Activation and inactivation of cAMP-response element-mediated gene transcription in cardiac myocytes

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Abstract

Objective: Chronic β-adrenergic stimulation of the cAMP-dependent signalling pathway is implicated in functionally relevant expression changes in congestive heart failure. We studied activation and inactivation of the cardiac gene transcription mediated by the cAMP-response element (CRE) and the CRE-binding protein (CREB) as an important mechanism of a cAMP-dependent gene regulation.

Methods: We investigated the transcriptional activation by forskolin, an activator of the adenylyl cyclase, in chick embryonic cardiomyocytes transfected with a CRE-controlled luciferase construct in comparison to the phosphorylation and expression of CREB determined on immunoblots.

Results: Forskolin (10 μmol/l; 8 h) increased CRE-mediated transcription and phosphorylation of CREB 13- and 1.5-fold, respectively. The phosphorylation was further elevated in combination with cantharidin, an inhibitor of type 1+2A protein phosphatases. The transcriptional response to forskolin was desensitized by pretreatment with forskolin (1 μmol/l; 24 h) while CREB phosphorylation was increased. In forskolin-pretreated cells, total CREB protein levels were decreased. Cantharidin did not restore the attenuated transcriptional response.

Conclusions: In cardiomyocytes, there is an activation of the CRE-mediated gene transcription by forskolin that is attenuated after prolonged stimulation, and this attenuation is not dependent from a dephosphorylation of CREB. We suggest that attenuation of the CRE-mediated transcription through chronic stimulation of the cAMP-pathway, e.g. by elevated catecholamines, contributes to the altered expressional regulation in congestive heart failure.

Keywords: Adrenergic (ant)agonists; Gene expression; Heart failure; Myocytes; Protein phosphorylation

1. Introduction

Congestive heart failure (CHF) is the final stage of various cardiac diseases, e.g. ischemic or idiopathic dilated cardiomyopathy, and is a major cause of cardiovascular morbidity and mortality in Western countries [1]. The molecular mechanisms underlying the pathophysiology of CHF are not understood in detail, however, an increased β-adrenergic stimulation due to elevated catecholamines is thought to play a role in the pathophysiology of the disease [2,3]. Consistent with this, long-term treatment with β-blockers improves prognosis and cardiac performance in patients with CHF [4,5] whereas therapy with β-adrenoceptor agonists is associated with increased mortality [6]. Moreover, chronic infusion of the β-adrenoceptor agonist isoproterenol in rats led to similar changes in cardiac function and gene expression as observed in CHF [7,8]. Therefore, chronic stimulation of the cAMP-dependent signalling pathway might be involved in the induction of deleterious expressional changes in CHF.

The transcriptional regulation mediated by the cAMP response element binding protein (CREB) and the cAMP-response element (CRE) is an important mechanism of gene regulation in response to stimulation of the cAMP-dependent signalling pathway (for review see Refs. [9,10]). CREB, a 43-kDa basic leucine zipper (bZip) transcription factor, binds to the CRE, an 8-bp palindromic consensus element (TGACGTCA) present in various gene
promoters. There are several alternatively spliced isoforms of CREB of which CREB341 and CREB327 (lacking 14 amino acids) are expressed in most tissues. CREB binds to the CRE either as a homodimer or as a heterodimer in combination with other members of the CREB/ATF and AP-1 families of transcription factors [11,12]. The phosphorylation of a critical serine in the kinase inducible domain of CREB by the cAMP-dependent protein kinase or the calcium-calmodulin-dependent protein kinase leads to a transcriptional activation through an interaction of CREB with the CRE-binding protein [13]; although there are conflicting results the binding of CREB to the DNA seems not to be affected by the phosphorylation [14]. Other phosphoacceptor sites within the kinase-inducible domain and a possible role of other kinases were reported [9,10]. However, phosphorylation at serine133 in CREB341 — which is serine119 in CREB327 — is both sufficient and necessary for transactivation [15].

