Cyclic Adenosine Monophosphate-Dependent Phosphorylation of Mammalian Mitochondrial Proteins: Enzyme and Substrate Characterization and Functional Role†

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ABSTRACT: A study is presented on cyclic adenosine monophosphate- (cAMP-) dependent phosphorylation of mammalian mitochondrial proteins. Immunodetection with specific antibodies reveals the presence of the catalytic and the regulatory subunits of cAMP-dependent protein kinase (PKA) in the inner membrane and matrix of bovine heart mitochondria. The mitochondrial cAMP-dependent protein kinase phosphorylates mitochondrial proteins of 29, 18, and 6.5 kDa. With added histone as substrate, PKA exhibits affinities for ATP and cAMP and pH optimum comparable to those of the cytosolic PKA. Among the mitochondrial proteins phosphorylated by PKA, one is the nuclear-encoded (NDUFS4 gene) 18 kDa subunit of complex I, which has phosphorylation consensus sites in the C terminus and in the presequence. cAMP promotes phosphorylation of the 18 kDa subunit of complex I in myoblasts in culture and in their isolated mitoplast fraction. In both cases cAMP-dependent phosphorylation of the 18 kDa subunit of complex I is accompanied by enhancement of the activity of the complex. These results, and the finding of mutations in the NDUFS4 gene in patients with complex I deficiency, provide evidence showing that cAMP-dependent phosphorylation of the 18 kDa subunit of complex I plays a major role in the control of the mitochondrial respiratory activity.

Although different hormones and neurotransmitters utilizing the cAMP[1] cascade activate the same enzyme, protein kinase A (PKA), they still trigger distinct physiological processes. It follows that different effectors, each capable of raising intracellular cAMP, can result in the preferential phosphorylation and functional modulation of specific target proteins. A breakthrough in understanding this selectivity was provided by the recognition of the existence of intracellular gradients of cAMP (*1*) and the discovery of a new family of proteins (protein kinase A anchor proteins, AKAP), which specifically anchor PKA to different cellular structures (*2, 3*). These acquisitions indicate that the selectivity of the diverse effects mediated by the PKA pathway, once the system is activated in a cell by a given neurohormonal effector−receptor interaction, is determined by specific changes in the cAMP level in various cell compartments and by the subcellular sites where the PKA holoenzyme is localized or where the catalytic subunits, once released from the cytosolic holoenzyme upon binding of cAMP to the R-subunits, migrate (*4*).

In the resting state PKA exists as an inactive tetrameric complex consisting of two regulatory subunits (R-PKA), which have a high affinity for cAMP, and two catalytic subunits (C-PKA). Four R-PKA isoforms (*5*) and three C-PKA isoforms (*6*) have been identified. R-PKA isoforms can serve as a cytoplasmic reservoir, sequestering C-PKA in an inactive state (*7*) until the holoenzyme dissociates in response to cAMP and C-PKA subunits are released in an active state. R-PKA isoforms can also be associated by AKAPs in various cellular structures (*2, 3*) and this complex can serve to localize the PKA holoenzyme in different subcellular compartments, thus determining the physiological substrates to be phosphorylated.

After contradictory reports (*8*−*12*), more recent results of biochemical (*13*−*16*) and immunochemical investigations (*17*), have provided evidence showing the existence of PKA in mitochondria. Two mammalian AKAPs, binding both RI

