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Review

Role of soluble adenylyl cyclase in mitochondria[☆]Q1 Federica Valsecchi¹, Csaba Konrad¹, Giovanni Manfredi^{*}

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ABSTRACT

The soluble adenylyl cyclase (sAC) catalyzes the conversion of ATP into cyclic AMP (cAMP). Recent studies have shed new light on the role of sAC localized in mitochondria and its product cAMP, which drives mitochondrial protein phosphorylation and regulation of the oxidative phosphorylation system and other metabolic enzymes, presumably through the activation of intra-mitochondrial PKA. In this review article, we summarize recent findings on mitochondrial sAC activation by bicarbonate (HCO_3^-) and calcium (Ca^{2+}) and the effects on mitochondrial metabolism. We also discuss putative mechanisms whereby sAC-mediated mitochondrial protein phosphorylation regulates mitochondrial metabolism. This article is part of a Special Issue entitled: The role of soluble adenylyl cyclase in health and disease.

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1. Introduction

In 1975, Braun and Dods described for the first time the presence in the cytosol of a “soluble” adenylyl cyclase (sAC), distinct from the transmembrane adenylyl cyclase (tmAC), which could be stimulated by Mn^{2+} and potentiated by calcium (Ca^{2+}). These initial findings were made in rat seminiferous tubules and epididymal sperm [1,2]. Indeed, the gene encoding for sAC is highly expressed in testis, where the protein mostly consists of a shorter fragment (48 kDa) of the full-length sAC (187 kDa) [3]. Functionally sAC is a cyclase that converts ATP in cyclic AMP (cAMP), the ubiquitous second messenger responsible for many signaling cascades in the cells. The presence of a soluble form of adenylyl cyclase allows cAMP to be produced in defined subcellular compartments, where it serves local signaling functions [3]. The catalytic active portions of sAC (C1 and C2) are conserved in cyanobacteria and myxobacteria, suggesting an evolutionary continuity, between the bacterial and mammalian sAC–cAMP signaling systems.

The enzymatic activity of sAC can be directly stimulated by bicarbonate (HCO_3^-) *in vivo* and *in vitro* [4] and by Ca^{2+} , in a concentration dependent manner [5,6]. Furthermore, it has been shown that sAC reflects alterations in intracellular ATP fluctuation in HEK293 cells [7]. Taken together, these findings suggest that sAC can be considered as a putative metabolic sensor. Recently the crystal structure of the human sAC apo state was described [8,9], including the mechanism of catalysis

and the binding site of the HCO_3^- activation and regulators. The structure shows how HCO_3^- binds and activates sAC and how sAC can be inhibited by a drug [8,9].

sAC was shown to localize to various subcellular compartments, including mitochondria, centrioles, mitotic spindles, mid-bodies and nuclei [10], suggesting that each one of these cell compartments contains a cAMP target. In particular, the role of sAC in mitochondria started to emerge in the recent years. Several laboratories have demonstrated that cAMP is generated in the mitochondrial matrix, where it regulates mitochondrial metabolism, coupling the CO_2 generation in the Krebs cycle with the activity of the oxidative phosphorylation machinery [11–13]. It needs to be noted, however, that sAC and PKA, as well as the phosphodiesterase PDE2, are not reported in the MitoCarta compendium of the mitochondrial proteome [14]. The lack of predicted mitochondrial localization on the basis of canonical import signals in the primary protein sequences suggests that sAC and PKA enter mitochondria through alternative, still poorly understood, mechanisms. Alternatively, there could be unidentified splice isoforms with mitochondrial targeting. In the following sections, some of the issues raised around the presence of these proteins in mitochondria will be discussed in more detail.

Once generated by sAC, cAMP inside the cell stimulates its cellular effector protein kinase A (PKA) responsible for protein phosphorylation. In mitochondria, the activity of many enzymes and carriers is modulated by phosphorylation [15,16]. The mechanisms underlying the regulation of these post-translational modifications are emerging, but are yet to be described in complete detail. Our current understanding of this process is based on the evidence that cAMP is locally produced inside mitochondria by sAC, where it activates a kinase, presumably PKA, which then phosphorylates mitochondrial proteins, thereby regulating their function [12,17,18]. Here, we review and discuss

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current evidence on the localization of sAC in mitochondria and on the role of sAC-generated cAMP in mitochondria in the regulation of energy metabolism.

