Cyclic AMP Produced inside Mitochondria
Regulates Oxidative Phosphorylation, Rebeca Acín-Perez et al, Cell Metabolism 9, 265–276, March 4, 2009
Regulation of OXPHOS by cAMP-PKA agonists and antagonists

A) Respiration (coupled and uncoupled) in HeLa cells after 8Br-cAMP (n=6), 8CPT methyl-cAMP (8CPTm-cAMP) (n=3), H89 (n=6), for 0.5 hours or with forskolin plus IBMX (fsk-IBMX) (n=3). Control, untreated cells (n=33). Values are fmoles O2 per min per cell. B) Cellular cAMP levels after stimulation of tmAC with fsk-IBMX. Values are pmol cAMP per 106 cells. C-E) OXPHOS in mouse liver mitochondria. State III (phosphorylating) mitochondrial respiration driven by glutamate/malate (C); succinate (D) or TMPD/ascorbate (E). Compounded number of replicas were: control (n=21); 8Br-cAMP (n=9); cAMP (n=3); 8CPTm-cAMP (n=3); forskolin+IMBX (fsk+IBMX, n=3); H89 (n=9); PKI 14-22 (1µM n=3); 8Br-cAMP+PKI (n=3); KH7 (n=9); KH7.15 (n=3) and 8Br-cAMP+KH7 (n=3). F) ATP synthesis with 8Br-cAMP (n=3), cAMP (n=3), H89 (n=3), PKI 14-22 (PKI, n=3). Values are nmoles ATP per min per mg of mitochondrial protein. G) Safranin-O fluorescence curves showing changes in mitochondrial membrane potential (ΔΨm) driven by glutamate/malate (G+M) and inhibited by rotenone (Rot), or driven by succinate (Succ) and blocked by the complex III inhibitor antimycin A (AA). Values are relative fluorescent units (RFU). The plots are representative of three independent determinations, which showed similar responses. H) COX Vmax (IU per milligram of protein, n=9). *, p<0.05; **, p<0.001; ***, p<0.0001.
sAC is localized and generates cAMP in mitochondria

A) Mitochondria isolated from mouse liver after two rounds of Nycodenz gradient purification. Lanes 2, 3, 4, and 5 were from the first round. Fractions 4.2, 4.3, 4.4 and 4.5 were from a second round of purification of fraction 4. Tim 23, mitochondrial marker. PDI, protein disulfide isomerase, ER marker. GAPDH, cytoplasmic marker. Hom, homogenate. B) Expression of sACt-HA in total cell homogenate of transiently transfected COS cells and in crude non-treated (n.t) mitochondrial fractions detected by R21 and HA antibodies. PK, Proteinase K; T+PK (Triton X-100 and PK). Hsp60 and Tim23 are markers of the matrix and the inner membrane, respectively. C) cAMP levels in intact mouse liver mitochondria in the presence of bicarbonate (HCO$_3^-$) or KH7. Values are pmol cAMP per mg mitochondrial protein (n=3). D) Mitoplasts purity tested using protein markers for the different mitochondrial compartments: Hsp60 (matrix), COXI (inner membrane), cytochrome c (Cyt c, intermembrane space), Tom40 (outer membrane). M (intact mitochondria), Mp (mitoplasts), P-Mp (post-mitoplast fraction). E) Residual cAMP levels after 50 pmol cAMP were added to a reaction mixture containing sonicated mitoplasts (mitoplast) or no mitoplasts (control), with and without PDE plus calmodulin (PDE), IBMX, or KH7. n=3 for each reaction. *** p<0.0001.
CO2-TCA & sAC Modulation regulates Ox-Phos

A) COX Vmax in isolated mitochondria treated with increasing concentration of HCO3−, from 5mM to 40mM (n=6). B, C) Isolated mitochondria from mouse liver incubated with bicarbonate (HCO3−) (n=4), or NaCl (n=3) or KH7, (n=9), KH7.15, (n=3), KH7 plus 8Br-cAMP (n=3). ATP synthesis (B) was increased by HCO3− treatment whereas KH7 inhibited it. COX Vmax was increased by HCO3−, whereas KH7 diminished it (C). COX Vmax decrease was not observed with KH7.15, and was rescued by 8Br-cAMP.

D) A representative trace of respiration driven by COX using TMPD/ascorbate mouse liver mitochondria. AA is added to mitochondria before TMPD/ascorbate to block electron transfer upstream of COX. KCN is added at the end to inhibit COX. Each addition is marked by downward arrowheads. E) Quantification of the experiments shown in D (n=9). F) KH7 (n=6) reduced respiration in intact HeLa cells and this inhibition was prevented by 8Br-cAMP (n=3), but not by forskolin plus IBMX (n=3). Control, untreated cells (n=33). G) COX activity in intact or sonicated mitochondria with or without the inhibitory anti-sAC antibody R21. HCO3− stimulation of COX was antibody insensitive in intact mitochondria, whereas it was abolished in sonicated mitochondria by R21 (n=3). H) COX activity in intact or sonicated mitochondria with or without PDE. HCO3− stimulation of COX was PDE insensitive in intact mitochondria, whereas it was abolished in sonicated mitochondria by PDE. IBMX prevented the PDE-mediated decrease in COX activity (n=3). I) cAMP levels in sonicated mitochondria were increased by HCO3− and reduced by PDE. IBMX prevented the decrease in cAMP. Values expressed as pmol cAMP per mg mitochondrial protein (n=5). *, p<0.05; **, p<0.001; ***, p<0.0001.
Physiological role of the intramitochondrial mito-sAC pathway in the regulation of OXPHOS and ROS production

A) COX Vmax in mouse liver mitochondria with CAI (n=9), CAI plus HCO3− (n=9) and CAI plus 8Br-cAMP (n=9) and pyruvate and malate as substrates. CAI decreased COX activity, which was rescued by HCO3− or 8Br-cAMP. AA (n=6), blocked CO2-production by the TCA cycle and decreased COX activity, which was rescued by HCO3− or 8Br-cAMP.

B) cAMP levels in mitochondria decreased when CA or the TCA cycle were blocked with CAI and AA, respectively. HCO3− reverted the effects of CAI or AA (n=8).

C) CAI (n=6) decreased respiration in HeLa cells and this inhibition was prevented by 8Br-cAMP (n=3). Control, untreated cells (n=33).

D) Respiration in cells grown in glucose or galactose medium (black bars) (n=6) for 48 hours. The calcium ionophore A23187 (dashed bars) increased respiration in control (untreated) cells in glucose.

E) ROS production in cells grown in glucose or galactose and treated as in D (n=6). Galactose resulted in decreased ROS production in control cells (black bars). All other comparisons are between treated and untreated cells grown under the same conditions (i.e., glucose or galactose medium). *, p<0.05; **, p<0.001; ***, p<0.0001.
Diagram of the proposed intramitochondrial CO2-HCO3--sAC-cAMP-PKA regulatory pathway of OXPHOS
Activators and inhibitors of the various steps of the CO2-HCO3--sAC-cAMP-PKA pathway are indicated. Abbreviations: PM, plasma membrane. OM, outer mitochondrial membrane. IMS, inter membrane space. IM, inner mitochondrial membrane. PKA, protein kinase A. sAC, soluble adenylyl cyclase. CA, carbonic anhydrase, KH7, inhibitor of sAC. H89 and PKI (PKI 14-22), inhibitors of PKA. PDE, phosphodiesterase. I through V indicate respiratory chain complexes (I–IV) and Complex V (ATPase).