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Soluble adenylyl cyclase accounts for high basal cCMP and cUMP concentrations in HEK293 and B103 cells

Alan Hasan [a], Kerstin Y. Danker [a,1], Sabine Wolter [a], Heike Bähre [a,b], Volkhard Kaever [a,b], Roland Seifert [a,*]

[a] Institute of Pharmacology, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany
[b] Research Core Unit Metabolomics, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

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ABSTRACT

Intact HEK293 cells and B103 neuroblastoma cells possess high basal concentrations of the established second messengers cAMP and cGMP and of the emerging second messengers cCMP and cUMP. We asked the question which nucleotidyl cyclase accounts for the high basal cNMP concentrations. Activators and inhibitors of soluble guanylyl cyclase had no major effects on cNMPs, and the activator of membranous adenylyl cyclase forskolin increased only cAMP. Addition of bicarbonate to medium increased, whereas removal of bicarbonate decreased levels of all four cNMPs. The inhibitor of soluble adenylyl cyclase, 2-(1*H*-benzo[*d*]imidazol-2-ylthio)-*N*′-(5-bromo-2-hydroxybenzylidene) propanehydrazide (KH7), reduced bicarbonate-stimulated cNMPs. In conclusion, bicarbonate-stimulated soluble adenylyl cyclase plays an important role in the regulation of basal cellular cNMP levels, most notably cCMP and cUMP.

1. Introduction

NCs convert NTPs to the corresponding cNMPs and PP_i [1–4]. Mammalian cells express nine mACs (isoforms 1–8 are activated by the diterpene FSK) and a bicarbonate-stimulated sAC [2,5,6]. Both FSK and bicarbonate activate their target enzymes by an evolutionary conserved allosteric mechanism [5,7]. Moreover, mammalian cells express seven pGCs and a NO-stimulated sGC [3,4]. It is generally assumed that NCs are base-specific, i.e. ACs produce cAMP, whereas GCs produce cGMP [1–4]. However, over the past decade, it has become increasingly clear that NCs exhibit a rather broad base-specificity, i.e. they are potently inhibited by 2′,3′-N-methylanthraniloyl- and 2′,3′-(2,4,6)-trinitrophenyl-substituted purine- and pyrimidine nucleotides [8–10]. Based on these data, the question arose whether NCs are also promiscuous with respect to their substrates. In fact, the bacterial "AC" toxins edema factor from *Bacillus anthracis* and CyaA from *Bordetella pertussis* were the first NCs unequivocally demonstrated to generate cCMP and cUMP, in addition to the "cognate" product cAMP [11,12]. Subsequently, sGC was shown to generate cCMP and cUMP, in addition to cGMP, in a NO-dependent manner both at the level of the purified enzyme and the intact cell [13,14]. Intriguingly, generation of individual cNMPs by NCs is differentially regulated by divalent cations [11–14]. The development of highly sensitive and specific HPLC–MS/MS and HPLC–MS/TOF methods was very important for proofing cCMP and cUMP formation by sGC and toxins [11–14]. In marked contrast to sGC and bacterial AC toxins, the pGC isoforms A, E and F do not generate cCMP and cUMP [12]. With regard to mACs and sAC, to the best of our knowledge, it has not yet been examined whether these enzymes produce only cAMP or also other cNMPs.

During the course of our studies on NCs we serendipitously observed that HEK293 cells possess high basal cCMP and cUMP levels [12]. In a survey study of various mammalian cell types we noticed that B103 neuroblastoma cells possess high basal cCMP and cUMP levels as well [15]. Intriguingly, both HEK293 cells and B103 cells are derived from the neuronal cell lineage [16]. sAC

