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Stimulation of oxidative phosphorylation by calcium in cardiac mitochondria is not influenced by cAMP and PKA activity

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ABSTRACT

Cardiac oxidative ATP generation is finely tuned to match several-fold increases in energy demand. Calcium has been proposed to play a role in the activation of ATP production via PKA phosphorylation in response to intramitochondrial cAMP generation. We evaluated the effect of cAMP, its membrane permeable analogs (dibutyryl-cAMP, 8-bromo-cAMP), and the PKA inhibitor H89 on respiration of isolated pig heart mitochondria. cAMP analogs did not stimulate State 3 respiration of Ca^{2+}-depleted mitochondria (82.2 ± 3.6% of control), in contrast to the 2-fold activation induced by 0.95 μM free Ca^{2+}, which was unaffected by H89. Using fluorescence and integrating sphere spectroscopy, we determined that Ca^{2+} increased the reduction of NADH (8%), and of cytochromes b_H (3%), c_1 (3%), c (4%), and a (2%), together with a doubling of conductances for Complex I + III and Complex IV. None of these changes were induced by cAMP analogs nor abolished by H89. In Ca^{2+}-undepleted mitochondria, we observed only slight changes in State 3 respiration rates upon addition of 50 μM cAMP (85 ± 9.9%), dibutyryl-cAMP (80.1 ± 5.2%), 8-bromo-cAMP (88.6 ± 3.3%), or 1 μM H89 (89.7 ± 19.9%) with respect to controls. Similar results were obtained when measuring respiration in heart homogenates. Addition of exogenous PKA with dibutyryl-cAMP or the constitutively active catalytic subunit of PKA to isolated mitochondria decreased State 3 respiration by only 5–15%. These functional studies suggest that alterations in mitochondrial cAMP and PKA activity do not contribute significantly to the acute Ca^{2+} stimulation of oxidative phosphorylation.

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1. Introduction

The heart is capable of balancing the rate of mitochondrial ATP production with utilization continuously over a wide range of activity [1]. For instance, in large animals, the heart has a dynamic range of ATP production that exceeds 10-fold, in contrast with other organs such as the liver, which has a dynamic range of less than 2-fold [2]. Remarkably, the heart maintains a constant phosphorylation potential despite this large change in metabolite turnover [1,3]. However, the molecular mechanisms responsible for generating this energy homeostasis are poorly understood. Given the importance of oxidative phosphorylation and the balance of energy metabolism in the heart, the potential regulatory effect of mitochondrial function by classical signaling events that lead to phosphorylation of mitochondrial proteins is of interest. Although it has become appreciated over the last several years that protein phosphorylation within the cardiac mitochondrial matrix and respiratory complexes is extensive [4], little is known about the kinase/phosphatase system responsible for most mitochondrial phosphorylation events, and the vast majority of phosphorylation sites in mitochondrial proteins are located in regions that lack consensus recognition motifs for known kinases. Furthermore, most phosphorylation sites identified so far in mitochondrial enzymes have required the use of ultrasensitive mass spectrometers and phosphopeptide enrichment techniques, which suggests a very low molar fraction of phosphorylation in any given enzyme pool and casts doubt on their functional relevance [4].

An important body of evidence suggests that calcium (Ca^{2+}) is a key element in the signaling pathway responsible for the activation of mitochondrial oxidative phosphorylation (reviewed in [5]). Mitochondria maintain a large Ca^{2+} gradient across their inner membrane that is influenced by cytosolic Ca^{2+} concentrations by virtue of the proximity of mitochondria to Ca^{2+} release sites [6,7]. Ca^{2+} activates matrix dehydrogenases to accelerate NADH generation [8,9], and in parallel increases the conductance of the respiratory complexes [10], as well as the specific activity of Complex V in the inner membrane [2,11,12]. However, the precise mechanism of activation by Ca^{2+} of most of its mitochondrial effectors remains unknown, with the only elucidated case being that of pyruvate dehydrogenase, which is dephosphorylated by a Ca^{2+}-sensitive phosphatase [13,14] to attain its catalytically competent conformation [15]. Regulation of oxidative phosphorylation by Ca^{2+} might not be as important in small animals such as the mouse, where knocking out the putative mitochondrial Ca^{2+} transporter (MCU) had a very modest exercise phenotype [16]. However, it is well

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known that the dynamic range of the mouse heart is very narrow, and mitochondrial complexes are almost fully activated at rest [2]. Thus, the MCU knockout results suggest that mitochondrial activation in the mouse may not be as sensitive to Ca^{2+} as in large animals, consistent with in vitro studies of mitochondrial activity.

Recent studies have pointed to the generation of cAMP by a Ca^{2+}-sensitive soluble adenylyl cyclase within the mitochondrial matrix of the liver and HeLa cells [17,18]. The same authors reported a modest increase in respiration and cytochrome oxidase activity in the presence of permeable analogs of cAMP, as well as a more dramatic decrease in respiration after the addition of PKA inhibitors. Furthermore, rises in matrix Ca^{2+} have been reported to induce cAMP increases inside mitochondria in cardiomyocyte cultures [19], which could lead to the conclusion that Ca^{2+} might stimulate oxidative phosphorylation, at least partially, through PKA activation and subsequent phosphorylation of target enzymes involved in ATP generation. However, a recent work using cAMP and PKA activity reporters targeted to the mitochondrial matrix in HeLa cells found no evidence of PKA activity even under conditions that increased the intramitochondrial cAMP concentrations [20]. Moreover, the addition of a permeable cAMP analog together with a phosphodiesterase inhibitor to permeabilized rat heart fibers resulted in an inhibition of respiration [21], in contrast to what was found in liver mitochondria [17]. Therefore, the participation of cAMP and PKA in the acute regulation of mitochondrial metabolism remains a controversial issue. This is because the precise localization of PKA, and the distinction between the effects of PKA acting inside or on the outside of mitochondria have proven difficult to resolve [4,22]. Even more speculative is the involvement of mitochondrial PKA in mediating the stimulation of oxidative phosphorylation by Ca^{2+}.

Since the heart has a significantly larger dynamic range of ATP turnover than the liver or cultured cells, especially in larger animals [2], the proposed role of cAMP and PKA activity in the acute modulation of mitochondrial respiration, including its suggested role in mediating activation by Ca^{2+}, should be more evident in heart mitochondria. To address this issue, we have determined the effect of Ca^{2+} on pig heart mitochondrial respiration and electron distribution within the respiratory chain in the presence of widely used membrane permeable analogs of cAMP or a potent PKA inhibitor, and even after addition of exogenous PKA. Our results do not support the assumption, which could be extrapolated from a superficial interpretation of results obtained using cAMP analogs [17,22], that matrix cAMP concentrations and/or PKA activity are involved in the Ca^{2+} activation of oxidative phosphorylation in the heart or in any other acute change in ATP generation capacity.

