Reduced CREB phosphorylation after chronic lithium treatment is associated with down-regulation of CaM kinase IV in rat hippocampus

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Abstract

Lithium is widely used in the treatment of bipolar disorder, although its mechanism of action is not fully clear. This study was undertaken to assess the effects of prolonged lithium administration on cAMP responsive element-binding protein (CREB) phosphorylation and CaM kinase IV (CaMKIV), one of the main kinases phosphorylating CREB in neurons following synaptic activation. CREB total protein expression and phosphorylation (Ser133), as well as CaMKIV enzymatic activity, phosphorylation of Thr196 (the activator residue) and kinase expression level were assessed in total homogenates and nuclei from the hippocampus and prefrontal/frontal cortex following 5 wk lithium treatment. Whereas no significant effects were found in prefrontal/frontal cortex, lithium administration reduced CREB phosphorylation and at the same time down-regulated CaMKIV (enzymatic activity, phospho-Thr196 and protein expression level) in cell nuclei from the hippocampus. These data suggest for the first time the involvement of CaMKIV in the mechanism of action of lithium.

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Introduction

After almost 50 years since its discovery as an anti-manic agent, lithium is still one of the mainstays for treatment of bipolar disorder, both for the acute manic phase and for prophylaxis of recurrent manic and depressive episodes (Manji et al., 2001; Quiroz et al., 2004). However, despite its proven efficacy, the specific mechanism(s) by which lithium exerts its therapeutic actions still remains poorly understood. As the latency of clinical action of lithium suggested plastic changes mediated by intracellular signalling processes, a great effort was made to investigate its effects on transcriptional mechanisms as well as on several components of the intracellular signalling pathways. It was reported that lithium induces changes in expression of different genes, including neuromodulators, receptors, enzymes, and other signalling molecules, probably regulating the levels or activity of specific transcription factors (Gould et al., 2004; Manji et al., 2000). Among these, the best characterized and studied in relation to neuroplasticity, cellular resilience, cognition and mood disorders, is the cAMP responsive element-binding protein (CREB) (Carlezon et al., 2005; Tardito et al., 2006). Several studies showed that phosphorylation and transcriptional activity of CREB are modulated by lithium (Chen et al., 1999; Einat et al., 2003; Kim et al., 2004; Ozaki and Chuang, 1997; Wang et al., 1999). However, while some preclinical studies reported an up-regulation of phosphor-CREB (pCREB) and its DNA-binding activity after lithium treatment (Einat et al., 2003; Kim et al., 2004; Ozaki and Chuang, 1997) others showed a down-regulation of pCREB after lithium treatment (Chen et al., 1999; Wang et al., 1999). Moreover, a decrease in the phosphorylated form of CREB was observed in post-mortem brain tissue of
affective patients treated with lithium (Stewart et al., 2001; Young et al., 2004). CREB function is activated by a vast array of physiological stimuli, conveyed through a number of signalling pathways acting in concert (Lonze and Ginty, 2002; Tardito et al., 2006; West et al., 2002). Although the cAMP–protein kinase A (PKA) cascade was originally suggested to play a central role in the action of antidepressants/mood stabilizers on CREB transcriptional activity, a growing body of data showed recently that other major pathways, such as CaM kinase IV (CaMKIV) and MAP kinase cascades, have a primary role in CREB phosphorylation and in the regulation of activity-dependent neuronal gene expression (Bito et al., 1996; Ghosh et al., 1994; Kasahara et al., 2001). Indeed, it was recently reported that CaMKIV is the main kinase phosphorylating CREB in neurons following synaptic activation (Lonze and Ginty, 2002; West et al., 2002) and we have recently found that chronic antidepressant treatments significantly activate CaMKIV, but not PKA (Tiraboschi et al., 2004). Thus, lithium effects on CREB are controversial, and although a few studies have investigated the role of the MAP kinase cascades in lithium effects (Eina et al., 2003; Pardo et al., 2003), to the best of our knowledge no data are available on the possible involvement of CaMKIV in the lithium mechanism of action. Therefore, the aim of this study, developing and extending our previous research on antidepressants, was to investigate the effects of chronic lithium treatment on the signalling mechanisms regulating phosphorylation and transcriptional activity of CREB, by assessing CaMKIV activation and expression in the hippocampus and prefrontal/frontal cortex of treated rats.

