Soluble adenylyl cyclase
A regulator of intrinsic cellular functions
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Cyclic AMP and alkaline pH downregulate carbonic anhydrase 2 in mouse fibroblasts

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Abstract

Background: The hydration of CO₂ 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⁻/⁻ 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−/−) 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.
Introduction

In mammals, the synthesis of bicarbonate catalyzed by carbonic anhydrases plays a pivotal role in circulating CO\textsubscript{2} transport, acid-base physiology, gastric acid secretion, bone resorption, and vectorial transport of fluid and electrolytes in various epithelia\textsuperscript{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)\textsuperscript{5,6}. Mutations in carbonic anhydrase 2 have been linked to renal tubular acidosis with osteopetrosis and cerebral calcification in humans\textsuperscript{7} and a similar, albeit milder, phenotype is observed in mice with a chemically induced null mutation of Ca2\textsuperscript{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\textsuperscript{9}, parietal cells\textsuperscript{5} and osteoclasts\textsuperscript{10}; whereas human hepatocytes and HepG2 cells do not show this acute cAMP stimulation of CA2 transcription\textsuperscript{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\textsuperscript{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\textsuperscript{11}. In contrast, the same region is unresponsive to forskolin in HepG2 cells\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsubscript{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)\textsuperscript{18,19}. 


During the analysis of gene expression in murine fibroblasts with a null mutation of the most important isoforms of \( \text{Ae2} \), \( \text{Ae2}a \) and \( \text{Ae2}b \), we found that \( \text{Ca2} \) was strongly repressed, which correlated with chronic intracellular alkalization and increased cAMP synthesis. We hypothesized that stimulation of cAMP synthesis may be derived from the bicarbonate-stimulated activity of soluble adenylyl cyclase (Adcy10) and could result regulation of \( \text{Ca2} \) gene expression in mouse fibroblasts.

In this study, we report that \( \text{Ca2} \) 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 \( \text{Ca2} \) mRNA levels, pointing to complex and divergent roles of cAMP synthesis in the activation and repression of the \( \text{Ca2} \) 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 \( \text{CA2} \).

**Materials and Methods**

**Materials**

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

**Methods**

**Cell culture and treatments**

Fibroblasts were isolated from the peritoneal wall of male \( \text{Ae2}_{a,b}^{+/+} \) and \( \text{Ae2}_{a,b}^{-/-} \) 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 \( \text{L-glutamine} \), 100 U/ml penicillin, 100 μg/ml streptomycin under 10% \( \text{CO2} \). Medium pH was modified by adding 20 mM HEPES, together with proper amounts of \( \text{HCl} \) or \( \text{NaOH} \) to normal DMEM.
Incubations at different $pH_o$ were carried out for 24 hours. 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.

$\textit{pH}_i$ measurements

Half a million cells were seeded on collagen-coated round coverslips and cultured for 48 hours (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 seconds in the 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).

$cAMP$ measurements

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

Quantitative RT-PCR

Five micrograms 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’-TGGGGATACAGCAAGCACAA-3’; reverse primer: 5’-CTTTCAGCACTGCATTG TCC-3’) and $Gapdh$ (forward primer: 5’-TCAATGAAGGGGTCGTGAT-3’; reverse primer: 5’- CGTCCCCGTAGACAAAAATGATG-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\textsuperscript{22}. Results are expressed either as relative expression of ratios to \textit{Gapdh} mRNA levels in arbitrary units or as percentage of the control value, which is the ratio of \textit{Ca2} mRNA to \textit{Gapdh} mRNA in \textit{Ae2}\textsubscript{a,b}\textsuperscript{+/+} fibroblasts cultured at pH\textsubscript{0} 7.4.

\textbf{Cell fractionation and immunodetection of CA2}

Ten million cells were seeded in 10-cm tissue culture plates 48 hours before the experiment. Nuclear, membrane, and cytosolic fractions were prepared as described elsewhere\textsuperscript{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.

\textbf{Statistics}

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

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

pH, homeostasis relies largely on proton and bicarbonate transport at the plasma membrane, which allows cells for rapid adaptation to changes in pH produced by normal metabolism or pathophysiological conditions. In most cells, an important source of these transport substrates is the hydration of CO2 catalyzed by CA2. Fibroblasts obtained from mice lacking the most prominent variants of AE2 (Ae2a,b−/−) develop chronic intracellular alkalosis in culture, due to the absence of AE2-mediated bicarbonate extrusion.

Quantitative RT-PCR analysis shows that Ca2 expression is markedly reduced in Ae2a,b−/− fibroblasts (Figure 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 (Figure 1B). This finding led us to investigate a potential association between alkaline cellular pH and the downregulation of Ca2 observed in Ae2a,b−/− fibroblasts. The pattern of Ca2 mRNA expression in Ae2+/+ fibroblasts cultured at different pH for 24 hours reveals that medium alkalization is a stimulus for Ca2 repression, whereas the opposite is true upon medium acidification (Figure 1C). Immunoblots against CA2 on cytosolic fractions from wild type and knockout cells confirm the pH-dependent expression of Ca2 (Figure 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. Ae2a,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 Ae2a,b−/− cells (Figure 1D).

