Biochimica et Biophysica Acta 1840 (2014) 1765–1770

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Cyclic AMP and alkaline pH downregulate carbonic anhydrase 2 in mouse fibroblasts

Pablo Mardones [1], Jung Chin Chang [1], Ronald P.J. Oude Elferink *

Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 12 July 2013
Received in revised form 6 December 2013
Accepted 10 December 2013
Available online 19 December 2013

Keywords:
Carbonic anhydrase
Intracellular pH
Soluble adenylyl cyclase
Chloride/bicarbonate exchanger
Bicarbonate
Cyclic AMP

ABSTRACT

Background: The hydration of CO_2 catalyzed by the ubiquitous carbonic anhydrase 2 (Ca2) is central for bicarbonate transport, bone metabolism and acid–base homeostasis in metazoans. There is evidence that in some tissues Ca2 expression can be acutely induced by cAMP, whereas in other cell types it is unresponsive to cAMP-mediated transcriptional activation.
Methods: We isolated fibroblasts from wild type and mice lacking the ubiquitous chloride/bicarbonate exchanger ($Ae2^{-/-}_{a,b}$ mice). In these cells the regulation of carbonic anhydrase 2 by cAMP was studied.
Results: We show that Ca2 expression is strongly inhibited by chronic incubation with dibutyryl-cAMP, forskolin or alkaline pH in cultured mouse fibroblasts. Furthermore, fibroblasts obtained from anion exchanger 2 deficient ($Ae2^{-/-}_{a,b}$) mice, which display intracellular alkalosis and increased cAMP production, express less than 10% of control Ca2 mRNA and protein. Surprisingly, inhibition of the bicarbonate-sensitive soluble adenylyl cyclase (sAC) was found to reduce CA2 expression instead of increasing it.
Conclusions: CA2 expression is strongly regulated by intracellular pH and by cAMP, suggesting a role for soluble adenylyl cyclase. Regulation occurs in opposite directions which may be explained by an incoherent feedforward loop consisting of activation by pCREB and repression by ICER.

1. Introduction

In mammals, the synthesis of bicarbonate catalyzed by carbonic anhydrases plays a pivotal role in circulating CO_2 transport, acid–base physiology, gastric acid secretion, bone resorption, and vectorial transport of fluid and electrolytes in various epithelia [1–4]. Carbonic anhydrase 2 (CA2), in particular, is especially important for renal transport of acid–base equivalents and the activity of acid secreting cells of the gastric epithelium (parietal cells) and bone (osteoclasts) [5,6]. Mutations in carbonic anhydrase 2 have been linked to renal tubular acidosis with osteopetrosis and cerebral calcification in humans [7] and a similar, albeit milder, phenotype is observed in mice with a chemically induced null mutation of *CA2* [8], underscoring the conserved role of this enzyme in mammalian physiology.

The ubiquitous expression and tissue-specific nature of CA2 functions seem to require diverse mechanisms of regulating its expression. For instance, signaling through cyclic AMP (cAMP) induces an acute upregulation of *CA2* expression in avian erythroid cells [9], parietal cells [5] and osteoclasts [10]; whereas human hepatocytes and HepG2 cells do not show this acute cAMP stimulation of *CA2* transcription [11,12].

Promoter analysis of murine, rat, and human *CA2* genes has revealed the presence of a number of regulatory regions, namely consensus sequences for binding of AP-1, AP-2 and Sp1 transcription factors [11,13,14]. It has also been demonstrated that part of the 5′ AP-2 sequence of the mouse *Ca2* promoter can function as a non-canonical CRE site and it is required for in vitro transcriptional activation by forskolin in NIH-3T3 cells [11]. In contrast, the same region is unresponsive to forskolin in HepG2 cells [11,12]. This cell type-specific discrepancy has yet to be characterized in detail, however it is an indication of the level of tissue-specificity underlying *Ca2* transcriptional regulation.

Bicarbonate transport is an important component of intracellular and whole-body pH homeostasis [15,16]. In this context, CA2 not only is capable of direct bicarbonate synthesis, but it can also interact with proton and bicarbonate transporters at the plasma membrane as part of a 'transport metabolon', in which the product of an enzymatic reaction is locally funneled into the active site of transport [17]. A C-terminal consensus sequence for CA2 interaction is found in all members of the SLC4 family of anion exchangers, comprised of the erythrocyte and kidney-specific anion exchanger 1 (SLC4A1, AE1), the ubiquitous pH_i regulator anion exchanger 2 (SLC4A2, AE2), the anion exchanger 3 (SLC4A3, AE3) in muscle and brain tissue, and the electrogenic sodium/bicarbonate cotransporter (eNBC1, SLC4A4) [18,19].

During the analysis of gene expression in murine fibroblasts with a null mutation of the most important isoforms of *Ae2*, *Ae2a* and *Ae2b* [20], we found that *Ca2* was strongly repressed, which correlated with chronic intracellular alkalization and increased cAMP synthesis. We

* Corresponding author at: Tytgat Institute for Liver and Intestinal Research, Academic Medical Center S1-162, University of Amsterdam, Meibergdreef 69-71, 1105 BK, Amsterdam, The Netherlands. Tel.: +31 20 5663828; fax: +31 20 5669190.
E-mail address: r.p.oude-elferink@amc.uva.nl (R.P.J. Oude Elferink).
[1] These authors equally contributed to the manuscript.

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.12.015

hypothesized that stimulation of cAMP synthesis may be derived from the bicarbonate-stimulated activity of soluble adenylyl cyclase (Adcy10) and could result regulation of *Ca2* gene expression in mouse fibroblasts.

