Activation of Cyclic AMP Signaling in Ae2-deficient Mouse Fibroblasts

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Anion exchanger 2 (AE2, SLC4A2) is a ubiquitously expressed membrane solute carrier that regulates intracellular pH (pH_i) by exchanging cytosolic bicarbonate for extracellular chloride. We used fibroblasts from Ae2-deficient (Ae2a,b−/−) mice to study the effects of an alkaline shift in resting intracellular pH (pH_i) on the activation of cAMP signaling and gene expression. AE2a,b−/− fibroblasts showed increased pH_i (by 0.22 ± 0.03 unit) compared with wild type cells at extracellular pH (pH_e) 7.4 and 37 °C. This shift in resting pH_i is associated with an up-regulation of bicarbonate-activated soluble adenylyl cyclase expression, increased cAMP production, Creb phosphorylation, inducible cAMP early repressor 1 mRNA expression, and impaired activation of c-Fos transcription by forskolin. These results highlight the importance of bicarbonate transport via AE2 in maintaining pH_i homeostasis in cultured mouse fibroblasts and unveil the role of cAMP in the cellular response to chronic alkalization, which putatively includes an inducible cAMP early repressor 1-mediated attenuation of phosphorylated Creb activity.

Fluctuations of intracellular and extracellular pH are among the most frequent challenges that living cells must overcome during their life span. The first line of defense against potentially harmful pH_i changes, apart from the limited buffering capacity of the cytosol, is comprised of several electrolyte transporter proteins in the plasma membrane, which can import or export acid-base equivalents in a pH_e-dependent manner (1). Among these, the sodium-independent anion exchanger family of transporters (AE, SLC4A1–3) mediates the extrusion of base equivalents in the form of bicarbonate, coupled with the electroneutral import of chloride (2). In particular, AE2 (SLC4A2) is considered to be a housekeeping regulator of pH_i because of its broad pattern of expression (3, 4), combined with the ability to respond to intracellular alkalization by increasing its transport activity (5). These properties distinguish AE2 from the closely related transporters AE1 and AE3. AE1 (SLC4A1) expression is highly restricted to the erythroid cell lineage and acid-secreting cells in the kidney, and its activity is not regulated by pH. AE3 (SLC4A3) is mostly expressed in excitable cells from cardiac and neural tissue.

The intrinsic pH-sensing properties of most acid-base transporters allow for rapid cellular adaptation to transient pH_e changes. Chronic pH_e or pH_i changes, on the other hand, may trigger alternative long term responses as to maintain pH_i homeostasis or adapt to a new pH_i set point. Even though changes in gene expression in response to acid and/or alkaline challenges in several cell types have been reported (6, 7), the signaling pathways and molecular targets leading to these adaptive responses remain largely unknown.

It has been proposed that soluble adenylyl cyclase (sAC), a distinct form of adenylyl cyclase activated by bicarbonate could function as both a metabolic and a pH_i sensor (8). sAC is insensitive to G-protein-coupled receptors and is therefore fundamentally different from the membrane-bound adenylyl cyclases. In this way, sAC would constitute a second line of response to pH_i disturbances, one that could have an impact on cell signaling, protein trafficking, and gene expression (8–10). sAC is abundantly expressed in male germ cells, where it plays an essential role in bicarbonate-mediated spermatozoa maturation and capacitation (11, 12). sAC expression has also been detected in many other tissues and cell lines of both murine and human origin (13, 14). Because of its bicarbonate responsiveness, sAC appears as a suitable candidate for initiating at least part of a cAMP-dependent component of pH_i regulation (15). If so, it would imply that changes in intracellular bicarbonate concentration, ultimately controlled by the rates of bicarbonate/proton generation, consumption, and/or transport, are the primary signals in this pathway.