We measured intracellular pH as a function of changes in extracellular pH in both wild type and Ae2a,b−/− fibroblasts. Figure 2 shows that intracellular pH moderately increased with extracellular pH in both genotypes and in line with our previous findings the intracellular pH was 0.2 pH unit more alkaline in Ae2a,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 Ae2a,b−/− cells was much more dramatic than one might expect on the basis of intracellular pH alone.
Ca2 is downregulated in mouse fibroblasts by chronic cAMP stimulation

Besides an alkaline shift in resting pHo, Ae2αb−/− fibroblasts also display increased total cAMP levels when compared to their wild type counterparts25. This could be due to bicarbonate-mediated activation of the soluble adenylyl cyclase25,26. We tested this hypothesis by analyzing the effect of KH7, a specific inhibitor of sAC that does not affect other adenylyl cyclases27, on cellular cAMP levels in Ae2αb−/− fibroblasts. Figure 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,
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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-hour exposure of wild type mouse fibroblasts to the membrane permeable cAMP analog dibutyryl-cAMP resulted in a 90% reduction of Ca2 mRNA levels (Figure 4A), together with an 86% reduction in protein (Figure 4B). Direct activation of the G protein receptor-coupled adenylyl cyclase with 25 μM forskolin for 24 hours led to a comparable 75% decrease in Ca2 protein expression (Figure 4C). These results show that Ca2 is susceptible to downregulation via a cAMP-dependent pathway in mouse fibroblasts.

Figure 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 for 6.5, the measured intracellular pH in Ae2a,b−/− cells was significantly higher than in wild type cells.
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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-hour treatment with 25 μM forskolin in wild type and Ae2-ab-/- fibroblasts. As shown in Figure 5, Ca2 mRNA expression in Ae2+/+ cells is reduced to 30% of its starting value after 1 hour of treatment, and continues to decline over the course of the experiment for at least 12 hours, after which it appears to undergo a recovery phase reaching 50% of its starting value at 24 hours of treatment. This pattern of response is similar in Ae2-ab-/- fibroblasts, although starting mRNA levels of Ca2 are much lower than in wild type cells and there is no recovery phase at 24 hours, 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.

Figure 3. The effect of KH7 on cellular cAMP content.

Ae2-ab-/- fibroblasts were cultured in the presence of the indicated concentrations of the soluble adenylyl cyclase inhibitor KH7 for 24 hours. 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.
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Paradoxical effect of soluble adenylyl cyclase (sAC) inhibition on the expression of Ca2

Given that bicarbonate-sensitive sAC is upregulated and that cellular cAMP levels are indeed increased in Ae2a,b−/− fibroblasts, we hypothesized that sAC may be the mediator of Ca2 repression in response to elevated pH/\[\text{HCO}_3\] 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 fibroblasts comparable or even lower than those observed in Ae2a,b−/− fibroblasts under normal culture conditions (Figure 6A).

The transcriptional behavior of Ca2 in wild type cells is particularly striking because on the one hand a 24-hour period of elevated cAMP (by incubation with dibutyryl-cAMP 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.
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(Figure 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.

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. In contrast, relatively fewer examples of gene repression by cAMP signaling have been reported in detail. 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.
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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\textsuperscript{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\textsuperscript{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. \textit{Ae2\textsubscript{a,b}} fibroblasts present a marked increase in steady state levels of \textit{Icer-1} mRNA\textsuperscript{25}, suggesting that this repressor might indeed be involved in regulating CA2 expression.

Figure 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 hours) or vehicle. Total RNA was prepared and real-time RT-PCR for \textit{Ca2} and \textit{Gapdh} were performed. Results represent the relative expression presented as the mean of \textit{Ca2}/\textit{Gapdh} mRNA ratios for each group ± SD. (n = 3, **P < 0.01, ***P < 0.001).
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 dibutyryl-cAMP 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 Ae2a,b−/− fibroblasts, as well as in response to alkaline pH0 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.

In apparent contradiction with this model, sAC inhibition with KH7 did not abate Ca2 repression in Ae2a,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 dibutyryl-cAMP 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.

Figure 7. Titration of wild type and Ae2a,b−/− fibroblasts with KH7.

Cells were cultured with the indicated concentration of KH7 for 24 hours. Subsequently mRNA was isolated and quantified by real-time RT-PCR. Results represent the relative expression presented as the mean of Ca2/Gapdh mRNA ratios for each group ± SD (n = 3).
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 dibutyryl-AMP does not reach sites where sAC-derived cAMP has its opposite effect. This is the case for mitochondria. Thus, forskolin and dibutyryl-AMP 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, dibutyryl-AMP 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 dibutyryl AMP 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 (Figure 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.
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