In this study we report that *Ca2* expression is downregulated in cultured wild type mouse fibroblasts by extracellular alkalization, dibutyryl cAMP or forskolin. However, specific chemical inhibition of the bicarbonate-sensitive soluble adenylyl cyclase also reduced *Ca2* mRNA levels, pointing to complex and divergent roles of cAMP synthesis in the activation and repression of the *Ca2* gene.

Our results suggest that the effects of chronic cAMP exposure may play a previously unrecognized role in cellular physiology through the cell-type- and tissue-specific regulation of CA2.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents and chemicals were purchased from Sigma (St. Louis, MO).

2.2. Cell culture and treatments

Fibroblasts were isolated from the peritoneal wall of male $Ae2^{+/+}_{a,b}$ and $Ae2^{-/-}_{a,b}$ mice of the same genetic background. The peritoneal wall was excised and cut into small pieces (< 1 mm). These pieces were incubated with trypsin for 30 min at 37 °C. Subsequently, tissue debris was removed by low speed centrifugation (50 ×*g*) and cells in the supernatant were spun down at 1000 ×*g*. Cells were cultured in DMEM (Cambrex, Verviers, Belgium) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin under 10% CO_2.

Medium pH was modified by adding 20 mM HEPES, together with proper amounts of HCl or NaOH to normal DMEM. Incubations at different pH_o were carried out for 24 h.

Forskolin, dibutyryl-cAMP, the soluble adenylyl cyclase (sAC) inhibitor KH7, or vehicle (0.01% DMSO) were applied at the indicated doses and times to wild type and/or $Ae2^{-/-}_{a,b}$ cells, diluted in normal DMEM.

2.3. pH_i measurements

5×10^5 cells were seeded on collagen-coated round coverslips and cultured for 48 h (20 mm diameter). Subsequently, they were washed with Hank's balanced salt solution (HBSS; Cambrex, Verviers, Belgium) and incubated for 10 min with 5 μM BCECF-AM (Molecular Probes, Eugene, OR) in HBSS at 37 °C. BCECF loaded cells were mounted in a custom-made perfusion chamber and perfused at 0.7 ml/min with Tyrode's buffered solution (composition in mM, KCl: 2.5; NaCl: 145; HEPES: 10; glucose: 10; $MgCl_2$: 1.2; $CaCl_2$: 1.5; pH 7.4) at 37 °C. After equilibration, fluorescence (535 nm excitation, 490 nm and 440 nm emission) was monitored every 30 s in a Novostar multiplate reader (BMG Labtechnologies, Offenburg, Germany). At the end of the experiment a single-point calibration was performed by perfusing cells with 10 μM nigericin in high-K^+ buffer at pH 7.0 [21]. pH_i was calculated by interpolating normalized 490 nm/440 nm ratios in a standard curve obtained by perfusing cells with 10 μM nigericin, high-K^+ buffers at 9 different pH_o between 5.8 and 8.2. The standard curve was adjusted to pass through the point [fluorescence ratio 1.0, pH 7.0], and least squares non-linear fitting was performed as described elsewhere [21]. All calculations were done in Prism v4.0 (GraphPad Software, San Diego, CA).

2.4. cAMP measurements

Total cAMP was measured in adherent fibroblast cultures with a commercial enzyme-linked immunoassay kit (Amersham Biosciences, Piscataway, NJ).

2.5. Quantitative RT-PCR

5 μg of total RNA, isolated from $Ae2^{+/+}_{a,b}$ and $Ae2^{-/-}_{a,b}$ fibroblasts with Trizol reagent (Invitrogen, Carlsbad, CA), was subjected to reverse transcription. Quantitative real-time PCR for *Ca2* (forward primer: 5′ TGGG GATACAGCAAGCACAA 3′; reverse primer: 5′ CTTTCAGCACTGCATTGTCC 3′) and *Gapdh* (forward primer: 5′ TCAATGAAGGGGTCGTTGAT 3′; reverse primer: 5′ CGTCCCGTAGACAAAATGGT 3′) was performed on 50 ng of template cDNA in a LightCycler apparatus (Roche Diagnostics, Mannheim, Germany). Initial RNA concentrations were calculated by linear regression using LinReg v. 9.16 software [22]. Results are expressed either as relative expression of ratios to *Gapdh* mRNA levels in arbitrary units or as percentage of the control value, which is the ratio of *Ca2* mRNA to *Gapdh* mRNA in $Ae2^{+/+}$ fibroblasts cultured at pH_o 7.4.

2.6. Cell fractionation and immunodetection of CA2

1×10^7 cells were seeded in 10 cm tissue culture plates 48 h before the experiment. Nuclear, membrane, and cytosolic fractions were prepared as described elsewhere [23]. Total lysates or cytosolic fractions were blotted as indicated with anti-CA2 (1:1000, CHEMICON, Temecula, CA), and anti-β-actin (1:2000, Sigma, St. Louis, MO) antibodies. Appropriate secondary antibody-peroxidase conjugates (Bio-Rad, Hercules, CA) were detected in a Lumimager (Roche Diagnostics, Mannheim, Germany) after incubation with chemiluminescent substrate (Roche Diagnostics, Mannheim, Germany). Immunoblot signals were normalized using β-actin as loading control. Protein staining on the blots was quantified with the LumiAnalyst 3.1 program using Lumi-Imager F1 (Roche, The Netherlands) equipment. The range of linearity was determined by a titration of a standard lysate with a fixed dilution of the primary antibody. The background signal in an empty lane was subtracted from all other signal intensities.