2. Material and methods

2.1. Pig heart homogenate and mitochondrial preparation

All procedures performed were in accordance with the Animal Care and Welfare Act (7 U.S.C. 2142 § 13) and approved by the NHLBI Animal Care and Use Committee. Hearts were harvested from anesthetized pigs after injection of KCl to induce arrest and perfused in situ with cold buffer A (0.28 M sucrose, 10 mM HEPES, 1 mM EDTA, 1 mM EGTA pH 7.1) to prevent warm ischemia and remove blood and extracellular Ca^{2+} as previously described [22]. Approximately 3 g of left ventricular free wall was dissected of all fat and connective tissues on ice, and minced with scissors in 15 ml of cold buffer A. This suspension was homogenized for 10 s in a 50 ml tube at 40% power using a tissue homogenizer (Virtis, Gardiner, NY). The rest of the free ventricular wall (~80 g) was processed as described previously to isolate mitochondria [23]. One modification was that 1 mM K_2HPO_4 was added to buffer A in all mitochondrial re-suspension steps to avoid phosphate depletion of the mitochondrial matrix [24]. Mitochondria were washed twice with this phosphate containing buffer A, once with buffer B (137 mM KCl, 10 mM HEPES, 2.5 mM $MgCl_2$, 0.5 mM K_2EDTA), and finally resuspended in buffer B.

Complex IV content in the heart homogenates and in the mitochondrial suspension was determined spectrophotometrically as previously described [25,26] using Triton X-100 solubilization followed by reduction with ascorbate in the presence of cyanide for tissue homogenates and dithionite in the case of mitochondria. For quantification in heart homogenates, approximately 0.1 ml of each sample was dissolved in 1 ml of 2% (vol/vol) Triton X-100 in 0.1 M potassium-phosphate buffer, at pH 7.0. After mixing, the suspension was centrifuged for 1.5 min at 13,000 *g* to remove any residual connective tissue and solid material. The difference in absorbance at 605 nm between the reduced and oxidized forms of the enzyme, after baseline correction, was used to calculate the cytochrome aa_3 (cyt *a*) content using extinction coefficients of 10.8 $mM^{-1}\ cm^{-1}$ for tissue homogenate (due to the presence of myoglobin) and 12 $mM^{-1}\ cm^{-1}$ for mitochondria.

2.2. Mitochondrial respiration

Mitochondrial oxygen consumption, NADH fluorescence, and membrane potential ($\Delta\Psi$) were measured simultaneously and continuously in a water-jacketed chamber maintained at 37 °C as described previously [12,27]. The standard experimental buffer used for mitochondrial respiration (buffer C) contained 125 mM KCl, 15 mM NaCl, 20 mM Hepes, 1 mM EGTA, 1 mM EDTA, and 5 mM $MgCl_2$ (pH 7.1) at 37 °C. To ensure that Ca^{2+} was absent from mitochondria, a small volume equivalent to 0.75 nmol of cyt *a* was incubated for 6 min in 1.5 ml of buffer C at 37 °C [12] before the addition of 5 mM K_2HPO_4 and 0.1 mM ADP to oxidize endogenous substrates. After a 1 min incubation, 5 mM glutamate + malate was added, followed 2 min later by either 0.8 mM total $CaCl_2$ (corresponding to 0.95 μM free Ca^{2+}) or 50 μM of 8-Bromoadenosine 3′,5′-cyclic monophosphate (Br-cAMP). Other concentrations and cAMP analogs were used where indicated. After 1 min, 2 mM ADP was added to induce State 3. For control experiments, a 3 min waiting period was allowed between the addition of glutamate + malate and that of 2 mM ADP. For some experiments, 1 μM of the PKA inhibitor N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89) was added 1 min after 0.8 mM Ca^{2+} and before 2 mM ADP. cAMP and its analogs, as well as H89, were purchased from Sigma-Aldrich Co. For each mitochondrial preparation, a titration was performed to determine the optimal Ca^{2+} concentration for maximal stimulation of State 3 respiration, which always peaked at ~0.95 μM free Ca^{2+}. The concentration of free Ca^{2+} was determined using the calculator programs of Fabiato and Fabiato [28] translated to Labview VIs (National Instruments Corp., Austin, TX) by Reitz and Pollack [29].

When maximal respiration rates were investigated, the 6 min preincubation of mitochondria was omitted, and additions were made to 1 ml of buffer C in the following order: 0.9 mM total $CaCl_2$ (corresponding to 1.57 μM free Ca^{2+}), 3 mM K_2HPO_4, 0.25–0.5 nmol cyt *a* of mitochondria (or 0.5 nmol cyt *a* of heart homogenate), the indicated concentration of cAMP, N^6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate (db-cAMP), or Br-cAMP. After an incubation period of 1 min, 5 mM glutamate + malate was added followed by 60 μM ADP, which was consumed within tens of seconds to reach State 4 respiration. 1.2 mM ADP was then added to obtain State 3 respiration rates. Alternatively, cAMP, db-cAMP, Br-cAMP or 1 μM of H89 were added after glutamate + malate and incubated for 10 min with the mitochondria before induction of State 3 with 1.2 mM ADP.

For some experiments, mitochondria were diluted to 10 nmol cyt *a*/ml with buffer C and incubated with 1 μM H89 for 30 min on ice before being added to the respiration chamber to discard the possibility of a slow binding of the PKA inhibitor. To determine the effect of exogenous PKA, addition of 300 U of purified bovine heart PKA holoenzyme or 100 U of the catalytic subunit of PKA (both from Sigma-Aldrich Co.) was made to respiration buffer containing 0.9 mM total $CaCl_2$ (corresponding to 1.57 μM free Ca^{2+}), 3 mM K_2HPO_4, 3 mM ATP, 0.5 nmol cyt *a*/ml of mitochondria, and 50 μM of db-cAMP. This mixture

was incubated for 1 min before addition of 1.2 mM ADP to induce State 3.

2.3. Integrating sphere spectroscopy

Electron distribution between and within respiratory complexes was determined in intact pig heart mitochondria using an integrating sphere (LabSphere) that minimizes light scattering effects as described previously [30]. Mitochondria were suspended at final concentration of 1 nmol cyt *a*/ml in 2 ml of buffer C, and incubated for 6 min at 37 °C to ensure Ca^{2+} depletion [12] before maximal oxidation of the respiratory chain was attained by adding 5 mM K_2HPO_4 and 0.1 mM ADP. After 1 min incubation, 5 mM glutamate + malate was added, and 2 min later either 0.95 μM free Ca^{2+} or 50 μM Br-cAMP was added. After 1 min, 2 mM ADP was added to induce State 3. A second addition of 2 mM ADP allowed induction of anoxia. For control experiments, a 3 min waiting period was allowed between the addition of glutamate + malate and that of 2 mM ADP, and when indicated, 1 μM H89 was added 1 min after 0.95 μM free Ca^{2+} and before 2 mM ADP. Spectra were collected every 0.1 s, and were averaged and fitted by linear regression to separately collected references for each chromophore as described before [30]. Respiration, $\Delta\Psi$, and NADH fluorescence data, collected in parallel, were used in conjunction with the redox state of cytochrome *c*, and cytochrome *a* (the species with an absorbance peak at 607 nm) to calculate conductances of Complex I + III and Complex IV as described previously [10].

