

cAMP and Mitochondria

Phosphorylation of mitochondrial proteins has emerged as a major regulatory mechanism for metabolic adaptation. cAMP signaling and PKA phosphorylation of mitochondrial proteins have just started to be investigated, and the presence of cAMP-generating enzymes and PKA inside mitochondria is still controversial. Here, we discuss the role of cAMP in regulating mitochondrial bioenergetics through protein phosphorylation and the evidence for soluble adenylyl cyclase as the source of cAMP inside mitochondria.

Mitochondria are traditionally viewed as the major source of cellular ATP and a hub of intermediate metabolism. Over the years, we have come to appreciate that mitochondria also perform several additional crucial functions in cell signaling and regulation (23, 50, 54, 55, 92, 106, 128), and mitochondrial dysfunction has been implicated in numerous human diseases. Genetic disorders affecting mitochondrial proteins encoded either by the nuclear or the mitochondrial genomes (primary mitochondrial disorders) are implicated in cancer, diabetes, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), and cardiomyopathy (98, 118, 119, 126, 127, 141).

To be able to respond to changes in substrate availability and bioenergetic demands, mitochondria require rapid, short-term, metabolic adaptation mechanisms. Over the past decade, numerous studies have linked posttranslational modifications of mitochondrial enzymes to the regulation of energy metabolism in mammalian cells. The molecular mechanisms underlying these regulations (i.e., how they are linked to metabolic needs) and their physiological significance are still under active investigation. Specifically, reversible phosphorylation of mitochondrial proteins has emerged as an important player in the regulation of mitochondrial oxidative metabolism. Yet, many of the protein kinases, their regulatory elements, and their molecular targets remain to be identified. Recently, a quantitative map of phosphoproteins in liver mitochondria was generated. This broad analysis identified 811 phosphosites (100 of which had not been described before) on 295 different mitochondrial proteins (58), implicating reversible phosphorylation as a fundamental regulatory mechanism of mitochondrial metabolism.

The ubiquitous second messenger cyclic AMP (cAMP) and its cellular effector protein kinase A (PKA) constitute one of the most widely studied signaling cascades, yet the roles of cAMP signaling and PKA phosphorylation of mitochondrial proteins in the regulation of mitochondrial metabolism remain

controversial issues. This is because the source of cAMP in mitochondria, the precise localization of PKA, and the distinction between the effects of PKA acting inside or on the outside of mitochondria are often difficult to resolve. In this review, we summarize current knowledge on the mechanisms for regulating cAMP levels in mitochondria and the role of cAMP effectors in regulating mitochondrial energy metabolism and other relevant aspects of mitochondrial signaling.

General cAMP Signaling System

Cyclic AMP is generated from ATP via adenylyl cyclases (ACs) and degraded via phosphodiesterases (PDEs). In mammals, there are two types of class III adenylyl cyclases: membrane bound and soluble. There are nine genes encoding transmembrane adenylyl cyclases (tmAC) and a single gene encoding multiple isoforms of soluble adenylyl cyclase (sAC). TmACs are regulated by heterotrimeric G proteins and the pharmacological activator forskolin, whereas sAC is directly regulated by calcium (70, 88), physiological changes in ATP (88), and bicarbonate (29), which means its activity also reflects local fluctuations in CO₂ and pH_i (136). TmACs are thought to be exclusively restricted to the plasma membrane, with the notable exception that they can also be found in internalized endosomes and may signal as they traffic from the plasma membrane (22, 53). There are reports of heterotrimeric G-protein alpha subunits found in the mitochondria (7, 89), but, to date, no one has demonstrated presence of the tmAC stimulatory Gs protein nor tmACs at mitochondria. In contrast, there are several reports demonstrating sAC at mitochondria (discussed below).

The cAMP generated by adenylyl cyclases is tightly regulated by phosphodiesterases (PDE), which catabolize cAMP into 5' AMP (12, 14, 32, 65). There are 11 known PDE families; some are specific for cAMP (PDEs 4, 7, 8), others for cGMP (PDEs 5, 6, 9), whereas the remainder (PDEs 1–3, 10, 11) catabolize both cAMP and cGMP. Each of

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the PDE families includes multiple isoforms with distinct enzymatic characteristics, modes of regulation, expression patterns, and distribution throughout the cell (14, 31, 76, 96).