Several studies suggested CREB as an important regulator of gene expression in cardiomyocytes with possible relevance for the pathophysiology of CHF. CREB is expressed in chick embryonic cardiomyocytes [16] and in rat and human cardiac ventricular tissue [17,18]. Although the role of a CRE-mediated transcriptional regulation has not been confirmed for all examples, CREs were found in the promoters of various regulatory genes which are differentially expressed in CHF or which are involved in cardiac gene regulation, e.g. β1- and β2-adrenoceptors [19,20], phospholamban [21,22], the protein phosphatase type 2A [23], the potassium channel Kv1.5 [24], the protooncogene c-fos [25] or the transforming growth factor β3 [26]. Transgenic mice with heart-specific overexpression of a non-phosphorylatable dominant-negative CREB-mutant (serine133 replaced by alanine) developed four chamber-dilatation and severe heart failure within a few weeks after birth [27]. These results suggest that an inhibition of the CRE-mediated gene transcription is associated with the phenotype of CHF and lead to the hypothesis that this mechanism contributes to the altered gene regulation in human heart failure. However, these results were obtained with a protein that is not naturally expressed and little is known about a transcriptional activation mediated by the CRE and the cAMP-dependent signalling pathway in cardiomyocytes and conditions leading to alterations of this regulation.

Here, we studied the effects of an activator of the adenyl cyclase, forskolin, on the CRE-mediated transcriptional regulation in chick embryonic cardiomyocytes. Forskolin activated CRE-mediated transcription and increased phosphorylation of CREB and the transcriptional response was attenuated by prolonged stimulation with forskolin for 24 h. This attenuation did not require a dephosphorylation of CREB at serine119/133 and was also observed after prestimulation with the β-adrenoceptor agonist isoproterenol in combination with isobutylmethylxanthine (IBMX), an inhibitor of phosphodiesterases. We hypothesize that a desensitization of the transcriptional activation mediated by the cAMP-response element contributes to the regulation of expression changes in the failing human heart.

2. Methods

2.1. Isolation and culture of cardiomyocytes

Primary chick embryonic cardiomyocytes were prepared and kept at a density of 200,000 cells per cm² according to published protocols [16,28] and to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). There were >90% beating cardiomyocytes visible 24 h after plating. Cells were stimulated as indicated with forskolin (Sigma) and cantharidin (Sigma), an inhibitor of type 1+2A protein phosphatases [29]. Forskolin was dissolved in dimethylsulfoxide (DMSO; Sigma), and the final concentration of DMSO was 0.1% with 10 μmol/l forskolin. In another set of experiments cells were pretreated with the β-adrenoceptor agonist (-)-isoproterenol (+)-bitartrate salt (Sigma) and 3-isobutyl-1-methylxanthine (IBMX, Sigma), an inhibitor of phosphodiesterases. Here, the final concentration of DMSO was 1%.

2.2. Transient transfection and luciferase assays

Transfection of cardiomyocytes was performed using 3 μl Superfect-reagent per μg DNA (Qiagen, Hilden, Germany). Cells were transfected with 0.4 μg per 400,000 cells of 4xSomCRE, a firefly luciferase construct controlled by four cAMP-response elements [30] which was kindly provided by Dr. E. Oetjen (Göttingen). Transfection efficiency was controlled by cotransfection of 0.1 μg per 400,000 cells of pRL-CMV (Promega, Piscataway, NJ, USA) for expression of Renilla reniformis luciferase. The activities of both luciferase isoenzymes were separately determined using the dual luciferase assay kit (Promega). Firefly luciferase activity was normalized to the activity of Renilla reniformis luciferase and expressed relative to the control.