1.1. sAC localization in mitochondria

The first evidence that sAC is localized in mitochondria was shown in COS7 cells stained with both α N-term and α C-term sAC antisera and the mitochondrial marker MitoTracker green [10]. In this work, sAC was also detected in mitochondria of cardiac and skeletal muscle. Functionally, our group proposed for the first time that cAMP contained in the mitochondrial matrix is not generated in the cytosol and transported across the inner membrane, as previously suggested [19], because the latter is impermeant to cAMP. Instead, we showed that matrix cAMP originated inside the mitochondria by the action of sAC, in response to metabolically generated carbon dioxide [11]. Other groups have confirmed the presence of this signaling pathway in the mitochondrial matrix, using different approaches, such as cAMP specific FRET sensors [12,18]. It was also shown that sAC activation increases ATP production in cells and isolated mitochondria [12,17]. The oxidative phosphorylation (OXPHOS) activities, such as ATP production, O_2 consumption, and cytochrome oxidase (COX) activity, are decreased by sAC inhibition, whereas sAC activation by HCO_3^- and Ca^{2+} stimulates OXPHOS [12,17]. These authors concluded that the sAC–cAMP signaling pathway represents a metabolic sensor that modulates OXPHOS and ROS generation. Fig. 1 shows a schematic representation of the putative sAC–cAMP signaling pathway in mitochondria (Fig. 1).

1.2. sAC regulation by HCO_3^- in mitochondria

The role of sAC in energy metabolism started to emerge when the sensitivity to HCO_3^- was discovered [4]. HEK293 cell line expressing the full-length sAC cDNA accumulated cAMP upon sAC stimulation with $NaHCO_3$. On the contrary, tmsAC was insensitive to HCO_3^- . The

latest is generated inside the mitochondria via the action of the enzyme carbonic anhydrase that converts CO_2 derived from the Krebs cycle. Our group has investigated cAMP production in mouse liver isolated mitochondria in the presence of 30 mM HCO_3^- , which is in the range of the physiological intra-mitochondrial HCO_3^- concentration. Under these conditions, COX activity and ATP synthesis were significantly increased [11], indicating a stimulation of the OXPHOS system by cAMP generated within mitochondria. We noted that, since OXPHOS activity is increased by both HCO_3^- and by exogenously generated CO_2 , in a carbonic anhydrase dependent manner, these effects could not be due to pH changes (or ionic strength), because HCO_3^- addition increases, while CO_2 addition decreases the pH of the experimental medium, which (because the ΔpH is constant) is followed by the pH of the matrix [11].

Di Benedetto et al. recently showed that CHO cells transfected with the cAMP sensor 4mth30 targeted to mitochondria and treated with 50 mM $NaHCO_3$ displayed a biphasic kinetic of cAMP generation, with an initial rise followed by a plateau [12]. Cells pretreated with the sAC inhibitor hydroxyl-estradiol and stimulated with HCO_3^- showed a significant decrease in cAMP generation. Taken together, these data demonstrated a direct effect of HCO_3^- in activating sAC and cAMP production in mitochondria.

1.3. sAC regulation by Ca^{2+} in mitochondria

Jaiswal et al. showed that sAC in the cytosol is regulated by Ca^{2+} in mature human spermatozoa in a concentration dependent manner (between 10^{-8} and 10^{-4} M). They also showed sAC regulation by Ca^{2+} in HEK293 cells treated with a Ca^{2+} ionophore (which induces Ca^{2+} influx from the extracellular media) or carbachol (which recruits Ca^{2+} from intracellular stores) [5]. The recent development of targeted cAMP sensors [12,18] allowed for a better understanding of the cAMP responses inside mitochondria and for the investigation of Ca^{2+} and cAMP crosstalk in the regulation of energy metabolism.