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[1] Abbreviations: cAMP, cyclic adenosine monophosphate; PKA, cyclic adenosine monophosphate-dependent protein kinase; AKAP, protein kinase A anchor protein; PKI, cyclic adenosine monophosphate-dependent protein kinase inhibitor; IBMX, 3-isobutyl-1-methylxanthine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; H89, *N*-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide; CCCP, carbonyl cyanide (*m*-chlorophenyl)hydrazone; NAD(H), nicotinamide adenine dinucleotide (reduced); UQ, ubiquinone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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and RII isoforms, D-AKAP1 (*18*) and D-AKAP2 (*19*) have also been identified. The catalytic site of the mitochondrial PKA was shown to be localized at the matrix side of the inner membrane, where it phosphorylates proteins of 29, 18, and 6.5 kDa (*16*). The 18 kDa phosphoprotein has been identified as the nuclear-encoded 18 kDa AQDQ subunit of complex I of the respiratory chain (NADH−ubiquinone oxidoreductase, E.C. 1.6.5.3) (*20, 21*). This protein, which is highly conserved in the known sequences of mammals (*22*), is apparently located at the inner surface of the mitochondrial membrane at the junction between the peripheral mass protruding in the matrix and the membrane moiety of complex I (*23*). The mature 18 kDa protein consists of 133 residues; at positions 129−131 there is a PKA phosphorylation consensus site RVS (*24, 25*). The protein has a leader sequence, removed after import in mitochondria (*26*), which also presents a phosphorylation consensus site RSLS at positions −10 to −7.

In this paper a study is presented on the localization and functional characterization of PKA in mitochondria and on the role of cAMP-dependent phosphorylation of the 18 kDa subunit of complex I.

MATERIALS AND METHODS

Materials. C2C12 mouse myoblasts were from ATCC. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), trypsin (0.05%)−EDTA (0.02%), penicillin, streptomycin, calf serum, and fetal bovine serum were from EuroClone. Decylubiquinone, IBMX, antimycin A, rotenone, CCCP, PKI, catalytic subunit of cAMP-dependent protein kinase purified from bovine heart, dibutyryl-cAMP, protein A−Sepharose, and mouse monoclonal antibody against phosphoserine were from Sigma. Horseradish peroxidase conjugate goat anti-mouse IgG antibody, dodecyl maltoside, high-purity digitonin, okadaic acid, cholera toxin, and monoclonal antibody against Cα subunit of PKA were from Calbiochem. Polyclonal antibodies against RIβ and RIIβ subunits of PKA were from Santa Cruz Biotechnology. Polyclonal antibodies against 20-residue phosphorylated C-terminal and 20-residue N-terminal peptides of the 18 kDa protein of complex I were produced at our request by Neosystem. [γ-^{32}P]ATP, 3000 Ci/nmol, and Western blot chemiluminescence reagent were from NEN Life Science. Hyperfilm MP was from Amersham International. Histone H2B was from Boehringer-Mannheim. Nitrocellulose membrane was from Schleicher & Schuell. All other reagents were of the highest purity grade commercially available.

Sample Preparation. Cytosol, heavy mitochondria, and mitoplasts were isolated from beef heart following the procedure described in ref *27* with some modification. The ventricles, quickly excised, were placed in ice-cold 0.25 M sucrose, pH 7.4. The tissue was homogenized in 10 volumes of isolation medium containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, and 0.25 mM PMSF. The homogenate was centrifuged at 1200*g* for 10 min. The supernatant was centrifuged at 9500*g* for 10 min. The resulting supernatant constituted the cytosolic fraction, from which any residual particulate was removed by a second centrifugation at 10000*g*. The initial 9500*g* pellet of the homogenate was resuspended in the isolation medium and centrifuged at 14000*g* for 10 min. The final pellet was washed to remove loosely packed damaged mitochondria and resuspended in the isolation medium. Mitochondrial intactness was determined by comparing NADH oxidase activity before and after sonication of mitochondria (*27*); it amounted to 95%. For mitoplast preparation mitochondria, 3 mg of protein/mL, were incubated for 15 min at 0 °C, in a medium containing 0.08 M sucrose, 10 mM Tris-HCl, pH 7.4, 100 mM EGTA, 0.25 mM PMSF, and digitonin at a concentration of 0.5 mg/mg of mitochondrial protein. The suspension was then centrifuged at 11000*g* for 15 min. The pellet, washed gently to remove loosely packed damaged mitoplasts, was suspended in 0.08 M sucrose, 10 mM Tris- HCl, pH 7.4, 1 mM EGTA, and 0.25 mM PMSF. Mitochondria exhibited an adenylate kinase activity of 166 nmol min^{-1} (mg of protein)$^{-1}$, and mitoplasts, 23 nmol min^{-1} (mg of protein)$^{-1}$. Cytochrome *c*, 0.22 nmol/mg of protein in mitochondria, was absent in the mitoplast fraction.