Our group has generated mice with a targeted mutation in the Ae2 gene (16). This mutation abolishes the expression of the three major variants of Ae2, which are generated by a combination of alternative splicing and alternative promoter usage (3, 4): Ae2a, the most abundant and ubiquitous isoform, plus Ae2b1 and Ae2b2, whose expression is more restricted to epithelial cells of the gastric mucosa, kidney, and liver. The two remaining variants, Ae2c1 and Ae2c2, are almost exclusively

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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expressed at rather low levels in gastric epithelium, and the function of Ae2c2 as a bona fide bicarbonate transporter has recently been questioned (17). Because neither of the two c isoforms was targeted in our model, we termed these mice Ae2a,b−/−. Ae2a,b−/− mice display some overt phenotypes, namely male infertility, impaired gastric acid secretion, and osteopetrosis (16, 18).

In the present study we investigated the effects of the Ae2a,b null mutation on resting pH, sAC expression, and cAMP signaling in mouse fibroblasts, a relatively less specialized cell type, isolated from Ae2a,b−/− mice. Our results point to a novel pathway of pH-dependent gene regulation in murine fibroblasts and support a model in which sAC functions as a pH/bicarbonate sensor, generating significant and possibly highly localized signals in the form of cAMP, which in turn leads to Creb phosphorylation and strongly increased expression of the inducible cAMP early repressor protein 1 (Icer1), resulting in a marked attenuation of pCre-mediated transcriptional activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise indicated, all reagents and chemicals were purchased from Sigma.

**Cell Culture and Treatments**—Fibroblasts were isolated from the peritoneal wall of male Ae2a,b+/+ and Ae2a,b−/− mice of the same genetic background (FVB/N) (16). 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, the tissue debris was removed by low speed centrifugation (50 × g), and cells in the supernatant were spun down at 1000 × g. The cells were cultured in DMEM (Cambrex, Verviers, Belgium) containing 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin under 10% CO2.

Medium pH was modified as required by adding 20 mM HEPES, plus proper amounts of HCl or NaOH to normal DMEM. All incubations at different pH were carried out for 24 h. Forskolin or vehicle (0.01% Me2SO) were applied from 1000 μM forskolin, or vehicle (0.01% Me2SO) were applied for the experiment a single-point calibration was performed by perfusing chamber and perfused at 0.7 ml/min with standard Hanks’ balanced salt solution at pH 7.4.

**pH, Measurements**—5 × 105 cells were cultured on round coverslips (20-mm diameter), after which they were washed with Hanks’ balanced salt solution (Cambrex, Verviers, Belgium) and incubated for 10 min with 5 μM BCECF-AM (Molecular Probes, Eugene, OR) in Hanks’ balanced salt solution at 37 °C. BCECF-loaded cells were mounted in a custom-made perfusion chamber and perfused at 0.7 ml/min with standard HEPES-buffered solution (19) at 37 °C. After 1 h of equilibration, fluorescence (535-nm excitation, 490- and 440-nm emission) was monitored every 30 s in a Novostar multilplate 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 (19). pH 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, between 5.8 and 8.2. The standard curve was adjusted to pass through the point (fluorescence ratio, 1.0; pH 7.0) and subjected to least squares nonlinear fitting as described elsewhere (19). All of the calculations were done in Prism v4.0 (GraphPad Software, San Diego, CA).

**RT-PCR and Quantitative RT-PCR**—Total RNA was isolated from Ae2a,b+/+ and Ae2a,b−/− fibroblasts with TRIzol reagent (Invitrogen), and reverse transcription was performed on 5 μg of total RNA. 0.5 μg of the resulting cDNA was subjected to PCR for Ae2a, Ae2b1, Ae2b2, Ae2c1, Nhe1, Nhe2, Nhe3, sAC (truncated and full length), and Gapdh using the primer pairs listed in supplemental Table S1. Stomach, liver, kidney, testis, and small intestine cDNA samples were used as positive PCR controls (not shown).