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cCMP, cytidine 3′,5′-cyclic monophosphate; cNMP, 3′,5′-cyclic nucleoside monophosphate; cUMP, uridine 3′,5′-cyclic monophosphate; pGC, particulate guanylyl cyclase; CAH, carbonic acid anhydrase; mAC, membranous adenylyl cyclase; sAC, soluble adenylyl cyclase; sGC, soluble guanylyl cyclase; NO, nitric oxide; FBS, fetal bovine serum; PDE, phosphodiesterase; FSK, forskolin; KH7, 2-(1*H*-benzo[*d*]imidazol-2-ylthio)-*N*′-(5-bromo-2-hydroxybenzylidene) propanehydrazide; IBMX, 3-isobutyl-1-methylxanthine; NC, nucleotidyl cyclase; ODQ, [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; HPLC–MS/MS, high performance liquid chromatography tandem mass spectrometry; HPLC–MS/TOF, high performance liquid chromatography quadrupole time of flight mass spectrometry; SNP, sodium nitroprusside; NTP, nucleoside 5′-triphosphate.

* Corresponding author. Fax: +49 511 532 4081.
E-mail addresses: hasan.alan@mh-hannover.de (A. Hasan), kerstin.danker@evotec.com (K.Y. Danker), wolter.sabine@mh-hannover.de (S. Wolter), baehre.heike@mh-hannover.de (H. Bähre), kaever.volkhard@mh-hannover.de (V. Kaever), seifert.roland@mh-hannover.de (R. Seifert).
[1] Present address: Evotec AG, Essener Bogen 7, D-22419 Hamburg, Germany.

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was cloned from HEK293 cells [17], and astrocytes, developmentally related to neuroblastoma cells [18], express sAC at high levels, too [19]. Additionally, 2′,3′-N-methylanthraniloyl- and 2′,3′-(2,4,6)-trinitrophenyl substituted purine- and pyrimidine nucleotides inhibit sAC with similar potency [9]. Based on these data, in the present study, we asked the question whether sAC plays a role in the regulation of basal cCMP and cUMP concentrations in intact cells.

2. Materials and methods

2.1. Materials

IBMX, FSK and M7403 medium were purchased from Sigma–Aldrich (Seelze, Germany). DMEM high glucose 4.5 g/L, MEM, penicillin, streptomycin, L-glutamine, and Dulbecco's PBS were purchased from PAA (Pasching, Austria). SNP (Merck, Darmstadt, Germany) was freshly dissolved as 100 mM stock solution in 100 mM sodium acetate, pH 5.0, using a light-protected brown tube. KH7 (4.8 mM, Biozol, Eching, Germany) and FSK (10 mM) were prepared as stock solutions in dimethyl sulfoxide. Dimethyl sulfoxide up to 1% (v/v) had no effect on cNMP levels in the short-term experiments conducted in this study (data not shown). Fetal bovine serum was obtained from Lonza (Verviers, Belgium). HPLC-grade acetonitrile, methanol and water were supplied by Baker (Deventer, The Netherlands). Tenofovir was used as internal standard for HPLC–MS/MS experiments and was a kind gift from the National Institutes of Health, AIDS Research and Reference Program, Division of AIDS (Bethesda, MD, USA).

2.2. Cell culture

HEK293 cells were grown in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2 at 37 °C in DMEM high glucose (4.5 g/L) supplemented with 10% (v/v) fetal bovine serum, 200 μg/mL L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin up to 80% confluency. For B103 cells, MEM medium was used instead; all other medium components were like for HEK293 cells. Cells were harvested by aspirating the cell culture medium followed by addition of 300 μL cold extraction solution (4 °C) consisting of acetonitrile/methanol/water (2:2:1, (v/v/v)) and 25 ng/mL tenofovir. Cells were scraped off and the suspension was heated for 20 min at 96 °C. After cooling, cell suspension was centrifuged at 20,000×*g* for 10 min. The supernatant fluid was evaporated completely under nitrogen atmosphere at 40 °C. Residue was dissolved in 150 μL water and analyzed as described in analysis of cNMPs in intact cells. For determination of protein concentration, cell pellets were dried at room temperature. Dried cell pellets were dissolved in 0.1 M sodium hydroxide at 96 °C for 20 min. Ten microlitres of protein solution were taken for quantitation of protein concentration by means of bicinchoninic acid protein assay. Transfection of HEK293 cells with pcDNA 3.0 plasmids encoding for pGCs C, D or G was performed as described recently for sGC [13].

