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The Bcl-2 Gene Polymorphism rs956572AA Increases Inositol 1,4,5-Trisphosphate Receptor–Mediated Endoplasmic Reticulum Calcium Release in Subjects with Bipolar Disorder

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Abstract

Background—Bipolar disorder (BPD) is characterized by altered intracellular calcium (Ca^{2+}) homeostasis. Underlying mechanisms involve dysfunctions in endoplasmic reticulum (ER) and mitochondrial Ca^{2+} handling, potentially mediated by B-cell lymphoma 2 (Bcl-2), a key protein that regulates Ca^{2+} signaling by interacting directly with these organelles, and which has been implicated in the pathophysiology of BPD. Here, we examined the effects of the Bcl-2 gene single nucleotide polymorphism (SNP) rs956572 on intracellular Ca^{2+} dynamics in patients with BPD.

Methods—Live cell fluorescence imaging and electron probe microanalysis were used to measure intracellular and intra-organelle free and total calcium in lymphoblasts from 18 subjects with BPD carrying the AA, AG, or GG variants of the rs956572 SNP. Analyses were carried out under basal conditions and in the presence of agents that affect Ca^{2+} dynamics.

Results—Compared with GG homozygotes, variant AA—which expresses significantly reduced Bcl-2 messenger RNA and protein—exhibited elevated basal cytosolic Ca^{2+} and larger increases in inositol 1,4,5-trisphosphate receptor–mediated cytosolic Ca^{2+} elevations, the latter in parallel with enhanced depletion of the ER Ca^{2+} pool. The aberrant behavior of AA cells was reversed by chronic lithium treatment and mimicked in variant GG by a Bcl-2 inhibitor. In contrast, no differences between SNP variants were found in ER or mitochondrial total Ca^{2+} content or in basal store-operated Ca^{2+} entry.

Conclusions—These results demonstrate that, in patients with BPD, abnormal Bcl-2 gene expression in the AA variant contributes to dysfunctional Ca^{2+} homeostasis through a specific ER inositol 1,4,5-trisphosphate receptor–dependent mechanism.

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Dr. Manji is now Global Therapeutic Area Head, Johnson & Johnson Pharmaceuticals, and a full time employee of Johnson & Johnson. Dr. Zarate is listed as a co-inventor on a patent for the use of ketamine in major depression. Dr. Zarate has assigned his patent rights on ketamine to the US Government. All authors declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

Keywords

Bcl-2; bipolar disorder; calcium; electron probe micro-analysis; endoplasmic reticulum; inositol 1;4;5-trisphosphate; mania; mitochondria

Impaired regulation of calcium (Ca^{2+}) signaling is considered the most reproducible cellular abnormality in bipolar disorder (BPD) research(1), but the mechanisms underlying this abnormality remain poorly understood. Studies using peripheral cells from individuals with BPD have shown elevated Ca^{2+} levels, suggesting increased vulnerability to injury or impaired cellular resilience, broadly defined as the ability of cells to adapt to insults and maintain their original form and function (2,3). Furthermore, these abnormalities have been associated with dysfunction in both endoplasmic reticulum (ER) and mitochondrial activity (2,4,5).

The antiapoptotic protein B-cell lymphoma 2 (Bcl-2) is a globally important signaling protein known to regulate diverse neurobiological processes such as neurogenesis (6), morphogenesis (7), and synaptic plasticity (8). Interestingly, behavioral studies (9) suggest that Bcl-2 plays a potential role in BPD, and mood stabilizers have been found to elevate Bcl-2 expression (10). A significant decrease in Bcl-2 protein and messenger RNA (mRNA) levels was recently described in the frontal cortex of individuals with BPD (11). In addition, a Bcl-2 gene single nucleotide polymorphism (SNP) (rs956572 variant AA) was shown to significantly reduce Bcl-2 levels and mRNA expression in human lymphoblasts and decrease grey matter volume in healthy subjects (12). Preclinical models have also demonstrated that 4 weeks of lithium treatment upregulates Bcl-2 in the dentate gyrus and hippocampus (13).

At the cellular level, Bcl-2 and its family members control Ca^{2+} dynamics through direct interaction with cytosolic targets, mitochondria, and/or the ER (14,15). On the ER, Bcl-2 is known to interact with the inositol 1,4,5-trisphosphate receptor (InsP_3R), an ER Ca^{2+} channel that either elevates cytosolic Ca^{2+} directly by releasing intra-ER Ca^{2+} , or indirectly by stimulating “capacitative” Ca^{2+} influx upon store depletion (16). Recent evidence suggests that Bcl-2 suppresses InsP_3 -mediated Ca^{2+} release; however, there are conflicting reports regarding the effect of Bcl-2 on luminal ER Ca^{2+} (reviewed in 17,18). This is a significant issue, because the basal state of ER Ca^{2+} stores might play an important role in cellular resilience, in that enhanced steady-state Ca^{2+} levels and continuous Ca^{2+} overload can increase cellular vulnerability to diverse stressors and apoptotic signals (19). Controversy notwithstanding, these observations support the idea that lowered Bcl-2 expression might be related to the dysfunctional Ca^{2+} regulation implicated in BPD (1). This hypothesis is further supported by the observation that chronic lithium treatment attenuates Ca^{2+} mobilization in lymphoblasts from individuals with BPD (20), an effect associated with inositol depletion (21) and Bcl-2 and ER chaperone up-regulation (22,23).