Quantitative real time PCR for Ae1, Ae3, Nbc1, Nke1, Slc26a1, Slc26a2, Slc26a6, Ca2, T-sAC, Icer1, and Gapdh was performed on 50 ng of template cDNA in a LightCycler apparatus (Roche Applied Science). Most primer pair sequences for quantitative PCR were obtained from the mouse q PrimerDepot data base (supplemental Table S1) (20). Initial RNA concentrations were calculated by linear regression using LinReg v. 9.16 software (21). The results are expressed either as relative expression of ratios to Gapdh mRNA in arbitrary units or as a percentage of the control value, which in all cases is the ratio of mRNA to Gapdh mRNA in Ae2a,b−/− fibroblasts cultured at pH 7.4.

**Cell Fractionation and Immunodetection of sAC, Creb, pCreb, and Ca2**—1 × 107 cells were seeded 48 h before the experiment. Nuclear, membrane, and cytosolic fractions were prepared as described elsewhere (22). 30 μg of total protein from each fraction were separated by SDS-PAGE and electrotransferred to nylon membranes. Blots of total lysates or appropriate subcellular fractions were incubated as indicated with anti-sAC (R51, 1:1000, kindly provided by Dr. J. Buck), anti-Creb (1:500; Cell Signaling Technology, Danvers, MA), anti-Ser(P)133-Creb (1:500, Cell Signaling Technology, Danvers, MA), anti-Mrp4, or anti-Mrp5 (1:2000; both kindly provided by Dr. G. Scheffer), or anti-β-actin (1:2000; Sigma) antibodies. Proper secondary antibody-peroxidase conjugates (Bio-Rad) were detected in a Lumimager (Roche Applied Science) after incubation with chemiluminescent substrate (Roche Applied Science). Immunoblot signals were normalized using β-actin as a loading control. The results are expressed as fold change relative to Ae2a,b+/+ fibroblasts under normal culture conditions.

**cAMP Measurements**—Total, extracellular, and intracellular cAMP was measured in fibroblast cultures with an enzyme-linked immunosorbent assay kit (Amersham Biosciences). Extracellular cAMP was calculated as the difference between total and intracellular cAMP concentrations for each sample. Treatments with 10 μM rolipram, 25 μM forskolin, or vehicle (Me2SO) were applied from 1000X stocks 12 h after seeding and 12 h prior to the determination of cAMP. The results are expressed as fmol of cAMP/106 cells.

**Statistics**—The results are expressed as the means ± S.D. Sample size corresponds to the number of independently processed cell preparations. 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 of the data are representative of at least two independent experiments.
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RESULTS

Ae2 Expression in Murine Fibroblasts—We performed RT-PCR analysis on total RNA samples from wild type and knockout fibroblasts to assess the expression of Ae2 isoforms. Of the five Ae2 variants, only Ae2a, Ae2b1, and Ae2b2 mRNAs were detected in wild type fibroblasts (Fig. 1A). Ae2a appears to be the predominant variant in this cell type, which is in good agreement with the general pattern of expression of Ae2 isoforms (4). On the other hand, Ae2a,b−/− cells express low levels of a mutant Ae2 transcript, which yields a PCR product of expected smaller size (Fig. 1A, top panel). This mutant transcript lacks a region comprising three exons (2, 1a, and 1b), which contains the start codons for Ae2a, b1, and b2 (16). Exon 1 constitutes only part of the 5′-untranslated region of Ae2 mRNA; therefore this mutant mRNA is not translated into a functional polypeptide (18). Ae2c1, the only c isoform with significant anion exchanger activity (17), could not be detected in Ae2a,b−/− cells (data not shown).