2.7. Statistics

Quantitative results are expressed as mean ± SD. Differences between groups were tested for statistical significance ($p < 0.05$) using the two-tailed Student's *t*-test in Prism v4.0 (GraphPad Software, San Diego, CA). All data are representative of at least two independent experiments.

3. Results

3.1. *Ca2* expression is strongly repressed by AE2 deficiency and cellular alkalization in murine fibroblasts

pH_i homeostasis relies largely on proton and bicarbonate transport at the plasma membrane, which allows cells for rapid adaptation to changes in pH_i produced by normal metabolism or pathophysiological conditions [15,24]. In most cells, an important source of these transport substrates is the hydration of CO_2 catalyzed by CA2 [1,2]. Fibroblasts obtained from mice lacking the most prominent variants of AE2 ($Ae2^{-/-}_{a,b}$) [20] develop chronic intracellular alkalosis in culture, due to the absence of AE2-mediated bicarbonate extrusion [25].

Quantitative RT-PCR analysis shows that *Ca2* expression is markedly reduced in $Ae2^{-/-}_{a,b}$ fibroblasts (Fig. 1A). *Ca2* mRNA content amounts to less than 10% of the control value, with a concomitant 93% decrease in CA2 protein detected in total lysates by immunoblotting (Fig. 1B). This finding led us to investigate a potential association between alkaline cellular pH and the downregulation of *Ca2* observed in $Ae2^{-/-}_{a,b}$ fibroblasts. The pattern of *Ca2* mRNA expression in $Ae2^{+/+}$ fibroblasts cultured at different pH_o for 24 h reveals that medium alkalization is a stimulus for *Ca2* repression, whereas the opposite is true upon medium acidification (Fig. 1C). Immunoblots against CA2 on cytosolic fractions from wild type and knockout cells confirm the pH-dependent expression of *Ca2*

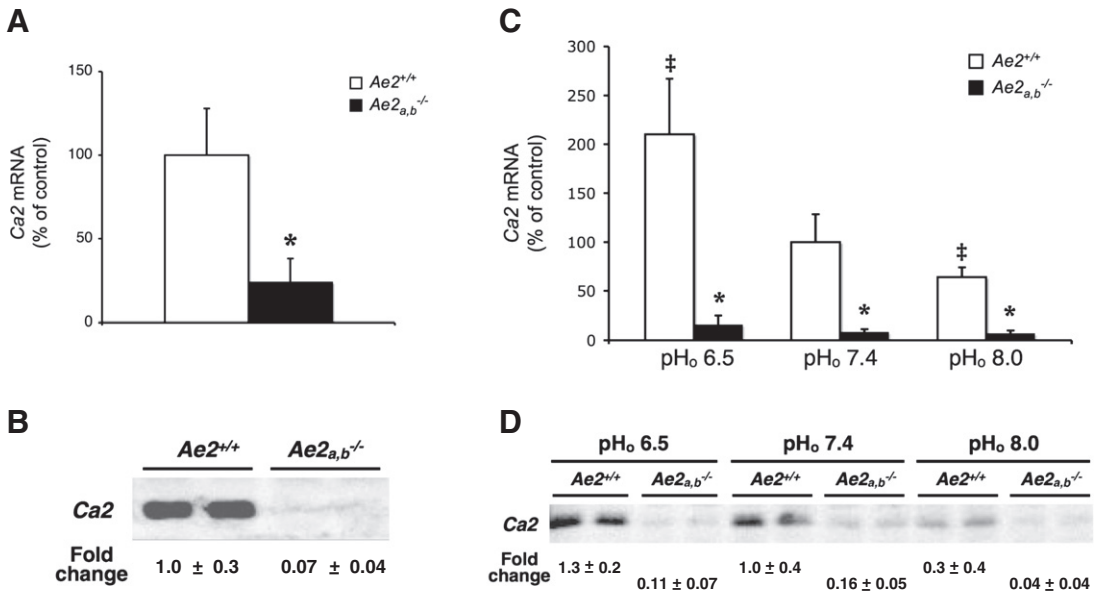

Fig. 1. Analysis of Ca2 expression in $Ae2^{+/+}$ and $Ae2^{-/-}_{a,b}$ fibroblasts. (A) Quantitative real-time RT-PCR for Ca2 was performed on total RNA samples from $Ae2^{+/+}$ and $Ae2^{-/-}_{a,b}$ fibroblasts. Results are normalized for *Gapdh* expression and expressed as mean percentage of control ratios ± SD ($n = 5$, $^*p < 0.0001$). (B) 30 μg of total protein from cell lysates was subjected to SDS-PAGE followed by immunoblotting with anti-Ca2 and anti β-actin (not shown) antibodies. The blot shows two separate preparations for each genotype. Quantitation is expressed as fold change with respect to the average Ca2/β-actin signal ratio in wild type fibroblasts ($n = 4$). (C) Fibroblasts were incubated at different pH_o for 24 h. Total mRNA was isolated followed by quantitative RT-PCR for *Ca2* and *Gapdh* ($n = 5$, $^*p < 0.0001$ compared to wild type cells under the same conditions, ‡ $p < 0.03$ compared to wild type cells at pH_o 7.4). Results are presented as percentage of *Ca2*/*Gapdh* mRNA ratio in wild type fibroblasts at pH_o 7.4 ± SD ($n = 5$). (D) Cytosolic fractions from fibroblasts incubated at different pH_o for 24 h (duplicate preparations of each condition) were immunostained for CA2 and β-actin (not shown). Results are presented as fold change with respect to the average CA2/β-actin signal ratio in wild type fibroblasts at pH_o 7.4 ($n = 3$).