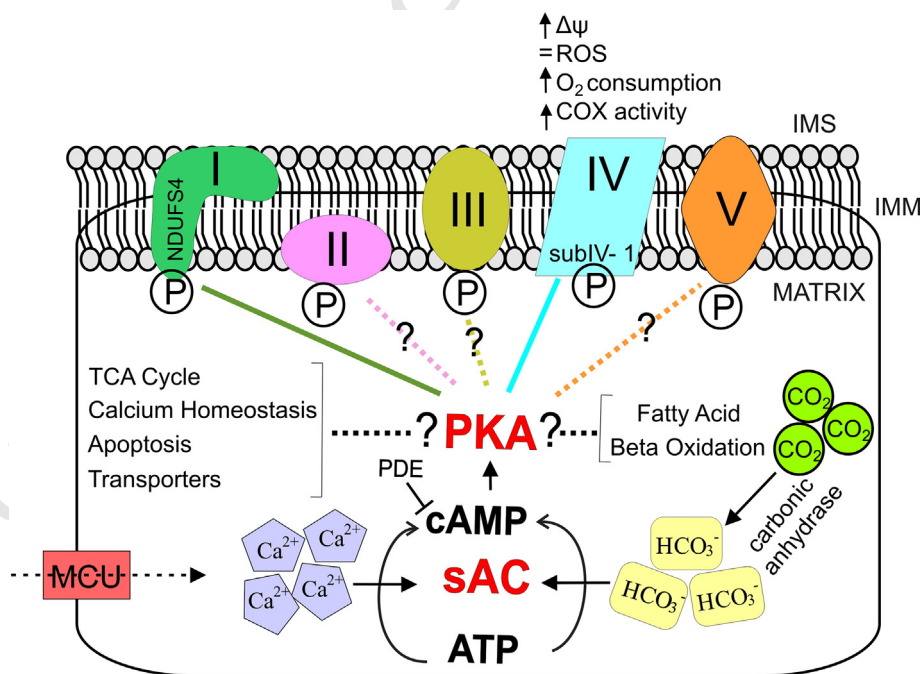


Fig. 1. sAC–cAMP–PKA pathway in mitochondria. sAC converts ATP in cAMP, the ubiquitous second messenger, which is degraded by phosphodiesterase (PDE). sAC is activated by Ca^{2+} (e.g. overexpression of the mitochondrial calcium uniporter, MCU) and HCO_3^- originated from the CO_2 derived from the Krebs cycle spontaneously or by the action of carbonic anhydrase. It was proposed that cAMP activates PKA, leading to phosphorylation of the NDUF54 subunit of complex I (CI, green line), thereby regulating CI activity. sAC also induces phosphorylation of subunit COXIV-1 of complex IV (blue line), leading to an increase in COX activity, membrane potential ($\Delta\psi$), O_2 consumption and ATP production, without altering ROS level. It needs to be noted that the evidence that PKA is the sAC effector in mitochondria is contradictory and other kinases may be involved. Other OXPHOS complexes are phosphorylated, but whether this involves the mitochondrial sAC–signaling pathway is still unknown. Furthermore, proteins related to TCA cycle, calcium homeostasis, apoptosis, transporters, fatty acid oxidation and β -oxidation are also phosphorylated.

Recently, Di Benedetto et al. demonstrated that cAMP is produced inside mitochondria using a mitochondrially targeted cAMP sensor (4mtH30) properly targeted to the mitochondria [12]. In order to assess if Ca^{2+} could stimulate cAMP inside mitochondria, CHO cells were transfected with 4mtH30, derived from CFP-Epac(δ DEP-CD)-YFP probe with a mitochondrial targeting signal added to its N-terminus [20]. Cytosolic Ca^{2+} increase from intracellular stores release, induced by the IP_3 -generating agonist ATP in combination with the SERCA pump inhibitor TBHQ, resulted in a marked increase in mitochondrial cAMP signal, supporting the notion that Ca^{2+} stimulates sAC inside mitochondria [12].

Overexpression of the mitochondrial Ca^{2+} uniporter (MCU) leads to an increase in mitochondrial Ca^{2+} accumulation [21,22]. Accordingly, Di Benedetto et al. showed that overexpression of MCU resulted in an increase in mitochondrial cAMP, while MCU silencing decreased mitochondrial cAMP [12]. Moreover, increased frequency and amplitude of mitochondrial Ca^{2+} oscillations triggered an increase of matrix cAMP in primary cultured neonatal rat cardiomyocytes transfected with mth30 sensor [12].