Relative Expression of Acid-Base Transporters and Resting pHi in Ae2+/+ versus Ae2a,b−/− Fibroblasts—The lack of Ae2 transport activity in knockout fibroblasts might lead to compensatory changes in the expression of other acid-base transporters. The electrogenic sodium/bicarbonate cotransporter 1 (NBCe1, Slc4a4) and the sodium/potassium/2 chloride cotransporter 1 (Nkcc1, Slc12a2) mediate sodium-driven bicarbonate and chloride import, respectively, in several tissues (23). In nonepithelial cells NBCe1 participates in pHi regulation by protecting cells from intracellular acid loads. Nkcc1, on the other hand, is responsible for maintaining intracellular chloride homeostasis and regulating cellular volume (23). Both transporters share overlapping substrates and opposing functions with Ae2. Other candidates that may compensate for the absence of Ae2 are the remaining members of the Slc4 family, the Slc9 family of sodium/proton exchangers (Nhe) (24), and the less specific sulfate-bicarbonate/chloride exchangers from the Slc26 family (25). We quantified relative mRNA expression levels of Slc4a1 (Ae1), Slc4a3 (Ae3), Slc4a4 (NBCe1), Slc26a1 (Sat1), Slc26a2 (Dtdst), and Slc26a6 (Pat-1) (Fig. 1C). Ae1 and Slc26a1 were barely detectable, as could be anticipated considering their rather tissue-specific expression. NBCe1 expression is relatively low in both cell lines (less than 5% of other prominent transporters), whereas Nhe1, Nkcc1, and Slc26a6 expression is comparable between wild type and knockout fibroblasts. Expression of Nhe2 and Nhe3 could not be observed in fibroblasts (Fig. 1B). Interestingly, Ae3 and Slc26a2 mRNA levels are up-regulated in Ae2a,b−/− fibroblasts, by 25 and 100%, respectively. Ae3 is normally expressed in excitable tissue (brain, muscle), and Slc26a2, also known as Dtdst (diastrophic dysplasia sulfate transporter), is expressed at particularly high levels in the intestine, cartilage, and osteoblasts, where it is mainly involved in sulfate import (25). Even though Ae3 is capable of mediating bicarbonate exchange, it is not as efficient as Ae2, nor has it a marked pH-responsive pattern of transport (26). Slc26a2, on the other hand, has no demonstrated bicarbonate transport activity, unlike other members of the Slc26 family (25). Therefore, the physiological significance of Ae3 and Slc26a2 expression, in terms of pHi regulation, is not clearly defined, and their up-regulation in Ae2a,b−/− fibroblasts could rather indicate a regulatory response to altered electrolyte homeostasis.

A more definite proof for the relative importance of Ae2 in pHi homeostasis was obtained by direct measurement of pHi in Ae2-deficient cells. We used the pH-sensitive fluorescent dye BCECF to determine intracellular pH under standard tissue culture-like conditions. Table 1 shows that Ae2a,b−/− fibroblasts display a significant 0.22 ± 0.03 unit increase in resting pHi compared with wild type cells, suggesting that Ae2a is indeed the primary acid loader at the cell surface in murine fibroblasts and that its absence cannot be sufficiently compensated by other bicarbonate extruders present in these cells, such as Ae3.

Soluble Adenylyl Cyclase Expression Is Induced in Ae2a,b−/− Fibroblasts—Recently, Sun et al. (27) reported that bicarbonate up-regulates sAC expression in corneal endothelial and Calu-3 cells. The higher pHi observed in Ae2a,b−/− fibroblasts, when maintained under high extracellular volume and open tissue culture conditions, is likely to be associated with higher intracellular bicarbonate concentration. We therefore performed quantitative RT-PCR to determine sAC expression in mouse fibroblasts. As depicted in Fig. 2B, there is a 4.5-fold increase in mRNA levels for the most active (28), truncated form of sAC (T-sAC) in Ae2a,b−/−, as compared with wild type cells. This is

Table 1: Intracellular pH in mouse fibroblasts

| Genotype      | pHi            |
|---------------|----------------|
| Ae2+/+        | 7.45 ± 0.04    |
| Ae2a,b−/−     | 7.67 ± 0.03*   |

* p < 0.002 (n= 6 cell preparations).
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produced a comparable 7–8-fold increase in total cAMP levels in both Ae2+/+ and Ae2a,b−/− fibroblasts (Fig. 3C), with a much greater fraction of cAMP accumulating in the extracellular medium from Ae2a,b−/− cultures when compared with wild type cells (Fig. 3, D and E). It has been shown that the organic anion transporters Mrp4 and Mrp5 can mediate the active secretion of cAMP and/or cGMP from cells (30). However, immunoblots stained for Mrp4 and Mrp5 indicate that the expression of these proteins in Ae2a,b−/− fibroblasts is comparable with that observed in wild type cells (not shown).