Methods

Animal treatment and preparation of homogenate and nuclear fraction


Male Sprague–Dawley rats (mean initial weight 150 g) were housed two per cage with free access to food and water. Twenty controls and 20 treated animals were given a regular diet or pellets containing 0.24 % (w/w) lithium carbonate for 5 wk as described elsewhere (Mori et al., 1998). Hypertonic saline was available for preventing dehydration. Serum lithium levels were determined by atomic absorption spectrometry (mean lithium levels ± s.d. were 0.82 ± 0.14 mequiv/l). An additional group of eight controls and eight treated rats receiving regular diet or a lithium carbonate-supplemented diet (0.24% w/w) for 6 d was used for the acute study. Rats that received chronic lithium treatment gained weight at a slower rate than controls, as previously observed (Mori et al., 1998).

Animals were sacrificed after 5 wk of treatment; hippocampus (HC) and the whole frontal lobe, referred to as the prefrontal/frontal cortex (P/FC) (see Tiraboschi et al., 2004), were quickly excised on ice and homogenized 1:10 (w/v) by a loose-fitting Potter in homogenization buffer, 0.28 M sucrose, 10 mM Hepes (pH 7.4), 0.1 mM EDTA, 5 mM Na3PO4, 1 mM Na2VO4, and 5 μl/ml protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Total homogenates were centrifuged for 5 min at 1000 g, and the resulting pellets, enriched in nuclei, were resuspended in lysis buffer, 120 mM NaCl, 20 mM Hepes (pH 7.4), 0.1 mM EGTA, 0.1 mM DTT, protease and phosphatase inhibitors as in the homogenization buffer.

CaMKIV immunoprecipitation

CaMKIV was immunoprecipitated from 150 μg of proteins from total homogenate or nuclear fraction using a polyclonal antibody, as previously described (Kasahara et al., 1999; Tiraboschi et al., 2004). Samples were incubated with antibody in 50 mM Tris (pH 7.5), 500 mM NaCl, 10 mM EDTA, 4 mM EGTA, 1 mM Na3VO4, 20 mM Na3P2O7, 5 mM NaF, 1 mM DTT, 0.5% Triton X-100, 100 μM calyculin A, protease inhibitor cocktail, protein A-sepharose (Sigma-Aldrich) for 4 h at 4 °C. The beads were washed with 50 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, 1 mM Na3VO4, 20 mM Na3P2O7, 5 mM NaF, 100 μM calyculin A, and centrifuged. Immunoprecipitates were used for assay of CaMKIV activity.

Assay of CaMKIV activity

Enzymatic activity of immunoprecipitated CaMKIV was measured by assaying phosphate incorporation in the selective substrate peptide-γ (Primm, Milan, Italy) (Kasahara et al., 1999). CaMKIV enzymatic activity was expressed as cpm/μg.min of [γ-32P] phosphate incorporated in peptide. The reactions were carried out in standard phosphorylation buffer (50 mM Hepes, 10 mM Mg acetate, 100 mM calyculin A, 1 mM Na3VO4, 100 mM peptide-γ, 0.5 mM CaCl2, 0.3 mM calmodulin (CaM) (Biomol, Plymouth Meeting, PA, USA), 100 mM [γ-32P]ATP (200–500 cpn/pmol, Amersham Biosciences, Milan, Italy) for 10 min at 30 °C and stopped by ice-cold TCA (final concentration 5%). After centrifugation, 25 μl supernatant was
spotted on phosphocellulose P81 paper (Whatman, Maidstone, Kent, UK). Filters were washed in 75 mM phosphoric acid, dried and counted for liquid scintillation. Blanks were incubated in the absence of peptide.

Western analysis

Western analysis was carried out as previously described (Tiraboschi et al., 2004), by incubating polyvinylidene difluoride membranes, containing electrophoresed proteins (10 μg per lane) from either total homogenates or nuclear enriched fractions, with monoclonal antibodies for α-CaMKIV (Transduction Laboratories, Lexington, KY, USA) 1:1000, CREB and phospho-Ser133 CREB 1:1000, β-actin 1:5000 (Sigma-Aldrich), polyclonal antibody for phospho-Thr196 α-CaMKIV 1:500 (Kasahara et al., 1999). Following incubation with peroxidase-coupled secondary antibodies, protein bands were detected by using ECL (Amersham Biosciences, Piscataway, NJ, USA). Standard curves were obtained by loading increasing amounts of samples on gels. All protein bands used were within the linear range of standard curves, and normalized for actin level in the same membrane. Standardization and quantitation was as previously reported (Tiraboschi et al., 2004).