ATP production increases when sAC is overexpressed in cells. Di Benedetto et al. demonstrated that ATP concentration increases in mitochondria with targeted sAC expression (mtsAC cells) [12], using a luciferase sensor, mtLUC, targeted to mitochondria [23]. Mitochondrial ATP dynamics was measured upon Ca^{2+} stimulation in control and mtsAC HeLa cells. Under this condition both the rate and the extent of ATP concentration were upregulated, demonstrating that Ca^{2+} stimulated-ATP depends, at least in part, on sAC, as Ca^{2+} regulates directly some of the Krebs cycle dehydrogenases, having per se a stimulatory effect [24]. To further investigate if this mechanism is PKA-dependent, a PKA inhibiting peptide was targeted to the mitochondria (mt-PKI). The overexpression of mt-PKI induced a decrease in ATP concentration in both control and mtsAC overexpressing cells. These data confirm that the target of mitochondrial cAMP is PKA or a PKA-like kinase.

Taken together the work described above showed that Ca^{2+} modulates components of the cAMP pathway within mitochondria, including sAC and PKA. It is certainly an interesting finding that the physiology of the two major cell regulators Ca^{2+} and cAMP takes place inside the mitochondria with an interconnected pathway [25].

1.4. sAC–cAMP effectors in mitochondria

A number of protein families have been identified as cAMP effectors in mammalian cells. Upon cAMP binding, exchange proteins directly activated by cAMP (Epac) 1 and 2 serve as guanine nucleotide exchange factors for the small GTP-ases Rap1 and Rap2 [26,27]. A large family of cyclic-nucleotide-activated ion channels have been described, which can be divided into three major subgroups; hyperpolarization-activated cyclic-nucleotide-modulated (HCN) channels, voltage independent cyclic-nucleotide-gated (CNG) channels and K^+ channels called EAG and EAG-like channels; however, modulation of the latter by cAMP is less well established [26].

The best understood regulatory effects of cAMP are mediated by activation of the cAMP-dependent protein kinases (PKAs). PKA exists primarily as an inactive tetrameric holoenzyme, consisting of two of each catalytic (C) and regulatory (R) subunits. cAMP binding on the regulatory subunits triggers the release of the active C subunits, which then phosphorylate specific serine and threonine residues of target proteins [28]. There is no single consensus sequence that predicts phosphorylation sites; however, the Arg-Arg-X-Ser is common and highly similar domains are contained within the vast majority of known sites [29]. The cellular distribution of PKA largely depends on the R subunit isoforms. Type II α and II β are mostly bound to anchor proteins, while type I α and I β are considered being more diffusely cytosolic, but unspecific anchoring of both types (see below) and type I specific anchor sites have also been described to play important roles

in signaling [30–33]. Depending on cell type and subcellular localization of the enzyme, the cAMP dependent protein phosphorylation by PKA is responsible for a wide variety of cellular responses [34–36]. Investigations of subcellular fractions revealed the existence of mitochondrial protein phosphorylation activity [37,38] in different sub-mitochondrial fractions [39]. Nevertheless, especially for the matrix compartment the nature of the cAMP effector is controversial. The evidence both supporting and undermining the idea of PKA being the effector of cAMP in different mitochondrial compartments are reviewed below.

Evidence of A-kinase anchoring proteins (AKAPs) targeted to mitochondria may be a good indication that PKA is the effector of cAMP in these organelles. AKAPs are often part of heterogeneous complexes together with the R subunit of PKA, including mRNAs, tyrosine phosphatase(s) and tyrosine kinase(s) [40]. Their role is the control of spatial distribution and specificity of PKA activity throughout the cell, allowing for efficient transduction of the cAMP signal. The AKAP1 gene translates into several splice variants, of which three were found to be associated to mitochondria. Both S-AKAP 84 [41] and AKAP 121 [42] bind RII subunits, while D-AKAP1 [43] binds both RI and RII; furthermore, all three variants share a targeting sequence on their N terminals, directing them to the outer mitochondrial membrane (OMM). AKAP 121 was also demonstrated to be present in the matrix by immunoelectron microscopy [44] and to regulate oxidative phosphorylation through COX [45]. Other AKAPs, such as D-AKAP2, co-localize with cytochrome c upon immunostaining [46]. The mechanism by which D-AKAP2 is targeted to mitochondria is still unclear. Recently, it was also shown that AKAP79, known to be associated to the plasma membrane and lacking a mitochondrial targeting sequence [47], is present in mitochondria isolated from human placenta [48]. Finally, two proteins were discovered to act as AKAPs, as a secondary function. The Rab subfamily of Ras proteins regulates membrane dynamics upon activation by GTP. Rab 32, uniquely in the subfamily, is an important player in mitochondrial fission and also binds PKA [49]. The sphingosine kinase interacting protein (SKIP), which interestingly specifically binds the RI (mostly cytosolic) subunit, was shown to be present in the intermembrane space (IMS) and the mitochondrial matrix [50,51].