2.3. Analysis of cNMPs in intact cells

cNMP quantitation was performed via HPLC–MS/MS as described using a QTrap 5500 triple quadrupole mass spectrometer (ABSCIEX, Foster City, CA, USA) [13]. Ion source settings and collision gas pressure were manually optimized regarding ion source voltage, ion source temperature, nebulizer gas, and curtain gas (ion source voltage of 5500 V, ion source temperature of 400 °C, curtain gas of 30 psi, collisionally activated dissociation gas of 9 psi). Nitrogen was used as collision gas. Chromatographic data were collected and analyzed with Analyst 1.5.1 software (ABSCIEX).

2.4. Statistics

Data are presented as means ± SD, and are based on 3–6 independent experiments. GraphPad Prism software version 5.01 software (San Diego, CA, USA) was used for calculation of mean and SD, as indicated. *p*-Values were calculated by means of ANOVA Dunnett's multiple comparison test with $^{***}p < 0.001$; $^{**}p < 0.01$; and $^{*}p < 0.05$.

3. Results

3.1. Studies with HEK293 cells

As reported before [12], HEK293 cells exhibited considerable basal cNMP levels in the order cAMP > cUMP > cCMP ∼ cGMP (Fig. 1). The non-selective PDE inhibitor IBMX [20] by itself had no stimulatory effect on cNMP levels (data not shown). Addition of the sAC stimulator $NaHCO_3$ (40 mM) [6] to HEK293 cells cultured in DMEM medium supplemented with 10% (v/v) FBS increased levels of all cNMPs by about 3–4-fold. The sGC inhibitor ODQ [13,14] had no inhibitory effect on basal cNMP levels (but was rather slightly stimulatory), and the sGC activator SNP [13,14] showed only the expected increase in cGMP, but not a stimulatory effect on cAMP, cCMP and cUMP.

The exchange of DMEM medium against Sigma M7403 resting medium without $NaHCO_3$ (medium composition listed in

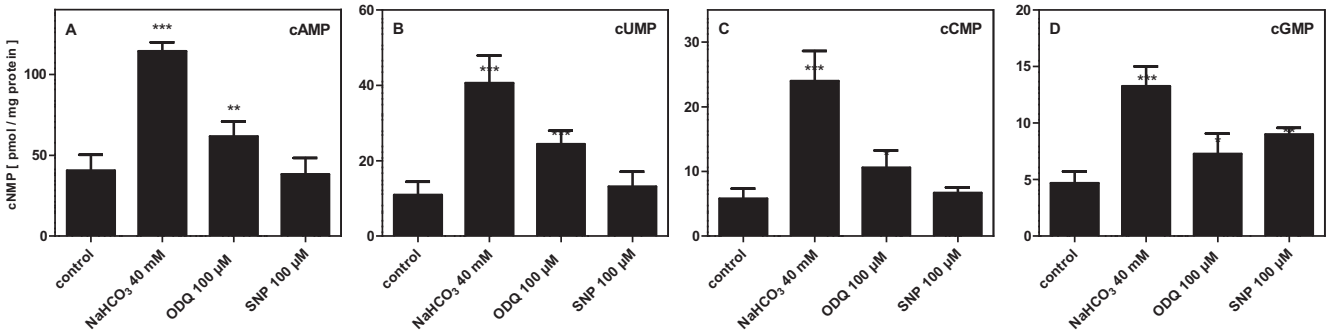

Fig. 1. Regulation of cNMP levels in HEK293 cells by SNP, ODQ and $NaHCO_3$ (A–D). HEK293 cells were pre-incubated with IBMX (100 μM) for 30 min followed by addition of ODQ (100 μM; 30 min pre-incubation), $NaHCO_3$ (40 mM) and SNP (100 μM). After additional 60 min cell metabolism was stopped and cNMP concentrations were analyzed by HPLC–MS/MS (n = 3–6 ± SD). Cells were seeded in 6-well plates for 24 h with 4×10^5 cells per well before analysis. Control is composed of DMEM + 10% FBS + IBMX 100 μM. Please note the different scales of the *y*-axes in all panels. $^{***}p$-value < 0.001; $^{**}p$-value < 0.01 (n = 3 ± SD).

