

Article

Long-Term Lithium Treatment Increases cPLA₂ and iPLA₂ Activity in Cultured Cortical and Hippocampal Neurons

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Abstract: Background: Experimental evidence supports the neuroprotective properties of lithium, with implications for the treatment and prevention of dementia and other neurodegenerative disorders. Lithium modulates critical intracellular pathways related to neurotrophic support, inflammatory response, autophagy and apoptosis. There is additional evidence indicating that lithium may also affect membrane homeostasis. Objective: To investigate the effect of lithium on cytosolic phospholipase A₂ (PLA₂) activity, a key player on membrane phospholipid turnover which has been found to be reduced in blood and brain tissue of patients with Alzheimer's disease (AD). Methods: Primary cultures of cortical and hippocampal neurons were treated for 7 days with different concentrations of lithium chloride (0.02 mM, 0.2 mM and 2 mM). A radio-enzymatic assay was used to determine the total activity of PLA₂ and two PLA₂ subtypes: cytosolic calcium-dependent (cPLA₂); and calcium-independent (iPLA₂). Results: cPLA₂ activity increased by 82% (0.02 mM; $p = 0.05$) and 26% (0.2 mM; $p = 0.04$) in cortical neurons and by 61% (0.2 mM; $p = 0.03$) and 57% (2 mM; $p = 0.04$) in hippocampal neurons. iPLA₂ activity was increased by 7% (0.2 mM; $p = 0.04$) and 13% (2 mM; $p = 0.05$) in cortical neurons and by 141% (0.02 mM; $p = 0.0198$) in hippocampal neurons. Conclusion: long-term lithium treatment increases membrane phospholipid metabolism in neurons through the activation of total, c- and iPLA₂. This effect is more prominent at sub-therapeutic concentrations of lithium, and the activation of distinct cytosolic PLA₂ subtypes is tissue specific, *i.e.*, iPLA₂ in hippocampal neurons, and cPLA₂ in cortical neurons. Because PLA₂ activities are reported to be reduced in Alzheimer's disease (AD) and bipolar disorder (BD), the present findings provide a possible mechanism by which long-term lithium treatment may be useful in the prevention of the disease.

Keywords: lithium; neuronal cell culture; iPLA₂ activity; cPLA₂ activity

1. Introduction

Lithium is a first-line drug for the acute and long-term treatment of bipolar disorder (BD). More recently, evidence derived from experimental models, along with data from epidemiological, neuroimaging and a few clinical studies, has reinforced the potential use of lithium for the treatment and/or prevention of dementia and related neurodegenerative conditions [1]. Lithium has been reported to play a role in neuronal homeostasis [2], stimulation of neuronal growth cones [3], up-regulation of neurotrophins brain-derived neurotrophic factor (BDNF) [4] and vascular

endothelial growth factor (VEGF) [5,6], inhibition of glutamatergic excitotoxicity [7], down-regulation of autophagy [8], inhibition of β -amyloid production [9] and toxicity [3], and glycogen synthase kinase 3 β -mediated Tau pathology [10].

Early studies suggested that lithium might negatively affect membrane homeostasis through the inhibition of phospholipase A₂ (PLA₂) [11], with relevant downstream effects on signal transduction and eicosanoid production [12]. However, a recent study from our group indicates that lithium stimulates hippocampal neurogenesis [13], an effect that apparently depends on the integrity of PLA₂ function [14]. The independent assessment of distinct PLA₂ subtypes, including activity and regional distribution, is probably the key to understanding the discrepancies of the enzymatic activity in Alzheimer's disease (AD) and BD brain.

PLA₂ is a superfamily of enzymes that are central to membrane phospholipid metabolism and can be divided into three main groups: secretory PLA₂ (sPLA₂); calcium-dependent PLA₂ (cPLA₂); and calcium-independent PLA₂ (iPLA₂) [15–18]. These enzymes generally regulate the release of lipid mediators from the cell membrane, playing important roles in signal transduction and regulation of inflammatory response [17–19]. Arachidonic acid (AA) is the most important and abundant free fatty acid released from membrane phospholipids through the catalytic activity of PLA₂ [20,21]. Both c- and iPLA₂ are highly expressed in the central nervous system, with distinct roles and regional specificities, in addition to a distinct sensitivity and pattern of response to the effect of regulatory cascades. Previous work from our group found that PLA₂ activity is reduced in AD [22], and that lithium treatment reduces the risk for AD [23]. In the present study, we further develop this hypothesis by analyzing the effect of chronic lithium treatment at different working concentrations (0.02, 0.2 and 2 mM) on PLA₂ activity in primary cultures of cortical and hippocampal neurons.