Treatment with 10 μM rolipram, a phosphodiesterase inhibitor, resulted in a 2.8-fold accumulation of total cAMP in both wild type and knockout cells (Fig. 3B), which suggests that cAMP degradation is not significantly compromised by the absence of Ae2 activity. Taken together, these results show that cAMP synthesis is increased in Ae2a,b−/− cells, resulting in stimulated cAMP secretion.

Creb, pCreb, and Icer1 Expression in Ae2+/+ versus Ae2a,b−/− Fibroblasts—The data presented so far suggest that Ae2-deficient fibroblasts are capable of maintaining normal steady state intracellular cAMP levels by sequestering the excess into the extracellular medium (Fig. 3A). However, it is not clear whether this could prevent the activation of cAMP downstream targets, such as the cAMP-dependent protein kinase (PKA), especially if the source of cAMP production is localized to subcellular compartments or discrete microdomains. Immunoblots against Creb, a well known target of PKA (31), and its Ser133 phosphorylated form (pCreb) show, respectively, a 48% and a 70% increased staining of nuclear fractions from Ae2a,b−/− fibroblasts (Fig. 4A), a good indication of PKA activation.

To evaluate the effectiveness of Creb phosphorylation in Ae2a,b−/− cells, we quantified mRNA levels of Icer1, a truncated form of the cAMP response element modulator protein (Crem) transcript, which translates into a potent transcriptional repressor induced by pCreb and involved in the feedback attenuation of pCreb activity through nonproductive binding to CRE sites (32). Besides its more general role in feedback regulation, Icer1 is involved in a number of physiological processes that require precise transient activation of cAMP signaling, such as spermatogenesis and circadian protein expression, by effectively suppressing waves of gene activation generated by pCreb (33, 34).

Fig. 4B shows that Icer1 mRNA concentration is 6-fold higher in Ae2a,b−/− fibroblasts compared with wild type cells at pH 7.4. In addition, Icer1 shows a pH-dependent pattern of expression in wild type cells, where it is significantly induced upon medium alkalinization (Fig. 4B). A similar tendency is observed in knockout fibroblasts, but Icer1 mRNA expression remains significantly higher than controls under all experimental conditions (Fig. 4B). Collectively, these results suggest that known targets of cAMP signaling via PKA are activated in Ae2-deficient cells and may play an important role in regulating pH-dependent gene expression in murine fibroblasts.

Kinetics of Icer1, Crem, and c-Fos mRNA Expression in Response to Forskolin—Based on the increase in CAMP production and the strong induction of Icer1 mRNA in Ae2a,b−/− fibroblasts, we hypothesized that the activation of gene transcription through the cAMP-PKA-Creb pathway could be

also apparent in a conventional RT-PCR (Fig. 2A). Similar results were obtained for the full-length form of sAC (data not shown). Immunoblots of subcellular fractions show that sAC protein levels are also higher in knockout fibroblasts, albeit not to the same extent as mRNA (Fig. 2C). The majority of sAC immunoreactivity was found in nuclear fractions, in good agreement with previous reports in COS7 cells and other cell types (10, 29). A small fraction of sAC co-purified with cellular membranes (Fig. 2C), and an even smaller amount, per μg of total protein, was found in cytosolic fractions (not shown). Nuclear sAC protein levels are 40% higher in Ae2a,b−/− when compared with wild type fibroblasts.