Cyclic AMP effector molecules contribute to the complexity and specificity of cAMP signaling. PKA, a well studied cAMP downstream effector, is a tetrameric enzyme consisting of two catalytic domains (C) and two regulatory domains (R). In mammals, there are three known isoforms of catalytic subunits (C α , C β , C γ) and four isoforms of regulatory subunits (RI α , RI β , RII α , RII β). cAMP binding to R releases active C subunits, which phosphorylate key substrates (77, 130, 131). A structurally diverse group of proteins called A kinase anchoring proteins (AKAPs) direct PKA to distinct subcellular sites (46, 144, 147). In addition to tethering PKA to its particular subcellular location, AKAPs also act as scaffolds to PKA's substrates, cyclases, PDEs, and protein phosphatases, facilitating regulation of the cAMP cascade (10, 47, 48, 82, 129, 146). Cyclic AMP can also mediate its effects via other effectors; cAMP modulates two types of cyclic nucleotide-regulated channels plus a family of guanine-nucleotide exchange factors (GEFs) called exchange proteins activated by cAMP (EPAC) (17, 64, 124). Cyclic nucleotide regulated channels include cyclic nucleotide-gated channels (cNGC) (15, 18, 36, 72) and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (16, 108, 140, 155); however, neither has been reported to be found at mitochondria (34). There are two EPAC isoforms, EPAC1 and EPAC2 (also called cAMP-GEF1 and cAMP-GEF2), which are expressed in different tissues (113). EPACs, when bound to cAMP, activate the small GTPases Rap1 and Rap2 proteins and function via PKA-independent mechanism to stimulate phosphoinositide-hydrolysing phospholipase C ϵ (PLC ϵ)-generating inositol-1,4,5-trisphosphate that regulates calcium intracellular signaling pathways (40, 41, 73, 85, 99, 100, 121).

Compartmentalization of cAMP Signaling

Cyclic AMP had long been assumed to be a freely diffusible second messenger (11, 67). However, the idea that cAMP signaling was compartmentalized first emerged in the late 1970s, when it was appreciated that, in a single cell type, different hormones or neurotransmitters stimulated cAMP production but resulted in different physiological outcomes. In heart, both PGE₁ and isoproterenol elevated cAMP, but only isoproterenol caused activation of phosphorylase kinase and inhibition of glycogen synthase (60–62, 74, 75). Yet cAMP compartmentalization did not gain widespread acceptance until Förster resonance energy

transfer (FRET based) (45, 107, 151, 152) and biophysical methods (71, 111) for measuring cAMP in situ in living cells revealed that cAMP levels are not uniform within cells. Now it is widely accepted that intracellular membranes and/or PDEs targeted to discrete cellular locations limit the diffusion of cAMP pools (31, 150). Compartmentalization of cAMP signaling into a variety of independently regulated functional domains explains how a single second messenger can mediate a wide variety of physiological processes within a cell (FIGURE 1). In this context, cAMP functional compartments can be defined as pools of cAMP, which are confined in a restricted region of the cell, such as an organelle, and which are locally generated and regulated from other distinct parts of the cell. Immunocytochemistry revealed sAC to be distributed throughout the cytoplasm, at the centrioles, inside nuclei and mitochondria, on the mitotic spindle, and in the mid-body (153), suggesting it could be a local source of cAMP at these sites (21, 153, 154). In fact, sAC inside mitochondria now represents one of the best-characterized cAMP signaling domains.

cAMP Signaling in Mitochondria

There appear to be at least two independent cAMP domains that signal in mitochondria, one associated with the cytosolic-mitochondrial interface and a second one wholly contained inside the mitochondrial matrix. In regard to the source of cAMP in the mitochondrial matrix, two contradicting theories have emerged. The first, based on results obtained with mitochondrially targeted cAMP reporters, postulates that cAMP is generated in the cytosol and is then translocated into the mitochondrial matrix through yet unidentified transport mechanisms (45). The second, based on functional results, but not on direct identification of cAMP, affirms that the inner mitochondrial membrane is impermeable to cAMP and that matrix cAMP is generated locally by resident adenylyl cyclases, such as sAC (1, 2, 4). The availability of more sensitive and specific cAMP reporters correctly and exclusively targeted to the mitochondrial matrix will likely help resolve this issue. The pools of cAMP in mitochondria are postulated to activate PKA that resides in the various mitochondrial compartments. Although the presence of PKA on the mitochondrial outer membrane was extensively demonstrated, PKA was also found in the intermembrane space (see *cAMP effectors in mitochondria*) and in the matrix (29). The mechanisms that allow PKA to enter mitochondria, however, are still unknown, since the protein lacks apparent targeting signals. The following sections will outline some of the key aspects of cAMP signaling in mitochondria.