(Fig. 1D), although the reduction in CA2 protein levels is more pronounced at alkaline pH than the upregulation observed under extracellular acidification, suggesting post transcriptional/translational effects on CA2 expression in this cell type. $Ae2^{-/-}_{a,b}$ fibroblasts show a very low amount of *Ca2* mRNA and protein regardless of any change in extracellular pH, although alkaline pH appears to have a further repressor effect over CA2 protein in $Ae2^{-/-}_{a,b}$ cells (Fig. 1D). We measured intracellular pH as a function of changes in extracellular pH in both wild type and $Ae2^{-/-}_{a,b}$ fibroblasts. Fig. 2 shows that intracellular pH moderately increased with extracellular pH in both genotypes and in line with our previous findings [25] the intracellular pH was 0.2 pH unit more alkaline in $Ae2^{-/-}_{a,b}$ fibroblasts compared to wild type (except at an extracellular pH of 6.5). Although *Ca2* expression in wild type cells was significantly pH-dependent, the reduction of *Ca2* expression in $Ae2^{-/-}_{a,b}$ cells was much more dramatic than one might expect on the basis of intracellular pH alone.

3.2. Ca2 is downregulated in mouse fibroblasts by chronic cAMP stimulation

Besides an alkaline shift in resting pH_i, $Ae2^{-/-}_{a,b}$ fibroblasts also display increased total cAMP levels when compared to their wild-type counterparts [25]. This could be due to bicarbonate-mediated activation of the soluble adenylyl cyclase [25,26]. We tested this hypothesis by analyzing the effect of KH7, a specific inhibitor of sAC that does not affect other adenylyl cyclases [27], on cellular cAMP levels in $Ae2^{-/-}_{a,b}$ fibroblasts. Fig. 3 shows that at 25 μM KH7 and higher the total cellular cAMP content was significantly decreased. It must be stressed that other adenylyl cyclases are not inhibited; hence, full depletion of cellular cAMP is not to be expected.

We subsequently tested whether Ca2 expression is regulated by cAMP in fibroblasts. We found that a 24 h exposure of wild type mouse fibroblasts to the membrane permeable cAMP analog dibutyryl-cAMP

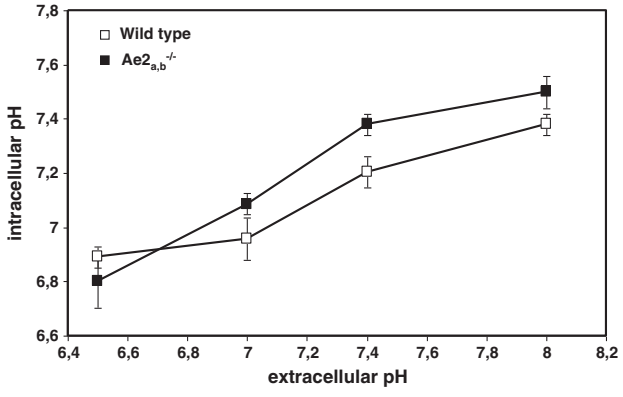

Fig. 2. Intracellular pH at various extracellular pH values in wild type and $Ae2a,b^{-/-}$ fibroblasts. Intracellular pH was measured after loading of the cells with BCECF as described in Materials and Methods. The extracellular pH was varied with buffered HEPES/TRIS and the cells were allowed to equilibrate until a constant value for intracellular pH was measured. At all pH values except 6.5 the measured intracellular pH in $Ae2^{-/-}$ cells was significantly higher than in wild type cells.

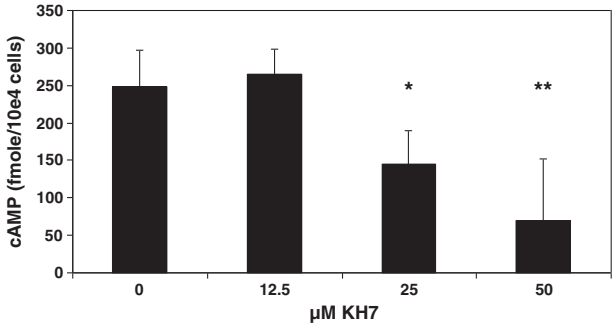

Fig. 3. The effect of KH7 on cellular cAMP content. $Ae2^{-/-}_{a,b}$ fibroblasts were cultured in the presence of the indicated concentrations of the soluble adenylyl cyclase inhibitor KH7 for 24 h. After this period the cells were harvested and cAMP levels were measured as described in Materials and methods. The data represent measurement in three preparations ± S.D. $^*p < 0.05$, $^{**}p < 0.01$.

resulted in a 90% reduction of *Ca2* mRNA levels (Fig. 4A), together with an 86% reduction in protein (Fig. 4B). Direct activation of the G protein receptor-coupled adenylyl cyclase with 25 μM forskolin for 24 h led to a comparable 75% decrease in Ca2 protein expression (Fig. 4C). These results show that *Ca2* is susceptible to downregulation via a cAMP-dependent pathway in mouse fibroblasts.

