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Is phosphoadenosine phosphate phosphatase a target of lithium's therapeutic effect?

G. Shaltiel¹, J. Deutsch^{2,3}, S. I Rapoport³, M. Basselin³, R. H. Belmaker^{1,*}, and G. Agam¹

¹ Psychiatry Research Unit, Ben-Gurion University of the Negev, and Mental Health Center, Beersheva, Israel

² Department of Medicinal Chemistry, School of Pharmacy, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

³ Brain Physiology and Metabolism Section, National Institute on Aging, National Institutes of Health, Bethesda, MD, USA

Abstract

Lithium, which is approved for treating patients with bipolar disorder, is reported to inhibit 3'(2')-phosphoadenosine-5'-phosphate (PAP) phosphatase activity. In yeast, deletion of PAP phosphatase results in elevated PAP levels and in inhibition of sulfation and of growth. The effect of lithium on PAP phosphatase is remarkable for the low K_i (~0.2 mM), suggesting that this system would be almost completely shut down *in vivo* with therapeutic levels of 1 mM lithium, thereby elevating PAP levels. To test the hypothesis that lithium inhibition of PAP phosphatase is pharmacologically relevant in bipolar disorder, we fed rats LiCl for 6 weeks, and assayed brain PAP levels after subjecting the brain to high-energy microwaving. We also measured PAP phosphatase mRNA and protein levels in frozen brain tissue of lithium-treated mice. Brain adenosine phosphates were extracted by trichloroacetic acid and assayed by HPLC with a gradient system of two phases. PAP phosphatase mRNA was measured by RT-PCR, and PAP phosphatase protein was measured by Western blotting. Brain PAP levels were below detection limit of 2 nmol/g wet wt, even following lithium treatment. Lithium treatment also did not significantly change brain PAP phosphatase mRNA or protein levels. These results question the relevance of PAP phosphatase to the therapeutic mechanism of lithium. A statistically significant 25% reduced brain ADP/ATP ratio was found following lithium treatment in line with lithium's suggested neuroprotective effects.

Keywords

Lithium; bipolar; rat; brain; adenosine; 3'(2')-Phosphoadenosine-5'-phosphate(PAP); phosphatase; microwaving

Introduction

Lithium is an effective treatment of manic-depressive illness and has numerous biochemical effects, some of which may be related to its therapeutic action and others to its side effects (Shaldubina et al. 2001). Among biochemical effects described for lithium is its inhibitory effect on 3'(2')-Phosphoadenosine-5'-phosphate (PAP) phosphatase activity (Lopez-Coronado

*Correspondence should be addressed to: RH Belmaker, MD, Ben Gurion University of the Negev, Beer-Sheba Mental Health Center, PO Box 4600, Beer-Sheba, ISRAEL., Phone: (972 7) 640-1602. Fax: (972 7) 640-1621, belmaker@bgu.ac.il.

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et al. 1999, Yenush et al. 2000). PAP phosphatase is a ubiquitous enzyme highly conserved during evolution (Spiegelberg et al. 1999, Yenush et al. 2000). PAP phosphatase specifically catalyzes the hydrolysis of the 3' or 2'-phosphate from PAP. PAP is obtained when phosphoadenosine phosphosulfate (PAPS) is used in sulfation reactions (Klaassen & Boles 1997). PAP phosphatase converts PAP to adenosine 5'-phosphate (AMP) and inorganic phosphate (Lopez-Coronado et al. 1999, Yenush et al. 2000). Thus, PAP phosphatase plays a role in sulfation processes. In yeast, deletion of PAP phosphatase results in elevated PAP levels, inhibits sulfation (Dichtl et al. 1997) and causes growth inhibition (Spiegelberg et al. 2005). Based on the assumption that Li's inhibition of PAP phosphatase leads to elevated PAP levels in mammals as found in yeast, resulting in inhibition of sulphation we have previously examined the effect of chronic Li treatment on serum and brain DHEA and DHEA-S levels in rats (Maayan et al. 2004) and found that Li administration lowered frontal cortex and hippocampus DHEA and DHEA-S levels. Additionally, we have previously found 24% significantly lower PAP phosphatase protein levels in postmortem brain of bipolar patients with no change in PAP phosphatase mRNA levels or in enzymatic activity (Shaltiel et al. 2002) suggesting that abnormality of PAP phosphatase may play a role in bipolar disorder etiology (Agam & Shaltiel 2003, Agam et al. 2003).