For fractionation of mitochondria, 30 mg of proteins was suspended in 1.5 mL of 0.25 M sucrose, 1 mM Tris-HCl, pH 7.5, 1 mM EGTA, and 250 μM PMSF at 0 °C and disrupted with glass beads to obtain the inner membrane (IM) and supernatant matrix (S) fractions as described in ref *15*. The presence of cytosol in the fractions was estimated from measurement of lactate dehydrogenase, matrix from measurement of aconitase, and inner membrane from spectrophotometric determination of cytochrome $a+a_3$ as in refs *14* and *15*.

Protein Kinase Assay. Protein phosphorylation was assayed by incubating, at 30 °C under stirring, the various preparations (as specified in the figure captions) in 450 μL of 10 mM Tris-HCl, pH 7.5, (0.25 M sucrose, pH 7.4, for mitoplasts), 8 mM $MgCl_2$, 20 mM NaF, 0.25 mM PMSF, 3 μg of rotenone, 3 μg of oligomycin, and [γ-^{32}P]ATP at the concentrations specified in the figure captions. To stop the reaction, 150 μL of the incubation mixture was mixed with 40 μL of 0.2 M Tris-HCl, pH 6.8, 30% glycerol (w/v), 7% SDS, and 15% (v/v) β-mercaptoethanol, and the mixture was boiled for 3 min.

Gel Electrophoresis, Autoradiography, and Immunodetection. SDS−PAGE of mitoplasts and IM and S fractions was carried out on boiled samples as described in ref *14*. Two-dimensional blue-native/SDS−PAGE of the mitoplast fraction from myoblasts was performed as described in refs *28* and *29*). Radioactive bands were visualized by exposure to Hyperfilm and radioactivity was measured as in ref *14*. For immunodetection of phosphoserine, C-PKA, RI-PKA, RII-PKA, and phosphorylated C- and N-termini of the 18 kDa subunit of complex I, proteins resolved by SDS−PAGE were electrotransferred, by use of a semidry Pharmacia apparatus, to nitrocellulose membranes (*30*). The nitrocellulose membrane was washed in 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.05% Tween 20 (TTBS) and 5% fatty acid-free dry milk (except for phosphoserine detection) for 12 h. The nitrocellulose membranes were then incubated with the antibody against phosphoserine (diluted 1:1000) for 3 h, antibody against C-PKA (diluted 1:1000), antibodies against RI-PKA and RII-PKA (diluted 1:200) for 2 h, antibody against the 18 kDa subunit of complex I phosphorylated C-terminus (diluted 1:100) for 12 h, and antibody against the 18 kDa subunit of complex I phosphorylated N-terminus (diluted 1:200) for 2 h. After being washed in TTBS, the

membranes were incubated for 60 min with anti IgG peroxidase-conjugated antibody (diluted 1:5000). Immunodetection was then performed, after further TTBS washes, with the enhanced chemiluminescence (ECL) Dupont/NEN kit.

Immunoprecipitation. For immunoprecipitation, 20 μg of mouse antibody against cytochrome oxidase subunit IV were added to 20 mg of protein A−Sepharose; this was washed twice in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet-P40, 0.1% sodium cholate, and 0.1% SDS and resuspended in 50 μL of the same buffer. After overnight incubation the unbound antibody was washed out with the buffer. IM proteins (50 μg) were added to the protein A−Sepharose−antibody complex, and the mixture was brought with the buffer to 100 μL and incubated overnight. The supernatant and the protein A−Sepharose immunocomplex, separated by centrifugation at 14000*g*, were both analyzed by SDS−PAGE.