Generation of cAMP in Mitochondria

The earliest studies using sAC-specific antisera demonstrated co-localization with mitochondria by immunocytochemistry and subcellular fractionation (153). Parallel studies also showed bicarbonate-regulated (i.e., sAC-like) adenylyl cyclase activity enriched in mitochondrial fractions. Ladilov and coworkers confirmed these findings in primary cells, demonstrating sAC localization at mitochondria of coronary endothelial cells (83) and cardiac myocytes (9). In these cell types, mitochondrially localized sAC plays a role in apoptosis; in fact, in coronary endothelial cells, acidotic stress caused a translocation of sAC from the cytosol to mitochondria in what appears to be an early step in ischemia-induced apoptosis (83). The precise localization in relationship with mitochondria was not addressed, but it is likely to involve association with the outer membrane surface. sAC-generated cAMP plays at least one other role within mitochondria. Studying regulation of oxidative phosphorylation (OXPHOS), we found a difference between extra-mitochondrial cAMP and membrane-permeable cAMP analogs. In whole cells, membrane-permeable analogs of cAMP stimulated oxygen consumption, whereas forskolin stimulation of tmACs in concert with the broadly specific PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) had no effect (4). In contrast, it

must be noted that in a different report the strong activation of tmAC by cholera toxin promoted serine phosphorylation in a subunit of complex I (CI), leading to increased respiration and CI activity (104, 117, 133) but no effect on the activity of cytochrome oxidase. Despite the partial discrepancy in the findings on the effects of extramitochondrial cAMP on respiratory activity, taken together, the data suggest that cAMP plays a role on mitochondrial respiration. In the matrix space, we proposed that the source of cAMP could be sAC, based on Western blot results and by demonstrating that cAMP generation in intact mitochondria could be stimulated by a sAC-specific activator (i.e., bicarbonate) and inhibited by a sAC-specific inhibitor (i.e., KH7). Other experiments independently confirmed this sAC-defined pathway resided within a membrane-protected compartment inside mitochondria. The pathway was sensitive to exogenously added PDE or an anti-sAC inhibitory monoclonal antibody, but only in sonicated mitochondria; intact mitochondria remained insensitive (4). We subsequently identified an isoform of PDE2A as a component of this intramitochondrial, sAC-defined domain. The unique NH₂ terminus of isoform 2 of PDE2A targets the enzyme to the mitochondrial matrix, where it opposes the action of sAC in regulating OXPHOS (2).

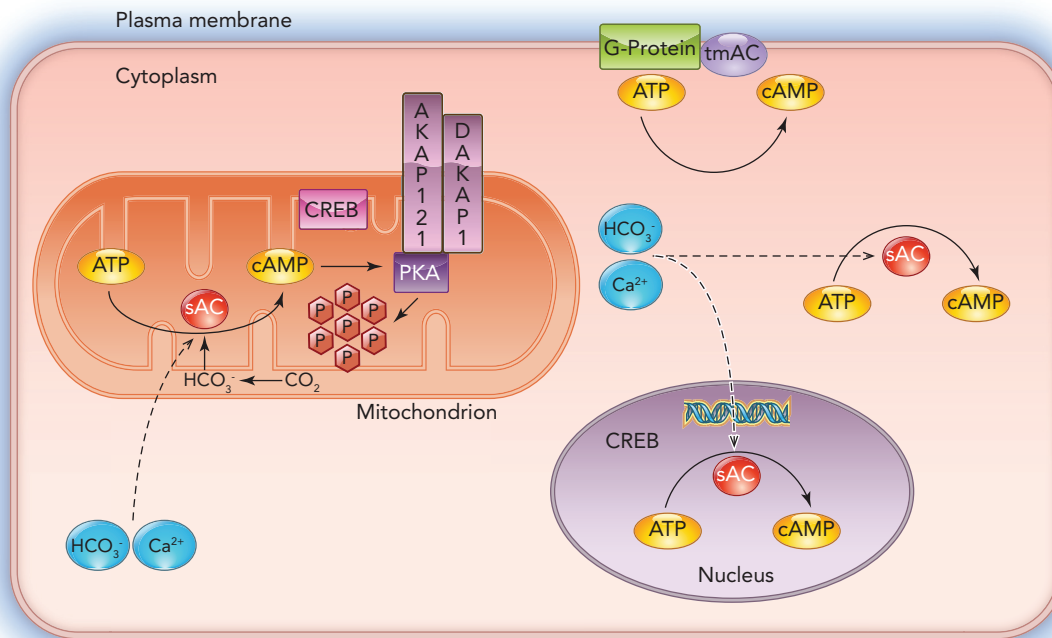


FIGURE 1. cAMP-PKA-sAC compartmentalization

In mammalian cells, cAMP is generated from ATP via the action of the transmembrane adenylyl cyclase (tmsAC) and G protein located in the plasma membrane or via the action of soluble adenylyl cyclase (sAC) located in the cytoplasm, in the nucleus, and in the mitochondrial matrix. sAC can be activated by Ca²⁺ and HCO₃⁻ derived from the extracellular space. In mitochondria, sAC can be activated by HCO₃⁻ generated from the CO₂ derived from the Krebs cycle via the action of carbonic anhydrase. CREB is not only localized in the nucleus but is also attached to the mitochondrial inner membrane. Specific AKAP mitochondrial proteins (AKAP121 and DAKAP1) interact with PKA to induce phosphorylation of mitochondrial substrates.