CAMP Production Is Increased in Ae2a,b−/− Fibroblasts—We hypothesized that the combination of higher pH (or [HCO3−]) and up-regulated sAC expression in Ae2a,b−/− cells might result in increased rates of cAMP synthesis and higher steady state levels of cAMP. As shown in Fig. 3, there is a significant 30% increase in total cAMP in Ae2a,b−/− fibroblasts. Interestingly, this difference was accounted for by a 100% increase in extracellular cAMP (Fig. 3A), suggesting that cAMP export pathways are more active in knockout cells. This was corroborated by treating cells with 25 μM forskolin, which does not affect sAC activity but activates adenylate cyclase at the plasma membrane to produce maximal amounts of cAMP. Forskolin

FIGURE 2. Analysis of sAC expression in Ae2+/+ and Ae2a,b−/− fibroblasts. RT-PCR (A) and quantitative RT-PCR (B) were performed on total RNA samples from fibroblasts. The results are expressed as percentages of control sAC/Gapdh mRNA ratio and presented as the means ± S.D. (n = 5; *, p < 0.0001). After cell fractionation, nuclear and total membrane fractions were immunostained for the most active truncated variant of sAC and β-actin (C). The results are expressed as fold change with respect to TsAC/β-actin signal ratios in Ae2+/+ fibroblasts (n = 4; *, p < 0.01).
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more widely affected in these cells. To test this hypothesis, we followed the expression of Icer1, Crem, and c-Fos (a prominent target of pCreb) in time, upon treatment with the PKA agonist forskolin. Forskolin activates membrane-bound adenylate cyclase and not sAC; as will be clear from Fig. 3, forskolin induces maximal intracellular cAMP production. In this way forskolin was applied to cells for 12 h and total (Fig. 3A), extracellular (Fig. 3B), and intracellular (Fig. 3C) cAMP was determined (n = 5; *, p < 0.002 with respect to Ae2+/+/fibroblasts under the same culture conditions; †, p < 0.004 with respect to control Ae2+/+/fibroblasts). The results are expressed as fmol of cAMP/10^6 cells. All of the data are represented as the means ± S.D.

DISCUSSION

The exclusive expression of Ae2α, Ae2β1, and Ae2β2 in mouse fibroblasts provides a tractable cellular model of complete Ae2 deficiency, derived from Ae2a,b−/− mice. In the present study, we used this model to investigate the role of an altered alkaline pH set point on signal transduction through cAMP.

The 0.22-unit shift toward a higher resting pH in knockout fibroblasts is consistent with Ae2 functioning as an acid loader at the plasma membrane. The relative importance of Ae2 for pH homeostasis in murine fibroblasts is underscored by the inability of other acid-base transporters to counterbalance intracellular alkalosis in the absence of Ae2.

Since the identification of a soluble form of adenyl cyclase activated by bicarbonate binding (36), it has been proposed that cAMP could participate as a signaling molecule in response to alkalosis and bicarbonate concentration and/or pH (8, 9). This has been partly confirmed in some cellular models (15), but to this date there is little evidence of direct involvement of cAMP signaling in cellular acid-base homeostasis.
Here we demonstrate a direct link between chronic intracellular alkalization and significantly increased cAMP synthesis and signaling, not only in Ae2-deficient cells, but also in wild type fibroblasts incubated in alkaline medium (Figs. 3A, 4, and 5D). We hypothesize that sAC plays a key role in this process. Indeed, it is unlikely that the observed higher intracellular pH and increased sAC expression (Table 1 and Figs. 2 and 3) would result in unaltered cAMP production through SAC, inasmuch as an increase in intracellular pH would favor the dephosphorylation of phosphorylated Creb, and increased sAC expression (Table 1 and Figs. 2 and 3) would result in unaltered cAMP production through SAC, inasmuch as an increase in intracellular pH would favor the dephosphorylation of phosphorylated Creb, and the increased sAC expression would result in increased cAMP production through SAC.

A thorough analysis of the kinetics of cAMP production in Chinese hamster ovary cells stimulated with a number of β-adrenergic agonists (37) has revealed that extracellular cAMP accumulation in time is a distinctive feature of increased cAMP synthesis, especially under prolonged adenylyl cyclase stimulation. In the same study, it was shown that the transcriptional activity of phosphorylated Creb is strongly induced even in the absence of significant changes in intracellular cAMP concentration upon treatment with weak β-adrenergic agonists. The authors concluded that a high turnover of newly synthesized cAMP is a distinctive feature of increased cAMP synthesis, especially under prolonged adenylyl cyclase stimulation.