Cell Culture and Mitoplast Preparation. C2C12 mouse myoblasts were maintained in culture with Dulbecco's modified Eagle's medium with 10% calf serum at 37 °C and, where indicated, treated for 3 h with 1 μg/mL cholera toxin + 100 μM IBMX. The cells were harvested from 150 mm Petri dishes with 2 mL of 0.05% trypsin, 0.02% EDTA, and phosphatase inhibitors (5 mM NaF, 500 nM okadaic acid, and 1 mM sodium orthovanadate). After addition of 50 mL of phosphate-buffered saline (PBS), pH 7.4, with 5% calf serum, cells were collected by centrifugation at 500*g*. For mitoplast preparation, myoblasts suspended in PBS were exposed for 10 min on ice to 0.2 mg of digitonin/mg of cellular protein. With this procedure, 90−95% of the cytoplasmic proteins are released (*31*). The mitoplast fraction was pelleted by centrifugation of the suspension at 14000*g* for 10 min. For fractionation the mitoplast suspension in PBS was diluted 40-fold in distilled water. This osmotic shock treatment released mitochondrial matrix proteins, leaving an inner membrane fraction pelleted by centrifugation of the suspension at 14000*g* for 15 min (*32*).

Measurement of Respiratory Activity. Respiration was monitored by oxygen polarography (*29*); 0.5 mg of mitochondrial or mitoplast proteins was suspended in a medium containing 50 mM KCl, 75 mM sucrose, 30 mM Tris-HCl, pH 7.4, 1 mM potassium phosphate, 0.5 mM EDTA, and 1 mM $MgCl_2$, final volume 1.6 mL, 25 °C. For measurement of respiratory activity of cells these were suspended in PBS.

Enzymatic Assays. For measurements of the NADH−UQ oxidoreductase activity, cells or mitoplasts were exposed to ultrasound energy for 15 s at 0 °C. The NADH−UQ oxidoreductase activity of cells or isolated mitoplasts was determined at 25 °C with NADH as reductant and decylubiquinone as oxidant, following the oxidation of NADH at 360−374 nm, by use of a $\Delta\epsilon$ value of 2.01 mM^{-1}.

RESULTS

cAMP-Dependent Protein Kinase and Protein Phosphorylation in Bovine Heart Mitochondria. Immunoblot analysis with specific antibodies revealed the presence of both the regulatory subunits (RI-PKA and RII-PKA) and the catalytic subunit (C-PKA) in bovine heart mitochondria (Figure 1). RI-PKA appeared to be exclusively associated with mitochondria, where it was particularly enriched in the

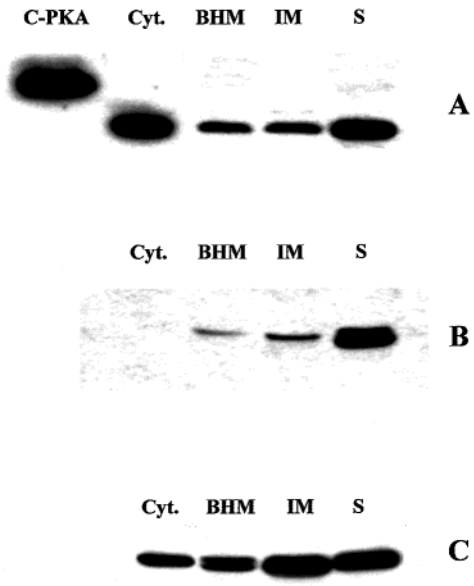

FIGURE 1: Localization of the catalytic (C-PKA) and regulatory subunits (RI-PKA and RII-PKA) of cAMP-dependent protein kinase in bovine heart mitochondria. Cytosol, heavy mitochondria, and submitochondrial fractions were prepared from beef heart as described under Materials and Methods. Cytosolic contamination, estimated from measurement of lactate dehydrogenase activity, amounted to 8% of the total proteins in mitochondria, 0.4% in inner membrane, and 5% in matrix fractions. Proteins—(Cyt) cytosol, 10 μg; (BHM) mitochondria, 30 μg; (IM) inner membrane fraction, 30 μg; (S) soluble matrix fraction, 30 μg—were separated by SDS−PAGE, transferred to nitrocellulose membrane, and immunodetected with antibodies against C-PKA (A), RI-PKA (B), and RII-PKA (C). For other details see under Materials and Methods.