Table 1
Regulation of cNMP levels by $NaHCO_3$, KH7 and FSK in HEK293 cells. (A) HEK293 cells were pre-incubated under resting medium (Sigma M7403 without $NaHCO_3$) with IBMX (100 μM) and KH7 (10 μM, 30 μM) for 30 min followed by stimulation with $NaHCO_3$ (40 mM). After additional 60 min cell metabolism was stopped and cNMP concentrations were analyzed by HPLC–MS/MS ($n = 3 \pm SD$). Cells were seeded in 6-well plates for 24 h with 4×10^5 cells per well before analysis. cNMP levels under DMEM + 10% FBS conditions are shown as well. (B) HEK293 cells were pre-incubated with IBMX (100 μM) for 30 min followed by stimulation with FSK (10 μM, 100 μM). After additional 60 min, cell metabolism was stopped and cNMP concentrations were analyzed by HPLC–MS/MS ($n = 3 \pm SD$). Cells were seeded in 6-well plates for 24 h with 6×10^5 cells per well before analysis.

		cAMP [pmol/mg protein]	cUMP [pmol/mg protein]	cCMP [pmol/ mg protein]	cGMP [pmol/mg protein]
(A)					
	DMEM + 10% FBS	40.51 ± 4.01	11.48 ± 1.79	5.38 ± 0.92	2.42 ± 0.53
Resting medium + IBMX 100 μM	Control	12.85 ± 3.13	<LOD	<LOD	<LOD
	$NaHCO_3$	72.97 ± 5.81 ***	<LOD	6.03 ± 0.03 ***	5.62 ± 1.58 ***
	$NaHCO_3$ + KH7 10 μM	30.28 ± 8.30 **	<LOD	4.02 ± 0.16 **	2.18 ± 1.34 **
	$NaHCO_3$ + KH7 30 μM	22.65 ± 5.83 ***	<LOD	<LOD	0.64 ± 0.15 ***
(B)					
DMEM + 10% FBS + IBMX 100 μM	Control	62.41 ± 3.89	12.41 ± 0.92	7.33 ± 0.88	5.02 ± 0.29
	FSK 10 μM	1230 ± 130 **	12.12 ± 2.13	6.25 ± 1.28	3.99 ± 0.75
	FSK 100 μM	15,396 ± 1,004 ***	11.82 ± 1.04	6.97 ± 0.63	4.44 ± 0.62

LOD = limit of detection.
** p-value < 0.01.
*** p-value < 0.001.

Table S1) resulted in a dramatic decrease in cAMP level and complete disappearance of cGMP, cUMP and cCMP (Table 1A). Re-addition of $NaHCO_3$ restored high levels of cAMP, cCMP and cGMP, whereas cUMP did not increase. When DMEM medium was exchanged against fresh DMEM medium, no decrease in cNMP levels was observed (Fig. S1). The effect of exchange of DMEM medium against resting medium was rapid in onset and reached a maximum after 10 min (Fig. S2). Within 20 min after re-addition of $NaHCO_3$, basal cNMP levels were completely (cAMP, cGMP and cCMP) or largely (cUMP) restored (Fig. S3). Reconstitution of $CaCl_2$ had no stimulatory effect on cNMP levels in the absence or presence of extra $NaHCO_3$ (Fig. S4). Likewise, the addition of FBS (10%, v/v) to DMEM medium or resting medium had no effect on cNMP levels (data not shown).