Although a few of the AKAPs mentioned above were reported also in the mitochondrial matrix, evidence best supports that they are associated to the cytosolic surface and IMS of mitochondria, where small molecules of the cytosol can diffuse without constraint; therefore, theoretically both tmAC and sAC can produce cAMP affecting PKA in these domains. In the mitochondrial matrix however, sAC is the only source of cAMP described to date. There are numerous reports of matrix PKA activity revealed by protein phosphorylation upon radiolabeling [39, 52–60], by immunoblotting and immunocytochemistry [44,61] and by immuno-electron microscopy [62]. Sardanelli et al. estimated that ~90% of total mitochondrial PKA activity was present in the matrix [44]. More recently, a novel method to assess PKA activity specifically was developed by applying coumarin-derived fluorescent peptides as PKA substrates [63]. In this work, the relative PKA activities of the outer surface, IMS and matrix of isolated bovine heart mitochondria were measured to be 9:6:85 percent, respectively. Our group was the first to examine the impact of pharmacological effectors of PKA on mitochondrial parameters, and found that oxidative phosphorylation was activated by the agonist 8Br-cAMP; and inactivated by inhibitors (H89, myristoylated PKI) of PKA, while 8CPT methyl-cAMP, a specific activator of Epac had no effect [11]. The inhibitory effects of H89, PKI and additionally Rp-8-CPT-cAMP on matrix ATP levels were confirmed by Di Benedetto et al.; however, they did not achieve activation of matrix ATP by 8Br-cAMP or 8-CPT-6-Phe-cAMP [12]. It is worth to note, that matrix ATP concentration is only one of the numerous factors that contribute to the regulation of the kinetics of ATP production in mitochondria [64], and may very well not be the only one affected by protein phosphorylation; furthermore, elevated matrix ATP may not always correlate with elevated ATP output through the adenine nucleotide translocase [65].

The fact that PKA subunits are devoid of discernible mitochondrial targeting sequences undermines the idea of their presence in the matrix. Di Benedetto et al. reported failure to show mitochondrial localization of GFP tagged PKA subunits (unpublished results in [25]). Furthermore, PKA activity could not be measured by matrix targeted A-kinase activity reporter (AKAR) 3 and 4 [18]. AKARs are FRET based sensors containing a PKA specific substrate, which upon phosphorylation, due to conformational changes, increase their energy transfer [66,67]. To confirm that the negative result was not due to the sensor, AKAR 4 was shown to respond to genetically engineered PKA targeted to the matrix, which could be inhibited by co-expression of mito-PKI. These results suggest that a yet undiscovered cAMP effector exhibiting PKA-like responses to pharmacological manipulation could be responsible for phosphorylation events in the mitochondrial matrix, as proposed by Di Benedetto et al. [25]. Fig. 2 summarizes the genetically encoded probes to study the function of sAC–cAMP–PKA inside mitochondria (Fig. 2), as reported in recent publications by Di Benedetto and Lefkimiatis [12,18].

1.5. Degradation of cAMP in mitochondria

In order for an autonomous cAMP signaling cascade to operate properly in mitochondria, mechanisms for degrading the cAMP generated by sAC must also exist. Phosphodiesterase (PDE) activity, indicated by cAMP hydrolyzing activity in the matrix, which was sensitive to a non-selective PDE inhibitor, IBMX, was first reported in mitochondria along with the presence of sAC [11]. More recently, isolated mouse brain and liver mitochondria were shown to lose cAMP and cGMP hydrolysis, when treated with BAY 60, an inhibitor of the PDE2A isoform. The mitochondrial localization of the second splice variant of PDE2A to the mitochondrial matrix was also demonstrated [68,69]. The inhibition of PDE2A increases oxygen consumption and ATP production in isolated mitochondria [68]. Hydrogen sulfide at low levels was proposed as a physiological effector of PDE2A in mitochondria [69]. These findings may offer new intriguing approaches in the treatment of a wide variety of afflictions, in which bioenergetic incompetence

contributes to pathology, by developing specific and safe inhibitors of mitochondrial PDE2A2.