matrix fraction (cf. ref *17*). RII-PKA was present in both the cytosol and mitochondria, where it was distributed between the inner membrane and matrix fraction. As expected, a large amount of C-PKA was present in the cytosol. A significant level of C-PKA was also detected in mitochondria, where it was particularly enriched in the matrix fraction. It can be noted that the C-PKA in the cytosol and mitochondrial fractions migrated in SDS−PAGE faster as compared to the commercial purified sample. C-PKA has four sites that are (auto)phosphorylated in purified samples (*33*, *34*), with consequent reduction in its electrophoretic mobility (*35*, *36*). Of these sites, phosphorylation of Thr-197 is stable against phosphatases and is essential for the catalytic activity (*33*, *34*); phosphorylation of Ser-10 and Ser-139 can be removed by phosphatases without loss of catalytic activity (*33*). This might be the case for the C-PKA in our preparations.

Figure 2 shows the PAGE pattern of protein phosphorylation by [γ-^{32}P]ATP in the bovine heart mitoplast fraction, which consisted of inner membrane vesicles and matrix with removal of the outer membrane, intermembrane space, and cytosol contaminants. In the absence of added cAMP the only significant phosphorylation by [γ-^{32}P]ATP that could be detected was that of the protein band of 42 kDa, where the α subunit of pyruvate dehydrogenase migrates (*37*). The addition of cAMP or of dibutyryl-cAMP resulted, as already observed in intact mitochondria (*16*), in the phosphorylation of three other protein bands of 29, 18, and 6.5 kDa.

The pattern of mitochondrial protein phosphorylation obtained with ^{32}P labeling (Figure 2; see also refs *15* and *16*) was substantiated by immunochemical detection in the

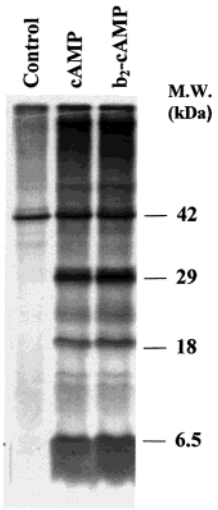

FIGURE 2: Autoradiograms of ^{32}P-labeled protein bands in bovine heart mitoplast fraction. Bovine heart mitoplast fraction (200 μg of protein) were incubated for 10 min at 30 °C, as specified under Materials and Methods", with 150 μM [γ-^{32}P]ATP (1000 cpm/pmol) in the absence or in the presence of 50 μM cAMP or 50 μM dibutyryl-cAMP (b_2-cAMP). Incubation was terminated and electrophoresis samples were prepared as described under Materials and Methods. Molecular weights of labeled protein bands were determined from standard proteins run on the same gels and detected by Coomassie blue. For other details see under Materials and Methods.

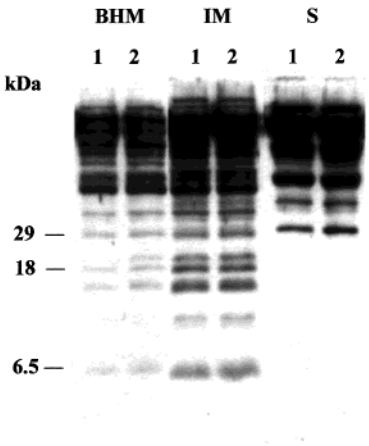

FIGURE 3: Immunodetection of phosphoserine in bovine heart mitochondria (BHM) and in the inner membrane (IM) and matrix (S) fractions. Frozen BHM, IM, or S fractions were incubated with 150 μM [γ-^{32}P]ATP in the absence (lanes 1) or in the presence (lanes 2) of 25 μM cAMP as described under Materials and Methods. After 20 min at 30 °C, samples were subjected to SDS−PAGE according to ref *14*. After electrophoresis, proteins were electrotransferred to nitrocellulose membrane and phosphoproteins were immunodetected with the monoclonal antibody against phosphoserine. For other details see under Materials and Methods.