3. Results and discussion

3.1. Cardiac mitochondrial respiration and NADH in the presence Ca^{2+} cAMP analogs and PKA inhibitor

Stimulation of mitochondrial respiration has been reported in liver mitochondria and cultured cells by addition of membrane permeable cAMP analogs or induction of cAMP synthesis inside the mitochondrial matrix by activation of a soluble adenylyl cyclase [17]. Conversely, it was found that the PKA inhibitor H89 decreased respiration by up to 50%. These results were interpreted as indicative of increased activity of individual mitochondrial complexes, in particular cytochrome *c* oxidase (Complex IV), by phosphorylation catalyzed by PKA associated with, or even located inside mitochondria [31]. Ca^{2+} is known to activate soluble adenylyl cyclases [32], one of which appears to be present in mitochondria [33], where matrix Ca^{2+} and cAMP concentration increases are reported to parallel each other [19]. This has led to the suggestion that the well-known stimulatory effect of Ca^{2+} on oxidative phosphorylation [5] is mediated or potentiated by increases in cAMP concentration and PKA activity inside mitochondria [19]. However, as shown in Fig. 1A, pig heart mitochondrial respiration was insensitive to the presence of the membrane permeable Br-cAMP at a concentration 1000 times higher than that reported to activate PKA [34], and under conditions in which Ca^{2+} matrix concentration was minimal. A similar insensitivity was obtained after incubating the mitochondria for 40 min with up to 1 mM Br-cAMP, conditions under which it is known that Br-cAMP can reach the mitochondrial matrix when added to cultured cells [19], or with the 10-fold more lipophilic cAMP analog db-cAMP used at 0.1 mM (Supplementary data, Fig. S1). As expected, replenishment of Ca^{2+} resulted in a 2-fold increase in respiration linked to ATP synthesis. Notably, the PKA inhibitor H89 did not prevent or decrease this Ca^{2+} stimulation of oxidative phosphorylation, indicating that Ca^{2+} activation is independent of PKA activity.

A distinguishing feature of the Ca^{2+} activation of respiration linked to ATP synthesis is a slight but significant increase in the NADH concentration [35]. As shown in Fig. 1B, we observed that Ca^{2+} caused an increase of 8% in the State 3 NADH level relative to the Ca^{2+}-depleted condition. This mild increase in NADH reflects the activation of matrix dehydrogenases by Ca^{2+} [9]. NADH levels were unaffected by Br-cAMP or H89 consistent with the lack of effect on net flux of electrons through the cytochrome chain. NADH reductions levels at State 4 (before addition of ADP) were increased from ~50% in the absence of Ca^{2+} to ~75% upon Ca^{2+} addition, an effect not observed with db-cAMP, Br-cAMP, and not impeded by H89 (data not shown).

Another membrane permeable compound, 8-CPT-6-Phe-cAMP, has the highest lipophilicity of all reported cAMP analogs (~400 times higher than cAMP), and has been recently reported to activate cytosolic PKA when added to the growth media of cultured cells [19]. We determined the effect of incubating mitochondria for 40 min with various concentrations of this highly permeable analog on the State 3 respiration rate of isolated cardiac mitochondria, as shown in the representative experiment of Fig. S2A in Supplementary data. Contrary to the reported stimulation of respiration and ATP synthesis with less permeable cAMP analogs [17], 8-CPT-6-Phe-cAMP inhibited mitochondrial respiration, an effect that was also observed in the presence of Ca^{2+}. This inhibition of respiration was at least partly due to a decrease in the availability of electrons entering the respiratory chain, as indicated by the lower NADH levels at State 3 obtained in the presence of 8-CPT-6-Phe-cAMP, as shown in Fig. S2B of Supplementary data. Similar results were obtained when this compound was

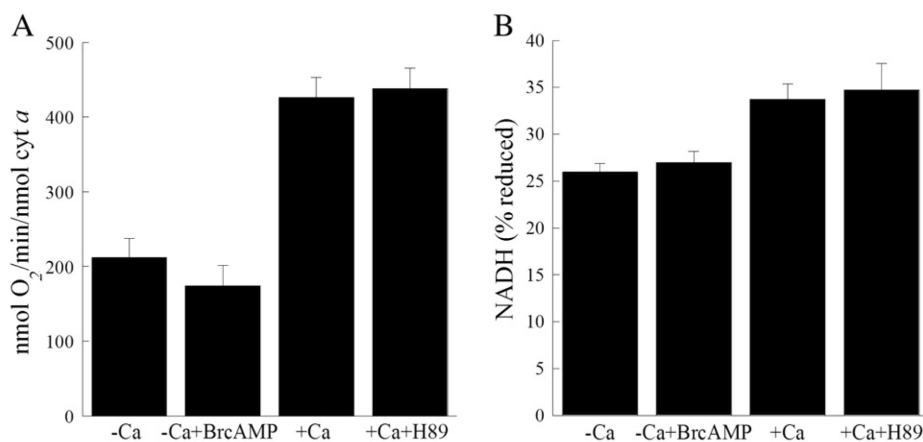

Fig. 1. Cardiac mitochondrial State 3 respiration and NADH levels in the presence of Ca^{2+}, Br-cAMP, or a PKA inhibitor. Pig heart mitochondria (0.5 nmol cyt *a*/ml) preincubated for 6 min at 37 °C to minimize matrix Ca^{2+} and respiring on 5 mM glutamate + malate were exposed or not to 0.95 μM free Ca^{2+} or 50 μM Br-cAMP before addition of 2 mM ADP. Alternatively, 1 μM H89 was added after Ca^{2+}. State 3 respiration (A) and NADH levels (B) were increased 2-fold and 8% respectively, by Ca^{2+}. Br-cAMP and H89 did not replicate or abolish the effect of Ca^{2+}. The average of 6 experiments ± S.E. is shown for 0 and 0.95 μM free added Ca^{2+} and of 3 experiments for Br-cAMP and Ca^{2+} + H89.

added directly to the mitochondria during a respiration assay without any preincubation (not shown). Therefore, great care should be exercised when using and interpreting results with highly lipophilic cAMP analogs because of unspecific inhibitory effects unrelated to PKA activation.

3.2. Effect of Ca^{2+} on the electron distribution in the respiratory chain of cardiac mitochondria

Even though respiration was not influenced by membrane permeable cAMP analogs, it could still be argued that cAMP, as a downstream effector of Ca^{2+}, is able to change the redox poise of the system by altering the activity of individual elements such as Complex IV, as previously proposed [18,36]. Thus, we sought to characterize for the first time the effect of Ca^{2+} on the redox poise of the cytochromes in intact porcine heart mitochondria. To accomplish this task, we used a center sample-mounted integrating sphere coupled to a scanning spectrophotometer to minimize light scattering. This approach has been extensively described in the determination of the redox poise between and within respiratory complexes under various conditions, including Ca^{2+} activation of skeletal muscle mitochondria [10] and post-reperfusion injury in heart mitochondria [30]. For the present studies, after incubation at physiological temperature in the presence of Na-containing medium to facilitate the exit of matrix Ca^{2+} [12], mitochondria were oxidized by adding phosphate and a small amount of ADP to remove any residual reducing equivalent substrates in the matrix [10]. This provided an estimate of the fully oxidized state, while anoxia or dithionite addition was used at the end of the experiment to generate the fully reduced condition for calculation of free energies.