Future studies will have to determine the mechanisms of how sAC becomes localized in the mitochondrial matrix and of PKA import into mitochondria.

cAMP Effectors in Mitochondria

PKA has been proposed to be associated with, or in, mitochondria since the 1970s (43, 79, 123, 139). The sub-mitochondrial distribution of PKA appears to depend on the tissue, cell type, and method of detection utilized. Recently, Ma et al. showed that PKA is present in human placental mitochondria (91), and sucrose density gradient separation showed that the catalytic (C) subunit of PKA is predominantly found in the mitochondrial outer membrane. There is also considerable evidence that PKA is localized in the mitochondrial matrix (116, 123, 132). Schwach et al. found a two-fold increase in PKA-specific activity in the mitochondrial matrix fraction relative to the whole mitochondrion, and they demonstrated that both forms of regulatory subunit (RI and RII) reside on the matrix side of the inner mitochondrial membrane in a variety of rat tissues, including liver, skeletal muscle, pancreas, and kidney (123). PKA was independently found in the inner mitochondrial membrane fraction from bovine heart (116, 132, 134). Although their abundances differed, both RI and RII subunits were found in the inner membrane and matrix of mitochondrial preparations, and the C subunit was found to be enriched in the matrix (116, 123, 132). These authors proposed the existence of a cAMP-PKA signaling cascade, stimulating the enzymatic activity of complex I, thereby increasing cellular respiration. In another study, in heart, hypoxia and ischemia increased mitochondrial PKA activity. The PKA-dependent phosphorylation of COX subunits (I, IVi1, and Vb) was postulated to reduce COX activity, leading to mitochondrial dysfunction in myocardial injury (109).

These studies, addressing PKA functions in different sub-compartments of mitochondria (outer, inner membrane, and matrix) utilized standard purification methods (i.e., density centrifugation, digitonin treatment), which suffer from specific drawbacks; for example, in studying intermembrane space, the use of digitonin could disrupt the inner membrane, resulting in matrix contamination of the intermembrane space. Recently, a fluorescent sensor of PKA actions has been developed to characterize cAMP-dependent PKA activity in intact mitochondria (5). The PKA sensor and quencher are small enough to move into and measure PKA activity in the intermembrane space. Addition of cAMP (together with the PKA sensor and quencher) to intact mitochondria measures PKA activity at both the outer membrane and the intermembrane space, whereas addition of cAMP to trypsin-treated mitochondria eliminates outer membrane activity and reveals PKA

activity only within the intermembrane space. Finally, total PKA activity measured after sonication of untreated mitochondria includes matrix-localized PKA. Using these treatments, the relative distribution of PKA activity in the matrix, intermembrane space, and outer membrane was estimated to be 79%:8%:13%, respectively. These results were comparable with earlier reports, which found that 90% of PKA activity in mitochondria is located in the matrix/intermembrane space (115). A recent analysis of mitochondrial phosphopeptides identified over 100 potential PKA targets in mitochondrial proteins (58).

PKA localization can also be inferred from, or measured in conjunction with, mitochondrial AKAPs. AKAPs direct PKA to subcellular sites, and many AKAPs have been localized to mitochondria. There are numerous splice variants of AKAP1 (S-AKAP84, AKAP121, D-AKAP1, and AKAP149), which share an NH₂-terminal core but have distinct COOH termini, identified in mitochondria. S-AKAP84 binds RII and a known mitochondrial target sequence (87); AKAP121 binds and targets RII α to the cytoplasmic surface of mitochondria (52, 115); D-AKAP1, which is a novel type of AKAP interacting with both type I and type II regulatory subunits of PKA, contains the same mitochondrial targeting signal sequence as S-AKAP84 (66); and AKAP79 was found in placental mitochondria (91). In addition, localization of a second dual-specific AKAP (D-AKAP2), recognizing both RI and RII forms of PKA, showed predominantly mitochondrial immunostaining (142).

A mitochondrial GTP-binding protein, Rab 32, was shown to function as an AKAP (6). Although it does not have a prototypical mitochondrial targeting sequence, Rab32 has a pair of COOH-terminal cysteines directing it to mitochondria. Rab32 has an important role in the regulation of mitochondrial fission.