3.3. Time course of Ca2 repression by forskolin

cAMP-mediated transcriptional activation normally occurs at a very early time point, therefore it is often studied shortly after stimulation. In order to establish whether an early activation event preceded *Ca2* downregulation we performed a time-course experiment, and followed the expression of *Ca2* mRNA at different time points during a 24 h treatment with 25 μM forskolin in wild type and $Ae2^{-/-}_{a,b}$ fibroblasts. As shown in Fig. 5, *Ca2* mRNA expression in $Ae2^{+/+}$ cells is reduced to 30% of its starting value after 1 h of treatment, and continues to decline over the course of the experiment for at least 12 h, after which it appears to undergo a recovery phase reaching 50% of its starting value at 24 h of treatment. This pattern of response is similar in $Ae2^{-/-}_{a,b}$ fibroblasts, although starting mRNA levels of *Ca2* are much lower than in wild type cells and there is no recovery phase at 24 h, a possible indication that in knockout cells intracellular alkalization and endogenous cAMP production are sufficient for maintaining a very low level of *Ca2* mRNA, and forskolin can only contribute to further repression.

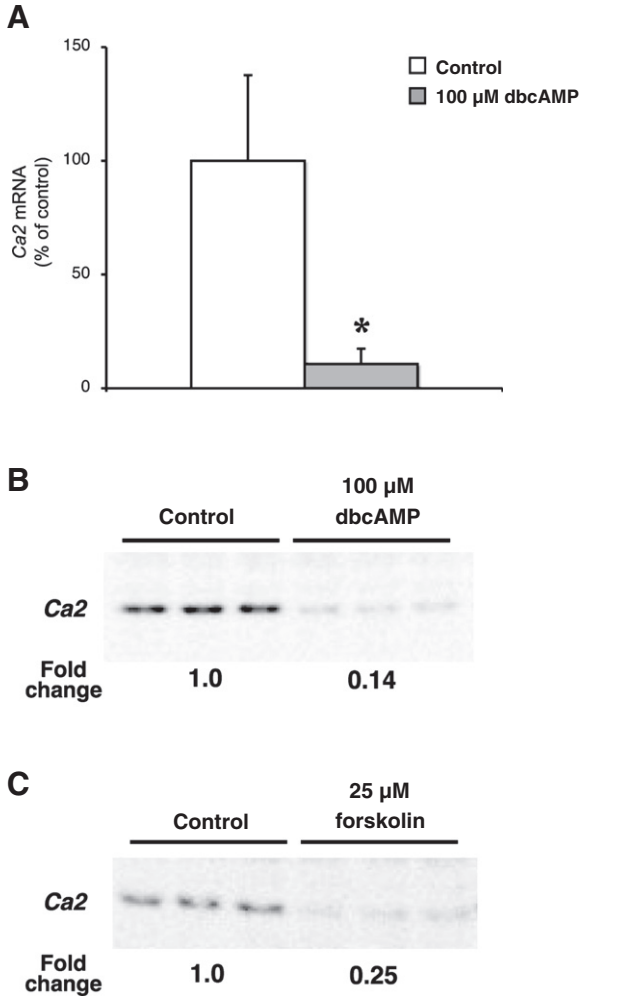

Fig. 4. Dibutyryl-cAMP (dbcAMP), and forskolin effect on Ca2 expression. $Ae2^{+/+}$ fibroblasts were treated with 100 μM dibutyryl-cAMP (dbcAMP), 25 μM forskolin, or vehicle (0.01% DMSO) for 24 h and total RNA and cell lysates were prepared. (A) Quantitative RT-PCR for Ca2 and Gapdh was performed on total RNA samples ($n = 5$, $^*p < 0.005$) and the ratios of normalized Ca2 mRNA levels are presented as percentage of the control ratio in wild type fibroblasts ± SD. (B) 30 μg of total protein from cell lysates were blotted with an anti-Ca2 antibody. The effect of dbcAMP (B) and forskolin (C) treatment on Ca2 protein levels ($n = 3$) are presented as fold-change with respect to vehicle treated cells (control) after normalizing Ca2 specific bands with the corresponding β-actin signals (not shown).

3.4. Paradoxical effect of soluble adenylyl cyclase (sAC) inhibition on the expression of Ca2

Given that bicarbonate-sensitive sAC is upregulated [25] and that cellular cAMP levels are indeed increased in $Ae2^{-/-}_{a,b}$ fibroblasts ([25]), we hypothesized that sAC may be the mediator of *Ca2* repression in response to elevated $pH_i/[HCO_3^-]$ as well as the main source of cAMP accumulation in these cells. Surprisingly, incubation with the specific sAC inhibitor KH7 for 24 h resulted in a steep downregulation of *Ca2* mRNA to near undetectable levels in wild type cells, comparable or even lower than those observed in $Ae2^{-/-}_{a,b}$ fibroblasts under normal culture conditions (Fig. 6A).

The transcriptional behavior of *Ca2* in wild type cells is particularly striking because on the one hand a 24 h period of elevated of cAMP (by incubation with dbcAMP or with forskolin) leads to downregulation of *Ca2* expression, whereas inhibition of sAC (leading to decreased cAMP) also downregulates *Ca2* expression. Several mechanisms may be proposed for this apparent paradox. One of these is an incoherent feed forward loop (see discussion). If this is the case one would expect that there is an optimum in the dependency of *Ca2* expression on cellular cAMP. To test this hypothesis, we incubated cells with increasing concentrations of KH7 and measured *Ca2* expression (Fig. 7). We observed that at 12.5 μM KH7 there was a 50% increase in Ca2 expression whereas at 50 μM KH7 *Ca2* expression dropped by 56% compared to the control situation.

4. Discussion

The role of CREB phosphorylation by PKA on the transcriptional activation of target genes has been profusely documented in the literature as a central part of various physiological processes [28]. In contrast, relatively fewer examples of gene repression by cAMP signaling have been reported in detail [29–31]. These studies generally link cAMP-mediated gene repression to either primary events such as phosphorylation of transcription factors (e.g. CIITA) by PKA, or secondary phenomena like sequestration of limited amounts of the coactivator CBP by phosphorylated CREB, leading to transcriptional inhibition.