In this study, we examined the potential role of the functional Bcl-2 SNP on intracellular Ca^{2+} dynamics in human carriers of the A and G alleles in subjects with BPD.

Methods and Materials

Subjects and Samples

The potential functional role of the Bcl-2 SNP (rs956572) in cellular Ca^{2+} dynamics was studied in BPD patients who met Structured Interview for DSM-IV criteria (SCID-I) for BPD-I. Most of these individuals also had a first-degree relative with mood disorders ($n = 9$). Eighteen BPD-I subjects were included in this study, with equal numbers of individuals

with variant AA (mean age 36.5 ± 4.4 years), AG (age 41.8 ± 5.4 years), and GG (age 35 ± 7.8 years). An average of 20 cells/patient were analyzed/sample in each experiment. All experiments were carried out in duplicate. No significant differences among genotype were identified for age, gender, or family history of BPD. This study was approved by all local institutional review boards, and informed consent was obtained from all participants.

Cell Culture and Establishment of Immortalized Cell Lines

The Bcl-2 gene consists of one large intron; rs956572 is at the 3' end. To understand the potential functional influence of rs956572, 72 human lymphoblast cell lines were genotyped. Of these, B-lymphoblastoid cell lines (BLCL) cultures from subjects carrying the Bcl-2 SNP rs956572 were selected to determine the influence of genotype on Bcl-2 levels and Ca^{2+} dynamics. The BLCL were cultured in Roswell Park Memorial Institute (RPMI) 1640 media in a 5% carbon dioxide humidified atmosphere at 37°C ; media were supplemented with 10% fetal bovine serum, L-glutamine, antibiotics, and antimycotics. All samples were cultured under the same standard procedures. Cultures were split at a ratio of 1:5 every 3–5 days. To control for potential confounding effects of cell passage and culture conditions, all cell lines on the same passage were recovered together and cultured in media from the same bulk preparation. Media were changed at the same time for all lines 24 hours before cell harvest. Cells were collected by centrifugation at the same time and washed three times with phosphate-buffered saline before being frozen and stored at -160°C . Propidium iodide staining was used to determine cell viability. All cultures presented more than 85% of viable cells.

Cells were thawed and used within 2 months. A previous study showed that the BLCL of the three different genotypes had no significant effects on Epstein-Barr Virus nuclear antigen induction (24).

Genotyping of rs956572

Genotyping was performed at Illumina (La Jolla, California), with Golden Gate Chemistry as part of a larger candidate-gene study. Genotypes displayed good clustering, Hardy-Weinberg Equilibrium in parents ($p > .5$), perfect Mendelization (no Mendelian errors were detected in 318 parent-offspring trios), 99.3% completeness among 1288 genotyped samples, and 100% agreement among 24 blind duplicates. The genotyping assay was designed by Illumina on the basis of the following reference sequence:
 TAACCTCCCTGAGCCTAAATTTATGAATTCATAAAACAGAGAAAAATAAAAGGC
 TGTAGTTGTGCGAGTGGTCAGGAGGATGAAATGACTTCTGTGAAGTGCCTGGCAG
 CAAAGGCACTAA[A/
 G]ATTCAGTCATGACTCCCTCTCATTCTCTCATTCTTTGCATTGAACTGTCAGA
 GATGGCAATTTTCCTTTCCCTAGGTACCTTCTTTATTACAAGTAGCTT.

The frequency of the minor (A) allele in the total sample was .42, which closely agrees with HapMap (.425–.442 in 120 CEU individuals).

Calcium Imaging and Analysis

Cells ($1 \times 10^6/\text{mL}$) adhered to poly-L-lysine-treated 15-mm coverslips were pre-incubated for 45–60 min at 37°C in an extracellular buffer (ECB) [HBSS plus 20 mmol/L HEPES, .5% bovine serum albumin, and 1 mmol/L calcium dichloride] with 1 $\mu\text{mol/L}$ acetoxymethyl esters of the fluorescent Ca^{2+} indicators fura-2 AM or fura-2FF (Invitrogen, Carlsbad, California). After incubation, cells were washed with ECB for 45 min at room temperature to permit dye de-esterification. Coverslips were mounted in a perfusion chamber on the stage of a Zeiss Axiovert 200 inverted microscope equipped with a $40\times$ Fluor objective (Zeiss, Thornwood, New York). Images and dual excitation (340/380 nm) fluorescence

records were continuously acquired with EasyRatioPro hardware and software (PTI, Birmingham, New Jersey). Experiments were carried out at room temperature (22°–25°C) and ambient atmospheric conditions. Cells were randomly selected for each experiment. The 340/380 ratios were transformed to Ca^{2+} concentrations with established procedures (25).

