$) and healthy subjects ($N=17$) were regrown from frozen stock and incubated with 0.75 mM lithium or vehicle for 24 h (acute) or 7 days (chronic). $[Ca^{2+}]_B$, lysophosphatidic acid (LPA)-stimulated Ca^{2+} mobilization ($[Ca^{2+}]_S$), and thapsigargin-induced store-operated Ca^{2+} entry (SOCE) were determined using ratiometric fluorometry with Fura-2. Compared with vehicle, chronic lithium exposure resulted in significantly higher $[Ca^{2+}]_B$ ($F=8.47$; $p=0.006$) in BLCLs from BD-I and healthy subjects. However, peak LPA-stimulated $[Ca^{2+}]_S$ and SOCE were significantly reduced ($F=11.1$, $p=0.002$ and $F=8.36$, $p=0.007$, respectively). Acute lithium exposure did not significantly affect measured parameters. In summary, the effect of chronic lithium to elevate $[Ca^{2+}]_B$ in BLCLs while attenuating both receptor-stimulated and SOCE components of intracellular Ca^{2+} mobilization in BLCLs suggests that modulation of intracellular Ca^{2+} homeostasis may be important to the therapeutic action of lithium.
Neuropsychopharmacology (2004) **29,** 759–769, advance online publication, 18 February 2004; doi:10.1038/sj.npp.1300400

Keywords: lithium; bipolar disorder; intracellular calcium mobilization; signal transduction; B lymphoblast; pathophysiology

INTRODUCTION

Lithium has been used in the management of bipolar disorder (BD) for over 50 years (Goodwin and Jamison, 1990), and despite its proven efficacy (Schou, 2001), the specific mechanism(s) by which it exerts its therapeutic effects in BD is still poorly understood (reviewed in Lenox *et al*, 1998; Manji *et al*, 2001). As the latency in onset of lithium's clinical action implicated plastic changes mediated through intracellular signaling processes, transcription regulation, and accompanying alterations in abundance and function of target proteins, intracellular signal transduction systems have come under closer scrutiny in search of the molecular basis of its therapeutic action in BD (Manji *et al*, 2001). Among these, the Ca^{2+} signaling system is of particular interest given its critical role in numerous cellular functions (for reviews see Ghosh and Greenberg, 1995; Berridge *et al*, 2000 and references therein).

Intracellular Ca^{2+} signaling and homeostasis are maintained by an intricate array of processes acting in concert (Berridge *et al*, 2000; Putney *et al*, 2001) including, for example, inositol trisphosphate (IP_3)- and ryanodine-stimulated release of Ca^{2+} from endoplasmic reticulum (ER) storage pools (Berridge, 1995; Barritt, 1999; Putney and Ribeiro, 2000), voltage- and ligand-gated ion channel mediated Ca^{2+} influx (Ghosh and Greenberg, 1995), store-operated Ca^{2+} entry (SOCE) (Putney *et al*, 2001), plasma membrane and sarcoplasmic/ER Ca^{2+}-ATPase pumps (PMCAs and SERCAs) (Brini and Carafoli, 2000), and mitochondrial Ca^{2+} uptake, storage, and release (Brini and Carafoli, 2000; Fall and Keizer, 2001). Disturbances of intracellular Ca^{2+} signaling can critically affect cellular function due to calcium's essential role in vital cellular processes including gene expression (Ghosh and Greenberg, 1995), neurogenesis and plasticity (Mattson, 2000), and cell death (Szalai *et al*, 1999). It is of some interest then that

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Received 17 October 2003; revised 22 December 2003; accepted 31 December 2003
Online publication: 7 January 2004 at http://www.acnp.org/citations/Npp01070403477/default.pdf

lithium acts on several second messenger systems and intracellular signal transducing proteins (Manji *et al*, 1999; Williams and Harwood, 2000) that are involved in key cellular functions that affect neurogenesis, neuroplasticity, and cell death (Manji *et al*, 2000).

The notion that intracellular Ca^{2+} dynamics may be altered in BD has been suggested by studies using peripheral blood cells as surrogate models, as well as investigations on post-mortem brain. Elevated basal ($[Ca^{2+}]_B$) and agonist-stimulated ($[Ca^{2+}]_S$) intracellular Ca^{2+} levels in the platelets and lymphocytes of bipolar I disorder (BD-I) patients compared with healthy subjects (Dubovsky *et al*, 1989, 1992; Tan *et al*, 1990; Plenge *et al*, 1994; Berk *et al*, 1995; Emamghoreishi *et al*, 1997; Hough *et al*, 1999) are among the most widely replicated findings in psychobiological studies of BD. Several observations suggest that these abnormalities reflect intrinsic disturbances in cellular function. First, plasma ultrafiltrates from BD patients did not affect $[Ca^{2+}]_B$ in platelets from healthy subjects (Dubovsky *et al*, 1994). More important, elevated $[Ca^{2+}]_B$ (Emamghoreishi *et al*, 1997) and agonist-stimulated $[Ca^{2+}]_S$ (Wasserman *et al*, 2003) have been reported in B lymphoblast cell lines (BLCLs) from BD patients. Finally, these abnormalities have been observed independent of state of illness (Tan *et al*, 1990; Kusumi *et al*, 1994; Emamghoreishi *et al*, 1997).

Post-mortem brain studies have also revealed changes that may reflect possible 'signatures' of abnormal Ca^{2+} homeostasis in BD. These include a marked blunting of G-protein activated phosphoinositide (PI) hydrolysis (Jope *et al*, 1996) and altered mRNA expression levels of two candidate proteins, which may have important roles in Ca^{2+} homeostasis, inositol monophosphatase (IMPase) type II (Yoon *et al*, 2001a), and a transient receptor potential channel, TRPM2 (TRPC7 in earlier nomenclature) (Yoon *et al*, 2001b), a ligand-gated plasma membrane ion channel which also mediates Ca^{2+} entry into cells.

Substantial evidence also links lithium treatment to the modulation of intracellular Ca^{2+} homeostasis. Lithium attenuates agonist-stimulated intracellular Ca^{2+} responses in a number of cellular paradigms, including astrocytes (Helman *et al*, 1986), C6 rat glioma cells (Yamaji *et al*, 1997), rat cerebellar granule cells (Nonaka *et al*, 1998), GH3 pituitary cells (Varney *et al*, 1992), rat hippocampal slices (Okamoto *et al*, 1995), human neutrophils (Forstner *et al*, 1994), and platelets (Dubovsky *et al*, 1991). As well, lithium inhibits IMPase uncompetitively (Hallcher and Sherman, 1980), an effect suggested to diminish overactive signaling through PI-linked second messengers and, in turn, Ca^{2+} (Berridge *et al*, 1982).