2. Results and Discussion

The MTT results indicate that treatment with lithium increases neuronal viability in comparison with the control cells, with a marginally significant difference ($p < 0.05$). Samples of cortical neurons show an increase of 19%, 6% (** $p < 0.01$) and 47% in the treatments of 0.02, 0.2 and 2 mM, respectively. The hippocampal cell culture samples show an increase of 20%, 21% and 31% in treatments of 0.02, 0.2 and 2 mM, respectively (Figure 1).

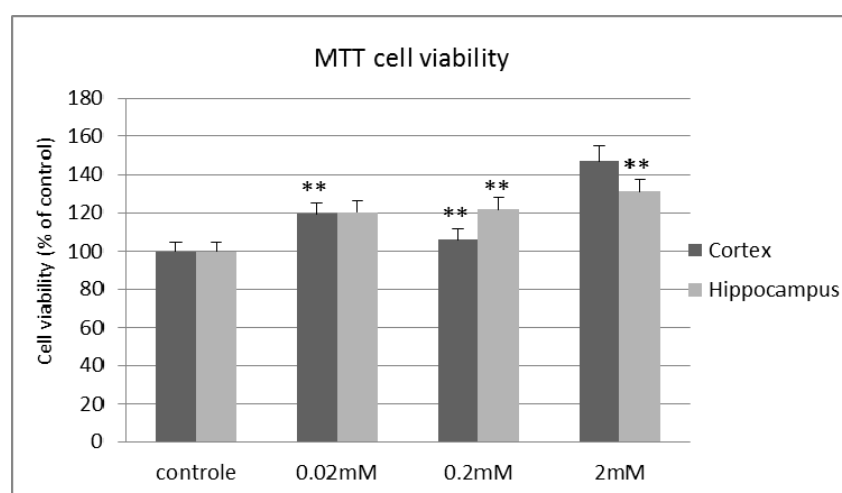


Figure 1. Viabilities of primary cultures of hippocampal and cortical neurons exposed to different concentrations of lithium chloride for 7 days ($n = 5$, ** $p < 0.01$).

In cortical neurons, lithium treatment significantly increases total PLA₂ activity by 25% (0.02 mM; $p = 0.04$) and 3% (0.2 mM; $p = 0.035$), when compared to control. cPLA₂ activity increases by 82% at 0.02 mM ($p = 0.05$) and 26% at 0.2 mM ($p = 0.04$), when compared to control (Figure 2).

Additionally, we found a 7% increase in iPLA₂ activity at 0.2 mM ($p = 0.04$) and a 13% increase at 2 mM ($p = 0.05$) (Figure 2).

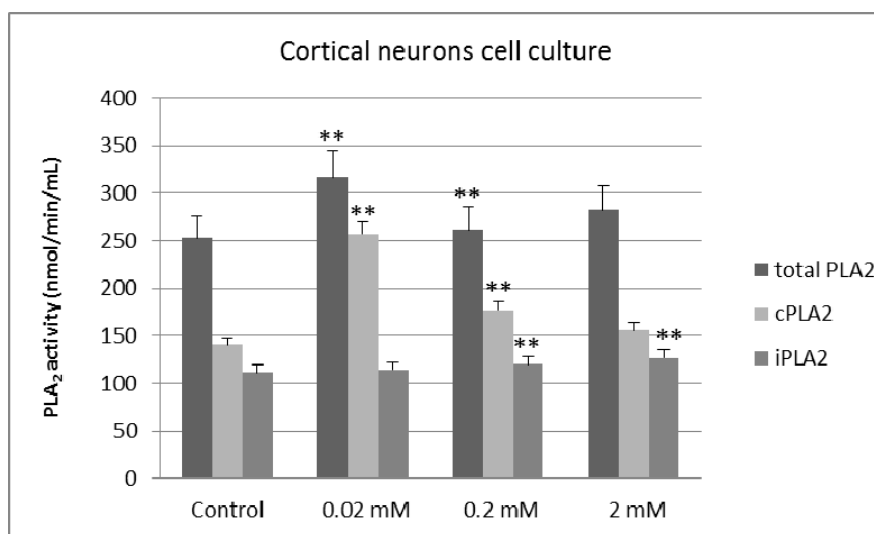


Figure 2. Effects of different concentrations of lithium chloride on the activities of total PLA₂, cPLA₂ and iPLA₂ in cultured cortical neurons ($n = 5$, ** $p < 0.02$).