Luminal ER Ca^{2+} measurements were performed according to Hofer (26) as adapted by Chen *et al.* (27). After incubation with 1 $\mu\text{mol/L}$ fura-2FF AM and de-esterification as described, cells mounted on the inverted microscope were perfused with an intra-cellular buffer (intracellular buffer: 125 mmol/L potassium chloride, 25 mmol/L sodium chloride, 10 mmol/L HEPES, 200 $\mu\text{mol/L}$ calcium dichloride, and 500 $\mu\text{mol/L}$ ethylene glycol bis-(β -aminoethyl ether)-*N,N'*-tetraacetic acid [EGTA], pH 7.25), supplemented with .5 mmol/L adenosine triphosphate. After selective permeabilization of plasma membrane with digitonin (10 $\mu\text{g/mL}$) for 40–60 sec, there was a strong decline in fluorescence excited at both 340 and 380 nm, but the residual signal had a higher 340/380-m ratio and reflected specific fluorescence from the ER lumen. After fluorescence reached steady-state, cells were perfused with the InsP_3R agonist adenophostin A (AdA) (75 nmol/L) for 20–30 sec. A further abrupt drop in the 340/380 ratio denoting Ca^{2+} release from the ER was confirmed by manganese quench of fluorescence after InsP_3R activation (27) (data not shown).

To estimate mitochondrial free Ca^{2+} levels, BLCL in ECB were loaded with dihydro-Rhod-2 (Invitrogen) for 60 min at 37°C. After being washed twice, cells were maintained at 37°C for an additional 60 min. Fluorescence (553/576 nm) was recorded with a Zeiss LSM 510 confocal microscope (Zeiss). After stabilizing the baseline, AdA (75 nmol/L) was added, and thereafter images were captured every 10 sec for 5 min. MitoTracker Green (Molecular Probes, Eugene, Oregon) was used to confirm mitochondrial localization. Average fluorescent intensity was determined, and F/F_0 ratios were calculated before and after AdA treatment with Metamorph software (MDS Analytical Technologies, Sunnyvale, California).

For the Bcl-2 inhibitor experiments, cells were incubated for 1 hour with BH3-I (100 $\mu\text{mol/L}$) in cell culture medium and then washed and incubated in Fura-2 AM as described in the preceding text. In the lithium experiments, cells were treated with lithium chloride (1 mmol/L) for 7 days.

Electron Probe Microanalysis

Samples of AA and GG BLCL were centrifuged to form a pellet that was placed on the tip of an aluminum pin and plunge-frozen in liquid ethane with a custom-designed freezing device. Pins were mounted in a Leica UC6 cryoultramicrotome precooled to -160°C , and ultrathin (approximately 100-nm-thick) cryosections were prepared and mounted on electron microscopy supports. Specimens were cryo-transferred to an analytical cryo-electron microscope (Zeiss) and freeze-dried, and digital images were recorded by means of a ProScan 2 kb $\times$ 2 kb slow-scan charge-coupled device camera controlled by AnalySIS software (Soft Imaging Systems, Lakewood, Colorado). The x-ray spectra were recorded at -110°C for 100 sec each with an approximately 5-nA focused probe with a diameter of 100 nm (mitochondria) or 63 nm (ER), as previously described (28). The x-ray spectra were recorded and processed with established procedures (29) to quantify total (i.e., free plus bound) elemental calcium concentrations. Concentrations are given in millimoles/kilogram dry weight.

Western Blotting and Quantitative Polymerase Chain Reaction (qPCR)

The BLCL were homogenized in an extraction buffer containing 20 mmol/L Tris-hydrogen chloride, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L ethylenediaminetetraacetic acid, 1

mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L α -glycerophosphate, protease inhibitor mixture (Sigma, St. Louis, Missouri) and phosphatase inhibitor mixture I and II (Sigma). Homogenates were centrifuged at $14,000 \times g$ for 15 sec, total protein content was adjusted to the same level for all samples, and equal amounts of protein were electrophoresed on 10% or 4% to approximately 20% tris-glycine gels. The blots were blocked with 5% dry milk for 1 hour at room temperature, followed by incubation with a pan-IP₃R antibody plus actin antibody (both Sigma, diluted 1:1000) or a Bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, California) (diluted 1:500) plus the actin antibody in blocking buffer overnight. After four washes with Tris-buffered saline containing .05% Tween 20, the blots were incubated with an horseradish peroxidase–conjugated anti-rabbit secondary antibody (diluted 1:2000; GE Healthcare, Piscataway, New Jersey) and then washed again four times. Finally, blots were developed with ECL (GE Healthcare) and visualized with Kodak x-ray film.