As shown in Fig. 2A, re-energization of heart mitochondria upon addition of glutamate and malate resulted in a modest reduction of all cytochromes (from 3% in cytochrome *a* to 30% in b_H relative to the total dithionite-reducible cytochrome pool). Ca^{2+} addition at State 4 resulted in subtle changes in the spectrum, which when fitted to individual components corresponded to a 6% reduction in flavin (consistent with an increase in NADH by activation of matrix dehydrogenases) and a 1% reduction in both cytochrome b_H and in the 607 nm species of cytochrome *a* (deconvoluted spectral fit not shown). These spectral changes induced by Ca^{2+} at State 4 were not mimicked by cAMP analogs nor were they abolished or prevented by addition of H89 either before or after Ca^{2+} (data not shown). Upon addition of ADP to induce State 3, further spectral changes were observed, which are more easily visualized in Fig. 2B, where the difference of spectra collected and averaged after addition of ADP minus those acquired at State 4 (before addition of Ca^{2+}) is shown. Both in the presence and absence of Ca^{2+}, increases were observed at the maximal absorbance wavelengths of cytochrome *c* (550 nm) and cytochrome *a* (607 nm), together with a decreased absorbance at 566 nm, corresponding to the absorbance maximum of cytochrome b_L of Complex III. However, in the absence of Ca^{2+} a more significant oxidation of flavin was observed (corresponding to an increased absorbance at 450 nm), and the cytochrome *c* absorbance increase appeared less prominent, similar to what was reported in skeletal muscle mitochondria [10].

Spectral fitting of the absorbance changes observed after induction of State 3 with or without Ca^{2+} addition for a representative experiment is presented in Fig. 3. Deconvolution of the spectral difference revealed that indeed oxidation of flavin was larger in the absence of Ca^{2+}, whereas reduction of cytochromes b_H, c_1, *c*, and *a* (the 607 nm species) was enhanced by Ca^{2+}. The absorbance decreases in cytochrome b_L and in the 580 nm species of cytochrome *a* were similar in the absence or presence of Ca^{2+}. As we have discussed elsewhere [30], the large b_L oxidation reflects the decrease in membrane potential that occurs upon opening of the F_O channel of Complex V upon initiation of ATP synthesis, which favors electrons to move from the b_L to the b_H heme within cytochrome *b* [37]. The fact that this b_L oxidation is of approximately the same magnitude regardless of the presence of Ca^{2+} is consistent with the lack of significant difference in membrane potential attained at State 3 (see below). Also as discussed before [30], the 580 nm cytochrome *a* species probably corresponds to the a_3–Cu_B intermediate that accepts and transfers the final electron for the reduction of oxygen to water [38], which is expected to decrease in concentration as the respiration rate increases upon induction of ATP synthesis. The larger increase in the 607 nm intermediate of cytochrome *a* in the presence of Ca^{2+} corresponds to the already reported linear correlation between the absorbance of this species and the rate of oxygen consumption [10,30], which was increased 2-fold by Ca^{2+}. The higher reduction levels of all other chromophores in the presence of Ca^{2+} are consistent with an activation of substrate oxidation, but this effect alone is not enough to account for the stimulation of oxidative phosphorylation, as has been reported in skeletal muscle mitochondria [10], and as will be discussed below for heart mitochondria. The spectral changes detected in the presence of Ca^{2+} cannot be attributed simply to changes in

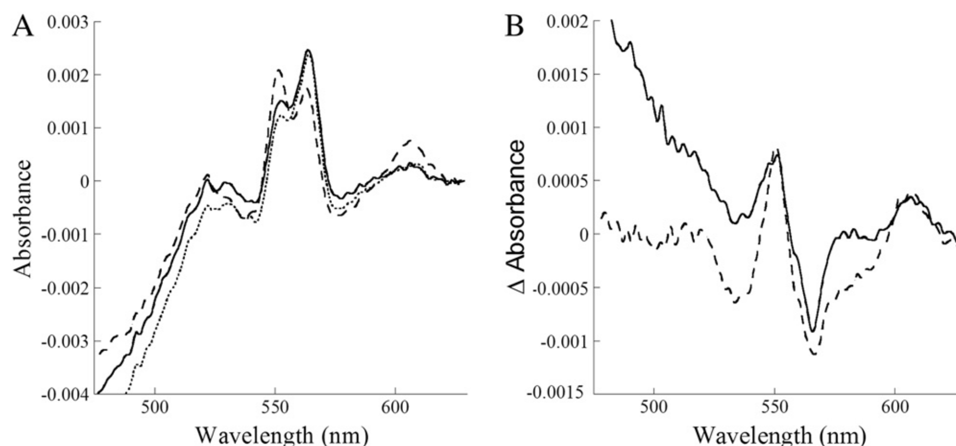

Fig. 2. Spectral changes of cardiac mitochondria associated with addition of substrate, Ca^{2+} and ADP. Pig heart mitochondria (0.5 nmol cyt *a*/ml) preincubated for 6 min at 37 °C to minimize matrix Ca^{2+} were oxidized in an integrating sphere chamber with 5 mM phosphate and 0.1 mM ADP to collect an oxidized reference averaged spectrum (A) that was subtracted from the averaged spectrum after addition of 5 mM glutamate + malate (solid line), followed by 0.95 μM free Ca^{2+} (dotted line), and 2 mM ADP (dashed line). Using the glutamate + malate reduced averaged spectra as the reference (B), the spectral changes induced by the addition of 2 mM ADP without (solid line) and with addition of 0.95 μM free Ca^{2+} (dashed line) added after substrate allow a clearer visualization of the effects of Ca^{2+} activation.