Current understanding of the molecular actions of lithium and other mood stabilizers has been gleaned almost entirely from preclinical work using tissue and cellular models of nonhuman origin, which do not express the disease phenotype. BLCLs from BD patients appear to stably express underlying abnormalities in Ca^{2+} homeostasis that may be more intimately linked to the pathophysiology of this disorder (Emamghoreishi *et al*, 1997; Yoon *et al*, 2001a, b; Wasserman *et al*, 2003). As such, they afford a particularly pertinent species and disease relevant model in which to explore the mechanisms by which lithium modifies these intracellular Ca^{2+} disturbances and the relevance of such effects to its therapeutic action. Accordingly, in this study we have exploited this model to determine whether lithium, at a therapeutically relevant concentration, modulates intracellular Ca^{2+} homeostasis abnormalities identified in BLCLs from BD-I patients, and whether this effect requires chronic exposure. Towards this end, two key components of intracellular Ca^{2+} mobilization were scrutinized: (1) receptor-G-protein coupled agonist (lysophosphatidic acid (LPA)) stimulation of Ca^{2+} mobilization through the PI/IP_3 signaling pathway, and (2) SOCE. We report here that chronic (7 days), but not acute (24 h), treatment of BLCLs from both BD-I patients and healthy subjects with lithium, at a therapeutically relevant concentration, significantly elevated $[Ca^{2+}]_B$ and attenuated LPA-stimulated Ca^{2+} mobilization and SOCE. These results suggest that the modulation of intracellular Ca^{2+} homeostasis may be important to the therapeutic action of lithium.

MATERIALS AND METHODS

Subjects

BD-1 patients and healthy subjects were recruited and assessed as previously described (Emamghoreishi *et al*, 1997). Confirmation of diagnoses was performed using the Structured Clinical Interview for DSM-IV Axis I Disorders—Patient Edition (SCID-I/P) (First *et al*, 1995b) or Nonpatient Edition (SCID-I/NP) (First *et al*, 1995a) administered by a research psychiatrist or a trained psychiatric assistant. Subjects had no concurrent medical illness, diabetes or hypertension, no recent (>3 months) drug or alcohol abuse (BD-I patients), and were competent to provide informed written consent. Healthy subjects had no family history of psychiatric disorders in first-degree relatives. All subjects were screened with a systems review and a physical examination, where necessary, to rule out the presence of any chronic physical illness. The study was approved by the Human Subjects Review Board of the University of Toronto.

Cell Culture Materials, Reagents, and Drugs

Characterized fetal bovine serum (FBS) was from HyClone (Logan, UT). RPMI-1640, L-glutamine and trypan blue were obtained from Invitrogen Life Technologies (Burlington, ON). Penicillin (10 000 U/ml), streptomycin (10 mg/ml), pyruvate, dimethylsulfoxide (DMSO), $MgCl_2$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), LPA (1-oleoyl-sn-glycero-3-phosphate), and ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetra-acetic acid (EGTA) were from Sigma (Oakville, ON). NaCl, KCl, $CaCl_2$, and D-glucose were of analytical grade. Polyethylene glycol-8000 was from Mallinckrodt (Point-Claire, QC). Triton X-100 and lithium chloride (LiCl) were from JT Baker (Phillipsburgh, NJ). Thapsigargin (TG) was obtained from Calbiochem (La Jolla, CA) and Fura-2 acetoxymethyl ester (Fura-2 AM) from Molecular Probes (Eugene, OR).

Establishment of BLCLs

Epstein–Barr virus (EBV) transformed BLCLs were established using standard techniques (Emamghoreishi *et al*, 1997). The BLCLs for this study were selected from frozen

stocks established from a cohort of BD-I patients and healthy comparison subjects participating in ongoing studies of signal transduction mechanisms in mood disorders in this laboratory (Emamghoreishi *et al*, 1997; Yoon *et al*, 2001a, b). BLCLs were initially expanded in culture for 13–16 passages in a 95% air/5% CO_2 humidified incubator at 37°C (2–3 days/passage) prior to freezing and storage in the vapor phase of liquid nitrogen.

BLCL Regrowth Conditions

BLCLs from BD patients and healthy controls were regrown in paired groups from subjects matched on the basis of age and sex. Cells were cultured for 6–10 passages (4–7 weeks) prior to initiating treatment with LiCl or vehicle. During regrowth, B cell media (BCM: RPMI 1640, 20% FBS, 2 mM L-glutamine, 1 mM pyruvate, 100 μg/ml streptomycin, 100 U/ml penicillin) was replenished every 2 days. Cell counts, and viability, as determined by trypan blue exclusion (Emamghoreishi *et al*, 1997), were estimated at each feeding.

Drug Treatment Protocol

Once BLCLs reached the required cell counts ($>9\times10^7$ cells, >95% viability), they were divided equally into three groups: two (A and B) were fed with aqueous control medium consisting of BCM to which isotonic NaCl vehicle solution of the same volume as the lithium solution, (10 ml/l BCM) was added, while the other (C) was treated with BCM containing LiCl (final concentration 0.75 mM). Media was completely replaced by gentle sedimentation (50 *g*, 10 min) and resuspension of cells every other day for the first 6 days. At day 6, cells were gently sedimented, the supernatant was removed, and the cells were resuspended in RPMI-1640 containing either vehicle solution as above (A) or 0.75 mM LiCl (B). Cells that had been treated with BCM containing LiCl (C) for the first 6 days were also washed and taken up in 0.75 mM LiCl in RPMI-1640. After a further 24 h incubation, cells were harvested and endpoint Ca^{2+} mobilization assays were performed.