For qPCR of Bcl-2 mRNA, a PRISM 7900 Sequence detector system (ABI, Foster City, California) and the Platinum qPCR SuperMix-UDG w/ROX (Invitrogen) were used. Detection was performed in triplicate with a pretested optimized cycling program for primers.

Reagents

The AdA was obtained from Calbiochem (Gibbstown, New Jersey). All other reagents were purchased from Sigma. When dimethyl sulfoxide was used as a drug solvent, the final concentration in the recording medium did not exceed .1%.

Statistics

All experiments with human lymphoblasts were performed in duplicate. The average value/patient was used for statistical analysis, but similar results were obtained when data were analyzed according to the total number of cells/group, with higher significance than per-patient. Data were evaluated with Student *t* test (matched values, two-tailed, paired) for two groups or one-way analysis of variance plus Bonferroni post hoc test for multiple comparisons. The χ^2 test was used to test differences among genotypes in gender and family history. Pearson's correlation analysis was performed to evaluate potential associations between Ca²⁺ levels and other numerical variables. Data are expressed as mean $\pm$ SEM; *p* values of < .05 were considered statistically significant.

Results

Basal Cytosolic Ca²⁺ Was Elevated in Lymphoblasts From BPD Subjects Carrying the Bcl-2–Deficient SNP Variant AA

We first evaluated the effects of the Bcl-2 SNP rs956572 on basal cytosolic Ca²⁺ concentrations ([Ca²⁺]_i). Figure 1A presents baseline cytosolic Ca²⁺ measurements on a per-patient basis for all BLCLs assayed, distributed according to the three patient groups (AA, AG, and GG) (*n*_{total} = 18). A significant increase in [Ca²⁺]_i was observed in the Bcl-2 SNP variant AA compared with the variant GG (AA = 56.6 ± 5.1 , AG = 50.3 ± 5.6 , GG = 27.9 ± 3.1 , *F* = 10.1; AA vs. GG *t* = 4.27, *p* < .01). Results were similar when data were analyzed on an individual cell basis (Figure 1B). Furthermore, basal [Ca²⁺]_i showed a significant inverse correlation with both Bcl-2 mRNA and protein levels (Figures 1C–1D) across the total sample. Interestingly, variant AA, which has the highest basal [Ca²⁺]_i level, expressed the least Bcl-2.

InsP₃R Activation Selectively Enhanced Cytosolic Ca²⁺ Elevations in the Bcl-2 SNP Variant AA

To evaluate the potential role of the ER and plasma membrane in the regulation of cytosolic Ca²⁺ changes associated with these Bcl-2 SNP variants, cells were treated with the sarco(endo)plasmic reticulum Ca²⁺-ATPase pump inhibitor thapsigargin (Tg) or with AdA, a selective InsP₃R agonist. For all three SNP genotypes, Tg only minimally increased [Ca²⁺]_i in Ca²⁺-free (EGTA-containing) medium (Figure 2A, lower traces) indicating that the mobilizable Ca²⁺ store was quite small in these cells; these results were further confirmed (Figure 3C). In contrast, Tg in the presence of 1 mmol/L external Ca²⁺ elicited robust but similar [Ca²⁺]_i elevations in all three groups (AA = 683 ± 105; AG = 757 ± 66; GG = 770 ± 87; *p* = .79) (Figures 2A–2B). This implies that store-operated Ca²⁺ entry (SOCE) is a dominant component of Ca²⁺ regulation in this cell type (30), albeit one that is not directly impacted by the Bcl-2 SNP genotype.

Similar to Tg, direct InsP₃R activation with AdA in a zero-Ca²⁺ medium induced only negligible [Ca²⁺]_i elevations, which were indistinguishable between genotypes (data not shown). In normal Ca²⁺ (1 mmol/L) medium, however, a significantly larger, presumably SOCE-mediated, increase in [Ca²⁺]_i in variant AA was observed compared with variant GG (AA = 958 ± 108, AG = 781 ± 58, GG = 585 ± 70, *F* = 5.03; AA vs. GG *t* = 3.17, *p* < .05 after Bonferroni correction) (Figures 2C–2D). These observations suggest that the Bcl-2 gene SNP variant AA might exert its effect by specifically modulating ER InsP₃Rs.