The sAC inhibitor KH7 [21] reduced $NaHCO_3$-stimulated cNMP levels in HEK293 cells in a concentration-dependent manner (Table 1A). Since the specificity of mAC inhibitors is very problematic [8,22], we did not study such compounds. Instead, we examined the mAC activator FSK [1,2,5,8]. FSK (10 μM and 100 μM) increased cAMP levels in HEK293 cells by about 20-fold and 240-fold, respectively (Table 1B), demonstrating endogenous expression of FSK-stimulated mAC isoforms in the cells. In marked contrast, FSK exhibited no stimulatory effects on cellular levels of cGMP, cCMP and cUMP. In agreement with these data, we did not observe stimulatory effects of FSK on formation of cGMP, cCMP and cUMP in Sf9 insect cell membranes expressing mAC isoforms 1, 2 or 5 (data not shown). We also transfected pGC isoforms C, D and G [3] into HEK293 cells, but found no evidence for a role of these pGCs in the regulation of cCMP and cUMP under various experimental conditions (data not shown). These data are in accordance with our previous data on pGCs A, E and F [12].

HEK293 cells endogenously express sAC at the mRNA and protein level [17]. In fact, HEK293 cells are such a rich sAC source that the enzyme was cloned from this system [17]. Since we experienced problems with the specificity of the sAC antibody R21 [19], we could not demonstrate sAC expression at the protein level (data not shown).

3.2. Studies with B103 neuroblastoma cells

Similar to HEK293 cells, B103 cells exhibited basal cNMP levels in the order cAMP > cUMP > cCMP ~ cGMP (Fig. 2A–D). The mAC activator FSK massively increased cAMP levels, but not the level of the other cNMPs. The sGC activator SNP had no stimulatory effect of cNMPs in B103 cells. In contrast, $NaHCO_3$ addition increased cNMP levels 2–3-fold. Note that the stimulatory effect of $NaHCO_3$ on cAMP was not statistically significant in the ANOVA analysis due to the exceedingly large stimulatory effects of FSK. When FSK was deleted from the ANOVA analysis, the effect of $NaHCO_3$ on cAMP was also significant with $p < 0.001$. Exchange of DMEM medium against resting medium without $NaHCO_3$ largely reduced cAMP levels and abrogated cCMP, cUMP and cGMP (Fig. 2E).

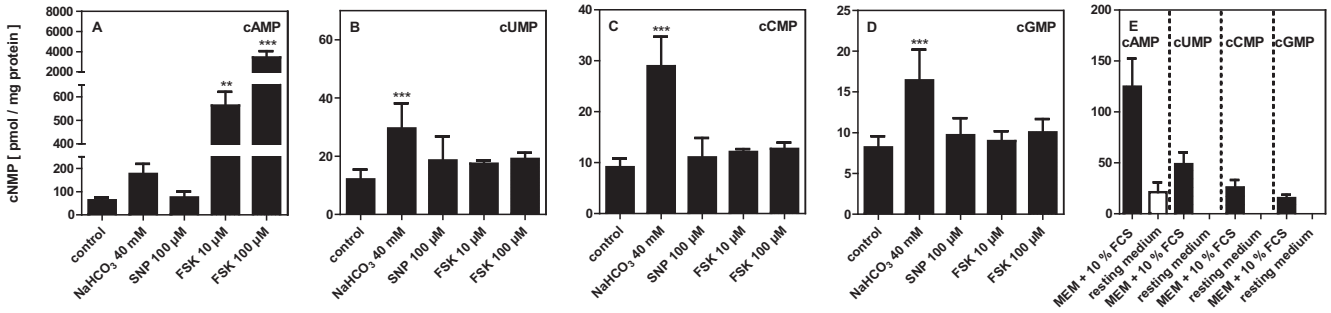

Fig. 2. Regulation of cNMP levels in B103 cells by $NaHCO_3$, SNP and FSK. (A–D) B103 cells were pre-incubated with IBMX (100 μM) for 30 min followed by stimulation with $NaHCO_3$ (40 mM), SNP (100 μM) and forskolin (10 μM, 100 μM). (E) Basal cNMP concentrations under MEM medium and under resting medium (Sigma M7403 without $NaHCO_3$) pre-incubated for 30 min. After additional 60 min, cell metabolism was stopped and cNMP concentrations were analyzed by HPLC–MS/MS ($n = 3–6 \pm SD$). Cells were seeded in 6-well plates for 24 h with 4×10^5 cells per well before analysis. Control is composed of MEM + 10% FBS + IBMX 100 μM. Please note the different scales of the y-axes in all panels. ***p-value < 0.001; **p-value < 0.01 ($n = 3 \pm SD$).