The effect of lithium on PAP phosphatase is remarkable for its low K_i (0.16 mM) (Spiegelberg et al. 1999), which supports a possible pharmacological effect of lithium in this system. For instance, at a therapeutically equivalent lithium level of 1 mM the lithium-inhibitable inositol monophosphatase would be inhibited by only 50% (Hallcher & Sherman 1980) and its residual activity might be sufficient for metabolic flow from inositol-1-phosphate to inositol. Glycogen synthase kinase-3, with a K_i of between 1 and 2 mM (Klein & Melton 1996) would likely be little affected by lithium *in vivo*. For PAP phosphatase, a K_i of 0.16 mM suggests that this system would be almost completely shut down *in vivo* at therapeutic levels of 1 mM or even at lower therapeutic levels of 0.5 mM. PAP levels would be expected to rise markedly if this system maintains even a moderate metabolic flow *in vivo*.

To test the hypothesis that lithium inhibition of PAP phosphatase is pharmacologically relevant in bipolar disorder we studied rodents treated chronically with lithium to produce a therapeutically-relevant plasma concentration, and attempted to measure brain PAP levels. In designing a PAP assay, we applied the approach of Chen et al. (Chen et al. 2007) for evaluating brain concentrations of ATP and its metabolites during ischemia. Since inhibition of relevant physiological systems often leads to compensatory increases in gene expression and in levels of the inhibited enzyme's protein, we also measured brain PAP phosphatase mRNA and protein levels in lithium-treated mice.

Methods

Materials

HPLC grade acetonitrile was obtained from Fisher scientific (Fair lawn, NJ, USA). Potassium phosphate (KH_2PO_4), AMP, ADP, PAP and ATP standards were from Sigma-Aldrich (St. Louis, MO, USA). Tetrabutylammonium hydroxide (TBA) and perchloric acid were purchased from Aldrich Chemicals (Milwaukee, WI, USA).

Animals

Two-month-old male Fischer F344 rats (Charles River Laboratories, Wilmington, MA, USA) were housed in a facility with a 12/12 light dark cycle. One group of rats was fed *ad lib* Purina rat chow (Harlan Teklad, Madison, WI, USA) containing 1.70 g LiCl per kg for 4 weeks, followed by chow containing 2.55 g LiCl per kg for 2 weeks. This feeding regimen produces plasma and brain lithium concentrations of about 0.7 mmol/L, 'therapeutically relevant' to

bipolar disorder (Chang and Jones 1998; Bosetti et al. 2002a). Control rats were fed lithium-free Purina rat chow for 6 weeks. Water and NaCl solution (0.45 mol/L) were available *ad libitum* to both groups. At the end of the 6 weeks, rats were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*) and subjected to focused high-energy microwave irradiation (5.4 kW, 6 sec, Cober Electronics, Stamford, CT, U.S.A) to stop enzymatic processes. Brains were removed and stored at -80°C until processed for PAP analysis. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23) and was approved by the Animal Care and Use Committee of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development. In addition, mice weighing 28–38 g were divided into two groups: one group was fed food containing lithium chloride 2.2 g/kg food leading to plasma lithium levels of $0.8 \text{ mM} \pm 0.4$ (S.D.) and the second group was fed regular food (without lithium), both for a period of 14 days. The mice were sacrificed by decapitation into liquid nitrogen and the brains were stored at -80°C until processed for PAP phosphatase mRNA and protein levels. The mice experimental procedures were approved by the Ben-Gurion University (Beer Sheva, Israel) Institutional Review Committee for the Use of Animals and carried out in compliance with the declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Determination of PAP levels

Extraction of tissue—Rat brain (0.1 g) was placed in 6% cold TCA solution (2 ml) on ice and sonicated for 50 sec, followed by vortexing for 2 min. Samples were centrifuged at 1000 g for 6 min. Aliquots of 600 μl were neutralized with 55 μl 4 M KOH and 50 μl was injected into an HPLC system. This is a variation of the assay reported by Deutsch et al (Deutsch et al. 2002).

HPLC separation—AMP, ADP, PAP and ATP were separated using an HPLC (Beckman, Fullerton, CA) with a Symmetry C-18, 5- μm column ($250 \times 4.6 \text{ mm}$; Waters-Millipore Corp., Milford, MA, USA), with a gradient system of two phases. UV absorbance was measured at 260 and 280 nm with a Hewlett Packard 1040 detector. Absorbance was monitored in two wavelengths to distinguish the peaks of PAP from those of other molecular species in the brain extract. Peaks that contain the adenosine moiety exhibit a 260/280 ratio of 4:1, respectively.