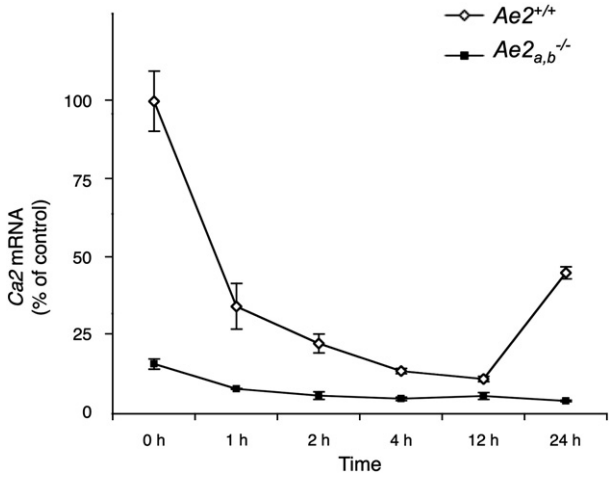

Fig. 5. Kinetics of *Ca2* mRNA expression in response to forskolin. Cells were treated with 25 μM forskolin for the indicated times, after which total RNA was collected and subjected to quantitative RT-PCR for Ca2 ($n = 4$). Results are expressed as relative mRNA expression, normalized using *Gapdh* mRNA as reference, in arbitrary units ± SD.

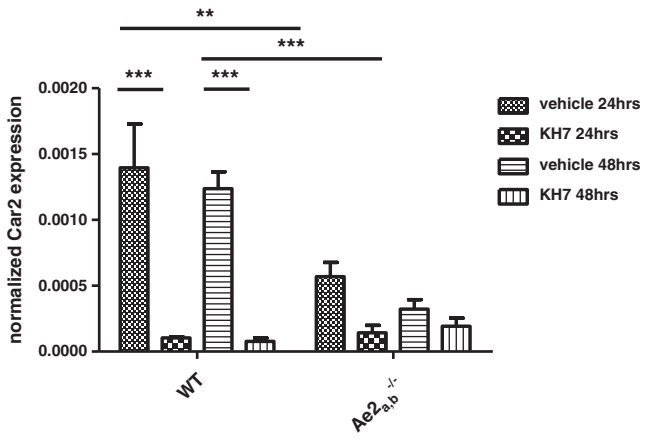

Fig. 6. Soluble adenylyl cyclase inhibition lowers *Ca2* mRNA levels in mouse fibroblasts. Cells were incubated with the specific sAC inhibitor KH7 (50 μM, for 24 h) or vehicle. Total RNA was prepared and real-time RT-PCR for *Ca2* and *Gapdh* were performed. Results represent the relative expression presented as the mean of *Ca2*/*Gapdh* mRNA ratios for each group ± SD. (n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$).

Even fewer cases have been reported where cAMP exerts a dual effect over transcriptional activity of the same gene depending on tissue or cell type. One example of this type of regulation is the enzyme nitric oxide synthase 2 (NOS2), which can be either upregulated or downregulated by cAMP in different cell-types [32].

A more general mechanism of cAMP-mediated gene repression takes place through the induction of ICER (inducible cAMP early repressor), a CREM isoform that functions as a strong transcriptional repressor by competing with phosphorylated CREB for binding to CRE sites [33,34]. Normally, ICER is activated as part of a negative feedback loop to attenuate the activity of phosphorylated CREB, therefore it only acts upon promoters containing one or more CRE sites, and it is not involved in repression of basal gene transcription. $Ae2^{-/-}_{a,b}$ fibroblasts present a marked increase in steady state levels of *Icer-1* mRNA [25], suggesting that this repressor might indeed be involved in regulating *Ca2* expression.

In what could be a novel case of dual cAMP regulation comparable to NOS2, here we show that *Ca2* expression is inhibited by prolonged exposure to the stable cAMP analog dbcAMP or forskolin in murine fibroblasts, whereas in a number of cell lines it has been shown to be either induced by cAMP or unresponsive. We believe this repression effect to be of physiological significance, since it is consistent with a model of homeostatic regulation of bicarbonate synthesis in response to intracellular alkalosis through the activation of soluble adenylyl cyclase in certain tissues or cell-types. Moreover, the strong reduction of *Ca2* expression in $Ae2^{-/-}_{a,b}$ fibroblasts, as well as in response to alkaline pH_o in wild type fibroblasts, suggests that *Ca2* repression occurs in circumstances that do not involve direct administration of cAMP agonists, and are quite possibly indicative of physiologically relevant adaptive cellular responses.

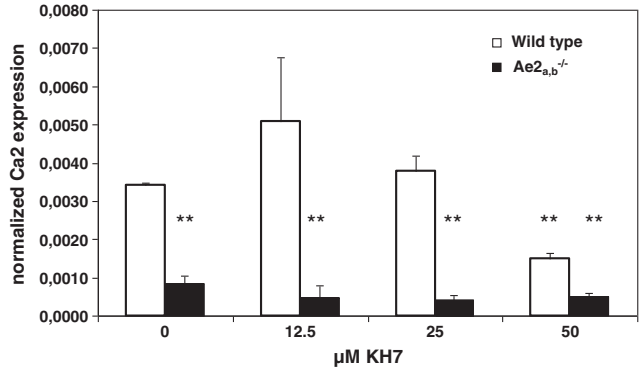

Fig. 7. Titration of wild type and $Ae2^{-/-}_{a,b}$ fibroblasts with KH7. Cells were cultured with the indicated concentration of KH7 for 24 h. Subsequently mRNA was isolated and quantified by realtime RT-PCR. Results represent the relative expression presented as the mean of *Ca2*/*Gapdh* mRNA ratios for each group ± SD (n = 3).