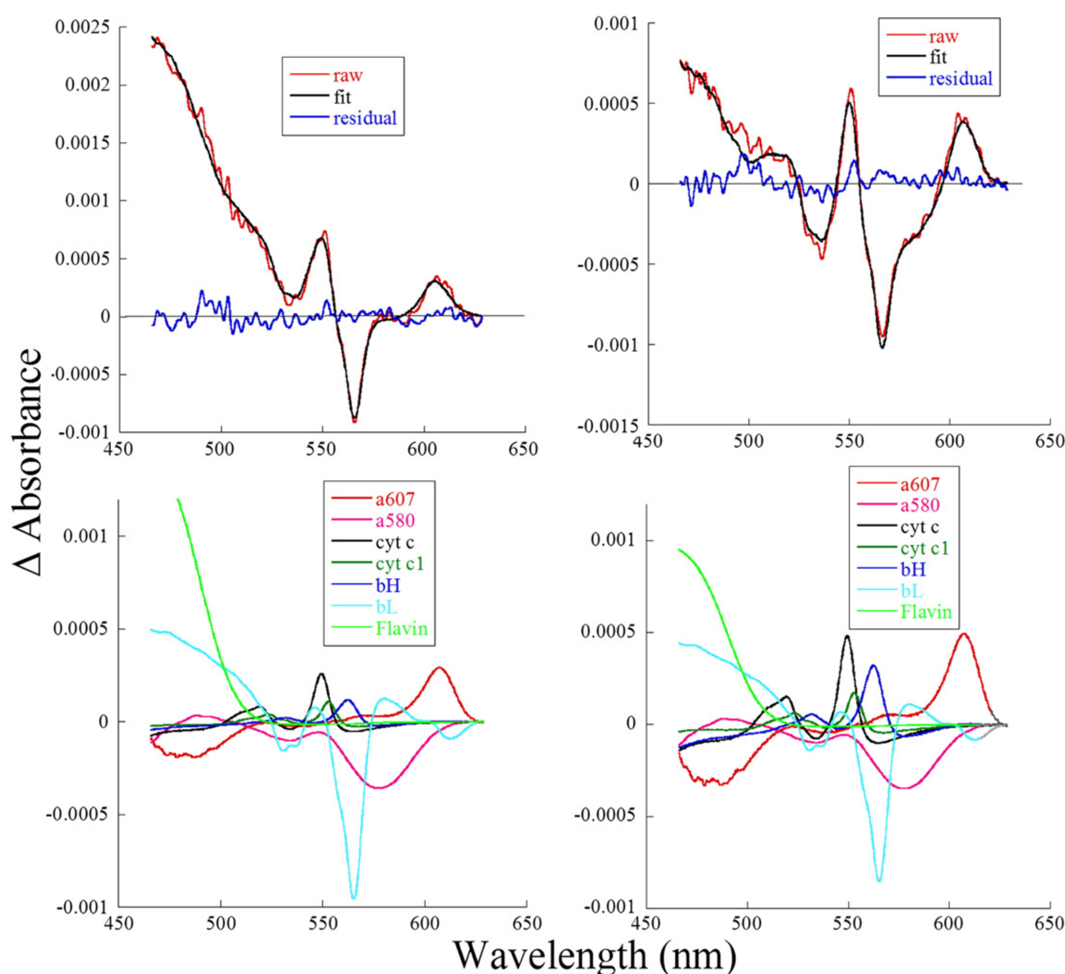

Fig. 3. Fitting and deconvolution of the spectral changes of cardiac mitochondria at State 3 associated with Ca^{2+} activation. The upper panels show the raw (red) and fitted (black) difference averaged spectra of State 3 (after 2 mM ADP) minus State 4 (after 5 mM glutamate + malate) either without Ca^{2+} addition (left) or upon addition of 0.95 μM free Ca^{2+} (right) after substrate to cardiac mitochondria, with fitting residuals also shown (blue). The lower panels show the contribution of each individual chromophore to the best fit with or without the addition of Ca^{2+}. Experimental conditions were the same as in Fig. 2.

mitochondrial volume that could generate variations in light scattering. As described elsewhere [30], integrating sphere spectroscopy cancels light scattering to a large degree. As a proof of this, we observed no variation in the spectra upon induction of mitochondrial shrinking after the addition of substrate by adding 100 mM KCl to make the assay medium hypertonic (data not shown). Also, variations in light

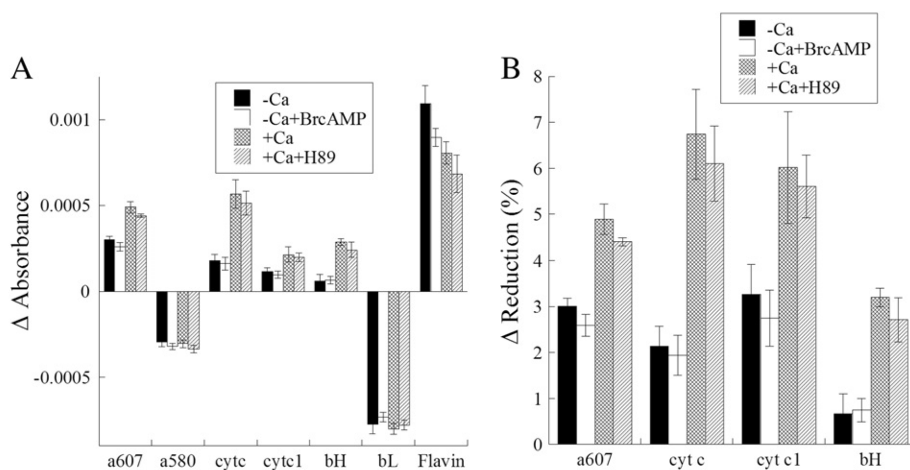

Fig. 4. Magnitude of spectral changes of cardiac mitochondria induced by ADP addition in the presence of Ca^{2+}, Br-cAMP, or a PKA inhibitor. Pig heart mitochondria incubated as in Fig. 2 were exposed or not to 0.95 μM free added Ca^{2+} or 50 μM Br-cAMP before addition of 2 mM ADP. Alternatively, 1 μM H89 was added after Ca^{2+}. Using the State 4 (after glutamate + malate addition) averaged spectra as a reference, the change in absorbance of the individual chromophores (A) was obtained after fitting and deconvoluting the averaged State 3 spectra. The reduction changes relative to the total concentration (determined by dithionite reduction) of each of the chromophores that varied significantly when comparing the addition of 0 or 0.95 μM free Ca^{2+} are also shown (B). The average of 6 experiments ± S.E. is shown for 0 and 0.95 μM free added Ca^{2+} and of 3 experiments for Br-cAMP and Ca^{2+} + H89.

scattering are very unlikely to generate increases precisely at the peak wavelengths of some chromophores and at the same time cause decreases in the peaks of others, as is observed when comparing the data obtained in the presence or absence of Ca^{2+}.

3.3. Electron distribution and relative conductances of respiratory complexes in the presence of Ca^{2+}, cAMP analogs and PKA inhibitor

The spectral differences in each mitochondrial chromophore upon induction of State 3 respiration as determined by integrating sphere spectroscopy are summarized in Fig. 4A. As already noted above, one of the major contributors in absolute absorbance to the spectral changes observed upon ADP addition was the oxidation of flavin, which was ~25% more reduced (lower absorbance at 450 nm) in the presence of Ca^{2+}, an effect that was almost statistically significant ($p = 0.051$). The addition of Br-cAMP seemed to decrease the oxidation of flavin, but this effect was not statistically significant ($p = 0.14$), and the PKA inhibitor H89 did not prevent the extra reduction of flavin induced by Ca^{2+}. The decrease in the absorbance of b_L and the 580 nm species of cytochrome *a* were not different in any of the four conditions tested. Relative to the changes that occurred in the absence of Ca^{2+}, the reduction of cytochromes b_H and *c* was 3-fold higher when Ca^{2+} was present, while that of cytochromes c_1 and *a* (at 607 nm) was increased by a factor of 2. These changes were not significantly different if Br-cAMP was added instead of Ca^{2+}, or if H89 was present together with Ca^{2+}. It must be noted that the increased reduction by Ca^{2+} is small when expressed in terms of the total concentration of cytochromes as determined by full reduction with dithionite, as shown in Fig. 4B. Ca^{2+} induced an additional reduction of only 3% in cytochrome b_H and c_1, 4% in cytochrome *c*, and 2% in the 607 nm species of cytochrome *a* over that attained in the absence of Ca^{2+}. Nevertheless, the fact that the relatively small effects induced by Ca^{2+} were measurable using integrating sphere spectroscopy demonstrates the sensitivity of this technique.