protein bands of phosphoserine, which is the residue specifically phosphorylated by PKA (*25*). Immunoblotting with a specific antibody revealed, in fact, in bovine heart mitochondria the presence of phosphoserine in various protein bands, including those of 29, 18, and 6.5 kDa (Figure 3). The phosphoserine-containing protein bands of 18 and 6.5 kDa were enriched in the inner membrane fraction of bovine heart mitochondria but were absent in the matrix fraction. The phosphoserine-containing protein of 29 kDa was enriched in the matrix fraction. It can be noted that the addition of cAMP failed to produce an immunodetectable increase in the phosphoserine level of the 29, 18, and 6.5 kDa protein bands. The presence of phosphoserine in the mitochondrial protein bands already in the absence of cAMP can be due to activation of PKA in the intact tissue as well as to the activity of cAMP-independent protein kinase(s). Still, the radioactive method allows detection of the cAMP-dependent ^{32}P labeling of even a small fraction of nonphosphorylated serine in the proteins (see Figure 2 and refs *15* and *16*).

Table 1: Kinetic Analysis of the Activity of PKA in the Inner Membrane Fraction (IM) of Bovine Heart Mitochondria[a]

V_{max} (pmol of ^{32}P min^{-1}) (mg of protein)$^{-1}$	K_m (μM ATP)	K_d (μM cAMP)	pH optimum
185 ± 7.4	108 ± 11.8	0.14 ± 0.007	7.0

[a] V_{max} and K_m were obtained from Lineweaver−Burk plots of mitochondrial PKA activity. IM proteins (50 μg) were incubated for 5 min at 30 °C with [γ-^{32}P]ATP (1000 cpm/pmol) and histone H2B (100 μg), as substrate, at different ATP concentrations (75−250 μM) in the presence of 1 μM cAMP in the mixture described under Materials and Methods. K_d for cAMP was obtained by incubation of 50 μg of IM proteins for 5 min at 30 °C with 150 μM [γ-^{32}P]ATP (1000 cpm/pmol) and histone H2B (100 μg), as substrate, at different cAMP concentrations (0.01−100 μM) in the mixture described under Materials and Methods. The pH optimum value was determined by measurement of PKA activity, by incubation of 50 μg of IM proteins for 5 min at 30 °C with 150 μM [γ-^{32}P]ATP, 100 μg of histone, and 1 μM cAMP in the mixture containing 5 mM Hepes, 5 mM Mes, and 5mM Tris adjusted at different pH values (5.0−8.5). Phosphorylated histone was separated by SDS−PAGE and radioactivity was measured on the isolated band, as described under Materials and Methods. The values reported in the table were corrected for ^{32}P incorporation in the presence of added PKI (0.25 μM) and absence of cAMP.

Kinetic activity analysis, with added histone H2B as substrate, showed that the PKA of the inner membrane fraction exhibits affinities for cAMP and the substrate ATP, as well as a pH dependence (Table 1), that are comparable to those of the cytosolic PKA (*38, 39*).

It has been reported that subunit IV of cytochrome *c* oxidase, which has a molecular mass of ≈17 kDa, is phosphorylated by an endogenous kinase in rat heart mitochondria (*40*). The inner membrane fraction of bovine heart mitochondria was incubated with ATP in both the presence and absence of added cAMP. Before PAGE, the inner membrane fraction was treated with a monoclonal antibody against subunit IV of cytochrome *c* oxidase. A cAMP-independent phosphoserine-labeled 18 kDa protein was recovered in the immunoprecipitated material, while the cAMP-dependent phosphoserine-labeled protein remained in the supernatant (Figure 4). These results thus show that although subunit IV of cytochrome *c* oxidase can contribute to the phosphorylated 18 kDa protein band, it does not account for the cAMP-dependent phosphorylation of the 18 kDa phosphoserine protein described in this paper.

cAMP-Dependent Protein Kinase, Protein Phosphorylation, and Activation of Respiration in Myoblasts. Analysis of subcellular fractions of cultured C2C12 mouse myoblasts showed the presence of one immunodetectable form of C-PKA in the cytosol and of two immunodetectable forms (possibly phosphorylated and dephosphorylated) of C-PKA in mitoplasts and their inner membrane and matrix fraction (Figure 5). The RII-PKA subunit was detected in both the cytosol and mitoplasts, where it was particularly enriched in the matrix fraction.