Another mitochondrial protein, sphingosine kinase interacting protein (SKIP), was subsequently found to have AKAP activity specific for type I PKA (93). Immunoblots of mitochondrial subfractions revealed that SKIP is found in both the intermembrane space and the matrix of murine heart mitochondria, where it presumably tethers PKA.

Another cAMP downstream effector, EPAC, has been identified in mitochondria (27, 110, 143). EPAC1-based fluorescent reporters have been targeted to the mitochondria to study the dynamics of cAMP signaling in the organelle (45, 94). Moreover, the GTP-binding protein Rap1 is also found in mitochondria (78, 135). To date, the function of EPAC in mitochondria is still unknown, and its association with this organelle requires further investigation; but, if confirmed, EPAC presence in mitochondria could suggest the existence of a coordinated cAMP-GTPase signaling.

Mitochondrial Regulation by cAMP: Extra-Mitochondrial Effects

Control of Mitochondrial Morphology and Mitophagy

Mitochondria are highly dynamic organelles that undergo reshaping via fission and fusion events. In humans, dysfunction of these processes causes neurological disorders. The dynamin-related GTPase proteins regulating mitochondrial morphology include two mitofusin isoforms [mitofusin 1 (Mfn1), mitofusin 2 (Mfn2)], optic atrophy 1 (OPA1), dynamin-related protein 1 (Drp1), and fission protein 1 (Fis1) (13, 145). Mitochondrial morphology is directly linked with the activity of the electron transport chain (145), but the mechanisms correlating form with function remain poorly understood.

Cyclic AMP can regulate mitochondrial morphology. PKA phosphorylation of Ser637 on Drp1 (also referred to as Ser656 in different splice variants) blocks its translocation to the mitochondrial surface. Phosphorylated Drp1 promotes mitochondrial elongation by allowing fusion to proceed unopposed (25, 26, 35); dephosphorylated Drp1 promotes fission. Phosphomimetic mutants of Drp1 or overexpression of PKA on the outer mitochondrial membrane induces mitochondrial fusion, forming a neuroprotective reticulum and increasing neuronal survival (95). Furthermore, PKA located in the outer mitochondria and the phosphatase PP2A modulates neuronal development inhibiting or activating, respectively, mitochondrial division (35, 42). The process of mitophagy (the autophagy of mitochondria) depends on mitochondrial morphology, because mitochondria must fragment before removal via the autophagosome (57). Certain autophagy signals, such as amino acid or serum starvation, elevate cytoplasmic cAMP, leading to PKA-mediated Drp1 phosphorylation. Phosphorylated Drp1 promotes mitochondrial fusion that spares the mitochondria increasing cell survival (56).

Regulation of Mitochondrial Biogenesis: cAMP Response Element-Binding Protein

The cAMP response element-binding protein (CREB) is a ubiquitous transcription factor that regulates the transcription of cAMP response element-regulated genes (125, 148). It belongs to the CREB/ATF1 family of cAMP/Ca²⁺ responsive transcription factors. PKA phosphorylation activates CREB. Nuclear CREB regulates PGC-1 α , the master regulator of mitochondrial biogenesis (30). Blocking CREB or PGC-1 α in mouse hepatocytes inhibited mitochondrial biogenesis induced by pyrroloquinoline quinone (PQQ), a redox cofactor and antioxidant compound.

In addition to its nuclear roles, CREB is also found bound to the mitochondrial DNA (24, 86). In

brain, mitochondrial CREB regulates mitochondrial gene expression and neuronal survival (86). CREB is thought to be transported to the mitochondria via chaperone molecules, such as Hsp70 (86). CREB uptake was promoted by the mitochondrial membrane potential and by TOM complex, responsible for the translocation of the proteins from the cytoplasm into the mitochondria (39). Once inside mitochondria, CREB interacts with cAMP response elements (CREs) in the mitochondrial DNA, thereby regulating expression of mitochondrial components of the electron transport chain. It appears to bind directly to the D-loop of mitochondrial DNA. Mitochondrial CREB phosphorylation was shown to regulate expression of mitochondrial DNA-encoded proteins, such as ND1, ND6, and COXIII/ATP6 (39). Based on experiments with either wild-type or Ser133 mutant mitochondrially targeted CREB, it was proposed that CREB can be phosphorylated by PKA within mitochondria (114). Nevertheless, these experiments did not exclude the possibility that CREB could be imported into mitochondria already in the phosphorylated state. Alteration in mitochondrial CREB activity affects the expression of mitochondrial genes ND2, ND4, and NDU5 (all subunits of CI), leading to impairment of related CI activity (86). CREB is also involved in redox state mechanisms, since phosphorylation of mitochondrial CREB at Ser133, in response to the antioxidant and the iron chelator deferoxamine, promoted survival in cortical neurons exposed to oxidative stress (114).