The ability to determine the redox levels of most components of the respiratory chain, together with membrane potential and oxygen consumption rates, allows the calculation of relative conductances of segments of the respiratory chain, which provide a direct measure of the intrinsic catalytic rate of respiratory complexes [30]. As shown in Table 1, respiratory complexes I + III and IV in heart mitochondria were activated in parallel by Ca^{2+}, with a 2 to 2.3-fold increase in their relative conductance. These values are similar to those determined in skeletal muscle mitochondria, where the respiratory chain was also found to be activated in parallel by Ca^{2+} along with matrix NAD-dependent dehydrogenases and ATP synthase [10]. Again, this effect was not mimicked by 8-BrcAMP nor abolished by H89. The failure of agents that activate or inhibit PKA in changing the intrinsic activities of respiratory complexes, including Complex I and Complex IV, which have been argued to be substrates (at least in vitro) of PKA phosphorylation [36,39], indicates the existence of a PKA-independent pathway for the activation of oxidative phosphorylation by Ca^{2+}. Furthermore, these results support the conclusion that the 2-fold stimulation of State 3 respiration by Ca^{2+} cannot be explained simply by matrix dehydrogenase activation, which generated only a ~8% higher NADH concentration, nor by exclusive activation of any individual respiratory complex, given the relatively small (2–4%) reduction increase observed in the cytochrome components (see Fig. 4B). Just as was demonstrated for the inhibition of respiration in mitochondria isolated from hearts exposed to ischemia–reperfusion injury [30], Ca^{2+} activation of respiration in the heart occurs by increasing the intrinsic activity of most of the enzymes comprising the oxidative phosphorylation pathway. The elucidation of this parallel mechanism of acute activation, which seems not to involve the cAMP–PKA signaling axis, remains an important missing piece to complete our understanding of metabolic homeostasis in the heart and other highly oxidative organs.

3.4. Cardiac mitochondrial respiration in the presence of cAMP analogs when preserving or adding PKA

The relatively long preincubation time at physiological temperature needed to maximize Ca^{2+} exit from the mitochondrial matrix, included in the previous experiments, could potentially deplete mitochondria from undetermined factors and metabolites, or even induce translocation and dissociation of proteins from the matrix or membrane compartments. To discard the possibility that this manipulation could be obscuring the effects of PKA activators and inhibitors, we first determined respiration in non-preincubated mitochondria under conditions that yielded maximal State 3 rates. This ensured that optimal mitochondrial function was being preserved, and also allowed us to evaluate if phosphorylation of mitochondrial complexes by activation of PKA could actually have an inhibitory effect, as has been proposed based on permeabilized rat heart fibers incubated with cAMP analogs [21]. As shown in Fig. 5A, mitochondrial respiration was largely insensitive to the presence of cAMP or the membrane permeable analogs db-cAMP and Br-cAMP, even at concentrations up to 30-fold higher than those known to activate PKA [40]. In these experiments, cAMP or its analogs were incubated with mitochondria for only 1 min before adding ADP to obtain State 3 respiration rates. As shown in Fig. 5B, even a 10 min incubation of mitochondria with cAMP or its analogs failed to stimulate respiration under conditions of ATP synthesis. Only a slight but statistically insignificant decrease in respiration was observed with all three compounds: cAMP (85 ± 9.9% of control State 3 respiration), db-cAMP (80.1 ± 5.2%), and Br-cAMP (88.6 ± 3.3%). A similar result was obtained when incubating mitochondria with the PKA inhibitor H89 (89.7 ± 19.9% of control respiration), which indicates that the lack of effect of the cAMP analogs cannot be attributed to preexisting high endogenous cAMP levels in the isolated mitochondria, which could have already activated fully the supposed PKA pool associated with the organelle. Even longer incubations of the H89 inhibitor (30–45 min) with mitochondria incubated on ice failed to affect mitochondrial respiration (data not shown), which eliminated the possibility of a very slow binding of H89 to PKA.

The mild unspecific inhibitory effect of db-cAMP could explain the results obtained in saponin-permeabilized rat heart fibers [21], in which an inhibition of up to 50% of respiration was obtained in the presence of 200 μM of this compound, four times higher than what we have used in most experiments of the present study. Furthermore, when using db-cAMP in the cited study, cardiac fibers were also exposed to theophylline to inhibit cytosolic phosphodiesterases that could deplete cAMP. However, we have determined that theophylline at the concentrations used in the cited study inhibited both State 3 and State 4 respiration by ~20% in the absence of cAMP analogs (data not shown), suggesting a direct mild inhibitory effect of theophylline

Table 1
Calculation of relative conductances for respiratory chain components in the presence of Ca^{2+}, Br-cAMP and H89.

Condition	State 3 respiration rate (VO_2) (nmol O_2/min/nmol cyt *a*)	ΔG (kcal/mol) NAD/NADH–cyt_*c*–ΔΨ	ΔG (kcal/mol) a607–O_2/H_2O–ΔΨ	Conductance of CI + CIII (VO_2/ΔG)	Conductance of CIV (VO_2/ΔG)
$-Ca^{2+}$	210.3 ± 25.9	−6.95 ± 0.17	−7.03 ± 0.4	30.3 ± 4.4	29.9 ± 5.1
$-Ca^{2+}$ + Br-cAMP	160.7 ± 27	−6.71 ± 0.21	−7.08 ± 0.12	23.9 ± 4.6	22.7 ± 4.1
$+Ca^{2+}$	426.4 ± 26.7	−6.27 ± 0.53	−7.39 ± 0.53	68.1 ± 9.3	57.7 ± 7.3
$+Ca^{2+}$ + H89	438.2 ± 27.2	−6.28 ± 0.58	−7.01 ± 0.55	69.7 ± 9.8	62.5 ± 8.1

The average of 3 experiments ± standard error is shown.