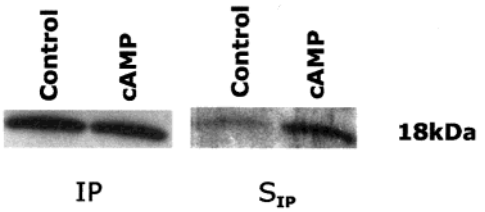

FIGURE 4: Immunodecoration with antibody against phosphoserine of the 18 kDa proteins of the IM fraction after immunoprecipitation with an antibody against subunit IV of cytochrome *c* oxidase. IM proteins (50 μg) were incubated with 150 μM ATP in the absence and in the presence of 25 μM cAMP, as described under Materials and Methods, and then immunoprecipitated with an antibody against subunit IV of cytochrome *c* oxidase. The immunoprecipitate (IP) and the supernatant (S_{IP}) were subjected to electrophoresis. Proteins were than transferred to nitrocellulose membrane and decorated with the antibody against phosphoserine. For other details see under Materials and Methods.

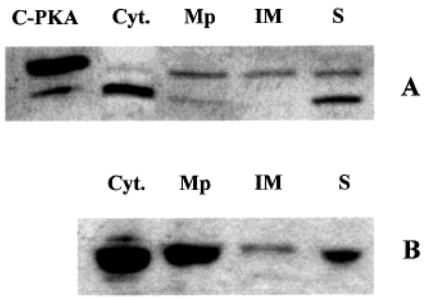

FIGURE 5: Subcellular localization of C-PKA and RII-PKA in C2C12 mouse myoblasts. Cytosol, mitoplasts, and submitochondrial fractions were obtained from C2C12 mouse myoblast as described under Materials and Methods. Proteins [cytosol (Cyt), 15 μg; mitoplast (Mp), 30 μg; inner membrane fraction (IM), 30 μg; soluble matrix fraction (S), 30 μg] were separated by urea−SDS−PAGE, transferred to nitrocellulose membrane, and immunodetected with antibodies against C-PKA (A) and RII-PKA (B) as described under Materials and Methods. For other details see under Materials and Methods.

The effect of cAMP on phosphorylation of mitochondrial proteins and respiration was analyzed in vivo in myoblast cultures. The intracellular level of cAMP was increased in these cells by their exposure to cholera toxin + IBMX (*41*). Western-blot analysis with the antibody against phosphoserine of the five complexes of oxidative phosphorylation, separated by two-dimensional gel electrophoresis of the mitoplasts isolated from myoblasts (*42*), showed that the cholera toxin treatment of the intact cells promoted specifically, under the experimental conditions used, serine phosphorylation in the 18 kDa subunit of complex I (Figure 6). In a series of experiments the effect of myoblast exposure to cholera toxin on the NADH−UQ oxidoreductase activity of complex I was tested. Table 2 summarizes data on the V_{max} and K_m values, obtained from typical Lineweaver−Burk plots, of the rotenone-sensitive NADH−UQ oxidoreductase in control and cholera toxin-treated myoblasts, which after treatment were exposed to ultrasonic energy to eliminate permeability barriers to exogenous NADH and decylubiquinone. It can be seen that cholera toxin treatment of myoblasts resulted in significant enhancement of the V_{max} of the rotenone-sensitive NADH−UQ oxidoreductase of complex I. The increase of the K_m produced by cholera toxin was not significant. The results presented in Table 2 show that the cholera toxin treatment of myoblasts resulted also in a significant stimulation of the overall respiratory activity