2.3. Protein analysis

Cardiomyocytes were harvested in 200 μl denaturation buffer [31] per 5 million cells. Electrophoresis on 10% polyacrylamide gels and transfer onto nitrocellulose membranes were performed as described [32]. Whole CREB was detected using an antibody recognizing CREB327 and CREB341 not discriminating between the non-phosphorylated and the phosphorylated forms (1:1000; Upstate Biotechnology Inc., NY, USA, #06-504). CREB phosphorylated at serine119/133 was measured using an
antibody specific for the phosphorylated, but not recognizing the unphosphorylated form (1:1000; Upstate Biotechnology, #06-519). Both antibodies were extensively characterized in baculovirus-expressed CREB [33], in chick embryonic cardiomyocytes [16] and in human cardiac ventricular tissue [18]. The troponin inhibitor (TnI) was determined for standardization using an antibody against the phosphorylated and the unphosphorylated forms (1:2000; [34]) which was a kind gift of Dr. G.S. Bodor (Department of Laboratories, Denver Health Medical Center, Denver, CO). It recognized two proteins of 28–30 kDa where the slower migrating protein likely represented phosphorylated TnI which was increased after stimulation with forskolin. We did not differentiate between both forms and used the sum of both signals for standardization, and TnI levels were not changed in the different groups. All signals were visualized and quantified with 125I-protein A and the PhosphorImager system (Molecular Dynamics, Sunnyvale, CA, USA). For all determinations 70 μl cell extract were loaded per lane which was within the linear range (Fig. 3). In the different groups investigated there was no change in cell viability as assessed by microscopy, the percentage of beating cells and the total protein content (data not shown) in accordance with published reports that neither isoproterenol nor forskolin stimulated protein synthesis in adult ventricular rat cardiomyocytes [35]. We normalized the immunological signals of phosphoCREB by the signals of CREB which were determined on a second set of identically loaded gels in order to obtain a parameter for the phosphorylation state of CREB. This parameter per se was not dependent on the amount of protein loaded. CREB signals were normalized by the signals for TnI as a myocyte-specific protein which were determined on the identical gels in order to exclude loading errors.

2.4. Statistical analysis

Data were presented as mean±S.E.M. of independent experiments performed in duplicates as indicated. Data were analysed by non-parametric variance analysis according to Kruskal–Wallis and statistical significance was tested using the Mann–Whitney test. A P<0.05 was considered significant.

3. Results

Forskolin produced a concentration-dependent increase in luciferase activity in cardiomyocytes transfected with the CRE-controlled luciferase construct 4xSomCRE with a maximal effect of 20-fold activation at 100 μmol/l (Fig. 1). There was no basal or forskolin-induced luciferase activity after transfection of the empty luciferase vector lacking the CREs used for the construction of 4xSomCRE. Transcriptional activation started after 1 h of stimulation with 10 μmol/l forskolin and reached a peak after 8 h with a subsequent decline to 50% and 20% of maximal activation after 24 and 48 h (data not shown). This indicates that there is a transcriptional regulation mediated by the CRE in cardiomyocytes which is inactivated after prolonged stimulation, even in the presence of the agonist.

In order to study both the activation and the inactivation of the CRE-mediated gene transcription, we investigated the effect of a pretreatment with forskolin (1 μmol/l) for 24 h on the peak transcriptional activation with 10 μmol/l forskolin for 8 h (Fig. 2). While in control cells (pretreated with solvent for 24 h), 10 μmol/l forskolin (8 h) increased luciferase activity 13-fold, the transcriptional response to 10 μmol/l forskolin was decreased to 2.4-fold in forskolin-pretreated cells. To study the role of protein phosphatases for this attenuated activation we investigated the effect of cantharidin, an inhibitor of type 1+2A phosphatases, on the attenuated transcriptional response to forskolin. The addition of cantharidin to forskolin treatment increased the ratio of phosphorylated to total CREB compared to forskolin treatment alone in DMSO-pretreated cells (Fig. 4, description later in the text). However, cantharidin treatment had no effect on transcriptional activity (Fig. 2). Whole CREB (phosphorylated and unphosphorylated) and CREB phosphorylated at serine119/133 (phosphoCREB) were determined using specific antibodies. Both antibodies detected proteins migrating as a
solvent-pretreated cells, stimulation with 10 μmol/l forskolin for 8 h increased CREB phosphorylation to 145% which was further increased to 190% by additional treatment with 30 μmol/l cantharidin (Fig. 4). In forskolin-pretreated cells the phosphorylation of CREB remained increased to 153% after 8 h treatment with DMSO and was maximally increased either with 8 h forskolin (10 μmol/l) alone (234%) or in combination with 30 μmol/l cantharidin (227%). Thus, attenuation of the transcriptional response to forskolin in forskolin-pretreated cells was not associated with a dephosphorylation of CREB. However, the expression of CREB (total CREB standardized to TnI) was decreased in solvent-pretreated cells after stimulation with 10 μmol/l forskolin in combination with 30 μmol/l cantharidin and in all conditions of forskolin-pretreated cells (Fig. 5). TnI did not change under the different conditions investigated (data not shown).

