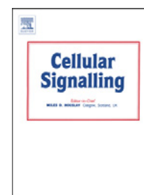
Cellular Signalling 25 (2013) 113–125

Contents lists available at SciVerse ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig



Lithium enhances CRTC oligomer formation and the interaction between the CREB coactivators CRTC and CBP — Implications for CREB-dependent gene transcription

Annette Heinrich [a,1], Anne Sophie von der Heyde [a], Ulrike Böer [a,2], Do Thanh Phu [a], Mladen Tzvetkov [b], Elke Oetjen [a,*]

[a] Department of Pharmacology, Georg-August-University Goettingen, Robert-Koch-Str. 40, 37075 Goettingen, Germany
[b] Department of Clinical Pharmacology, Georg-August-University Goettingen, Robert-Koch-Str. 40, 37075 Goettingen, Germany

ARTICLE INFO

Article history:
Received 13 August 2012
Received in revised form 11 September 2012
Accepted 13 September 2012
Available online 18 September 2012

Keywords:
Lithium
CREB
CRTC
Protein–protein interaction
cAMP
Transcriptional regulation

ABSTRACT

Lithium salts are important drugs to treat bipolar disorder. Previous work showed that lithium by enforcing the interaction between the transcription factor CREB and its coactivator CRTC1 enhanced cAMP-stimulated CREB-dependent gene transcription. Both CREB and CRTC have been implicated in neuronal adaptation, which might underlie lithium's therapeutic action. In the present study the mechanisms of lithium action on cAMP-induced CREB-dependent gene transcription were further elucidated. Transient transfection assays revealed that all three CRTC isoforms conferred lithium responsiveness to CREB whereas their intrinsic transcriptional activities remained unchanged by lithium, suggesting a conformational change of CREB or CRTC by lithium. In *in vitro* protein–protein interaction assays lithium enhanced the interaction between CREB and both coactivators CRTC and CBP. Furthermore, lithium enforced the oligomerization of CRTC, a prerequisite for CREB interaction. For further evaluation it was investigated whether lithium competes with magnesium, which coordinates the conformation of the CREB basic region leucine zipper (bZip). Mutational analysis of the magnesium coordinating lysine-290 within the bZip, *in vitro* and intracellular interaction assays and luciferase reporter-gene assays revealed that the effect of lithium on the CREB–CRTC interaction or on the transcriptional activity, respectively, was not affected by the mutation, thus excluding a magnesium–lithium competition. However, the CREB–CRTC interaction was strongly increased in lysine-290-mutants thereby extending the CRTC–CREB interaction domain. Taken together the results exclude a competition between lithium and magnesium at the bZip, but suggest that lithium by enforcing the CRTC-oligomer formation and the interaction of CREB–CBP–CRTC enhances cAMP-induced CREB-dependent gene transcription.

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1. Introduction

Lithium salts are clinically important drugs for the treatment of manic diseases and are still first choice for mood stabilization in the therapy of bipolar mood disorders. Although some biochemical targets of lithium are known including glycogen synthase kinase-3 (GSK3), adenylate cyclase, and inositol monophosphatase, the exact molecular mechanism of the clinical action of lithium is not completely understood. After initiation of treatment, the onset of mood stabilization by lithium is typically delayed for weeks or months, suggesting that lithium might induce neuronal adaptation defined by a new pattern of gene expression [1,2]. The cAMP-response element binding protein CREB has been demonstrated to play a crucial role in neuronal adaptation [3]. In addition, genetic variations in CREB may be associated with bipolar disorder and lithium response [4]. After homodimerization via its leucine zipper domain, CREB binds via its basic region to its DNA binding site, the cAMP responsive element (CRE), present in many genes. Thus, this ubiquitously expressed transcription factor mediates many and diverse functions from blood glucose control to brain adaptive responses including synaptic

Abbreviations: BDNF, brain derived neurotrophic factor; bZip, basic region-leucine zipper; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; CREB, cAMP-response element binding protein; CRTC, cAMP-regulated transcriptional coactivator; DBD, DNA-binding domain; EMSA, electrophoretic mobility shift assay; GSK3, glycogen synthase kinase 3; GST, glutathione S-transferase; HGF, hepatocyte growth factor; HIT-T15, hamster insulinoma tumor cells; KID, kinase-inducible domain; KIX, KID-interaction domain; PKA, protein kinase A; qPCR, quantitative real-time PCR; SCE, *somatostatin* CRE; TAD, transactivation domain; TORC, transducer of regulated CREB.