Ca^{2+} Mobilization Assays

Agonist (LPA) stimulation. The assay of LPA-stimulated Ca^{2+} mobilization was performed as previously described (Rosskopf *et al*, 1998) with modifications. Briefly, cells ($1–3\times10^6$/ml in RPMI-1640) were loaded with Fura-2-AM (1 μM) or treated with an equivalent volume of DMSO (1 μl/ml cell suspension; blank sample) as previously described (Emamghoreishi *et al*, 1997). Following loading, cells were placed on ice until spectrophotofluorometric quantitation to reduce compartmentalization and dye leakage. Immediately prior to analysis, cells were transferred to a spectrophotofluorometric cuvette and allowed to equilibrate in the temperature-controlled chamber of a Perkin-Elmer L50B fluorometer, with gentle stirring for 3 min at 37°C. Free- and Ca^{2+}-bound Fura-2 fluorescence intensities (excitation 340/380 nm, emission 500 nm) were determined as reported elsewhere (Emamghoreishi *et al*, 1997). After measuring a stable fluorescence ratio (R) for 30 s to establish a baseline level, a maximally stimulating concentration of LPA (100 μM, determined in preliminary concentration–response experiments) was injected directly into the sample as a bolus using a 50 μl Hamilton syringe. Subsequently, R was measured for an additional 270 s to determine peak response and initial decay phases of the stimulated $[Ca^{2+}]_i$ mobilization. Determination of and correction for autofluorescence was performed using the DMSO blank samples processed in parallel (Emamghoreishi *et al*, 1997). For calibration of Fura-2 fluorescence intensities, equivalent aliquots of cells were lysed with 0.05% Triton X-100 in a 1 mM $CaCl_2$ medium and R_{max} was measured. EGTA (6 mM, 30 mM Tris, pH 8.5) was then added and R_{min} was measured. $[Ca^{2+}]_i$ was then calculated using the Grynkiewicz equation (Grynkiewicz *et al*, 1985). Determinations were performed on duplicate aliquots of BLCLs from each subject and the mean values were used in all subsequent data analyses.

Thapsigargin stimulation. Determination of Ca^{2+} leakage from the ER and store depletion-induced SOCE was performed as previously described (Putney and Ribeiro, 2000), with modifications. This technique exploits the effects of TG to deplete TG-sensitive ER Ca^{2+} storage pools thereby providing a stimulus for opening of plasma membrane store-operated Ca^{2+} channels (SOCC) and influx of extracellular Ca^{2+}. Cells ($1–3\times10^6$/ml) were incubated with Fura-2 AM or DMSO vehicle, after which they were washed, resuspended in nominally Ca^{2+}-free HEPES (HEPES 10 mM, glucose 10 mM, NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1 mM) buffer and transferred to a spectrophotofluorometric cuvette, equilibrated, and free- and Ca^{2+}-bound Fura-2 fluorescence intensities determined as above. After measuring baseline R for 30 s, a maximally stimulating concentration of TG (200 nM, confirmed in preliminary experiments) was injected as a bolus into the sample. Subsequently, R was measured for an additional 360 s to ensure depletion of TG-sensitive ER Ca^{2+} storage pools and to allow Ca^{2+} levels to recede to a stable secondary baseline. Extracellular $[Ca^{2+}]$ was then rapidly raised to physiological concentrations by addition of $CaCl_2$ to a final concentration of 1 mM. R was measured for an additional 300 s to assess the degree of SOCE induced by the ER Ca^{2+} depletion stimulus. Correction for autofluorescence and calibration of Fura-2 fluorescence intensities were performed using equivalent aliquots of cells and $[Ca^{2+}]_i$ calculated as above. Determinations were performed on duplicate aliquots of BLCLs as above and the mean values were used in all subsequent data analyses.

Statistics

Data are presented as means ± standard error of the mean (SEM). Differences in the proportion of males to females in each diagnostic group were analyzed by a χ^2 test. After confirming homogeneity of variance (Levine's test), differences in dependent variables between subject groups were analyzed by repeated measures multivariate analysis of variance (MANOVA) with diagnostic group and drug treatment as factors. Subsequent repeated measures univariate analyses (ANOVA) were used to determine the differences in specific dependent variables. Dependent measures determined in the LPA-stimulated Ca^{2+}

mobilization paradigm were analyzed separately from those of the TG-stimulation protocol. This was done to avoid the reduced power of a single MANOVA, using all dependent cellular measures, resulting from exclusion of records with missing values: the cumulative loss of samples due to procedural difficulties resulted in a number of missing values among each set of experimental parameters. Differences between acute and chronic lithium treatment were analyzed by repeated measures ANOVA followed by simple contrasts to distinguish effects of time of exposure compared with vehicle control. Potential effects of other factors, including mood state and type of mood stabilizer treatment at the time of study, familial history of mood disorders, and psychiatric comorbidities, were assessed statistically using repeated measures ANOVA with the demographic characteristic as the between-subject factor, followed by Tukey's HSD test for *post hoc* comparisons. Statistical differences with two-tailed probability values of $p<0.05$ were taken as significant. Statistical analyses were performed using the SPSS (version 9.5, Chicago, IL) statistical package.

RESULTS

Subjects

Table 1 summarizes the demographic characteristics of the BD-I patients and healthy comparison subjects from whom BLCLs were used in this study. While BLCL samples were regrown from 26 BD-I patients and 16 healthy subjects, the actual numbers of subject samples analyzed for each parameter were less due to methodological difficulties resulting in sample loss during one or the other of the assay protocols used. Patient and healthy subject groups did not differ statistically in proportion of females to males ($\chi^2=1.27$, $p=0.26$) or in mean age ($t=0.994$, $p=0.33$).

Table 1 Demographic Characteristics

	Bipolar I patients	Healthy controls
Total	26	17
Sex(M/F)	16/10	7/10
Age (years)	42.2±2.3[a]	39.5±2.8
Age at onset of Illness	27.0±2.7 years	N/A
State at time of venipuncture (n)		
Euthymic	15	
Depressed	6	N/A
Hypomanic	3	
Manic	2	
Cotmorbidites (n)[b]		
Alcohol abuse/depedence	10	
Compulsive disorders	1	N/A
Eating disorders	2	
Panic disorder	1	
Family History of Mood Disorders (n)[c]		
Positive	12	
Negative	6	N/A
Unknown	8	
Mood stabilizer medication time of study (n)		
Lithium	11	
Valproate	5	
Carbamazepine	1	
Combination[d]	3	N/A
None	6	
Other Psychotropic medications at time of study (n)		
SSRIs	6	
Neuroleptics	4	N/A
Anxiolytics	4	
None	14	

[a]Data presented as mean ±SEM. N/A indicates not applicable.
[b]These categories are nonexclusive; an individual may meet more than one comorbid diagnosis.
[c]Familial mood disorders include bipolar disorder, major depressive disorder, dysthymia, seasonal affective disorder, and schizoaffective disorder (bipolar type).
[d]Combination thearapy refers to lithium plus valproate or carbamazepine.