Regulation of Apoptosis

Apoptosis initiated at mitochondria involves a family of Bcl-related proteins, including both anti-apoptotic (Bcl-2, Bcl_{XL}) and pro-apoptotic (Bax, Bak, BAD, BIM, Bcl_{XS}) members. Pro-apoptotic Bcl-related proteins modulate the release of cytochrome c from mitochondria. The cAMP signaling pathway can promote pro-apoptotic pathways in response to different stressors (69). In endothelial cells (84) and cardiomyocytes (9), acidosis during ischemia was proposed to induce sAC association with mitochondria and activation of PKA, presumably via local production of cAMP at the outer membrane. PKA-mediated Bax phosphorylation resulted in its translocation to mitochondria and activation of the apoptotic pathway leading to cytochrome c release and caspase-9 cleavage. In these experimental conditions, inhibition of sAC or PKA prevented the mitochondrial translocation of Bax, whereas inhibition or stimulation of tmAC had no effect, suggesting the direct involvement of locally generated cAMP through sAC. On the contrary, it was proposed that BAD phosphorylation by PKA anchored to the mitochondrial outer membrane leads to blockage of BAD IL-3-dependent

apoptotic activity (59). However, in these earlier reports, the source of cAMP at the mitochondria was not investigated. Therefore, cAMP-regulated phosphorylation of apoptotic proteins may have different effects, depending on cell types and environmental conditions.

Regulation of Mitochondria by cAMP: Intra-Mitochondrial Effects

Modern methods enable identification of many phosphorylated proteins, including membrane transporters, metabolic enzymes, and OXPHOS complexes (8, 33). These, along with the known kinase-phosphatase functions within the mitochondrial matrix, have recently been extensively reviewed (33). Unfortunately, the function of the vast majority of these posttranslational modifications on mitochondrial metabolism remains poorly understood. Here, we summarize the current understanding of the effects of sAC activation on mitochondrial energy metabolism and the effects of protein phosphorylation on complexes I and IV of the respiratory chain, two major components of the OXPHOS system.

Effects of Intramitochondrial sAC Activation on Mitochondrial Energy Metabolism

As introduced above, sAC and PKA may reside inside mitochondria and may regulate OXPHOS via phosphorylation of multiple substrates including subunits of cytochrome c oxidase (COX or complex IV) (1, 4). Carbonic anhydrases (CA) are ubiquitous enzymes that equilibrate CO₂ levels with bicarbonate and a proton, and two isoforms of type V CA have been shown to reside inside mitochondria (49, 63). The coincident localization of sAC, carbonic anhydrases, and the Krebs cycle suggested that the CO₂ produced by the Krebs cycle could be converted to bicarbonate by mitochondrial carbonic anhydrase and sensed by sAC. We demonstrated that sAC-PKA regulation of OXPHOS was sensitive to physiologically relevant intramitochondrial bicarbonate concentrations, metabolically generated CO₂, and carbonic anhydrase inhibition (4). Thus the intramitochondrial sAC-PKA pathway senses metabolic activity providing a mechanism linking respiratory activities to nutritional availability. As nutrients become available to the cell, local CO₂ levels increase and bicarbonate matrix concentration rises, stimulating the mitochondrial cAMP-PKA pathway. This leads to increased OXPHOS activity while simultaneously limiting ROS production. Another important implication of this local mitochondrial signaling pathway is that the changes in cAMP level in the

mitochondrial domain are not necessarily associated with the cAMP levels in the rest of the cell.

Mitochondrial Protein Phosphorylation: Complex I

The cAMP/PKA pathway plays an important role in the regulation of mitochondrial complex I (CI; NADH: ubiquinone oxidoreductase), the first entry point of the electron transport chain. CI is composed of 44 or 45 subunits, divided into core and accessory subunits, encoded by nuclear and mitochondrial DNA (80). Although several CI subunits are phosphorylated, the functional effect of phosphorylation is not completely clear yet. Phosphorylation has been postulated to play a role in CI assembly (19, 33, 101, 120), but further investigation is necessary to correlate protein phosphorylation with CI function. In particular, the relation between CI phosphorylation and function has been investigated in one of the accessory subunits, NDUFS4. This is a nuclear-encoded 18-kDa protein that regulates the assembly and activity of CI (81, 138). The NDUFS4 subunit is a hotspot of mutations in humans, associated with fatal neurological syndromes (20, 104, 105). As shown in whole fibroblasts and isolated mitochondria, NDUFS4 is phosphorylated by PKA in the mitochondrial matrix or in the cytosol before mitochondrial import, whereby phosphorylation could be regulating the protein import into mitochondria (28, 37, 102, 103). Although further experiments are needed to conclusively establish under which circumstances NDUFS4 phosphorylation takes place outside or inside mitochondria, intriguingly, fibroblasts from patients harboring mutant NDUFS4 displayed reduced levels of cAMP-dependent NDUFS4 phosphorylation and reduced activation of CI (104). In addition, cAMP-elevating agents, which led to NDUFS4 phosphorylation, were able to overcome CI inhibition in wild-type fibroblasts (38). Finally, the cAMP-PKA pathway and the 18-kDa accessory subunit of CI may play a role in the pathology of chromosome 21 trisomy (137). Human fibroblasts from patients with chromosome 21 trisomy display reduced cAMP levels and decreased cAMP-dependent phosphorylation of NDUFS4.