Elution was carried out with a gradient of 75 mM KH_2PO_4 in 5 mM TBA and acetonitrile. Conditions were set to a 1 ml/min gradient system composed of two solvents: A: 500 ml filtered solution of 75 mM KH_2PO_4 in 5 mM TBA and 3 ml of 4 M KOH, pH ~ 6.6 ; B: acetonitrile. The gradient started with 4% B, increased to 14% B at 10 min for 13 min, then returned to 4% B within one minute and continued for 6 min. AMP, ADP and ATP were identified according to the retention times of standards.

PAP phosphatase mRNA levels

RNA purification and concentration and mRNA levels were determined relative to the house-keeping gene β -actin, using first the Trizol reagent (Sigma, USA) with further purification of the RNA phase by the RNeasy Kit (QIAGene, USA). Purity and concentration of the purified RNA were determined spectrophotometrically (GeneQuant, Pharmacia Biotech, England).

RT reaction was preformed as previously described (Nemanov et al. 1999), using *PAP*-LOW (5'-CTTCAGCTGAAACCCAAAAG-3') and β -actin-LOW (5'-GGGCCGGACTC ATCGTACTC-3') as primers for the RT reactions. For the PCR reactions, the β -actin RT product was diluted 1:1000, and the *PAP* phosphatase - 1:250, dilution ratios of which 1–4 μl are within the linear range of amplification. Thus, 3 μl from each of the obtained first-strand synthesis solutions were further used. The PCR reactions were conducted as previously described (Shamir et al. 2003) and were comprised of *PAP*-UP (5'-CCT

GCCTCCTGGGGAAGTGG-3') and *PAP*-LOW or β -actin-UP (5'-GACGAGG CCCAGAGCAAGAG-3') and β -actin-LOW. Intensity of the bands on 2% agarose gel with ethidium bromide was quantified using densitometry software (AIDA-2D) and expressed as the ratio between PAP phosphatase and β -actin band intensity of each RNA sample. Each sample was measured twice, each time in duplicate.

PAP phosphatase protein levels

Sodium dodecyl sulfate/polyacrylamide gel electrophoretic separation, immunoblotting of PAP phosphatase, and band detection and quantification, were performed as described by us for GSK-3 β (Kozlovsky et al. 2000). Three micrograms total protein of mouse brain lysate, determined to be within the linear range of band densities and within the calibration curve of recombinant PAP phosphatase (kindly supplied by Spiegelberg and York, Duke University, Durham, NC, USA), were separated, blotted, and probed for 16 – 18 hours at 4°C with diluted (1:3000) anti-PAP phosphatase antibodies (primary antibodies - rabbit polyclonal, also kindly provided by Spiegelberg and York (secondary antibodies - anti-rabbit horseradish peroxidase, Amersham, Oakville, Ontario, Canada). To minimize effects of interblot variability, a calibration standard curve of 0.5 – 2 ng recombinant PAP phosphatase was run in each gel and band density was translated into absolute PAP phosphatase protein amount. Bands were visualized using Chemiluminescence Western blot detection kit (Amersham). Densities of the immunoreactive bands were quantified using AIDA-2D image analysis system (Dinco and Rehenium Marketing, Jerusalem, Israel).

Results

PAP levels in rat brain

Commercial adenosine phosphates (ATP, ADP and AMP) were resolved during 20-min gradient elution (Figure 1). ATP, ADP, AMP and other components that contain the adenosine moiety were separated when brain was extracted (Figure 2). The brain extract spiked with traces of PAP (20 nmol/g brain) showed a new peak just between the peaks of ATP and ADP (Figure 3). From the latter profile, we estimated a detection limit of PAP of 2 nmol/g wet wt brain.

Although, like Chen et al. (Chen et al. 2007), we were able to detect ATP and ADP signals, which resulted in 2 μ mol/g ATP and 1 μ mol/g ADP in control rat brain, we could not detect PAP in brain sampled from control or lithium-fed rats. This stems from the absence of a PAP peak in the brain samples at the retention time of PAP compared with the same samples spiked with PAP (Figure 4).

As illustrated in Table I, chronic lithium treatment resulted in a statistically significant decrease in the ADP/ATP ratio (Table I).

PAP phosphatase mRNA and protein levels in mouse brain

There was no difference in brain PAP phosphatase mRNA or protein levels between lithium-treated and control mice (Table II).

Discussion

Lithium has been used as a mood stabilizer for the last 6 decades, but its molecular mechanism of action is yet unresolved. The present study raised the question of the physiological relevance of lithium's inhibition of PAP phosphatase with respect to its therapeutic effect.