Effect of Chronic Lithium Treatment on Agonist-Stimulated Intracellular Ca^{2+} Mobilization

Figure 1a depicts a representative response to LPA stimulation in BLCLs incubated for 7 days with 0.75 mM LiCl or vehicle. LPA evoked a rapid rise in $[Ca^{2+}]_i$ from $[Ca^{2+}]_B$ reaching a peak response (peak $[Ca^{2+}]_S$) between 20 and 100 s after stimulation, following which it declined towards baseline levels. The decay phase reflects the restitution of Ca^{2+} towards baseline levels through homeostatic control mechanisms including plasma membrane and ER Ca^{2+} ATPase pumps (Brini and Carafoli, 2000) and Na^+/Ca^{2+} exchangers (Blaustein and Lederer, 1999). The parameters examined for lithium modulation in this paradigm included the $[Ca^{2+}]_B$, peak $[Ca^{2+}]_S$, the absolute difference ($\Delta[Ca^{2+}]_S$) between $[Ca^{2+}]_S$ and $[Ca^{2+}]_B$, and the slope of the initial rate of rise in LPA-stimulated $[Ca^{2+}]_S$ ($d[Ca^{2+}]_S/dt$).

The effect of chronic lithium treatment on the above parameters of Ca^{2+} mobilization in BLCLs from patients and healthy comparison subjects is presented in Figures 1a and 2. MANOVA revealed significant main effects of lithium treatment ($F=7.18$; $df=3,31$; $p=0.001$) and diagnosis ($F=3.42$; $df=3,31$; $p=0.03$), but no interaction ($F=0.243$; $df=3,31$; $p=0.87$) between these factors. Subsequent univariate ANOVAs revealed significant effects of lithium treatment on $[Ca^{2+}]_B$ ($F=8.47$; $df=1,33$; $p=0.006$), peak $[Ca^{2+}]_S$ ($F=9.94$; $df=1,33$; $p=0.003$), and $\Delta[Ca^{2+}]_S$ ($F=11.1$; $df=1,33$; $p=0.002$), but not $d[Ca^{2+}]_S/dt$ ($F=1.54$; $df=1,33$; $p=0.224$) in the LPA-stimulation protocol. While exposure of BLCLs to lithium for 7 days resulted in a modest but significant elevation in $[Ca^{2+}]_B$ in both the patient (11.1%) and the healthy subject

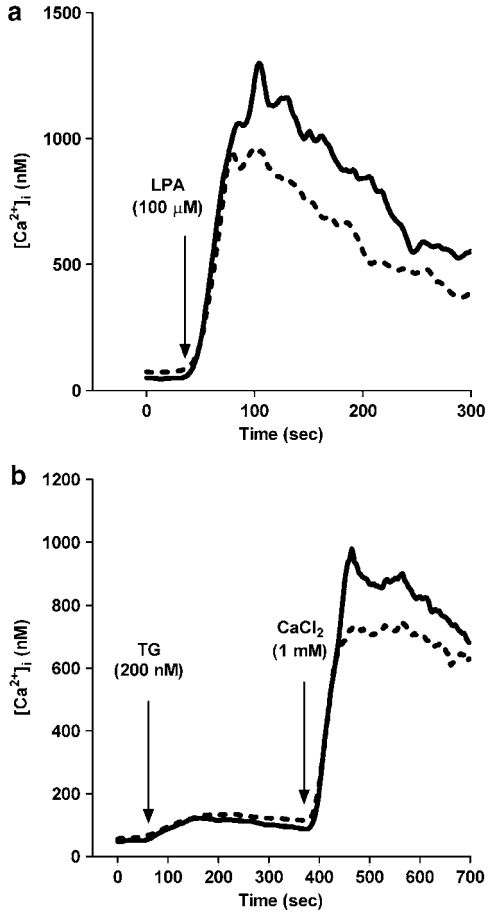


Figure 1 Effect of Chronic Lithium Treatment on Agonist-Stimulated Ca^{2+} Mobilization and ER Store-Depletion-Induced Ca^{2+} Entry in BLCLs. Cells were pretreated for 7 days with LiCl (0.75 mM) or vehicle. Cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) were measured using the Ca^{2+}-indicator Fura 2-AM as described in the Materials and methods. (a) Typical Ca^{2+} responses induced by 100 μM LPA in control (solid line) *vs* lithium-treated (broken line) cells from a healthy subject are shown. The arrow indicates the time of LPA administration. (b) Representative Ca^{2+} responses after the addition of 200 nM TG in Ca^{2+}-free medium, followed by the reintroduction of 1 mM extracellular Ca^{2+} in control (solid line) *vs* lithium-treated (broken line) BLCLs from a healthy subject is shown. Arrows indicate the time of TG/extracellular Ca^{2+} administration.

(10.0%) groups, the same treatment caused a significant attenuation in both $[Ca^{2+}]_S$ (12.2% lower in patients, 11.1% lower in healthy subjects) and $\Delta[Ca^{2+}]_S$ (14.1% lower in patients, 12.3% lower in healthy subjects) compared to vehicle treatment. The $d[Ca^{2+}]_S/dt$ was also reduced in lithium-treated samples (11.5%) but the difference was not statistically significant.

To test whether the above lithium-induced changes were dependent on the duration of exposure, BLCLs from a subset of subjects (eight patients, two healthy) were treated for 24 h or 7 days with 0.75 mM lithium or vehicle, and LPA-stimulated Ca^{2+} responses determined. As shown in Figure 4a and b, acute lithium treatment did not significantly influence $[Ca^{2+}]_B$ and peak $[Ca^{2+}]_S$ in BLCLs (simple contrasts: $F = 1.73$; $df = 1,9$; $p = 0.22$ and $F = 1.55$; $df = 1,9$; $p = 0.245$, respectively) in contrast to the chronic lithium treatment paradigm ($F = 4.28$; $df = 1,9$; $p = 0.068$ and $F = 21.0$; $df = 1,9$; $p = 0.001$, respectively).