1.6. Mitochondrial phosphoproteins and metabolism

Phosphorylation represents an important regulatory mechanism for mitochondrial protein function. In particular, as discussed above, reversible phosphorylation of mitochondrial proteins is a player in OXPHOS modulation. Many mitochondrial proteins are known to be phosphorylated [16,70–74], but the relation between phosphorylation and function is still not completely understood.

Contrasting results suggest that phosphorylation by PKA can both inhibit and activate OXPHOS. Papa et al. demonstrated that the NDUF54 accessory subunit of complex I (CI) is phosphorylated by PKA (Fig. 1). Patients harboring mutation in this subunit displayed decrease CI activity and decreased cAMP-dependent phosphorylation [75,76]. Bender and Kandenbach showed that the allosteric ATP inhibition of CIV in bovine heart is switched on by cAMP dependent phosphorylation of sub II and Vb by PKA, and switched off by protein phosphatase 1 [77]. Moreover, the cytochrome c oxidase results in a serine and threonine phosphorylation via PKA of subunit I, which was correlated with inhibition of the enzyme in the presence of ATP [78]. Our group proposed that PKA phosphorylates COXIV-I at serine 58 and enhances COX activity [79]. This COX regulation by phosphorylation of COXIV-1 subunit was due to prevention of COX allosteric inhibition by ATP (Fig. 1). Furthermore, overexpression of sAC targeted to the mitochondrial matrix of COXIV-I deficient cells dramatically improved mitochondrial function and stabilized a long-term metabolic adaptation. This was interpreted as the result of constitutive stimulation of cAMP production in mitochondria triggering adaptation mechanisms in COX deficient cells, which upregulated OXPHOS function and reduced the need for enhanced ROS-dependent OXPHOS biogenesis [79]. In a therapeutic perspective, mitochondrial sAC activation or cAMP degradation inhibition in COX deficient cells could be a viable strategy to improve COX deficiency *in vivo* [17].

Recent work investigated the correlation between phosphorylation and function in mitochondria from mouse liver and human skeletal