4. Discussion

The aim of this study was to examine the role of sAC in the regulation of basal levels of cAMP, cGMP, cCMP and cUMP in HEK293 cells and B103 cells. Fig. 3 provides a schematic presentation of the biochemical mechanisms analyzed in this study. sAC is activated by HCO_3^- [6,17] and inhibited allosterically by KH7 [21]. The stimulatory effects of $NaHCO_3$ addition and the inhibitory effects of $NaHCO_3$ depletion and KH7 strongly indicate that sAC is the major player in the regulation of basal cNMP levels in these cells. In marked contrast, mACs possess an exquisite specificity for cAMP formation, highlighted by massive cAMP increases upon FSK addition and contrasting lack of FSK on the other cNMPs. Our findings suggest that basal cNMPs (cAMP, cGMP, cCMP and cUMP) formed by sAC in distinct microdomains possess very different functions than stimulated cAMP formed by mACs.

As the next step, it will be very important to study the catalytic properties of purified sAC in terms of substrate-specificity. The very recent availability of the crystal structure of the sAC catalytic domain [7] will allow direct comparison with the catalytic domain of mAC [23] in terms of molecular dynamics simulations. Based on the present data we anticipate that sAC exhibits larger conformational flexibility than mAC so that the enzyme is capable of utilizing a wider variety of substrates than mAC. One approach to study the specific function of basal cNMPs formed by sAC is to add to cells membrane-permeable acetoxymethyl esters of cAMP, cGMP, cCMP and cUMP [24] at defined concentrations under resting medium conditions, i.e. $NaHCO_3$ depletion, and to analyze changes in cell functions. One sAC-regulated gene is the cystic fibrosis conductance regulator [25].

Our studies show that membranous NCs, be it mACs or pGCs, are not capable of generating cCMP and cUMP, whereas the soluble NCs are capable of such synthesis. We have already examined sGC at the level of the purified enzyme and at the level of intact cells with respect to basal and NO-stimulated cNMP formation and found striking differences between the two systems [13,14]. Moreover, cells overexpressing sGC behaved very differently from cells endogenously expressing sGC in terms of cNMP regulation [14]. To this end, we have only studied cells endogenously expressing sAC. By analogy to the sGC studies, we expect sAC-overexpressing cells to behave very differently than native HEK293 and B103 cells. Furthermore, it will be very difficult to predict the precise kinetic properties of purified sAC with respect to substrate-specificity, the type and concentration of divalent cation playing a role and the absence or presence of regulatory domains.

Our present study focused on neuronally derived HEK293 and B103 cells and found generally similar regulatory patterns of cNMP concentrations. We predict that cells with high sAC expression levels such as gut-derived Caco-2 cells and bone-derived osteoblasts also exhibit high basal levels of cUMP and cCMP [17]. In contrast, we expect that organisms that are devoid of sAC such as *Dictyostelium discoideum* [26] lack cCMP and cUMP. From our previous studies [12,14] and present report it is clear that cCMP and cUMP are not rare cNMPs. Rather, they can even surpass the levels of the established second messenger cGMP. Our data also urgently call for large efforts of the research community at identifying specific cCMP- and cUMP-binding proteins. Appropriate affinity chromatography and MS techniques are already in place [27]. Considering the differential substrate-specificities of membranous and soluble NCs, it is likely than any given cNMP also plays a specific role in signal transduction. In this context, it should also be noted that we observed subtle differences in the regulation of individual cNMPs by KH7 and bicarbonate and among the two related cell types, indicating that substrate-specificity of sAC can be specifically regulated by as yet unknown factors. Evidence for differential regulation of substrate-specificity was already obtained for sGC and bacterial "AC" toxins [11–13], here divalent cations being critical components of the system. We also observed experiment-to-experiment variations in the absolute cNMP concentrations. These differences may reflect the fact that it is experimentally impossible