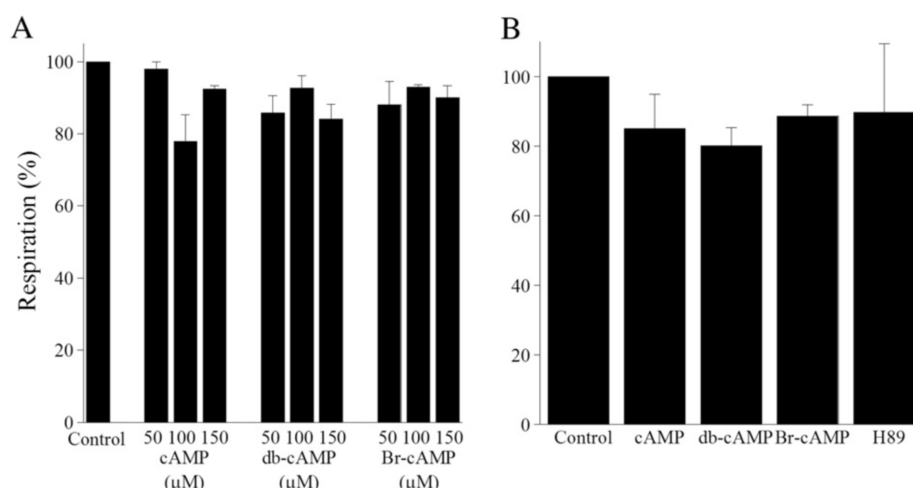

Fig. 5. Cardiac mitochondrial respiration in the presence of various concentrations of cAMP analogs and different incubation times with a PKA inhibitor. Pig heart mitochondria not preincubated to eliminate intramitochondrial Ca respiring on 5 mM glutamate + malate in the presence of 1.2 mM ADP and 1.57 μM free Ca^{2+} showed no activation by different concentrations of cAMP or its membrane-permeable analogs db-cAMP and Br-cAMP after a 1 min incubation (A), only a slight (and statistically insignificant, $p = 0.072$) inhibition with 100 μM db-cAMP was observed. Control respiration was 469 ± 21 nmol O_2/min/nmol cyt *a* (n = 3). For comparison, an independent set of additions of 50 μM of cAMP or its analogs (incubated for 10 min on ice) is shown (B). The PKA inhibitor H-89 (1 μM) also showed little effect on mitochondrial respiration. Control respiration in this set of experiments was 306 ± 16 nmol O_2/min/nmol cyt *a* (n = 4).

somewhere in the respiratory chain independent of PKA activation. If such inhibitory effect of theophylline is additive to that of high concentrations of db-cAMP, that could explain the ~50% inhibition observed without implying an inhibitory effect of respiratory complex phosphorylation by PKA. This would agree with the recent determination that PKA activity can only be detected in mitochondria associated with the outer mitochondrial membrane, but not in the mitochondrial matrix [20]. Indeed, we were not able to detect PKA activity in concentrated pig heart mitochondrial preparations even after solubilizing the mitochondrial membranes in the presence of high (0.1–1 mM) concentrations of Br-cAMP, db-cAMP or 8-CPT-6-Phe-cAMP using a PKA activity detection assay kit (Enzo Life Sciences), nor could we detect any of the subunits of PKA by mass spectrometry of isolated mitochondria, although the A-kinase anchor protein Akap1 was found (data not shown). This suggests that the association of PKA to the outside of mitochondria is probably weak and transient, and does not persist through mitochondrial isolation procedures.

Another possible explanation for the lack of effect of PKA activators or inhibitor on respiration is that mitochondrial isolation depleted the PKA pool associated with the organelle, especially if it is loosely bound to the outer mitochondrial membrane [20]. However, as shown in Fig. 6A, respiration in crude heart homogenates where mitochondria were not washed also failed to manifest any significant effect of cAMP or its permeable analogs on respiration (cAMP: 88.9 ± 2.4%; db-cAMP: 113.6 ± 13%; Br-cAMP: 101.1 ± 9.2% of control State 3 rate). Alternatively, isolated mitochondria were incubated with exogenous PKA, either the holoenzyme that can only be activated by cAMP and its analogs, or the catalytic subunit that is constitutively active. In all these experiments, 3 mM ATP was present to ensure that the exogenous PKA had enough substrate to phosphorylate any protein targets. As shown in Fig. 6B, only addition of PKA alone or in the presence of db-cAMP induced a very slight inhibition of respiration (PKA: 89.1 ± 1.2%; PKA + db-cAMP: 87.7 ± 1.3% of control State 3 rate), perhaps due to some contaminant present in the purified PKA. The addition of db-cAMP only or of the catalytic subunit of PKA showed identical respiration rates relative to control incubations (100% ± 1.2% and 100.5% ± 2.8%, respectively). The activity of the PKA holoenzyme and of the catalytically active PKA subunit added to mitochondria was verified separately by

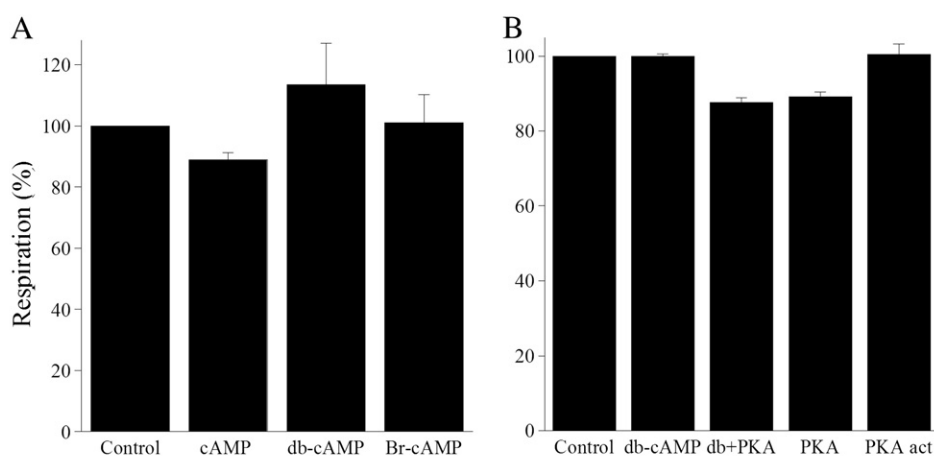

Fig. 6. Heart homogenate respiration in the presence of cAMP analogs and addition of exogenous PKA to mitochondria. To account for a possible loss of endogenous PKA during mitochondrial isolation, homogenized pig left ventricle myocardium was assayed in the presence of various cAMP analogs (A). Homogenates were allowed to respire by adding 5 mM glutamate + malate and 1.2 mM ADP after incubation for 1 min at 37 °C in the presence of 50 μM cAMP, db-cAMP, or Br-cAMP. Respiration in the absence of cAMP analogs (Control) was 311 ± 31 nmol/min/nmol cyt *a* (n = 3). Addition of 300 U of PKA purified from bovine heart with or without 50 μM dibutyryl-cAMP or 100 U of the constitutively active PKA subunit to isolated pig heart mitochondria (B) had little or no effect on glutamate + malate sustained respiration and membrane potential at State 3. Control respiration was 500 ± 50 nmol O_2/min/nmol cyt *a* (n = 3).

mass spectrometry identification of the phosphorylated peptide substrate GRTGRRNSI after mixing with the same amount of enzyme in buffer C for 5 min in the presence of 3 mM ATP (data not shown).