In hippocampal neurons lithium treatment significantly increased the activity of total PLA₂ by 80% (2 mM; $p = 0.04$); cPLA₂ by 61% (0.2 mM; $p = 0.03$) and 57% (2 mM; $p = 0.04$) and iPLA₂ by 141% (0.02 mM; $p = 0.0198$), 112% (0.2 mM; $p = 0.2$) and 97% (2 mM; $p = 0.3$) (Figure 3).

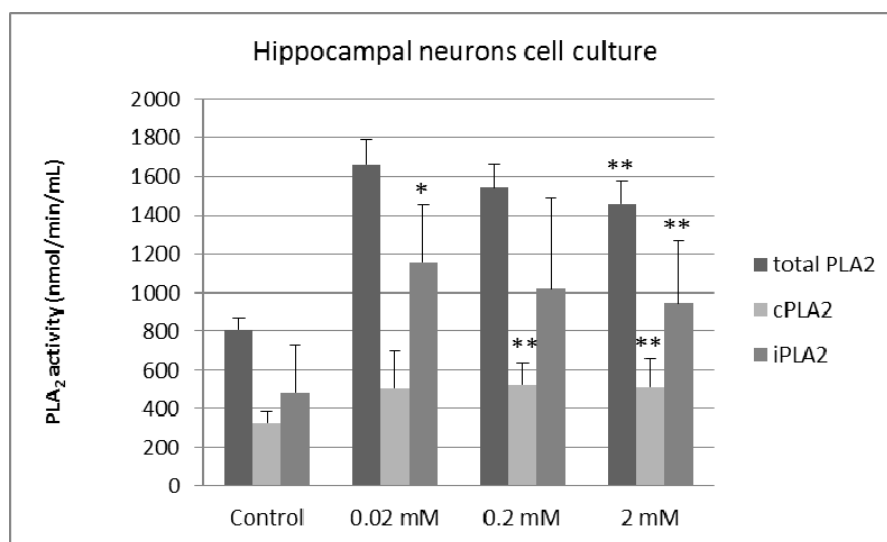


Figure 3. Effects of different concentrations of lithium chloride on the activities of total PLA₂, cPLA₂ and iPLA₂ in cultured hippocampal neurons ($n = 5$, * $p < 0.05$, ** $p < 0.02$).

Our results show that long-term lithium treatment increases both cPLA₂ and iPLA₂ activity in primary cultures of cortical and hippocampal neurons at therapeutic and sub-therapeutic concentrations of lithium chloride. This effect was more prominent at micromolar concentrations, with tissue-specific differences in the magnitude of the effect: treatment with 0.02 mM lithium yielded a potent activation of iPLA₂ in primary cultures of hippocampal neurons, with a 114% increase in

enzymatic activity, whereas the same treatment in cortical neurons resulted in a predominant effect on cPLA₂ (82% increase).

At first sight, the present findings seem to be in disagreement with the results from studies conducted in the late 1990's, which suggest that lithium might actually inhibit PLA₂ [11,12]. In the study by Chang and Jones [11] the effect of lithium on total PLA₂ activity was determined in rat brain homogenates, whilst Chang *et al.* [12] did not assess directly PLA₂ activity, but made rather an indirect assumption of PLA₂ inhibition by showing, *in vivo*, that chronic treatment of adult rats with therapeutic doses of lithium decreased the turnover of arachidonate within the total brain contents of phospholipids [7]. The methodological differences between these studies and ours are probably key to the interpretation of the current findings. First, we used a different experimental model (*i.e.*, primary neuronal cultures) to test the hypothesis. Second, we explored this effect using a wider therapeutic window of lithium, *i.e.*, ranging from sub-therapeutic (micromolar) to therapeutic (milimolar) concentrations, which proved to have distinct effects on the target enzymes. Third, we determined the activity of PLA₂ subtypes using a validated method that specifically reads c- and iPLA₂ [24]. Finally, we determined PLA₂ activity in primary cultures predominantly composed by hippocampal or cortical neurons; that is to say, we did not use a total brain model, in which the determination of PLA₂ activity represents the sum of neuronal and glial PLA₂, bearing in mind that the enzyme is more constitutively expressed and active in glial cell than in neurons.