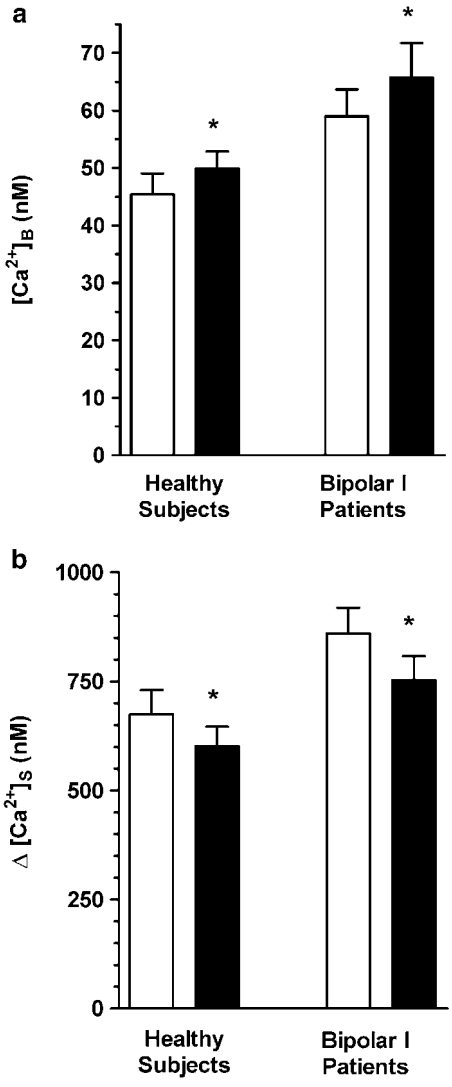


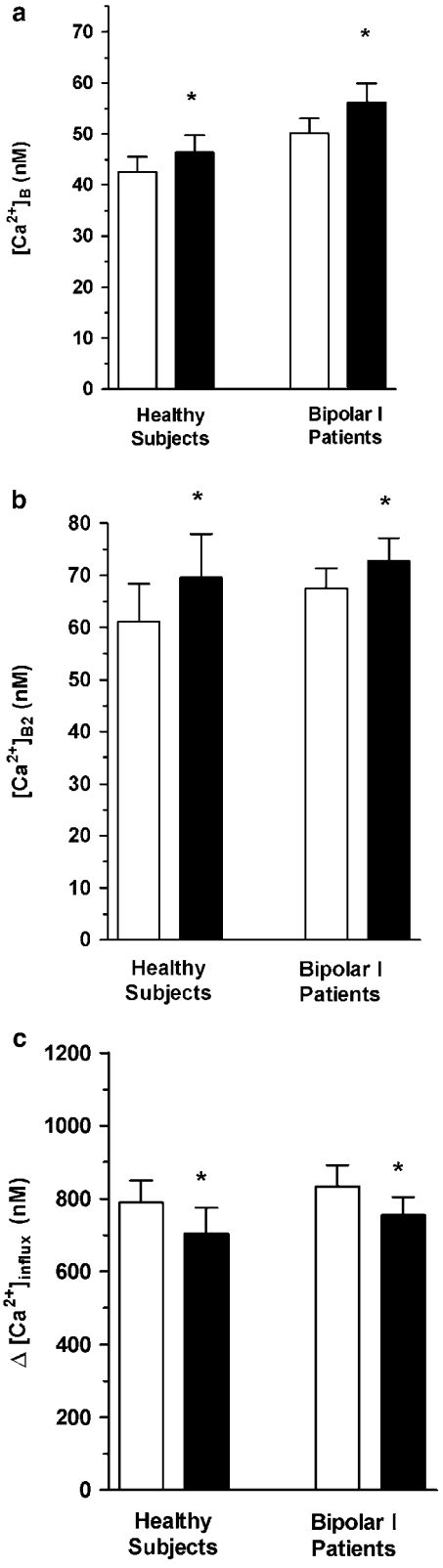
Figure 2 Effect of Chronic Lithium Treatment on Parameters of LPA-Stimulated Intracellular Calcium Mobilization. Cells were incubated for 7 days with LiCl (0.75 mM) or vehicle. The $[Ca^{2+}]_i$ was monitored prior to and following stimulation with 100 μM LPA as described in Figure 1a. Chronic lithium treatment increased basal intracellular Ca^{2+} levels ($[Ca^{2+}]_B$) (a) and attenuated the absolute difference between $[Ca^{2+}]_S$ and $[Ca^{2+}]_B$ ($\Delta[Ca^{2+}]_S$) (b) in response to LPA stimulation in BLCLs from both healthy subjects and BD-I patients. The open bars (□) represent vehicle-treated BLCLs, while the solid bars (■) represent BLCLs treated chronically with lithium. Values represent the mean and SEM of determinations made in 15 healthy subjects and 20 BD-I patients. *$p < 0.05$ compared to vehicle-treated BLCLs (ANOVA with Tukey's *post hoc* tests).

Effect of Chronic Lithium Treatment on Thapsigargin-Sensitive ER Ca^{2+} Release and Parameters of SOCE

Figure 1b demonstrates the time course of TG-induced depletion of intracellular Ca^{2+} stores and subsequent store depletion-stimulated influx of extracellular Ca^{2+} in BLCLs incubated for 7 days with 0.75 mM lithium or vehicle. In a Ca^{2+}-free medium, the addition of TG (200 nM) caused a slow increase in BLCL $[Ca^{2+}]_i$ to a new baseline ($[Ca^{2+}]_{B2}$). Subsequent addition of Ca^{2+} (1 mM) resulted in a rapid rise of $[Ca^{2+}]_i$ confirming the well-known observation obtained in other cell types that sustained Ca^{2+} entry is linked to

store depletion induced by TG (Putney *et al*, 2001). Parameters measured to characterize the TG-induced SOCE response include the $[Ca^{2+}]_B$, peak TG-sensitive $[Ca^{2+}]_i$ accumulation ($[Ca^{2+}]_{TG}$), the baseline plateau before reintroduction of Ca^{2+} to the external medium ($[Ca^{2+}]_{B2}$, the peak of Ca^{2+} influx ($[Ca^{2+}]_{influx}$), the difference between $[Ca^{2+}]_{influx}$ and $[Ca^{2+}]_{B2}$ ($\Delta[Ca^{2+}]_{influx}$), and the slope of the initial phase of rise in $[Ca^{2+}]_{influx}$ ($d[Ca^{2+}]_{influx}/dt$).