The lack of effect of PKA activators and inhibitor in isolated mitochondria even in the presence of added PKA, or in heart homogenates that contain endogenous cytosolic PKA, begs the question of why other studies have reported a role for PKA activation on ATP synthesis regulation [17,19]. First of all, most of the significant effects of PKA activation or inhibition have been described in cultured cells after exposure to cAMP analogs or PKA inhibitors during long periods of time, ranging from 0.5 to up to 3 h, suggesting that protein expression or other longer term changes could be occurring. Secondly, ATP concentrations or synthesis rates were determined using luciferase luminescence assays that do not seem to correlate well with more straightforward ATP synthesis measurements such as respiration rates of coupled mitochondria under phosphorylating conditions (State 3). For example, according to the initial studies proposing a role for PKA in the regulation of oxidative phosphorylation in liver mitochondria [17], Br-cAMP stimulated State 3 respiration weakly, a result that contradicts the reported doubling of the rate of ATP synthesis using a kinetic luminescence assay. Furthermore, when blocking PKA, State 3 respiration was found to be inhibited by 50% when using H89, but only ~20% when using other PKA inhibitors. Still, the rate of ATP synthesis was reported to be inhibited by up to 4-fold [17]. These inconsistencies suggest that some of the compounds used showed unspecific effects, especially when determining rates of ATP synthesis separately from respiration, implying that the effects of PKA activation and inhibition were probably overestimated.

In the study in which a correlation between cAMP increases in the matrix and Ca^{2+} activation of ATP synthesis was reported [19], the luminescence signal in HeLa cells expressing luciferase in the matrix inexplicably increased steadily even during unstimulated conditions, a drift that was strangely abolished altogether by transfecting cells with a PKA inhibitory peptide. ATP synthesis rates in this same study were compared during this luminescence drift after 2 h of having exposed cells to high outside Ca^{2+} along with an inhibitor of the sarcoplasmic reticulum Ca^{2+} ATPase in order to generate a large and sustained entry of Ca^{2+} from the medium. No evidence was provided of mitochondrial integrity under these highly unphysiological conditions, which could potentially induce Ca^{2+} overloading, swelling and permeability transition. Nevertheless, even using these questionable methods and conditions of measuring ATP synthesis rates, the alleged Ca^{2+} activation increased ATP synthesis rate by less than 50%, and exposure of cells to 1 mM Br-cAMP for 2 h failed to increase the rate of ATP synthesis at all. Casting further doubt on the luminescence-based assay is the result that incubation of cells for 2 h with 100 mM 8-CPT-6-Phe-cAMP, a concentration which we have presently found to strongly inhibit State 3 respiration in isolated mitochondria (see Fig. S2A in Supplemental data), was found to inhibit ATP synthesis only by ~20%. All these considerations suggest that the luciferase assays used were not accurate in determining ATP synthesis rates.

Samples from mitochondria and heart homogenate respired in the presence or absence of cAMP analogs, H89, and exogenous PKA analyzed by 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) failed to show any shifts in the position of protein spots (data not shown). Since this technique involves separation of proteins by their isoelectric point in the first dimension, the lack of visible protein shifts indicates that no significant post-translational modifications of abundant mitochondrial proteins (such as those belonging to respiratory complexes) occurred upon treatment with cAMP analogs, H89 or exogenous PKA. Acute regulation of respiration by PKA phosphorylation of mitochondrial complexes, as has been proposed in particular for Complexes I and IV [18,36,39,41] would imply modification of a significant mole fraction of those particular subunits so as to change the relative concentration of active enzyme [4]. The lack of protein shifts upon PKA activation, inhibition or addition agrees with our respiration data described above, and strongly suggests that PKA does not have a role in the acute regulation of oxidative phosphorylation in heart mitochondria. This conclusion is supported by the overall lack of PKA recognition sequences in mitochondrial respiratory proteins, with one exception being the transit peptide of subunit beta of ATP synthase [4], which has been reported to be irrelevant for protein import [42]. Other phosphorylation sites have been reported in Complex I subunits after in vitro incubation with PKA, such as Ser59 of NDUFS3 [39,43], Ser55 of NDUFA1 [44,45] and Ser20 of NDUFB11 [45]. However, the physiological relevance of those observations is questionable. Only in the case of Ser173 of NDUFS4 has a role of PKA phosphorylation been determined [39], but this is limited to protein import.

Our present results do not contradict other roles recently demonstrated for cAMP and PKA in the regulation of mitochondrial function. It has been found that cAMP concentration changes and concomitant PKA activation influence the control of mitochondrial morphology and mitophagy by Drp1 phosphorylation, regulation of mitochondrial biogenesis by phosphorylation of the CREB transcription factor, and induction of apoptosis by translocation of Bax to the outer mitochondrial membrane after phosphorylation by PKA [46]. However, a role of cAMP and PKA in the acute regulation of mitochondrial metabolism, even if Ca^{2+} causes an acute increase in mitochondrial matrix cAMP concentration [19], is not supported by the experimental evidence provided in the present study. Obviously, extrapolating isolated mitochondrial results to the intact heart function is problematic, even when using moderately diluted tissue homogenates or even an excess of exogenous PKA. Other unknown cytosolic factors might be depleted or diluted to the point in which they can no longer effectively mediate changes in cytosolic cAMP or PKA associated with the outer mitochondrial membrane, which is also an issue to consider in studies that use permeabilized cardiac fibers [21]. Nevertheless, our results strongly suggest that the cAMP variations observed in the mitochondrial matrix, which appear not to be associated with any detectable PKA activity inside the mitochondria (where the presence of this kinase is also highly disputed), are unrelated to the acute modulation of oxidative phosphorylation that Ca^{2+} is able to exert.

4. Conclusions

The large dynamic range of cardiac aerobic ATP production in a large animal is ideal for studying the acute regulation of oxidative phosphorylation. Here we have used the isolated pig heart mitochondrion as a model system to examine the possible role of cAMP and PKA in the activation by Ca^{2+} of the mitochondrial ATP synthetic capacity. We have determined for the first time in porcine heart mitochondria that the doubling of mitochondrial respiration linked to ATP synthesis upon addition of Ca^{2+} is characterized by a ~2-fold increase in the conductance of all respiratory chain complexes, along with subtle increases in the reduction level of most electron transferring chromophores. None of these effects associated with the parallel activation of oxidative phosphorylation by Ca^{2+} were influenced by membrane permeable analogs of cAMP or a PKA inhibitor. Even under conditions that tried to preserve or replenish PKA potentially associated with mitochondria, cAMP and its permeable analogs failed to significantly modify respiration. Only a minor inhibitory effect of db-cAMP was observed that is likely unrelated to PKA activation. These results suggest that matrix cAMP increases and putative matrix PKA activation do not play a major role in the rapid modulation of oxidative phosphorylation complexes by Ca^{2+} in porcine heart mitochondria.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.08.006.

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