Statistical analysis

Data were analysed with two-tailed Student’s t test, using \( p < 0.05 \) as the significance level. Data are expressed as mean ± S.E.M. (percent of control).

Results

Prolonged lithium treatment reduces phosphorylation of nuclear CREB in the hippocampus

In order to assess the effect of prolonged lithium treatment on CREB, its total protein expression levels and Ser133 phosphorylation were measured by Western analysis in nuclear enriched fractions from the HC and P/FC of treated rats. No changes in any of the mechanisms and effectors reported below were observed following acute treatment with lithium (not shown).

Upon neuronal activation, DNA-bound CREB is phosphorylated on Ser133 (West et al., 2001, 2002). As shown in Figure 1a, the 1000 g nuclear pellet we prepared is greatly enriched with the phosphorylated form of CREB (measured by using an antibody directed against pCREB), normally detected only in

![Diagram](http://ijnp.oxfordjournals.org/)

Figure 1. Chronic lithium treatment reduces CREB phosphorylation in the hippocampus (HC) of treated animals. (a) Western blot of actin and pCREB in total homogenate (H) and nuclear fraction (N) from HC and prefrontal/frontal cortex (P/FC) of control rats. Ten micrograms total protein per lane were loaded. Actin level was similar and slightly lower in nuclear fraction, whereas pCREB was seemingly concentrated in nuclei. Immunoblot representative of three experiments. Similar results were obtained from lithium-treated rats (not shown). (b) Total CREB protein levels were unaffected by prolonged lithium treatment in nuclear fractions from both the HC and P/FC (\( n = 3–5 \) experiments in triplicate). (c) Chronic lithium treatment reduces CREB phosphorylation in the HC, but not the P/FC of treated rats (\( n = 3–5 \) experiments in triplicate, \( * p < 0.01 \)). □, Controls; ■, lithium-treated rats.

the nuclear fraction. No significant changes were found in CREB total protein level after prolonged treatment with lithium, in both the HC and P/FC (Figure 1b). As illustrated in Figure 1c, a significant reduction (–26%, \( p < 0.01 \)) in CREB Ser133 phosphorylation was detected in nuclei from HC of lithium-treated animals (\( p < 0.01 \)). No significant effect was observed in pCREB immunostaining in the same fraction from the P/FC (Figure 1c).
Chronic lithium treatment modulates nuclear CaMKIV enzymatic activity and expression level

In light of the growing number of independent studies showing that CaMKIV is the major calcium/CaM-dependent kinase regulating the activity-dependent CREB phosphorylation in neurons (Bito et al., 1996; Ghosh et al., 1994; Kasahara et al., 1999; West et al., 2002), we assessed the effect of prolonged lithium administration on enzymatic activity, total protein level and phosphorylation state of CaMKIV. The kinase was immunoprecipitated from both total homogenate and nuclear fraction as previously described (Tiraboschi et al., 2004), and its enzymatic activity measured by an assay using selective peptide substrate (Kasahara et al., 2001).

Lithium did not significantly modify CaMKIV activity in total homogenate from both the HC and P/FC (data not shown). Lithium administration also had no effect on CaMKIV enzymatic activity in nuclei obtained from the P/FC of treated animals (Figure 2a). Conversely, a significant reduction (~30%, p < 0.01) of CaMKIV activity was observed in nuclei from the HC of lithium-treated animals with respect to control animals (Figure 2a).

In order to verify whether the observed modifications could be ascribed to changes in the phosphorylation of Thr196 by CaMK kinase (a mechanism responsible for activation of this enzyme) (West et al., 2002), and/or in the protein levels of the kinase itself these parameters were assessed in nuclei from control and lithium-treated animals. As shown in Figure 2b, we found a significant reduction in the phosphorylation state of CaMKIV on Thr196 in the HC, but not the P/FC. This finding was accompanied by a significant decrease of CaMKIV total protein level in nuclei from the HC (p < 0.01), whereas no changes were observed in the P/FC (Figure 2c).