* Corresponding author at: Department of Clinical Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. Tel.: +49 40741057252; fax: +49 40741055761.
E-mail addresses: annette.heinrich@uniklinik-freiburg.de (A. Heinrich), avdheyde@gmx.net (A.S. der Heyde), boeer.ulrike@mh-hannover.de (U. Böer), ntkdophu@yahoo.com (D.T. Phu), mtzvetk@gwdg.de (M. Tzvetkov), e.oetjen@uke.uni-hamburg.de (E. Oetjen).
[1] Present address: Department of Psychiatry, Section of Molecular Psychiatry, University Freiburg, Hauptstr. 5, 79104 Freiburg, Germany.
[2] Present address: GMP Model Laboratory for Tissue Engineering, Feodor-Lynen-Str. 31, 30625 Hannover, Germany.

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http://dx.doi.org/10.1016/j.cellsig.2012.09.016

plasticity, late-phase long-term potentiation, learning, and memory [5–8]. Phosphorylation of CREB at serine 119 (S119; in CREB-327, corresponding to serine-133 in CREB-341) induced by cAMP-dependent signaling and many other stimuli is a prerequisite for CREB transcriptional activity. Phosphorylation at this site results in the recruitment of the CREB binding protein CBP/p300 via the coactivators KIX domain [9,10]. Besides acting as a histone acetyltransferase CBP/p300 has its own C-terminal transactivation domain whose activity is stimulated by cAMP [11–13]. A second, equally important coactivator, the cAMP-regulated transcriptional coactivator (CRTC) also known as transducer of regulated CREB (TORC), has been recognized almost one decade ago [14–16]. Three different isoforms were identified in human and mouse. They are expressed at low levels in most tissues but expression pattern differs between isoforms [15,17]. CRTC transcriptional activity is dynamically regulated: under basal conditions phosphorylated CRTC is sequestered in the cytoplasm bound to 14-3-3 phospho-protein binding-proteins [18]. Increased levels of cAMP with an activation of the protein kinase A prevent the phosphorylation of CRTC and induce its nuclear translocation [14,16,18,19]. Once in the nucleus a CRTC tetramer interacts via its N-terminal amino acids with the coiled coil structure of the dimerized leucine zipper of CREB [20]. The association between the CREB leucine zipper and CRTC N-terminus may not require DNA binding and appears to be driven by electrostatic interactions [14] as a charged residue within the leucine zipper, arginine 300 (R300; R314 in CREB-341), was found to be involved [18]. Our previous studies showed that lithium enhances cAMP-stimulated CREB-dependent gene transcription [21]. Lithium was shown to increase the cAMP-induced CREB-mediated gene transcription by enhancing the binding of CRTC1 to CREB both *in vitro* and in cultured cells. This increase may be based on the action of lithium as a cation to boost directly the association of CRTC1 with CREB, once cAMP has shifted CRTC1 from the cytosol into the nucleus [21,22]. Thus, the CREB–CRTC1 complex constitutes a novel lithium target which might contribute to the drug's mood stabilizing effect [23]. The aim of the present study was to further elucidate the molecular mechanisms on how lithium enforces cAMP-stimulated CREB-dependent gene transcription.