![Graph](Image)

**Figure 4.** Creb phosphorylation and quantitative real time PCR of Icer1 mRNA in Ae2+/+ and Ae2+/− fibroblasts. Nuclear extracts were obtained from Ae2+/+ and Ae2+/− fibroblasts, and 30 μg of total protein was subjected to SDS-PAGE followed by immunoblotting with anti-Creb, anti-pCreb, and anti-β-actin antibodies (A). The results are expressed as fold change with respect to pCreb/β-actin signal ratios in wild type fibroblasts (n = 4; *, p < 0.05). Fibroblasts were incubated at different pH levels for 24 h, and total mRNA was isolated followed by quantitative RT-PCR for Icer1 and Gapdh (B). The results are expressed as percentages of control Icer1/Gapdh mRNA ratio and presented as the means ± S.D. (n = 5; *, p < 0.003, with respect to Ae2+/+ fibroblasts at the same culture conditions; †, p < 0.04 with respect to Ae2−/− fibroblasts at pH 7.4).

**Figure 5.** Kinetics of Icer1, Crem, and c-Fos mRNA expression in response to forskolin. Cells were treated with 25 μM forskolin for the indicated time, after which total RNA was obtained and subjected to quantitative RT-PCR for Icer1 (A), Crem (B), and c-Fos (C) (n = 4; *, p < 0.0001). The results are expressed as relative mRNA expression, normalized using Gapdh mRNA as reference, in arbitrary units. A separate set of cultured fibroblasts was incubated at pH 7.4 or pH 8.0 in normal DMEM for 24 h, total mRNA was prepared, and quantitative RT-PCR of c-Fos mRNA was performed (D). The results are expressed as percentages of control (pH 7.4) expression using Gapdh mRNA as reference for normalization (n = 4; *, p < 0.02 with respect to Ae2+/+ fibroblasts at pH 7.4; †, p < 0.002 with respect to Ae2−/− fibroblasts at pH 8.0). All of the data are presented as the means ± S.D.
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The Ae2 protein is more important for the activation of pCREB transcriptional activity than the absolute amount of intracellular cAMP. Similarly, we consider the extracellular accumulation of cAMP in Ae2-deficient fibroblasts (Fig. 3, A and D) a clear indication of chronic stimulation of cAMP synthesis and the apparently normal intracellular cAMP concentration (Fig. 3A) as a sign of compartmentalization and higher turnover of cAMP. In addition, Ae2<sup>−/−</sup> cells may try to counterbalance the chronically increased cAMP production by increased expression of a cAMP efflux system at the plasma membrane. This contention is strongly supported by the observation that maximal cAMP production with forskolin, which involves membrane-bound adenylate cyclase and not sAC, also results in higher cAMP excretion from Ae2<sup>−/−</sup> cells as compared with Ae2<sup>+/+</sup> cells.

It is generally accepted that cAMP production and activity can be spatially constrained to certain subcellular regions, mainly because of the assembly of molecular complexes of adenyl cyclases, phosphodiesterases, and protein kinase A anchoring proteins, that limit or direct the range of action of cAMP to neighboring targets (38, 39). In the case of sAC, a potential factor contributing to the limited range of cAMP action is its confinement to membrane-bound subcellular compartments. Although sAC was initially characterized as a “soluble” protein (i.e. not anchored to membranes), now it is known to localize to specific subcellular organelles, mainly nucleus and mitochondria (29). We observed predominant nuclear localization of sAC in murine fibroblasts (Fig. 2C) and therefore speculate that the bulk of cAMP synthesis in response to chronic intracellular alkalosis, as well as the most direct effector events, should take place in the nucleus of Ae2-deficient fibroblasts. A better understanding of the cross-talk between different pathways of cAMP generation could shed further light into the compartmentalized nature of cAMP signaling. In this regard, Ae2-deficient cells could represent an extreme case, in which a highly localized source of cAMP production, i.e. nuclear sAC, percolates into other canonical targets. The observed changes in forskolin-induced expression of Icer1, Crem, and c-Fos suggest that nuclear cAMP production might fine tune the kinetics of transcriptional regulation by forskolin-induced cAMP production at the plasma membrane. If true, this might be a good model for studying the interaction between different cAMP signaling pathways.