The effects of chronic lithium treatment on the parameters of the 'TG-Ca^{2+} add back protocol' in BLCLs from the BD patients and healthy comparison subjects are presented in Figures 1b and 3. Repeated measures MANOVA of these parameters revealed a significant effect of lithium treatment ($F = 8.36$; $df = 5,30$; $p = 0.00005$) but not diagnosis ($F = 1.28$; $df = 5,30$; $p = 0.30$), and no significant interaction ($F = 0.551$; $df = 5,30$; $p = 0.74$) between these two factors. Subsequent univariate ANOVAs revealed significant effects of chronic lithium treatment on $[Ca^{2+}]_B$ ($F = 9.42$; $df = 1,34$; $p = 0.004$), $[Ca^{2+}]_{B2}$ ($F = 10.1$; $df = 1,34$; $p = 0.003$), $[Ca^{2+}]_{influx}$ ($F = 8.36$; $df = 1,34$; $p = 0.007$), and $\Delta[Ca^{2+}]_{influx}$ ($F = 11.7$; $df = 1,34$; $p = 0.002$), but not $[Ca^{2+}]_{TG}$ ($F = 1.22$; $df = 1,34$; $p = 0.278$) or $d[Ca^{2+}]_{influx}/dt$ ($F = 2.49$; $df = 1,34$; $p = 0.124$), in TG-stimulated BLCLs. $[Ca^{2+}]_B$ and $[Ca^{2+}]_{B2}$were significantly higher in BLCLs treated with lithium compared with vehicle control in both the patient (12.0 and 8.8%, respectively) and healthy subjects (7.0 and 13.3%, respectively). As well, chronic lithium treatment modestly, but significantly, attenuated both $[Ca^{2+}]_{influx}$ (9.0% in BD-I, 7.9% in healthy subjects) as well as $\Delta[Ca^{2+}]_{influx}$ (10.6% lower in BD-I, 10.4% lower in healthy subjects). As with LPA-stimulated Ca^{2+} mobilization, there was no difference in $[Ca^{2+}]_B$ between vehicle and acute lithium-treated samples ($F = 1.45$; $df = 1,13$; $p = 0.25$; Figure 4c), whereas the difference between vehicle and chronically treated samples was statistically significant ($F = 4.36$; $df = 1,9$; $p = 0.05$). Similarly, while $[Ca^{2+}]_{B2}$ in BLCLs treated for 24 h with lithium were not significantly reduced ($F = 3.15$; $df = 1,13$; $p = 0.10$), those treated for 7 days demonstrated higher $[Ca^{2+}]_{B2}$ compared with vehicle (12.5%, $F = 8.65$; $df = 1,13$; $p = 0.01$; data not shown). Finally, while $[Ca^{2+}]_{influx}$ did not differ between vehicle and acute lithium treatment conditions ($F = 1.39$; $df = 1,13$; $p = 0.26$; Figure 4d), it was in fact reduced in chronic lithium-treated BLCLs compared with the vehicle-treated condition ($F = 5.17$; $df = 1,13$; $p = 0.04$).



Relationship Between Calcium Mobilization Parameters and Bipolar Patient Characteristics

Other than the observation of significantly higher $[Ca^{2+}]_B$ in BLCLs from bipolar patients on lithium monotherapy at the time of the study compared with healthy subjects ($F = 3.11$; $df = 3,24$; $p = 0.04$) there were no statistically

Figure 3 Effect of Chronic Lithium Treatment on Parameters of Thapsigargin-Induced Store Depletion and Store-Operated Ca^{2+} Entry. Cells were incubated for 7 days with LiCl (0.75 mM) or vehicle. Store-operated Ca^{2+} entry was monitored using the 'TG-Ca^{2+} add back' protocol as described in Figure 1b. Chronic lithium exposure increased the basal intracellular Ca^{2+} levels prior to ($[Ca^{2+}]_B$) (a) and following TG challenge ($[Ca^{2+}]_{B2}$) (b) and attenuated the absolute difference between $[Ca^{2+}]_{influx}$ and $[Ca^{2+}]_{B2}$ ($\Delta[Ca^{2+}]_{influx}$) (c) in response to the readdition of extracellular Ca^{2+}, in BLCLs from both healthy subjects and BD-I patients. Open bars (□) represent vehicle-treated BLCLs, while solid bars (■) represent BLCLs treated chronically with lithium. Values represent the mean and SEM of determinations made in 13 healthy subjects and 24 BD-I patients. *$p < 0.05$ compared to vehicle-treated BLCLs (ANOVA with Tukey's *post hoc* tests).

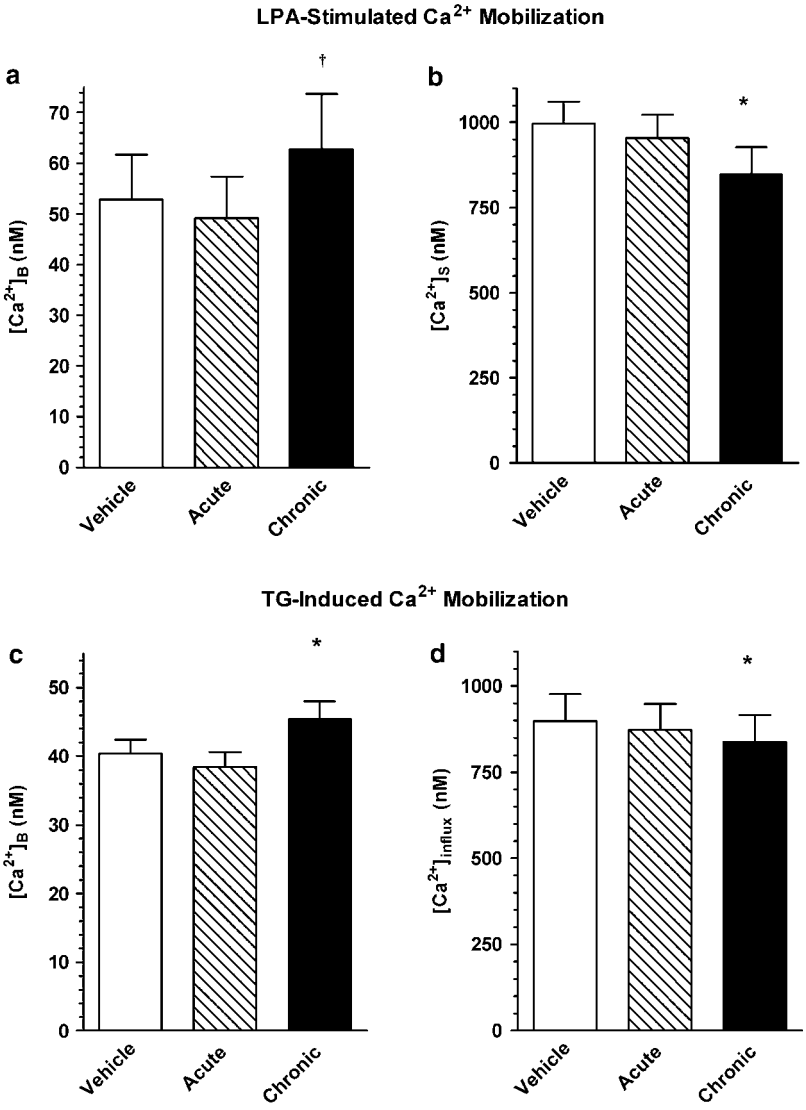


Figure 4 Effect of Acute *vs* Chronic Lithium Treatment on Basal Ca^{2+} levels, LPA-stimulated Ca^{2+} Mobilization, and Thapsigargin-Induced Store-Operated Ca^{2+} Entry. BLCLs were treated with vehicle (open bars) or with LiCl (0.75 mM) for 24 h (hatched bars) or 7 days (closed bars ■). Cells loaded with Fura-2 were challenged with LPA (a, b) or TG-induced SOCC (c, d) as described in Figure 1. Values represent the mean and SEM of determinations made in two healthy subjects and eight BD-I patients (a, b) or five healthy and 10 BD-I patients (c, d). $^{*}p < 0.05$ and $^{\dagger}p = 0.068$ compared to vehicle-treated BLCLs (repeated measures ANOVA with simple contrasts).

significant differences found in the LPA- and TG-induced Ca^{2+} mobilization indices measured within the patient group stratified on factors of mood state of illness at time of study (healthy subjects, and euthymic, depressed, and hypomanic/manic bipolar patients, F's = 0.06–1.69; df = 3,26; *p*'s > 0.2), psychiatric comorbidity (healthy subjects and bipolar patients with no comorbidity, alcohol abuse/dependence, or other comorbidity, F's = 0.41–1.63; df = 3,26; *p*'s > 0.21) and family history of mood disorders (healthy subjects, positive, negative, or unknown, F's = 0.26–2.56; df = 3,26; *p*'s $\geqslant$ 0.08) (data not shown).