In apparent contradiction with this model, sAC inhibition with KH7 did not abate *Ca2* repression in $Ae2^{-/-}_{a,b}$ fibroblasts and instead produced an unexpected strong downregulation of this enzyme in wild type cells. Particularly the transcriptional behavior of *Ca2* in wild type cells is striking. On the one hand, elevation of cAMP-dependent signaling with dbcAMP or forskolin dramatically inhibited *Ca2* expression, but on the other hand reduction of cAMP production from sAC with KH7 also reduced *Ca2* expression. Several mechanisms may be proposed to explain this apparent paradox. Firstly, production of cAMP by forskolin occurs at the plasma membrane whereas sAC is localized in the cytosol, mitochondria, nucleus and at the cytoskeleton [35]. These two, differently compartmentalized, cAMP pools may have opposite signaling functions. However, this explanation only holds true if dbcAMP does not reach sites where sAC-derived cAMP has its opposite effect. This is the case for mitochondria. Thus, forskolin and dbcAMP produce elevated cAMP levels in the cytosol but this does not reach mitochondria, whereas sAC is localized in mitochondria which have their own pool of cAMP and PKA [36,37]. Hence, it is possible that *Ca2* expression is inhibited by activation of PKA in the cytosol, but stimulated by the mitochondrial sAC-cAMP-PKA axis. Obviously, this would require an as yet to be identified transcriptional signal from the mitochondria to the nucleus.

Another possibility for the anomalous transcriptional behavior of *Ca2* is that cytosolic cAMP regulates Ca2 transcription via an incoherent feedforward regulatory loop [38], whereby cAMP has opposing effects on *Ca2* expression: stimulation mediated directly through p-CREB but indirect inhibition mediated by the inducible repressor ICER-1 [25]. If this is the case, a bell-shaped biphasic response of *Ca2* expression with regard to cAMP levels is expected, with maximal levels at intermediate cAMP concentrations. Inhibition of sAC might shift the system toward the initial part of the curve, where low concentrations of cAMP lead to lack of *Ca2* expression. On the other hand, dbcAMP and forskolin create cAMP levels that shift to the other side of the bell-shaped curve. In this regard it must be stressed that both forskolin and dbcAMP usually induce very high cAMP concentrations. AE2 dysfunction would induce intracellular accumulation of bicarbonate, increase cAMP signaling through sAC beyond the optimal cAMP concentration and repress *Ca2* expression. While our data in wild type cells seem to be in line with this scenario (Fig. 7), titration with KH7 did not uncover cAMP levels at which *Ca2* expression was de-repressed, so it is likely that in $Ae2^{-/-}_{a,b}$ fibroblasts another mechanism is operative which uncouples expression from its normal cAMP-dependency.

Regardless of the detailed molecular mechanisms of transcriptional repression, which warrant further investigation, this report establishes that cAMP can play a dual role in the regulation of *CA2* expression, and prompts to evaluate its modulating effect, especially in tissues where CA2 activity has proven critical for normal physiology.

Acknowledgements

This research was supported by a grant from the Netherlands Organization for Scientific Research (NWO; program grant 912-02-73).

References

[1] S. Breton, The cellular physiology of carbonic anhydrases, JOP 2 (2001) 159–164.
[2] R.P. Henry, Multiple roles of carbonic anhydrase in cellular transport and metabolism, Annu. Rev. Physiol. 58 (1996) 523–538.
[3] S. Lindskog, Structure and mechanism of carbonic anhydrase, Pharmacol. Ther. 74 (1997) 1–20.
[4] W.S. Sly, P.Y. Hu, Human carbonic anhydrases and carbonic anhydrase deficiencies, Annu. Rev. Biochem. 64 (1995) 375–401.
[5] V.W. Campbell, T. Yamada, Acid secretagogue-induced stimulation of gastric parietal cell gene expression, J. Biol. Chem. 264 (1989) 11381–11386.