We did not detect brain PAP in control or lithium treated rats. Our HPLC methodology provides a detection limit of about 2 nmoles/g wet wt tissue. This detection limit is in the same order of magnitude of rat PAP sulphate (PAPS) brain levels, previously reported to be about 7–10 nmoles/g wet wt tissue measured by an enzymatic assay (Brzezniczka et al. 1987; Kim et al. 1995).

PAPS is degraded via two pathways, one of which, catalyzed by PAPS sulfohydrolase, forms PAP (Klaassen and Boles, 1997). As of today no other pathway is known to produce PAP. It is therefore conceivable to assume that brain PAP levels are in the same order of magnitude as those of PAPS. Hence, a lithium effect of two fold increased PAP levels or higher should have been detected. The demonstration that analysis of brain extract spiked with 20 nmoles/g wet wt tissue PAP resulted in a quantifiable peak (Figure 3) favours this possibility. Inhibition of enzyme activity results in substrate accumulation and reduced product level. Since the product of PAP phosphatase is AMP, a metabolite obtained from multiple pathways, it was reasonable to expect that if lithium's effect on PAP phosphatase were involved in its therapeutic mechanism, the effect would be mediated by PAP accumulation. Taken together, our results suggest that lithium's inhibitory effect on PAP phosphatase, despite its low K_i within the therapeutic range of the drug (Spiegelberg et al. 1999), is not physiologically meaningful in brain. The lack of effect of lithium on mice brain PAP phosphatase mRNA and protein levels supports our conclusion. Yet, it may not be excluded that inhibition of PAP phosphatase resulting in PAP accumulation is involved in lithium's toxicity as suggested by Spiegelberg et al. (Spiegelberg et al. 2005).

As a by-product of our analysis of brain PAP levels, measures of brain ADP and ATP levels were obtained. Unlike its lack of effect on brain PAP level, lithium significantly reduced brain ADP/ATP ratio by about 25%. Our group and others reported that lithium treatment affects glucose metabolism and transport (Basselin et al. 2006, Macko et al. 2008) and a DNA microarray study found that lithium significantly affected expression of genes involved in ATP-binding and ATPase activity (McQuillin et al. 2007). A comprehensive assessment of brain energy requires measurement of multiple metabolic parameters including phosphocreatine/creatinine ratio, ADP/ATP ratio, glycogen content and lactate/pyruvate ratio (Folbergrova et al. 1981). However, the result of reduced brain ADP/ATP ratio is consistent with reports that lithium treatment enhances energy metabolism, in general, and glucose metabolism in particular (Basselin et al. 2006, McQuillin et al. 2007, Macko et al. 2008). A reduced ADP/ATP ratio is in line with lithium's suggested neuroprotective effects (Rowe & Chuang 2004). In yeast deletion of PAP phosphatase results in PAP accumulation leading to growth inhibition (Spiegelberg et al. 2005). The result of reduced brain ADP/ATP ratio following lithium treatment may, therefore, indirectly further support our conclusion that brain PAP levels are not affected by lithium treatment.

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Figure 2.
HPLC pattern of rat brain extract

Figure 3.
HPLC pattern of rat brain extract spiked with PAP

Figure 4.
HPLC pattern of lithium-treated rat brain extract

Table I

AMP/ATP and ADP/ATP ratios in brain of lithium-treated and control rats

Sample	ADP/ATP		AMP/ATP	
	Lithium	Control	Lithium	Control
1	0.503	0.760	0.133	0.265
2	0.377	0.520	0.161	0.149
3	0.344	0.547	0.081	0.107
4	0.506	0.676	0.153	0.531
5	0.605	0.547	0.215	0.127
Means	0.467*	0.610	0.148	0.235

* Student's t-test: $p=0.03$

The results were calculated from the HPLC recorded peaks and are the ratios of areas under the curves. Absolute ATP and ADP levels in control rat brain were 2 and 1 $\mu\text{mol/g}$, respectively.

Table II

PAP phosphatase levels in brain of chronic lithium-treated vs. control mice. Results are means $\pm$ S.D.

	Control	Lithium
Brain PAP phosphatase mRNA level (A.U.)	0.34 $\pm$ 0.25 n=14	0.30 $\pm$ 0.19 n=23
Brain PAP phosphatase protein level (ng/mg protein)	0.34 $\pm$ 0.18 n=16	0.31 $\pm$ 0.26 n=17

A.U. = Arbitrary Units (relatively to the house keeping gene β -actin)