InsP₃-Mediated ER Ca²⁺ Release But Not Basal ER or Mitochondrial Ca²⁺ Was Increased in the Bcl-2 SNP Variant AA

Given that InsP₃R activation induced dissimilar [Ca²⁺]_i elevations in different Bcl-2 SNP variants, InsP₃R-mediated ER Ca²⁺ regulation was studied in greater detail. Free and total basal ER luminal Ca²⁺ concentrations were evaluated directly by means of fluorescence microscopy and electron probe microanalysis, respectively. With a technique that selectively loads Ca²⁺-sensitive dye into the ER lumen of permeabilized cells (26), we found no difference in basal free ER Ca²⁺ between variants AA and GG (low-affinity fura-2FF, 340/380 ratios .65 ± .22 vs. .62 ± .23, ns) (Figures 3A–3B). Similarly, no significant difference in total ER luminal Ca²⁺ (AA = 5.5 ± .8, GG = 6.1 ± 1.5 mmol/kg dry weight) was observed (Figures 3C–3E). The low concentrations found here further suggest the small size of the basal ER calcium store. Because mitochondria generally play a significant role in Ca²⁺ regulation, we used electron probe microanalysis to assess the status of resting mitochondrial Ca²⁺ and found that total Ca²⁺ pools were small and similar in all SNP variants (Figures 3C–3E).

To determine the extent of ER Ca²⁺ release after InsP₃R activation, we used the permeabilization and ER loading strategy outlined in Figure 3A to directly measure the relative size of the luminal free Ca²⁺ pool after AdA exposure. A significant decrease was observed in the 340/380 fluorescence ratio in variant AA relative to variant GG (*p* < .05) (Figures 4A–4B), which implies reduced residual ER Ca²⁺ content and therefore increased luminal Ca²⁺ loss in SNP variant AA. Because no difference was observed in InsP₃R expression between Bcl-2 SNP variants as determined with load-normalized gels and a pan-reactive IP₃R antibody (Figure 4C), the most straightforward explanation for increased Ca²⁺ release from the ER pool of Bcl-2-deficient lymphoblasts is a quantitative reduction in the molecular interaction of Bcl-2 with InsP₃Rs (27,31). The InsP₃R-mediated Ca²⁺ rise was accompanied by an approximately threefold increase in free mitochondrial matrix Ca²⁺, as reported by rhod-2 fluorescence, but the three SNP variants did not differ significantly with respect to mean F/F₀ (AA, 2.68 ± .23; AG, 2.74 ± .31; GG, 2.603 ± .16; ns) (Figure 4D).

Lithium Reduced Abnormally High IP₃-Dependent ER Ca²⁺ Release in BPD Subjects Carrying the AA Variant

Treatment with lithium (1 mmol/L for 7 days) significantly increased Bcl-2 levels (to 128% of control, $p < .01$) in BLCL from BPD subjects expressing variant AA (Figure 5A). Lithium-treated AA cells showed a trend toward decreased basal cytosolic Ca²⁺ levels that did not, however, reach significance (untreated, 52.2 ± 7.9 nmol/L; lithium-treated, 37.3 ± 7.0 nmol/L; $t = 2.39$, $p = .07$). We also examined the effects of lithium on cytosolic and ER Ca²⁺ after stimulation in the functional Bcl-2 SNP. Lithium significantly decreased the abnormally high InsP₃-induced cytosolic Ca²⁺ elevations in the Bcl-2 SNP (rs956572) AA relative to untreated cells (451 ± 51 nmol/L with lithium vs. 588 ± 38 nmol/L in control subjects, $t = 2.82$; $p < .05$) (Figures 5B–5C); this suggests a rescue effect on Ca²⁺ dysregulation for this Bcl-2 SNP allele.

To determine whether lithium treatment affected basal and/or releasable luminal ER Ca²⁺ in an InsP₃- and Bcl-2-dependent manner, ER luminal Ca²⁺ was estimated before and after AdA exposure. Lithium did not significantly alter basal ER Ca²⁺ levels in variant AA (340/380 fluorescence ratios ($.59 \pm .11$ lithium-treated vs. $.51 \pm .18$ untreated; $t = 1.45$, $p = .2$) (Figure 5D) but did reduce the abnormally large loss of luminal ER Ca²⁺ after InsP₃R activation in variant AA compared with untreated cells (340/380 ratios $.47 \pm .10$ lithium-treated vs. $.38 \pm .07$ control subjects; $t = 3.14$, $p = .02$) (Figure 5D–5E).