Mitochondrial Protein Phosphorylation: Complex IV

Our group identified a specific residue (S58) of COX subunit IV-1 (COXIV-1) as one of the targets of the intramitochondrial sAC-PKA pathway (1, 4). This modification regulates COX allosteric inhibition by matrix ATP; when COXIV-1 is dephosphorylated, COX activity is inhibited by ATP (1). ATP inhibition may play a role in the transition to an energy storage state by facilitating accumulation of unused substrates, such as fat and glycogen. Other

subunits of COX are also subject to cAMP regulation, but their relationship with the intramitochondrial sAC-PKA pathway is still unclear. In hypoxia and myocardial ischemia/reperfusion, hyperphosphorylation of COX subunit I (COXI), IV-1, and Vb inhibit COX activity, increasing ROS production. This effect was completely prevented by the addition of the PKA inhibitor H89 (51, 109). Phosphorylation of COX subunit II (COXII) by mitochondrial c-Src resulted in increased COX activity in osteoclasts, and this regulation appeared to be dependent on cAMP. On the other hand, calcitonin downregulates COX activity via cAMP (97, 149). Furthermore, heart mitochondria isolated after heart failure exhibit increased cAMP-dependent phosphorylation of COX at threonine residues (112).

Increased cAMP signaling in mitochondria, presumably via at least a subset of these phosphorylation events, can partially rescue COX deficiencies. Cybrids harboring a mutation in the COXI gene exhibit increased ROS production and elevated PGC1 α and NRF1 expression, suggesting compensatory mitochondrial biogenesis. sAC targeted to the mitochondria (mt-sAC) of these cells improved the bioenergetic defects, including COX activity, respiration, and normalization of OXPHOS biogenesis (3).

Recently, Hüttemann and colleagues thoroughly reviewed the current evidence of COX subunit phosphorylation in multiple mammalian species and under physiological and pathological conditions such as inflammation and ischemia. A large number of phosphosites involved serine or threonine