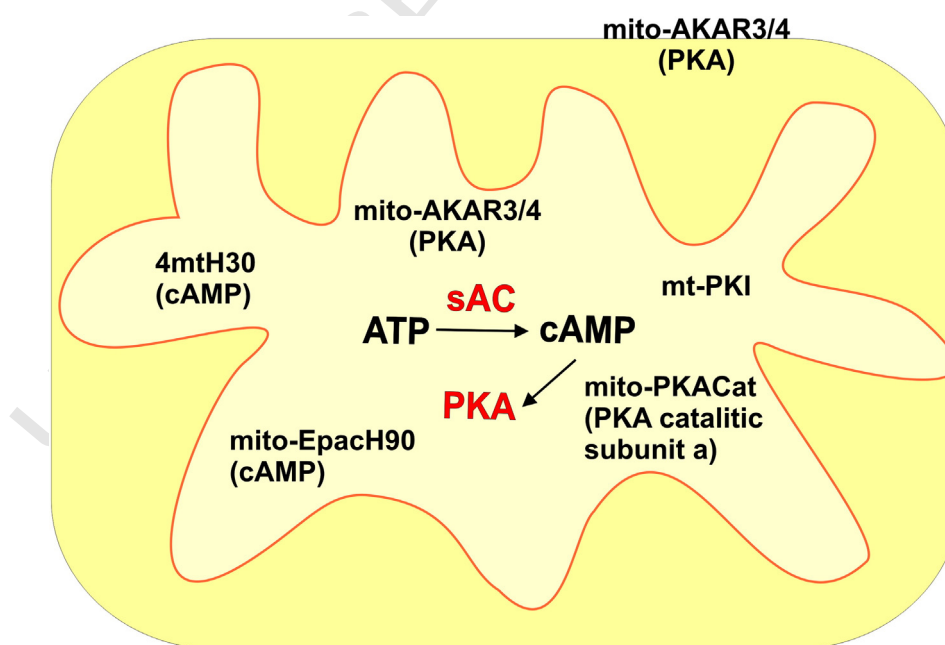


Fig. 2. Genetically-encoded tools to study the mitochondrial sAC–cAMP pathway. Recently, new tools were developed to investigate the mitochondrial sAC–cAMP pathway in living cells. The diagram schematically depicts the genetically encoded probes. To detect cAMP in the matrix H30 and H90 were targeted to the mitochondria matrix (4mtH30 and mito-Epach90) by adding 4 copies of the mitochondrial targeting sequence of subunit VIII of the human cytochrome oxidase (COX8) at the N-terminus of the Epac1–cAMP sensor. The same strategy was used to target AKAR3 and AKAR4, the FRET-based sensor to measure PKA activity, and PKI-mCherry (mt-PKI) and PKA-Cat-mCherry to the matrix [12,18]. To target PKA, AKAR3 and AKAR4 to the OMM the targeting peptide used was yTom70 [18].

muscle [73,80]. Not only OXPHOS proteins are included in these studies, but also metabolism related proteins indirectly connected with mitochondria. However, their relation with sAC-PKA remains unknown so far. Grimsrud et al. revealed that phosphorylation is a key mechanism in regulating ketogenesis (phosphorylation of Serine 456 on Hmgcs2) in obese and type 2 diabetes mice [80]. The acute mitochondria phosphorylation changes during fasting and re-feeding of mice. Together, these data show that Hmgcs2 S456, the enzyme that catalyzes the rate-limiting step in ketogenesis, is an important phosphorylation target, which enhances enzymatic activity during increased ketogenic demand.

Phosphorylation was shown in human skeletal muscle mitochondrial proteins involved in oxidative phosphorylation, TCA cycle, lipid metabolism, amino acid degradation, importers and transporters, calcium homeostasis, and apoptosis (Fig. 1) [73]. Using different phosphopeptide enrichment techniques and mass spectrometry, Zhao et al. identified 176 different phosphorylation sites in 86 mitochondrial proteins. The major proportion of the identified phospho-sites involves potential substrates of PKA, but also CKII, PKC or DNAPK. PKA activity has been shown in human mitochondria [15,81] regulating protein expression, biogenesis and apoptosis. As discussed above, the presence of PKA in mitochondria is still controversial. Detection of mitochondrial protein kinases is difficult due to their concentration relative to proteins. Moreover, the lack of a highly specific antibody and possible contamination from associated cell compartments limit the possibility of clearly dissecting the nature of the kinases involved [82].

1.7. Conclusions and future perspectives

The discovery of sAC within the mitochondria opens new avenues to study metabolic regulation dependent on HCO_3^- and Ca^{2+} stimulation via the sAC–cAMP pathway. It needs to be noted, however, that the studies of sAC function in mitochondria described above were conducted only in cell culture models or isolated mitochondria. Currently, there is no hard evidence on the relation between sAC function, mitochondrial protein phosphorylation, and metabolic regulation *in vivo*. Two different mouse models of sAC deletion are available [83,84]. In the first one, the catalytic domain C1 was knocked out (sAC C1KO), whereas in the second the C2 catalytic domain was abolished. Investigating mitochondrial function and protein phosphorylation in different tissues of these mice will shed new lights on the functions of sAC in mitochondria *in vivo*. Therefore, different approaches will need to be devised to specifically investigate the intra-mitochondrial effects of sAC modulation. Since total sAC KO will likely affect extra-mitochondrial function that may indirectly affect mitochondrial metabolism, models where sAC is exclusively depleted or increased in mitochondria would be very useful for a better understanding of mitochondrial and metabolic regulation. In addition, more work is necessary to investigate the effect of sAC on OXPHOS complexes and TCA cycle, fatty acid transporters, beta oxidation, amino acid degradation, calcium homeostasis, etc., [73] *in vitro* and *in vivo* under different relevant conditions that affect metabolism, such as high and low calorie intake or exposure to cold or in conditions where the energy metabolism is compromised by aging or disease. Such studies could provide insights on the potential significance of modulating sAC and cAMP in mitochondria as a therapeutic tool for metabolic and degenerative diseases that affect metabolism.

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