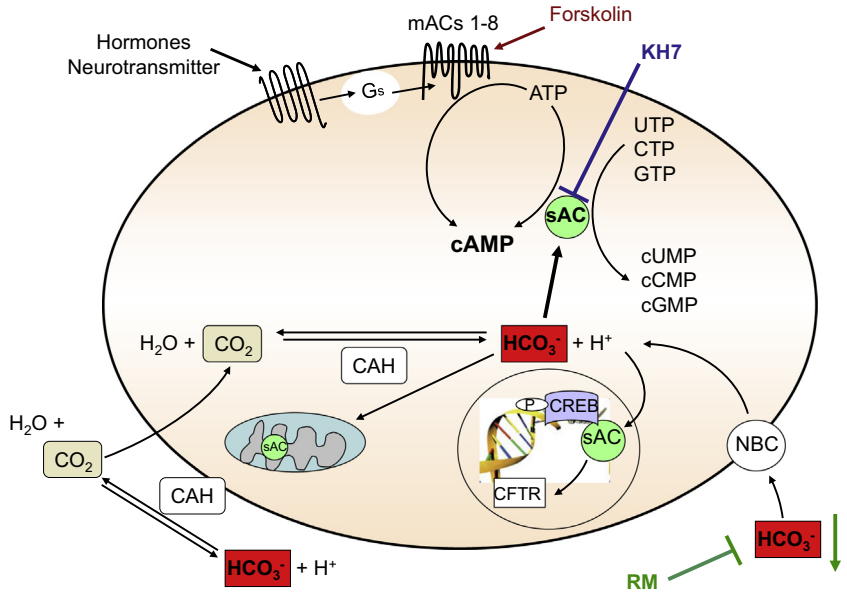

Fig. 3. Overview on regulation of cNMP formation by mACs and sAC in HEK293 and B103 cells. mACs are activated via G_s-protein-linked receptors for hormones and neurotransmitters. mACs 1–8 are directly activated by FSK. mACs are very specific for cAMP formation. In contrast, the HCO_3^- stimulated sAC exhibits broad substrate-specificity and generates cAMP, cGMP, cUMP and cCMP. sAC is localized in the cytosol, in mitochondria and in the nucleus where it controls the transcription factor CREB. HCO_3^- concentration is regulated by the activity of various CAH isoenzymes and Na^+/HCO_3^- cotransporter (NBC). sAC activity in intact cells (HEK293 and B103) can be manipulated by addition and removal of $NaHCO_3$ from the culture medium and by the sAC inhibitor KH7. Regulation of cNMP formation by NO-stimulated sGC is not shown here because this enzyme does not play a major role in the regulation of basal cNMP levels in HEK293 and B103 cells. pGCs are not shown either because there is no evidence that these enzymes generate cCMP and cUMP.

to precisely control the actual HCO_3^- concentration at the sAC enzyme and the metabolic state of the cell.

Another field of future investigation will be the analysis of CAH in the regulation of cNMPs. It is now appreciated that CAH is not a simple homeostatic enzyme but rather a complex family of enzymes with very different functions [28]. Thus, it is conceivable that different CAH isoforms play different roles in the regulation of basal cNMP levels. One approach to address this question will be the application of CAH isoform-specific inhibitors [28]. In addition, CAH knock-out and knock-down cells should be studied.

Last but not least, it is conceivable that total cellular and/or local NTP concentrations play a role in the regulation of basal cNMP concentrations. Specifically, in cells with low CTP and UTP levels, low basal cCMP- and cUMP concentrations are expected. Thus, availability of NTPs may provide a link between cell metabolism and second messenger-regulated signal transduction. Conversely, sAC may possess the function of an NTP sensor [29]. A link between NTPs and signal transduction was already made for P_{2Y}-receptors, the activity of which is regulated by ATP and UTP released from mechanically stressed cells [30].

In conclusion, we have provided evidence for an important role of sAC in the regulation of basal levels of cAMP, cGMP, cCMP and cUMP. sAC appears to play a distinct role in the regulation of cNMP levels compared to sGC, pGCs and mACs. An implication of our data is that cCMP and cUMP play unique functions as second messengers in homeostatic signaling.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.099.

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