We chose forskolin to study the CRE-mediated transcriptional regulation since it stimulates the cAMP-dependent signalling pathway bypassing the β-adrenoceptor. By this we could exclude possible effects on the receptor level (e.g. downregulation) which would complicate interpretation of data. However, without addressing to which extent receptor downregulation or other upstream mechanisms contribute to an altered transcriptional regulation we performed an additional experiment using the β-adrenoceptor agonist isoproterenol in the presence of IBMX, an alternative splicing isoforms CREB327 and CREB341 in accord with published data on the same antibodies in human cardiac tissue and chick cardiomyocytes [16,18]. In doublet at about 43 kDa (Fig. 3) likely representing the

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**Fig. 2.** Attenuation of the transcriptional response mediated by the CRE by pretreatment with forskolin. Cardiomyocytes were transfected with 4xSomCRE and pretreated for 24 h with either 1 μmol/l forskolin (filled bars) or solvent (DMSO, open bars) before cells were stimulated for 8 h with solvent (DMSO) or with 10 μmol/l forskolin alone (Fors) or in combination with 30 μmol/l cantharidin (Fors+Cant). Luciferase activities were corrected for the transfection efficiency and expressed relative to the solvent control (mean±S.E.M.; n=7–11). *P<0.05 versus pretreatment with DMSO.

**Fig. 3.** Linear range of the determination of whole CREB (CREB, ○) and CREB phosphorylated at serine119/133 (pCREB, ●) in regard to the amount of cell extract loaded (mean±S.E.M.; n=3), representative autoradiographies are shown as inset.

**Fig. 4.** Effect of 24 h pretreatment with 1 μmol/l forskolin (filled bars) or solvent (DMSO, open bars) and 8 h treatment with solvent (DMSO), 10 μmol/l forskolin (Fors) alone or in combination with 30 μmol/l cantharidin (Fors+Cant) on the phosphorylation of CREB. The signals for CREB phosphorylated at serine119/133 were divided by the corresponding signals of whole CREB and expressed relative to control (mean±S.E.M.; n=5–6). *P<0.05 versus pretreatment with DMSO, †P<0.05 versus treatment with DMSO, ‡P<0.05 versus treatment with Fors.
inhibitor of phosphodiesterases. The rationale of this experiment was to study whether stimulation of the \( \beta \)-adrenoceptor in principle can attenuate CRE-mediated transcriptional regulation. This might be of physiological relevance in patients with CHF where plasma catecholamines are elevated. The CRE-mediated transcriptional activation by 10 \( \mu \)mol/l forskolin (8 h) was studied in cardiomyocytes pretreated for 24 h with 1 \( \mu \)mol/l isoproterenol in the presence of 100 \( \mu \)mol/l IBMX and was compared to pretreatment with solvent. Similar to the experiment with forskolin pretreatment (Fig. 2), CRE-mediated transcription was about 10-fold activated by forskolin (10 \( \mu \)mol/l, 8 h) in solvent-pretreated cells (Fig. 6). Compared to pretreatment with forskolin the transcriptional response to forskolin was decreased to a similar extent (1.6-fold) by pretreatment with isoproterenol in combination with IBMX showing that prolonged stimulation of the \( \beta \)-adrenoceptor also may attenuate the CRE-mediated transcriptional response in the heart.