DISCUSSION

The principal findings of this study are that chronic, but not acute, treatment of BLCLs from both BD-I patients and healthy subjects with a therapeutically relevant concentration of lithium significantly attenuated LPA-stimulated Ca^{2+} responses (a G-protein-coupled receptor activated signaling pathway), and TG-evoked store-depletion-induced Ca^{2+} influx (a measure of SOCE). At the same time, $[Ca^{2+}]_B$ was significantly elevated by chronic, but not acute, lithium treatment. Taken together, these findings support the notion that lithium alters key processes regulating intracellular Ca^{2+} homeostasis, effects which may be particularly relevant to understanding the molecular and cellular actions responsible for its therapeutic effects.

As the pharmacological agents used to probe intracellular Ca^{2+} homeostatic control in this study act on cascades of interacting proteins and second messengers, the loci at which lithium acts to produce the observed effects can only be surmised in broad terms. It is unlikely, however, that the attenuating effects of lithium are related to factors that modify the rate of Ca^{2+} mobilization following agonist stimulation or the rate of Ca^{2+} influx induced by store

depletion as these changes occurred without statistically significant differences in $d[Ca^{2+}]_S/dt$ and $d[Ca^{2+}]_{influx}/dt$, the initial rates of activation of these responses. Furthermore, they do not appear to be related to an effect of lithium to reduce the size of the TG-sensitive ER stores, since neither acute nor chronic lithium treatment had any significant effect on $[Ca^{2+}]_{TG}$.

As SOCE may contribute to the net Ca^{2+} entry in the LPA (Warsh *et al*, unpublished data) as well as in the TG-stimulation paradigms (Putney *et al*, 2001), an effect of lithium on mechanisms regulating SOCE could be expected to attenuate both responses. As TG induces store depletion through an IP_3 receptor (IP_3R)-independent mechanism, the effect of lithium on this SOCE component is likely to be related to actions distinct from its well-established modulatory effect on PI signaling (Hallcher and Sherman, 1980; Berridge *et al*, 1982). The SOCE apparatus is complex, involving the orchestrated action of SOCC, IP_3Rs, and SERCAs (Barritt, 1999; Putney *et al*, 2001). Functional interactions between mitochondria and ER can also influence Ca^{2+} mobilization through SOCE (Fall and Keizer, 2001). While knowledge is sparse on the effects of lithium on these families of membrane proteins, the observed increase in $[Ca^{2+}]_B$ would not be expected if the attenuating effect of lithium on the stimulated Ca^{2+} mobilization were due to upregulation of the levels and/or function of PMCAs or SERCAs. Similarly, the lack of differences in $[Ca^{2+}]_{TG}$ argues against an effect of lithium on the functional activity of the SERCAs.

The potential involvement of TRPM2 or other members of the family of TRP proteins in the effect of lithium to attenuate LPA- and TG-stimulated Ca^{2+} mobilization also merits consideration. TRPM2 mRNA levels were significantly reduced in BLCLs from BD patients showing elevated BLCL $[Ca^{2+}]_B$ and varied inversely with $[Ca^{2+}]_B$ (Yoon *et al*, 2001b). Furthermore, in preliminary experiments, sustained elevation of BLCL $[Ca^{2+}]_i$ by ionomycin treatment suppressed TRPM2 mRNA levels (Yoon, 2002). While chronic lithium treatment suppresses TRPC3 protein levels in BLCLs from BD patients (Andreopoulos *et al*, 2003), the extent to which protein and mRNA levels of TRPM2 and other TRP proteins expressed in BLCLs are also down-regulated and the relationship of any such changes to the altered functional indices demonstrated in this work remain to be elaborated.

It is also possible that the effect of chronic lithium treatment to attenuate LPA-stimulated Ca^{2+} mobilization is mediated through a process distinct from that which affects the TG-evoked SOCE response. In addition to inhibiting IMPase (Hallcher and Sherman, 1980), lithium could attenuate LPA-stimulated Ca^{2+} mobilization by inhibiting $G_{\beta\gamma}$-mediated responses. Chronic lithium administration has been demonstrated to interfere with the dissociation of G_i into its active components, thereby removing the tonic inhibitory influence on adenylyl cyclase and diminishing signal transduction through the PI/IP_3 system (Masana *et al*, 1992; Wang and Friedman, 1999). Chronic lithium treatment has also been shown to reduce receptor G-protein coupling (Wang and Friedman, 1999) and decrease both receptor- and postreceptor-stimulated PI hydrolysis (reviewed in Hudson *et al*, 1993; Jope and Williams, 1994).

The extent to which the modulatory effect of lithium on intracellular Ca^{2+} mobilization in the current cellular paradigm represents a primary effect on one or more of the elements in the PI/Ca^{2+} signaling system, as opposed to a secondary one mediated through cross-talk mechanisms also merits consideration. For example, cAMP-mediated signaling promotes the inhibition of Ca^{2+} influx (Rasmussen, 1986), modulates IP_3Rs (Supattapone *et al*, 1988; Patel *et al*, 1999), and enhances the removal of Ca^{2+} from the cytosol by promoting the action of PMCA pumps (Helman *et al*, 1986). As well, protein kinase A (PKA) can modulate the PI/Ca^{2+} signaling system through the phosphorylation of several intracellular targets including receptors, G proteins, PI kinase, and phospholipase C (Supattapone *et al*, 1988; Hajnoczky *et al*, 1993; Galas and Harden, 1997). Abnormalities of the cAMP-PKA signaling cascade have also been implicated in the pathophysiology of BD and as a target of lithium action (Perez *et al*, 2000; Chang *et al*, 2002). Furthermore, the cAMP system is, in turn, subject to cross-regulation by Ca^{2+} signaling through regulation at a variety of points in the cAMP signaling cascade (Choi *et al*, 1993; Cooper *et al*, 1995). Thus, it may be that multiple actions of lithium on diverse signal transduction systems are essential to its therapeutic efficacy in BD.