These results suggest that the decreased CaMKIV enzymatic activity found in the HC of lithium-treated rats could be due to a reduced phosphorylation of the Thr196 residue of the enzyme itself, which, in turn, depends on a lower expression level of CaMKIV in lithium-treated animals.

Discussion

The results of this work may be summarized as follows:

1. Chronic lithium treatment significantly reduced nuclear CREB phosphorylation, without altering its total protein expression level, in the HC but not the P/FC.

2. CaMKIV enzymatic activity was reduced in nuclei from the HC after prolonged lithium administration. This decrease was accounted for by a...
significant reduction in the total protein expression level of the enzyme and in the phosphorylation state of CaMKIV Thr196, a mechanism responsible for activation of this enzyme.

Overall, our study, in line with previous reports of reduced CREB phosphorylation after chronic lithium treatment in both animals and cultured human neuroblastoma cells (Chen et al., 1999; Wang et al., 1999), adds a new element to the complex puzzle of lithium therapeutic effects in the treatment of bipolar disorder. Indeed, our results, showing for the first time a down-regulation of CaMKIV, suggest the involvement of this kinase in the down-regulation of pCREB after chronic lithium treatment. This hypothesis is consistent with the well-known role of CaMKIV as the major CREB kinase in activity-dependent neuronal gene expression (Lonze and Ginty, 2002; West et al., 2002). Indeed, CaMKIV has a marked nuclear localization, its kinetics of activation correlates with that of CREB phosphorylation, and blockade of its function was shown to inhibit CREB phosphorylation induced by depolarization (Bito et al., 1996; Ghosh et al., 1994; Tardito et al., 2006). Furthermore, in-vitro studies showed that CREB phosphorylation induced by long-term potentiation (LTP, a paradigm of synaptic plasticity) or electrical stimulation of neurons was mainly blocked by CaMK inhibitors and not by inhibition of other kinases (Bito et al., 1996; Kasahara et al., 2001). Therefore, CaMKIV appears to play a primary role in neuronal activity-dependent phosphorylation of CREB (Lonze and Ginty, 2002; West et al., 2002).

Our data showed that whereas lithium effects on CaMKIV enzymatic activity are mostly due to a decrease in the total protein expression level of the kinase, together with a reduction of its phosphorylation state in the activatory residue of Thr196, the effects on CREB only involve its phosphorylation on Ser133 with no change in total protein expression level. These findings suggest that the reduced CREB phosphorylation observed after chronic lithium could be due to the inhibition of one of the kinases responsible for its modulation. As previously discussed (see Introduction), neuronal activity stimulates CREB phosphorylation and activation via at least two major signalling pathways: a CaMKIV-regulated pathway and a Ras-ERK-dependent pathway (West et al., 2001). It was reported that 28 d lithium treatment increased CREB and Erk1/2 phosphorylation in the HC of treated rats (Einat et al., 2003). Our present data show that 5 wk lithium treatment reduced both CREB phosphorylation and CaMKIV activity in nuclei from the HC. The apparent discrepancy between these findings may arise from different lithium-treatment modalities, which are typically time- and concentration-dependent, and/or from the different cellular fraction analysed (i.e. total homogenate vs. cell nuclei). Indeed, we found no effect of lithium on CaMKIV in total hippocampal homogenate. However, one intriguing possibility is that, according to the hypothesis of Jope (1999), lithium has a bimodal model of action, resulting in balancing positive and negative regulators of signalling pathways involved in CREB phosphorylation. Based on this framework, it is plausible that CREB phosphorylation is differently modulated by lithium treatment according to the activation state of the signalling pathways. In this regard, we have recently found in human neuroblastoma SH-SY5Y cells that lithium treatment induced sustained Erk1/2 activation, and reduction of CaMKIV activation both in basal condition and after depolarization (Tardito et al., 2005).

In conclusion, our present data showed that chronic lithium administration reduces CREB phosphorylation in cell nuclei from the HC of treated rats. This effect is associated with a down-regulation of CaMKIV in the same cerebral area. These data add to the growing body of evidence suggesting the involvement of CaMKIV as a new target in the mechanism of action of psychotropic medications (Tardito et al., 2006).

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Statement of Interest
None.

References