4. Discussion

We have studied the phosphorylation and expression of CREB by activation of the CRE-mediated gene transcription via stimulation of the cAMP-dependent signalling pathway with forskolin in cardiomyocytes. Our data provides evidence that in cardiac myocytes (i) there is an activation of the CRE-mediated gene transcription by forskolin associated with increased phosphorylation of CREB, (ii) that this regulation is attenuated following prolonged stimulation and (iii) — though CREB protein levels were decreased — that neither CREB phosphorylation nor total CREB levels are directly related to the reduced CRE-mediated transcriptional activation. In cardiac myocytes, the maximal CRE-mediated transcriptional activation by forskolin is comparable to results from other cell types, e.g. HIT-T15 cells, with the same luciferase reporter [32]. However, the kinetics of the transcriptional response to forskolin is delayed compared to other cell types including PC12 pheochromocytoma and F9 teratocarcinoma cells. In the latter type of cells the peak activity is reached within 30 min followed by a gradual deterioration of the response within 4 to 6 h [10,36]. We investigated the phosphorylation of CREB in comparison to the transcriptional activation in cells pretreated with solvent for 24 h. Forskolin led to a 13-fold activation of CRE-mediated gene transcription within 8 h and to an increase in CREB phosphorylation to 145%. Therefore, as described for PC12 and F9 cells and many other cell types [10,36], CRE-mediated transcriptional activation is associated with increased phosphorylation of CREB at serine119/133 in cardiomyocytes. However, in comparison to cardiomyocytes, the extent of CREB phosphorylation and resulting CRE-mediated transcriptional activation (i.e. the signal amplification) was different in PC12 cells where
stimulation with 10 μmol/l forskolin resulted in a 3- to 4-fold increase in CREB phosphorylation but in a 4- to 5-fold induction in CRE-mediated transcription [14]. The degree of activation in DMSO-pretreated cardiomyocytes was the same as in nonpretreated cells (Fig. 1) showing that pretreatment with 0.01% DMSO for 24 h had no specific effect on transcription. Cantharidin in combination with forskolin did not further increase the transcriptional activity although it increased the phosphorylation state of CREB. This shows that 30 μmol/l cantharidin is sufficient to inhibit phosphatases (putatively of the type 1+2A) in cardiomyocytes in accordance with data on human cardiac tissue [29] and that these phosphatases dephosphorylate CRET at serine119/133 in cardiomyocytes. Moreover, it seems that in cardiac myocytes maximal activation of the CRE-mediated gene transcription does not require that all CREB molecules are phosphorylated at serine119/133. This is in accord with data on PC12 cells where only 40% of total CREB were phosphorylated in response to 10 μmol/l forskolin [14]. The inactivation of the CRE-mediated gene transcription was studied in cells pretreated with forskolin for 24 h since initial data showed a decline of transcriptional activity at that time point. Pretreatment with 1 μmol/l forskolin for 24 h led to a relative refractoriness of the CRE-mediated transcriptional response to forskolin. A similar attenuation was described in F9 cells and a protein phosphatase type 1-mediated dephosphorylation of CREB was suggested as the major mechanism for this regulation [36]. In contrast to this, inhibition of protein phosphatases type 1+2A by cantharidin did not restore the forskolin-induced transcription in forskolin-pretreated cardiomyocytes. Moreover, in forskolin-pretreated cardiomyocytes the phosphorylation state of CREB was maximally increased after 8 h stimulation with forskolin alone or in combination with cantharidin, although the transcriptional response was reduced. Therefore we conclude that attenuation of CRE-mediated gene transcription following prolonged stimulation with forskolin is not regulated on the level of a dephosphorylation of CREB in cardiomyocytes.