Bcl-2 Inhibition Elevated Basal and InsP₃R-Stimulated Cytosolic Calcium in the Variant GG

To confirm the role of Bcl-2 in facilitating InsP₃R-mediated Ca²⁺ dysregulation in the Bcl-2 SNP, variant GG lymphoblasts were pre-incubated with BH3-I, a Bcl-2 inhibitor (32), for 1 hour before fura-2 loading. The Bcl-2 downregulation significantly increased baseline [Ca²⁺]_i compared with control GG cells (32.1 ± 6.0 nmol/L vs. 20.5 ± 6.2 nmol/L, respectively; $p < .05$) (Figure 6A). Likewise, a significant increase in InsP₃R agonist-stimulated [Ca²⁺]_i elevations was observed after incubation (377.1 ± 49.1 nmol/L with inhibitor vs. 226.9 ± 29.9 nmol/L without; $p < .05$) (Figures 6B–6C). Thus, Bcl-2 inhibition seemed to transform the GG variant so that Ca²⁺ regulation in this genotype resembled that observed in the Bcl-2-deficient variant AA.

Discussion

The results of this study demonstrate that Bcl-2 gene expression regulated by the rs956572 SNP directly impacted intracellular Ca²⁺ dysregulation in individuals with BPD. Specifically, we found a selective and integrated control by Bcl-2 of both cytosolic Ca²⁺ levels and the InsP₃R-dependent releasable ER Ca²⁺ pool in A carriers, thus marking the ER as the main functional target of this Bcl-2-deficient variant in subjects with BPD. In variant AA, this control was reflected by increased baseline and InsP₃R agonist-stimulated cytosolic Ca²⁺ levels, in parallel with enhanced reduction of luminal ER Ca²⁺ after stimulation. The lower Bcl-2 levels present in the AA variant did not affect basal ER or mitochondrial Ca²⁺ content; nor did they modulate stimulated mitochondrial Ca²⁺ uptake. In addition, chronic lithium treatment reversed the abnormally high InsP₃R-dependent ER Ca²⁺ release and cytosolic Ca²⁺ elevations associated with the untreated A carriers. Basal ER Ca²⁺ content was unchanged after lithium treatment. Finally, pharmacological downregulation of Bcl-2 in the nondeficient Bcl-2 SNP variant GG increased basal and InsP₃R-dependent cytosolic Ca²⁺ levels, so that Ca²⁺ regulation in this variant resembled that observed in variant AA.

A recent study demonstrated that the lower Bcl-2 levels associated with the Bcl-2-deficient allele AA directly affects the brain by significantly reducing grey matter volume in the ventral striatum of healthy subjects (12). This area is known to play a critical role in the

neurobiology and pathophysiology of mood disorders, for example in reward processing and emotion regulation (33). Thus, the increased Bcl-2-modulated, ER-mediated cytosolic Ca^{2+} levels observed in the present investigation might be intrinsically associated with both increased risk for apoptosis (34) and decreased grey matter volume (12) in subjects carrying the AA allele. To our knowledge, this study is the first to directly and comprehensively quantify intracellular Ca^{2+} levels in the context of Ca^{2+} regulation by a BPD-related gene polymorphism.

Several lines of evidence support the involvement of ER stress proteins such as GRP78 and calreticulin in the pathophysiology and therapeutics of BPD (2). Mechanisms might well involve Bcl-2 modulation of InsP_3R -dependent ER Ca^{2+} release, because enhanced expression of GRP78 and calreticulin is regulated by Bcl-2 (14). As noted previously, the role of Bcl-2 in ER and cytosolic Ca^{2+} dynamics has received increasing attention in recent years, with conflicting results. Several studies have shown that Bcl-2 inhibits Ca^{2+} release from the ER and preserves ER luminal Ca^{2+} , whereas other reports suggest that Bcl-2 increases Ca^{2+} leak across ER membranes, thus decreasing luminal Ca^{2+} levels (17,18). Considering that we found no difference in InsP_3R expression levels among SNP variants, the present results are consistent with studies indicating that direct Bcl-2/ InsP_3R interactions reduce the permeability of this ER Ca^{2+} channel, thereby attenuating baseline cytosolic Ca^{2+} levels (27,31). Presumably, these same interactions facilitate the store depletion-mediated SOCE that accounts for regulatory Ca^{2+} elevations in BLCL. It remains to be determined whether the molecular mechanism(s) of SOCE in BLCL involves the stromal interaction molecule/Orai pathway, STIM/transient receptor potential (TRP) channel interactions (canonical TRPC3-like channels) are known to be present in this cell type (30), both, or perhaps other pathways. Ultimately, it will be important to clarify the mechanisms associated with InsP_3R sensitization in Ca^{2+} dysregulation, particularly because Ca^{2+} dysregulation seems to be an underlying characteristic of other disorders in addition to BPD (for instance, Alzheimer's disease) (35,36). It is also important to note that although mitochondria play a major role in Ca^{2+} regulation (37)—including in immune cells (38)—and that several studies support a role for mitochondrial dysfunction in the pathophysiology of BPD (39), the present study found no significant differences in either free or total mitochondrial Ca^{2+} at rest or after InsP_3R activation in any Bcl-2 SNP variant.