Even though our study focused on one general aspect of transcriptional regulation by cAMP, namely via CREB phosphorylation by PKA, the pleiotropic nature of cAMP action (40) leads us to propose that other cellular and molecular events such as kinase and phosphatase activities, ion channel function, and vesicular trafficking may also be affected by the absence of Ae2 in murine fibroblasts. Similarly, transcriptional activation mediated by phospho-CREB is likely to have a more extended impact on gene expression. The attenuated response of c-Fos to forskolin (Fig. 5C), and the effect of pH on c-Fos expression (Fig. 5D) is particularly interesting, considering the number of genes that are regulated by the AP-1 family of transcription factors (41). The possible consequences of c-Fos attenuation over cell growth and other signaling pathways in Ae2-deficient fibroblasts invite further investigation.

The CREM functions as a key regulator of cAMP signaling in many tissues and cell types, sometimes potentiating and other times counteracting the action of phospho-CREB (42). CREM expression has shown to be affected by cAMP signaling (34); therefore it was important to determine CREM mRNA levels in Ae2<sup>−/−</sup> fibroblasts to weigh its influence on downstream targets and, perhaps more importantly, to test the specificity of Icer1 activation, i.e. whether increased expression of Icer1 (a truncated form of CREM) could be accounted for by an up-regulation of full-length CREM transcripts rather than by the specific activation of Icer1 transcription. Our results clearly indicate that Icer1 induction is not secondary to CREM up-regulation, although the 2-fold increased CREM mRNA levels in Ae2<sup>−/−</sup> fibroblasts might partially contribute to the apparent “hypersensitization” of Icer1 transcription to forskolin treatment, provided that CREM mRNA availability was a limiting factor for the transcriptional activation of Icer1.

When analyzing Icer1, CREM, and c-Fos mRNA expression in cells under forskolin treatment, we clearly distinguished three different patterns of response. The most straightforward interpretation of these data is that chronic, alkalosis-driven cAMP stimulation in Ae2<sup>−/−</sup> cells leads to enhanced Icer1 transcription, causing a much stronger increase in Icer1 levels after treatment with forskolin. The expression of this potent repressor can, in turn, attenuate the induction of c-Fos and, presumably, other downstream targets of cAMP signaling in a way that pH regulation in murine fibroblasts but a common feature of cAMP signaling in other responsive cell types.

The in vivo relevance of the observations presented in this study remains to be carefully assessed; however, it is important to note that some of the phenotypes developed by Ae2-deficient mice, such as male infertility and osteopetrosis, could have disrupted cAMP signaling as an underlying, contributing, and/or aggravating factor. In particular, male germ cells, and cells forming bone structures are known to rely, at least in part, on cAMP signaling either for differentiation or proper activity. Suboptimal bicarbonate supply, sAC activity, or CREM expression in male germ cells can halt their differentiation into mature spermatozoa or hinder their capacitation (12, 35). The detailed molecular mechanisms of male infertility in Ae2<sup>−/−</sup> mice have yet to be elucidated; thus the results presented here provide a sound framework to foster future lines of research in this area.

Recently, a mouse model of sAC deficiency has been reported (12). Male infertility is the only overt phenotype of sAC knockout mice, and the consequences of sAC deficiency on acid-base or other aspects of physiology are presently unknown. The strong effect of Ae2 deficiency on cAMP levels and downstream targets, like Icer1 and c-Fos, in our model system suggests that subtler responses could be expected during transient changes in intracellular bicarbonate or pH, but to easily detect them in a physiologically relevant context, a major disruption of bicar-
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bonate transport may be needed. We suggest that tissue-specific Ae2 inactivation in sAC-deficient mice, or vice versa, could provide valuable insight into the role of cAMP in acid-base homeostasis in vivo.

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