2. Materials and methods

2.1. Plasmids

The GAL4-CREB-K290 mutants were generated by primerless PCR using the following primers (mutation underlined): for K290E 5′-GTCGTAGAAAGGAGAAAGAATATGTG-3′ (forward) and 5′-CACATATTCTTTCTCCTTTCTACGAC-3′ (reverse), for K290A 5′-GTCGTAGAAAGGCGAAAGAATATGTG-3′ (forward) and 5′-CACATATTCTTTCGCCTTTCTACGAC-3′, flanking primers and GAL4-CREB327 [21] as a template. For PCR cloning of GAL4-bZip-K290E and GAL4-bZip-K290A encoding the mutated bZip (CREB269–327), GAL4-CREB-K290E and GAL4-CREB-K290A, respectively, served as template. The products were subcloned into the GAL4 domain-coding vector pSG424 [24]. The expression vector for GST-CREB has been described before [22]. Correspondingly, the coding sequence for CREB-S119A containing the serine residue at position 119 mutated to alanine was subcloned into pGEX2T (GE Healthcare). The coding sequence of the CREB-TAD comprising amino acids 23–268 was subcloned into pGEX2T. GST-CREB-K290E and GST-CREB-K290A were prepared by PCR cloning using GAL4-CREB-K290E and GAL4-CREB-K290A as template. Following PCR the fragments were subcloned into pGEX2T (GE Healthcare). The reporter gene G5E1B-Luc [21,22] contains the luciferase gene under control of 5 repeats of the responsive element for the yeast transcription factor GAL4. The expression vector for human CRTC1 under control of a T7 promoter in pcDNA3 and the expression vector encoding N-terminally FLAG-tagged human CRTC1 have been described before [21,22]. For PCR cloning of $CRTC1_{\Delta 44}$ human CRTC1 served as template. The coding sequence of human CRTC2 and human CRTC3 was kindly provided by Marc Labow (Novartis Pharmaceuticals, Suffern, NY, USA) and subcloned into pcDNA3. Expression vector for GST-$CRTC1_{1-44}$ was generated by PCR cloning. The coding sequence of the N-terminal 44 amino acids of CRTC1 was subcloned into pGEX2T. The expression vector for His-CRTC1 was generated by cloning the PCR fragment of full length CRTC1 into the XbaI and XhoI sites of pET-28(b)+ (Novagen). For *in vitro* transcription/translation, the coding sequence of amino acids 553–679 comprising the KIX-domain of CBP was subcloned into pcDNA3 under control of the T7 promoter using *Hind*III and *Xho*I restriction sites. For expression as GST-fusion protein the coding sequence of the KIX domain was subcloned into pGEX6P1 (GE Healthcare). The correct sequence of all constructs was verified by sequencing.

S119 in the splice variant $CREB_{327}$ corresponds to serine 133 in human $CREB_{341}$. In this work the S119 nomenclature was used.

2.2. Cell culture, transfection, and treatments

Hamster insulinoma tumor cells (HIT-T15) [11] were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 5% horse serum, 100 units/ml penicillin and 100 μg/ml streptomycin. For ChIP-assays, HIT-T15 cells were transiently transfected with 4 μg of expression vector per 6-cm dish using Metafectene (Biontex) according to the manufacturer's instructions. For luciferase reporter-gene assays, the DEAE-dextran method [11] was used to transfect the cells with 2 μg of expression vector and 1 μg cytomegalovirus green fluorescent protein expression vector per 6-cm dish to control for transfection efficiency. Co-transfections were carried out with a constant amount of DNA, which was maintained by adding the empty pBluescript vector (Stratagene).

Treatment of cells for luciferase reporter-gene assay was with 1 mM or 2 mM 8-bromo-cAMP for 6 h and with 20 mM LiCl for 7 h prior to harvest. Luciferase activity was determined 48 h after transfection as previously described [11].

2.3. Chromatin immunoprecipitation (ChIP) assay and quantitative real-time PCR

Chromatin immunoprecipitation (ChIP) was performed as described before [22]. Briefly, HIT-T15 cells were transfected and treated as indicated. Proteins and DNA were crosslinked by use of 1% (v/v) formaldehyde. The cells were harvested, and protein–DNA complexes were precipitated. Immunoclearing, immunoprecipitation, and purification of DNA were performed as described [25].

Quantitative real-time PCR (qPCR) was performed using a TaqMan™ probe specific for the 5 repeats of the GAL4 binding site to evaluate the amount of G5E1B-Luc precipitated in ChIP assays. The following primers were used: 5′-GCAATAGCATCACAAATTTCACAAA-3′ (forward primer) and 5′-AATGCCAAGCTGGAATTCGA-3′ (reverse primer) combined with the TaqMan™ probe 5′-TAGAGGGTATATAATGATCCCCGGGTTACCGAG-3′ (modification: 5′-Fluorescein; 3′-TAMRA). For the qPCR, the reaction mix from Eurogentec, containing the AmpliTaq® DNA polymerase (Applied Biosystems), was used and prepared according to the manufacturer's instruction. Each sample out of 4 independent experiments was measured in triplicate on a 384 well plate in each 18 μl volume using the ABI Prism 7900HT Sequence Detection System and was analyzed with the SDS 2.1 Software (Applied Biosystems).

2.4. Expression and purification of GST-fusion proteins and His-tagged proteins from bacteria

Competent cells from *Escherichia coli* strain DH5α were transformed by heat shock with expression vectors coding for GST-CREB comprising amino acids 23 to 327 in wild type or mutants GST-CREB-K290E, GST-CREB-K290A, GST-CREB-S119A, or the truncated GST-CREB-TAD (from aa 23 to 268); GST-KIX, containing amino acids from 553 to 679

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