Previous studies have demonstrated that lithium increases Bcl-2 levels in several brain areas, and these effects are thought to underlie lithium's therapeutic mechanisms (40). Endoplasmic reticulum stress induces the formation of reactive oxygen species in part by upregulating Ca^{2+} signaling. Interestingly, an inverse correlation between Bcl-2 and reactive oxygen species levels was described in the lymphocytes of individuals with BPD (41), and altered Bcl-2 levels and increased oxidative stress have both been observed in BPD subjects, with rescue effects seen after lithium treatment (42,43). Given that the integrity of intracellular Ca^{2+} homeostasis is critical to cell survival, the effect of lithium on Ca^{2+} signaling might be mechanistically relevant to its neuroprotective actions, now thought to be key to the clinical effects of this agent (40).

Several technical aspects of this study merit comment, including the multiple advantages of using cultured lymphoblasts. These cells are easily accessible and manifest biological responses similar to central nervous system neurons (1). Furthermore, they are suitable for pharmacological studies both in vivo and in vitro, and the several passages necessary for culture limit the potential bias of environmental factors. Lastly, Epstein-Barr Virus transformation does not significantly alter the expression of key proteins (1). Most studies evaluating InsP_3 -dependent Bcl-2 regulation of ER Ca^{2+} release have been performed in cells overexpressing Bcl-2. A strength of the present investigation is that changes in Bcl-2 levels were endogenously expressed by a potential functional polymorphism without any

pharmacological or biological intervention aimed at regulating Bcl-2 levels; this is particularly important, because transient Bcl-2 overexpression might be toxic (44,45). In addition, this study is the first to quantitatively measure cytosolic and intra-organelle Ca^{2+} levels on an individual, cell-by-cell basis, which was possible because of the inherent advantages of microscopy-based techniques. Microscopy approaches have other advantages relative to methods that provide only population averages; for example, microscopy allows the exclusion of nonviable cells, the characterization of cell-to-cell variability, and the direct measurement of organelle Ca^{2+} levels under various experimental conditions.

It must be acknowledged that it is presently unclear how—or even whether—the SNP variants studied here relate to an increased risk for developing BPD. The results obtained regarding the AA variant provide evidence for Ca^{2+} dysregulation, which might be a candidate for a risk allele; however, similar genotype-specific Ca^{2+} dysregulation has been reported by others for the GG variant (46), implying that the rs956572 polymorphism is not causal. Thus, we do not consider the present effects to be specific to BPD. Although it is plausible that this Bcl-2 SNP plays some role in the pathophysiology of BPD, it remains unclear how such an intronic variant might operate to functionally influence Bcl-2 transcription and/or expression. Nevertheless, the evidence presented demonstrates that this SNP variant critically affects intracellular Ca^{2+} signaling in BLCL from BPD subjects by altering the “gain” of InsP_3R -dependent ER Ca^{2+} release. The results might provide new insights into the potential functional role of Bcl-2 in physiological and pathophysiological states.

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Figure 1.

Basal cytosolic calcium in B-cell lymphoma 2 (Bcl-2) single nucleotide polymorphism (SNP) variants. **(A, B)** Basal cytosolic free calcium (Ca^{2+}) concentrations measured with fura-2 in B-lymphoblastoid cell lines (BLCL) from individual BPD patients **(A)** and in individual BLCL cells **(B)** from 18 patients of three genotypes incubated in extracellular medium containing 1.0 mmol/L Ca^{2+} . Both patient/patient and cell/cell analysis indicated that $[\text{Ca}^{2+}]_i$ in variant AA was significantly higher than in variant GG ($p < .01$). **(C, D)** Plot of the inverse correlations between individual patient-averaged cytosolic Ca^{2+} concentrations and Bcl-2 messenger RNA expression **(C)** ($r = .60$, $p < .01$) and protein expression **(D)** ($r = .49$, $p < .05$) as a function of genotype. By both measures, reduced Bcl-2 expression in variant AA was associated with elevated basal $[\text{Ca}^{2+}]_i$. All statistical tests were one-way analysis of variance with post hoc Bonferroni correction. a.u., arbitrary unit.

Figure 2. Cytosolic calcium dynamics in Bcl-2 SNP variants. **(A)** Representative cytosolic Ca^{2+} traces (fura-2) from BLCL of the three variants after stimulation with thapsigargin (Tg) (1 $\mu\text{mol/L}$) in Ca^{2+} -free medium containing 200 $\mu\text{mol/L}$ ethylene glycol bis-(β -aminoethyl ether)- N,N' -tetraacetic acid (lower traces) or medium containing 1 mmol/L extracellular Ca^{2+} (upper traces). Tg induced significant $[\text{Ca}^{2+}]_i$ elevations only in the presence of extracellular Ca^{2+} . **(B)** Average (per-patient) $[\text{Ca}^{2+}]_i$ elevations in BLCL of the three different genotypes after Tg incubation in Ca^{2+} -containing medium did not significantly differ. **(C, D)** Typical Ca^{2+} traces **(C)** and average $[\text{Ca}^{2+}]_i$ elevations **(D)** in cells from the three Bcl-2 SNP variants after exposure to the InsP_3R agonist adenophostin A (AdA) (75 nmol/L) in a medium containing 1 mmol/L extracellular Ca^{2+} revealed a graded, genotype-dependent response. The $[\text{Ca}^{2+}]_i$ elevations in variant AA were significantly higher than in variant GG ($p < .05$). Other abbreviations as in Figure 1.