Therefore, our data indicate that lithium positively regulates the activity of these two cytosolic forms of PLA₂. Such an effect may be relevant to the understanding of the trophic effects of lithium, since both c- and iPLA₂ are implicated in processes related to neurodevelopment and neuroprotection [25]. In the developing human brain, iPLA₂ is mainly expressed in proliferative zones [26], which have been shown to be sensitive to the neurotrophic effect of lithium [21] including its ability to induce neurogenesis [13].

The reactivity of hippocampal iPLA₂ to the effect of lithium probably relates to the neurobiological functions of the enzyme in this important cerebral structure. The hippocampus is the brain structure where iPLA₂ displays its highest documented enzymatic activity [25]. In addition to a well-accepted role in signal transduction through the release of AA and other lipid mediators [27], regulating neurogenesis [26]. Evidence suggests that hippocampal iPLA₂ is also implicated in the long-term potentiation (LTP) of excitatory synaptic transmission [28,29] and subsequent mechanisms of memory formation [26,28]. Early studies from Clement *et al.* [30] showed that AA was released through PLA₂ catalytic activity at early stages of LTP induction in membranes prepared from the dentate gyrus. Drapeau and collaborators [31] also proposed that PLA₂ is involved in hippocampal LTP by increasing the production of AA, which acts as a trophic retrograde synaptic signal to increase transmitter release at glutamatergic synapses [32]. The hypothesis that PLA₂ facilitates transmitter release in LTP is further supported by the fact that iPLA₂ participates in membrane fusion, which is a process required for exocytosis [20,33]. Previous findings from our group indicate that the specific inhibition of c- and iPLA₂ impair neurite outgrowth and decrease the viability of cultured cortical and hippocampal neurons [34]. Accordingly, the inhibition of iPLA₂ in the rat hippocampus also impairs acquisition of short- and long-term memory [35]. Therefore, one must consider the possibility that the activation of iPLA₂ in the hippocampus may add to the myriad of neurobiological properties of lithium [36], particularly on the preservation of homeostatic mechanisms related to neuronal response to injury and memory formation [37–39].

The stimulatory effect of lithium on cPLA₂ and iPLA₂ is particularly interesting in the light of the involvement of PLA₂ in the pathophysiology of AD. Abnormalities in PLA₂ have been consistently described in AD patients, showing reduction of enzymatic activity both in the brain regions such as frontal and parietal cortex [40], hippocampus [41] and, peripherally, in platelets of patients with dementia and mild cognitive impairment (MCI) [39,40]. Recent findings from our group indicates that decreased iPLA₂ activity predicts the risk of conversion from MCI to dementia within the MCI-AD continuum, and decreased cPLA₂ predicts incident MCI in former cognitively unimpaired elders [37].

Interestingly, these abnormalities seem to respond to treatment with the antideementia drug donepezil, restoring homeostatic levels of enzymatic activity [42]. Therefore, the present findings suggest that lithium treatment may also modify a biological abnormality that is found in patients with, or at risk of AD.

3. Experimental Section

3.1. Establishment and Treatment of Primary Neuronal Cultures

Pregnant Wistar rats were sacrificed by cervical dislocation at gestational day 18 (E18), and the respective embryos were obtained by laparotomy. Whole embryonic brains were isolated and kept immersed in Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY, USA). Multiple fragments of cortical and hippocampal tissues were obtained by microdissection, followed by trypsinization (chemical dissociation) and mechanical dissociation. Single-cell suspensions were counted and re-suspended in Neurobasal medium containing B-27 supplement (Gibco), 2 mM glutamine, penicillin (100 I.U.), streptomycin (100 mg/mL), and 5% fetal calf serum (all Gibco). Cells were plated onto poly-D-lysine coated Petri dishes at a density of 1×10^7 cells per culture plate, and incubated for up to 10 days at 37 °C and 5% CO₂. On day 4 after *in vitro* plated, hippocampal and cortical neurons were incubated for 7 days (37 °C, 5% CO₂), with different concentrations of lithium (0.02 mM, 0.2 mM and 2 mM). Neuronal viability was microscopically ascertained prior to experimentation. All procedures involving laboratory animals were approved by the Ethics Committee and the Animal Care Committee of the University of São Paulo School of Medicine, in the city of São Paulo, Brazil, and were conducted in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (ISBN 0-309-05377-3, NIH publication No. 86-23, revised 1985; National Research Council 2011).