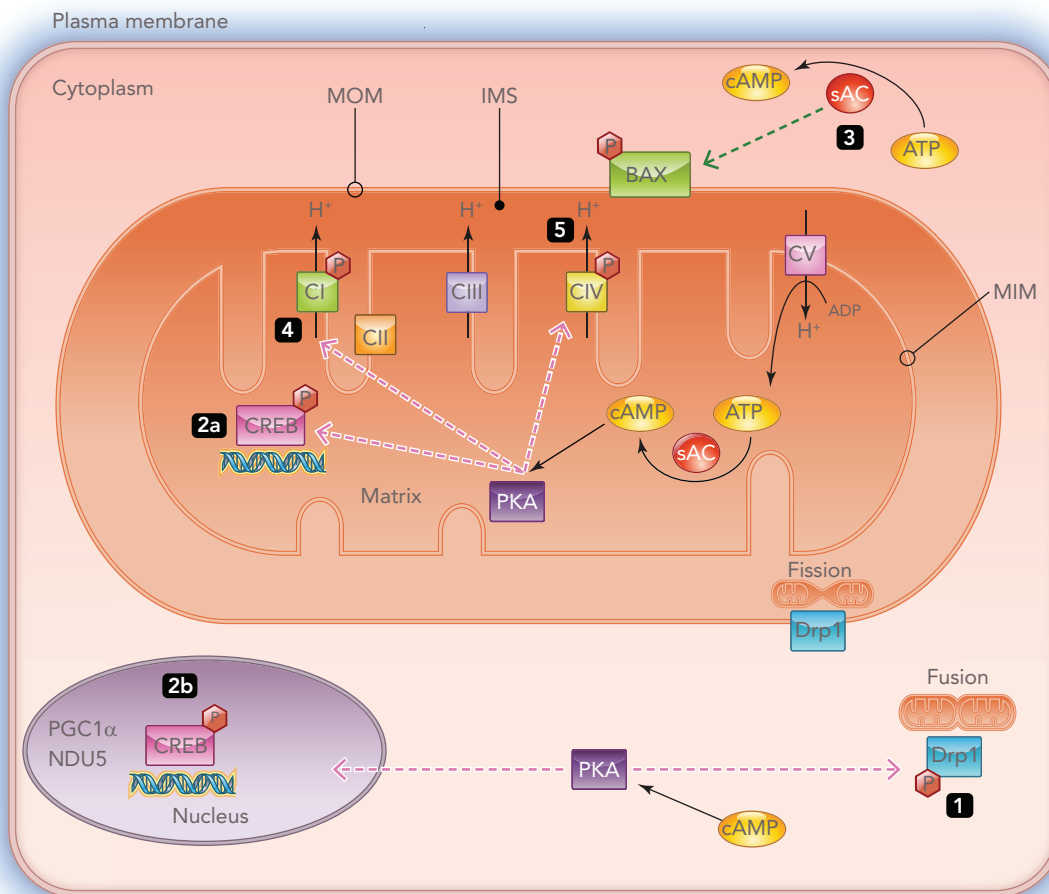


FIGURE 2. Extra- and intra-mitochondrial regulation of cAMP pathway

The cartoon shows the cAMP compartmentalization in cytoplasm and mitochondria and its effects on a variety of mitochondrial functions. 1: cAMP derived from the action of sAC on ATP induces the activation of PKA and the phosphorylation of Drp1, which prevents its translocation to mitochondria and is retained in the cytoplasm, thereby promoting mitochondrial fusion; in contrast, dephosphorylated Drp1 translocates to mitochondria, inducing mitochondrial fission (see section *Control of Mitochondrial Morphology and Mitophagy*). 2: CREB localized on mitochondrial DNA can be phosphorylated by PKA, residing in the mitochondrial matrix (2a) and induce mitochondrial biogenesis through the activation of PGC1 α or NDU5 transcription in the nucleus (2b) (see *Regulation of Mitochondrial Biogenesis: CREB*). 3: sAC in the cytosol leads to phosphorylation and translocation of BAX into mitochondria and activation of the pro-apoptotic pathway (see *Regulation of Apoptosis*). 4: PKA in the matrix activates complex I phosphorylation on the 18-kDa subunit (see *Mitochondrial Protein Phosphorylation: Complex I*). 5: PKA in the matrix regulates COX activity via COXIV-1 phosphorylation (see *Mitochondrial Protein Phosphorylation: Complex IV*). MOM, mitochondrial outer membrane; IMS, intermembrane space; MIM, mitochondrial inner membrane.

residues, but the responsible kinase remains to be identified in most cases. Nevertheless, this strongly suggests the existence of multiple potential targets of cAMP-PKA phosphorylation on COX, which is likely to play a significant role in regulating mitochondrial bioenergetics and ROS production (68). The broader picture suggests that different phosphorylation sites and their localization relative to the CIV holocomplex (e.g., matrix side vs. intermembrane space or transmembrane domains) have very different effects on the enzyme kinetics, activating in some cases and inhibiting in others. This type of complex regulation may all be responsive to cAMP-PKA signaling, but the latter could have different function in the different mitochondrial compartments. This possibility is reinforced by the complete separation of the intramitochondrial cAMP pool from the extra-mitochondrial one (3).

Conclusions

The discovery of a compartmentalized source of cAMP in mitochondria generated by sAC has led to new ideas on how this ubiquitous signaling system is able to provide short-term metabolic regulation for a discrete subcellular organelle. The integration of this signaling system in the microenvironment of mitochondria, where it can sense changes in pH, CO₂, calcium, and ATP, participates in the modulation of enzymatic activities by protein phosphorylation. The phosphorylation of CI and COX subunits described above are examples of many such events that lead to reversible activation or inhibition of OXPHOS in response to metabolic demands and environmental cues (FIGURE 2). These examples suggest that cAMP-dependent regulation of OXPHOS is involved in maintaining a balance between energy storage and energy consumption as well as ROS production in respiring mitochondria. It is also necessary to remember that several subunits of the respiratory chain contain transmembrane domains, which means they are exposed to both the intermembrane space and the matrix; these may be sensitive to different pools of cAMP, with potentially competing, additive, or synergistic effects on enzymatic activity. Nevertheless, we predict that, with the rapid expansion of the mitochondrial phosphoproteome and the repertoire of mitochondrial phosphodiesterases, kinases, and phosphatase involved in regulating protein phosphorylation and enzymatic function, we will soon be able to draw a more precise map of this complex signaling network. We also expect that the increasing knowledge of the downstream targets of cAMP signaling will shed new light on the mechanism of communication between mitochondria and other organelles that

regulate fundamental processes, such as mitochondrial biogenesis and turnover. ■

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