Figure 3.

Basal endoplasmic reticulum (ER) and mitochondrial Ca^{2+} stores are not altered in cells expressing the Bcl-2 SNP variant AA. (**A**, **B**) Typical Ca^{2+} traces (**A**) and per-patient averages (**B**) of low-affinity fura-2FF fluorescence emission (ratio 340/380 nm) from AA and GG BLCL permeabilized with digitonin (10 $\mu\text{g/mL}$) in an intracellular buffer (ICB) supplemented with 500 $\mu\text{mol/L}$ adenosine triphosphate. Fluorescence gradually increased to a steady-state maximum that reflects the level of luminal ER Ca^{2+} (23). Basal ER free Ca^{2+} was similar in Bcl-2 SNP variants AA and GG. (**C**) Total ER and mitochondrial matrix calcium concentrations, as measured by electron probe microanalysis, were similar in variants AA and GG, although the former was tenfold higher. (**D**, **E**) Electron micrographs of BLCL perinuclear regions. Left panel is a conventionally fixed and embedded specimen; right panel is a rapidly frozen, cryosectioned, and freeze-dried preparation as used for electron probe microanalysis. Both panels illustrate typical organelle abundance and distribution, including ER (arrows) and mitochondria (arrowheads). Bar = .5 μm for both panels. ECB, extracellular buffer; other abbreviations as in Figure 1.

Figure 4.

Inositol 1,4,5-trisphosphate (InsP₃)-induced ER Ca²⁺ release is enhanced in Bcl-2 SNP variant AA. (**A**, **B**) Inositol 1,4,5-trisphosphate receptor (InsP₃R)-dependent reduction in ER Ca²⁺ directly measured with fura-2FF in permeabilized cells. Traces in panel **A** are a continuation of Figure 3A, with the InsP₃ agonist AdA (75 nmol/L) added after a steady-state fluorescence ratio was reached; the sharp decline in fluorescence after AdA application corresponds to the decrease of ER luminal free Ca²⁺. Per-patient averages of residual ER Ca²⁺ after AdA (**B**) showed significantly greater Ca²⁺ release in the variant AA compared with GG ($p < .05$). (**C**) Representative Western blots and quantitative estimation of InsP₃R total protein show no differences between AA and GG cells. (**D**) Similarly, mitochondrial Ca²⁺ uptake, measured with the Ca²⁺-selective dye Rhod-2, detected no differences among the three variants after AdA application ($p = .77$). Other abbreviations as in Figures 1 and 3.

Figure 5.

Lithium reduces $InsP_3R$ -dependent ER Ca^{2+} release in Bcl-2 SNP variant AA. **(A)** Representative Western blots (top) and per-patient averages (duplicate analysis, $n = 6$ each) showed a significant increase in Bcl-2 expression in lithium-treated (1 mmol/L for 7 days) AA BLCL relative to untreated cells (** $p < .01$). **(B, C)** Representative cytosolic Ca^{2+} traces (fura-2) and per-patient averages showed reduced AdA-stimulated (75 nmol/L) $[Ca^{2+}]_i$ elevations in lithium-treated lymphoblasts (** $p < .01$). **(D, E)** In an experiment analogous to Figure 4A, typical traces of fura-2FF fluorescence emission (340/380 nm ratio, panel **D**) and per-patient averages (**E**) from digitonin-permeabilized, ER loaded cells showed reduced AdA-induced ER Ca^{2+} release in lithium-treated AA lymphoblasts (* $p < .05$). Abbreviations as in Figures 1 and 3.

Figure 6.

The Bcl-2 inhibition elevates InsP₃R-dependent cytosolic Ca²⁺ in the SNP variant GG. **(A)** One-hour pre-incubation with the Bcl-2 inhibitor BH3-I significantly increased fura-2-measured basal [Ca²⁺]_i in BLCL from the variant GG (**p* < .05 relative to control). **(B, C)** Representative cytosolic Ca²⁺ traces **(B)** and per-patient averages **(C)** show enhanced AdA-stimulated (75 nmol/L) [Ca²⁺]_i elevation in GG lymphoblasts after Bcl-2 inhibition (**p* < .05). Abbreviations as in Figures 1 and 3.