3.2. Assessment of Cell Viability

Cell viability was quantitatively assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, which estimates the percentage of living cells in a given substrate compared to controls [43]. Treatments were replicated at least 5 times with identical experimental conditions. Briefly, cells were plated in the same concentration by surface in a 96 well plate and followed the same treatments described above, after the last day (10 day in culture) of treatment, 50 µL of MTT solution (5 mg/mL in PBS) was added to each well (1×10^5 cells/mL) and the plates were incubated for 3 h, 37 °C and CO₂ 5%. Then, 500 µL of 10% SDS in 0.01 N HCl was added. After overnight incubation, the absorbance was measured by spectrophotometry at 570 nm.

3.3. Determination of PLA₂ Activity

To determine PLA₂ activity we used L- α -1 palmitoyl-2-arachidonyl-phosphatidylcholine (Perkin Elmer Life Science, New England Nuclear, Boston, MA, USA) labeled in position 2 with [114C] AA (PC-AA-[114C]) as enzyme substrate. The assay mixture contained 50 µL of 1.0 M Tris-HCl buffer pH 7.5, 200 µL of culture cell homogenates (1 mg of protein homogenate), 150 µL of PC-AA-(114C) (0.12 µCi). For cPLA₂ measurement we used an optimal concentration of CaCl₂ (30 µM) and the inhibitor bromoenol-lactone (BEL) to inactivate iPLA₂ activity (500 µM). Total PLA₂ was measured with CaCl₂ 5 mM and vehicle (DMSO). The solution was incubated for 30 min at 37 °C and the reaction was stopped by the addition of 700 µL isopropanol-hydrochloric acid. The ¹⁴C-labeled AA released by the cleavage of PC-AA-[114C] by PLA₂ was extracted with *n*-heptane, followed by adsorption of the unbroken phospholipids and the lysophospholipids on 60 mg of silica. The radioactivity of free ¹⁴C-labeled AA was measured in a Tri Carb Liquid Scintillation counter (Tri-Carb 2100TR; Packard, Meriden, CT, USA). The results were given in CPM (counts per minute) and converted to picomols per milligram of protein per minute using the equation: PLA₂ activity = CPM \times F/A \times E \times 2.22 \times B. The blank counts were subtracted from each sample count (where:

CPM = counts per minute; F = adjustment factor for protein concentration; A = specific activity of radioactive substrate in mCi/mmol; B = incubation time in minutes; E = equipment efficiency) [24]. iPLA₂ activity was inferred by calculating the difference between total PLA₂ and cPLA₂ [29].

3.4. Statistical Analysis

All experiments were conducted in quintuplicates, yielding mean values for PLA₂ subtypes (total, c- and i-PLA₂) in each treatment condition (vehicle or lithium chloride 0.02 mM, 0.2 mM and 2 mM) and model (cortical or hippocampal neurons). Independent sample Student's *t*-tests were carried out to test for the statistical significance of the difference between mean values of each treatment condition compared to the respective controls. Statistical significance was set at $p < 0.05$. Analyses were performed using the software package SPSS-18 (SPSS Inc., Chicago, IL, USA).

4. Conclusions

We provide evidence that long-term lithium treatment activates both forms of cytosolic PLA₂-cPLA₂ and iPLA₂- in primary cultures of cortical and hippocampal neurons. These effects were observed at therapeutic and sub-therapeutic concentrations of lithium chloride, with a more prominent effect at the micromolar range, but definitely without a dose-response pattern. We found that the effect of lithium on these two subtypes of PLA₂ depends on the brain area from which the primary neurons derive, *i.e.*, iPLA₂ being more sensitive to the effect of lithium in hippocampal neurons, and cPLA₂ in cortical neurons. Such differences are probably related to the distinct physiological roles and sensitivity to regulatory mechanisms of c- and iPLA₂ within the brain. The present findings may be relevant to the understanding of the neurobiological mechanisms of lithium related to neurotrophic response and neuroprotection.

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Author Contributions: V.J. De-Paula conceived, designed and performed the experiments, analyzed the data, wrote the paper. M.P.F. Carvalho designed and performed the experiments. E.L. Schaeffer designed and performed the experiments and analyzed the data. D.S. Kerr analyzed the data, wrote the paper. L.L. Talib designed and performed the experiments. W.F. Gattaz analyzed the data, wrote the paper. O.V. Forlenza conceived and designed the experiments, analyzed the data, wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: All samples were prepared according to the protocols described and are not available. All reagents are commercially available.



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