[6] A.V. Rousselle, D. Heymann, Osteoclastic acidification pathways during bone resorption, Bone 30 (2002) 533–540.
[7] W.S. Sly, D. Hewett-Emmett, M.P. Whyte, Y.S. Yu, R.E. Tashian, Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 2752–2756.
[8] S.E. Lewis, R.P. Erickson, L.B. Barnett, P.J. Venta, R.E. Tashian, N-ethyl-N-nitrosourea-induced null mutation at the mouse Car-2 locus: an animal model for human carbonic anhydrase II deficiency syndrome, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 1962–1966.
[9] S. Dragon, R. Baumann, Erythroid carbonic anhydrase and hsp70 expression in chick embryonic development: role of cAMP and hypoxia, Am. J. Physiol. Regul. Integr. Comp. Physiol. 280 (2001) R870–R878.
[10] J.P. David, M. Rincon, L. Neff, W.C. Horne, R. Baron, Carbonic anhydrase II is an AP-1 target gene in osteoclasts, J. Cell. Physiol. 188 (2001) 89–97.
[11] L.R. Marino, Characterization of the mouse carbonic anhydrase II gene promoter, J. Biol. Chem. 268 (1993) 7081–7089.
[12] X. Zhang, D.T. Odom, S.H. Koo, M.D. Conkright, G. Canettieri, J. Best, H. Chen, R. Jenner, E. Herbolsheimer, E. Jacobsen, S. Kadam, J.R. Ecker, B. Emerson, J.B. Hogenesch, T. Unterman, R.A. Young, M. Montminy, Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 4459–4464.
[13] L.W. Lai, R.P. Erickson, P.J. Venta, R.E. Tashian, Y.H. Lien, Promoter activity of carbonic anhydrase II regulatory regions in cultured renal proximal tubular cells, Life Sci. 63 (1998) 121–126.
[14] M.H. McGowan, J.A. Neubauer, C.A. Stolle, Characterization of the rat carbonic anhydrase II gene structure: sequence analysis of the 5′ flanking region and 3′ UTR, Gene 186 (1997) 181–188.
[15] S.L. Alper, Genetic diseases of acid–base transporters, Annu. Rev. Physiol. 64 (2002) 899–923.
[16] A. Pushkin, I. Kurtz, SLC4 base (HCO_3^-, CO_3^{2-}) transporters: classification, function, structure, genetic diseases, and knockout models, Am. J. Physiol. Renal Physiol. 290 (2006) F580–F599.
[17] D. Sterling, R.A. Reithmeier, J.R. Casey, A transport metabolon. Functional interaction of carbonic anhydrase II and chloride/bicarbonate exchangers, J. Biol. Chem. 276 (2001) 47886–47894.
[18] S.L. Alper, Molecular physiology of SLC4 anion exchangers, Exp. Physiol. 91 (2006) 153–161.
[19] M.F. Romero, Molecular pathophysiology of SLC4 bicarbonate transporters, Curr. Opin. Nephrol. Hypertens. 14 (2005) 495–501.
[20] J.F. Medina, S. Recalde, J. Prieto, J. Lecanda, E. Saez, C.D. Funk, P. Vecino, M.A. van Roon, R. Ottenhoff, P.J. Bosma, C.T. Bakker, R.P. Oude Elferink, Anion exchanger 2 is essential for spermiogenesis in mice, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15847–15852.
[21] G. Boyarsky, M.B. Ganz, R.B. Sterzel, W.F. Boron, pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO_3^-, Am. J. Physiol. 255 (1988) C844–C856.
[22] C. Ramakers, J.M. Ruijter, R.H. Deprez, A.F. Moorman, Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data, Neurosci. Lett. 339 (2003) 62–66.
[23] J. Sakai, E.A. Duncan, R.B. Rawson, X. Hua, M.S. Brown, J.L. Goldstein, Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment, Cell 85 (1996) 1037–1046.
[24] W.F. Boron, Regulation of intracellular pH, Adv. Physiol. Educ. 28 (2004) 160–179.
[25] P. Mardones, J.F. Medina, R.P. Oude Elferink, Activation of cyclic AMP signaling in AE2-deficient mouse fibroblasts, J. Biol. Chem. 283 (2008) 12146–12153.
[26] Y. Chen, M.J. Cann, T.N. Litvin, V. Iourgenko, M.L. Sinclair, L.R. Levin, J. Buck, Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor, Science 289 (2000) 625–628.
[27] K.C. Hess, B.H. Jones, B. Marquez, Y. Chen, T.S. Ord, M. Kamenetsky, C. Miyamoto, J.H. Zippin, G.S. Kopf, S.S. Suarez, L.R. Levin, C.J. Williams, J. Buck, S.B. Moss, The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization, Dev. Cell 9 (2005) 249–259.
[28] B. Mayr, M. Montminy, Transcriptional regulation by the phosphorylation-dependent factor CREB, Nat. Rev. Mol. Cell Biol. 2 (2001) 599–609.
[29] G. Li, J.A. Harton, X. Zhu, J.P. Ting, Downregulation of CIITA function by protein kinase a (PKA)-mediated phosphorylation: mechanism of prostaglandin E, cyclic AMP, and PKA inhibition of class II major histocompatibility complex expression in monocytic lines, Mol. Cell. Biol. 21 (2001) 4626–4635.
[30] G.C. Parry, N. Mackman, Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription, J. Immunol. 159 (1997) 5450–5456.
[31] J.D. Shepard, Y. Liu, P. Sassone-Corsi, G. Aguilera, Role of glucocorticoids and cAMP-mediated repression in limiting corticotropin-releasing hormone transcription during stress, J. Neurosci. 25 (2005) 4073–4081.
[32] E. Galea, D.L. Feinstein, Regulation of the expression of the inflammatory nitric oxide synthase (NOS2) by cyclic AMP, FASEB J. 13 (1999) 2125–2137.
[33] M. Lamas, P. Sassone-Corsi, The dynamics of the transcriptional response to cyclic adenosine 3′,5′-monophosphate: recurrent inducibility and refractory phase, Mol. Endocrinol. 11 (1997) 1415–1424.
[34] A. Molina, A. Segura, F. Garciaolmedo, Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens, FEBS Lett. 316 (1993) 119–122.
[35] M. Tresguerres, L.R. Levin, J. Buck, Intracellular cAMP signaling by soluble adenylyl cyclase, Kidney Int. 79 (2011) 1277–1288.
[36] R. Acin-Perez, E. Salazar, M. Kamenetsky, J. Buck, L.R. Levin, G. Manfredi, Cyclic AMP produced inside mitochondria regulates oxidative phosphorylation, Cell Metab. 9 (2009) 265–276.
[37] K. Lefkimmiatis, D. Leronni, A.M. Hofer, The inner and outer compartments of mitochondria are sites of distinct cAMP/PKA signaling dynamics, J. Cell Biol. 202 (2013) 453–462.
[38] Y. Hart, U. Alon, The utility of paradoxical components in biological circuits, Mol. Cell 49 (2013) 213–221.