Total CREB protein levels were decreased after pretreatment with forskolin and after treatment with forskolin in combination with cantharidin compared to solvent-pretreated and -treated cells. From this, it may be speculated that a reduction in total CREB protein might alter the balance of transactivators and repressors acting at the CRE and might contribute to the attenuated transcriptional response after pretreatment with forskolin. An increased expression of CREB was described for chick cardiomyocytes after 30 min stimulation with forskolin [16] whereas a 4-day infusion of isoproterenol in a rat model of chronic β-adrenergic stimulation decreased CREB mRNA in the heart [17]. There are cAMP-responsive elements present in the CREB promoter which mediate a transient positive autoregulation of CREB expression [37]. However, the attenuation of the transcriptional response after prolonged stimulation with forskolin is not directly related to a downregulation of CREB. For example, CREB levels are the same after pretreatment with forskolin/treatment with forskolin and after pretreatment with DMSO/treatment with forskolin in combination with cantharidin while the transcriptional response was different. Moreover, CREB levels after DMSO pretreatment/forskolin treatment were not different from the other groups treated or pretreated with forskolin while transcriptional activation was maximal. Although this discrepancy may be explained by the different time periods of exposition to forskolin (32 h vs. 8 h) it is possible that there are other mechanisms contributing to the reduced CRE-mediated transcriptional response. An upregulation of the inhibitory cAMP-responsive early repressor (ICER) was observed following stimulation of the cAMP-dependent signalling pathway [38]. Therefore, ICER or another CREM repressor might contribute to the attenuation of the CRE-mediated transcription in cardiomyocytes, either through direct action at the CRE of a CRE-controlled target gene and/or indirectly by a decrease in the expression of CREB. We immunoprecipitated different CREM isoforms in human cardiac ventricular tissue, of which one, the putative molecule CREM-IhΔC-X, is a repressor acting at the CRE [32]. This repressor has similar properties as the dominant-negative CREB mutant which led to CHF in a transgenic mouse model [27]. Unfortunately, using the commercially available antibodies against CREM we were not able to quantify CREM isoforms in chick cardiomyocytes. Although CREB and CREM were shown to be expressed in the human heart [18,32] little is known about the responsiveness of the CRE-mediated transcription and the interplay of CREB and CREM during both initiation and progression of CHF. Interestingly, CREB null mice with inactivation of all functional CREB isoforms die immediately after birth [39] suggesting that the tight regulation of the expression of CREB may be essential for the survival of the cell.

It was our aim to study whether CRE-mediated transcriptional regulation is altered after stimulation of the cAMP-dependent signalling pathway. In order to exclude possible effects upstream of the transcriptional regulation (e.g. receptor downregulation) and complicating the interpretation of data we chose forskolin as a tool for the receptor-independent stimulation of the adenylyl cyclase. Moreover, we present data that a 24 h prestimulation with the β-adrenoceptor agonist isoproterenol in combination with IBMX, an inhibitor of phosphodiesterases attenuates the transcriptional response to forskolin (8 h, 10 μmol/l) to a similar extent as pretreatment with 1 μmol/l forskolin. We conclude from the latter experiment that stimulation of the β-adrenoceptor in principle can also induce a reduction of the CRE-mediated transcriptional response suggesting that this process might be of physiological relevance. However, due to the presence of IBMX, this experiment does not address the question whether there are also
altered by carboxy-terminally upstream of the transcriptional regulatory region which contribute to the reduced transcriptional response after prolonged stimulation.

In summary, our data give evidence that prolonged stimulation of the cAMP-dependent signalling pathway either by forskolin or by isoproterenol in the presence of IBMX leads to desensitization of the CRE-mediated transcriptional response in cardiomyocytes which was thought to induce heart failure in transgenic mice overexpressing a dominant-negative CREB-mutant [27]. From this, it may be speculated that increased β-adrenergic stimulation by elevated plasma catecholamines contributes to the pathophysiology of CHF through an attenuation of the CRE-mediated transcriptional regulation with possible deleterious consequences.

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References