While the decrements in LPA-stimulated Ca^{2+} mobilization and TG-induced Ca^{2+} influx caused by chronic lithium treatment were modest, the true magnitude of the attenuating effects may have been underestimated. Fura-2, which has a K_d for Ca^{2+} in the order of 224 nM (at 37°C) (Nuccitelli, 1994), becomes increasingly saturated at high $[Ca^{2+}]_i$, such as attained with the LPA and TG protocols used in this study. Thus, at such high Ca^{2+} concentrations (eg 700–1000 nM), Fura-2 would be relatively less sensitive to changes in $[Ca^{2+}]_i$ providing only an approximation of the true magnitude of the Ca^{2+} changes. Although higher affinity fluorescence Ca^{2+} chelating dyes are available (Nuccitelli, 1994), their use would have been at the expense of accurately estimating the $[Ca^{2+}]_B$. Second, because the Ca^{2+} assay employed gives a 'static' measure, averaged across the entire population of cells in the light beam path of the fluorometer, it does not provide a dynamic picture of the effects that may occur in subpopulations of responding cells in which differences may be more pronounced. Finally, differences observed in this study may also have been affected by phenotypic heterogeneity of the BD patient population studied, a known confounding factor in genetic and molecular investigations of BD (Goodwin and Jamison, 1990; Lenox *et al*, 2002).

The attenuating effects of lithium on LPA- and TG-induced Ca^{2+} mobilization were only evident after 7 days but not 24 h of exposure suggesting that these effects are a result of chronic exposure to lithium. This duration of treatment, which has been widely used in studies of mood stabilizer actions in cell lines (Varney *et al*, 1992; Ozaki and Chuang, 1997; Yamaji *et al*, 1997; Nonaka *et al*, 1998; Wang *et al*, 2001), is one through which drug exposure can be managed without inducing significant cellular toxicity (BLCL viability after 7 days exposure $>95\%$). While this time interval is still shorter than that required for full clinical response to lithium, antimanic effects of lithium are evident at as early as 1 week of treatment (Schou, 1968). Also, unlike the clinical situation, in which pharmacokinetic

factors contribute, in part, to latency of onset of action, cells studied in the *ex vivo* paradigm here are directly and immediately exposed to a constant therapeutic concentration of lithium, thus presumably expediting the onset of biochemical actions. Finally, the lithium concentration used in this study (0.75 mM) falls in the mid-therapeutic range of serum concentrations (0.5–1.0 mM) used to guide lithium dosing clinically (Gelenberg *et al*, 1989) and within the range of those achieved in brain during therapeutic dosing (Kato *et al*, 1993; Plenge *et al*, 1994). However, the use of a single lithium concentration in this study precludes conclusions regarding whether the observed effects of lithium on parameters of Ca^{2+} mobilization were dose-dependent or maximal.

The lack of a differential effect of lithium on the Ca^{2+} mobilization in BLCLs from BD patients as compared with those from healthy subjects also merits consideration as it might question the therapeutic implications of lithium-induced Ca^{2+} mobilization changes observed. However, this could be related to several factors, including insensitivity to detect a small effect in a relatively small subject sample population, the limited sensitivity of the Ca^{2+} indicator used at high $[Ca^{2+}]_S$, as noted above, and the relatively large variance in the assay of LPA-stimulated Ca^{2+} mobilization. While the duration of exposure to lithium is sufficient to demonstrate certain chronic effects, as argued above, it may still be too short to capture cellular and molecular changes that evolve more slowly, in perhaps a diagnosis-specific fashion.

The alterations seen following chronic lithium treatment in this nonexcitable cell paradigm likely represent only one of a sequence of changes that, in neurons, act in conjunction to 'correct' disturbed Ca^{2+} homeostasis. In this regard, chronic lithium treatment also inhibits NMDA receptor-induced Ca^{2+} influx possibly by decreasing NR2B tyrosine phosphorylation (Hashimoto *et al*, 2002), effects that may underlie its protective actions against glutamate-induced excitotoxicity. Finally, the effects of lithium to attenuate stimulus-induced Ca^{2+} mobilization at the same time as elevating resting levels recalls the 'bimodal hypothesis' (Jope, 1999) that lithium normalizes the balance between positive and negative regulators of intracellular signal transduction processes, thereby stabilizing and maintaining their function within an optimal range. In this context, Ca^{2+} signaling modulates gene expression and protein function through spatially regulated changes in frequency and amplitude of intracellular Ca^{2+} waves (Ghosh and Greenberg, 1995). Stabilization of fluctuations in intracellular Ca^{2+} levels by lithium could significantly modify the transcriptional regulation and expression of key neuronal proteins that, in turn, correct aberrant neuronal activity in critical brain regions involved in mood regulation. As the integrity of intracellular Ca^{2+} homeostasis is critical to cell survival, these effects of lithium on Ca^{2+} signaling processes may also be relevant mechanistically to its neuroprotective effects, now thought to be important in the clinical action of this agent (Manji *et al*, 2000).

In conclusion, the findings of the current study suggest that chronic lithium treatment modifies processes that impact on intracellular Ca^{2+} signaling and homeostasis. Taken together with our recent report suggesting Ca^{2+} signaling may be hyperactive in BLCLs from patients with BD (Wasserman *et al*, 2003), a cellular model that may report disturbances particularly relevant to the pathophysiology of this disorder, these observations suggest that the regulation of intracellular Ca^{2+} homeostasis may be especially pertinent to the therapeutic action of lithium. In this regard, lithium's modulation of intracellular Ca^{2+} signaling, be it direct or indirect, could have important implications in terms of stabilizing gene expression, cellular communication, and perhaps excitotoxic or apoptotic cell death.

ACKNOWLEDGEMENTS

A preliminary report of this work was presented at the 57th annual meeting of the Society for Biological Psychiatry, Philadelphia, PA. This work was supported by the Canadian Institutes of Health Research (grants MOP12851 (JJW) and MOP53323 (JJW and PPL)). MJW is a recipient of a Research Studentship from the Ontario Mental Health Foundation and TWC was supported by a Natural Sciences & Engineering Research Council postgraduate scholarship and University of Toronto scholarship. We gratefully acknowledge the contributions of the physicians and research staff at the Centre for Addiction and Mental Health for their assistance in recruiting potential patient subjects for participation in these studies. Arvind Kamble and Kin Po Siu contributed to the technical laboratory aspects of this work and Bronwen Hughes in the clinical data collection and analysis.

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