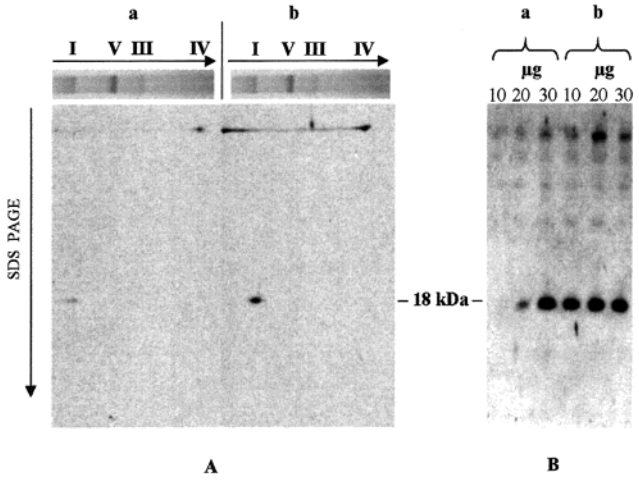

FIGURE 6: Immunodetection of phosphoserine in the 18 kDa subunit of complex I separated by two-dimensional gel electrophoresis of the mitoplast fraction of myoblast cultures. Mitoplasts separated from control mouse myoblasts (a) and from myoblasts treated 3 h with cholera toxin + IBMX (b) in the culture medium were subjected to blue native electrophoresis, which resulted in the separation of respiratory complexes I, III, IV and V (ATP synthase), detected by Coomassie blue staining (see refs *28* and *29*). After separation of the constituent subunits of the complexes by second-dimension SDS−PAGE, proteins were electrotransferred to nitrocellulose sheets and treated with the specific antibody for immunodetection of phosphoserine in the subunits (panel A). In panel B, after blue native electrophoresis, the protein band corresponding to complex I, from 10, 20, and 30 μg of mitoplast proteins from control (a) and from cholera toxin-treated myoblasts (b), was cut out and subjected to SDS−PAGE. Proteins were then electrotransferred to nitrocellulose sheets for immunodetection of phosphoserine in complex I subunits. For other details see under Materials and Methods.

when supported by glutamate plus malate. Succinate-supported respiration was, on the other hand, not stimulated by cholera toxin treatment. Cytochrome *c* oxidase activity, supported by ascorbate plus TMPD, was not significantly enhanced by cholera toxin treatment.

Figure 7 shows that direct treatment of the mitoplasts, isolated from myoblasts, with dibutyryl-cAMP promoted, while treatment with the H-89 inhibitor of PKA (*43*) depressed, serine phosphorylation of the consensus site in the C-terminal segment of the 18 kDa subunit of complex I. There was, on the other hand, no change in the immunodetectable level of the N-terminal segment of this protein (see Figure 7). Table 3 shows that mitoplast treatment with dibutyryl-cAMP stimulated, while treatment with H-89 inhibited, the rotenone-sensitive NADH−UQ oxidoreductase activity of complex I. Both stimulatory effects of dibutyryl-cAMP were abolished when it was added in the presence of H89 (Figure 7 and Table 3).

DISCUSSION

The present results show that the regulatory subunits (RI and RII) and the catalytic subunit of PKA are present in the inner membrane and matrix of bovine heart and mouse myoblast mitochondrial preparations (cf. ref *17*). In bovine heart mitochondria, RI is more abundant in the matrix than in the inner membrane. The opposite holds for RII. C-PKA is found to be distributed, both in bovine heart and in mouse myoblasts, between the inner membrane and matrix fraction.

Table 2: Effect of Cholera Toxin on Complex I and Respiratory Activities in C2C12 Mouse Myoblasts[a]

Enzymatic Activity				
	control		+cholera toxin	
	V_{max}[b]	K_m[c]	V_{max}	K_m
NADH−UQ oxidoreductase	7.75 ± 0.5	2.55 ± 0.58	14.3 ± 0.64 ($p < 0.01$)	4.27 ± 0.49 (NS)

Respiratory Activity[d]		
substrates	control	+cholera toxin
glutamate + malate	7.40 ± 2.78	12.51 ± 4.4 ($p < 0.01$)
succinate	5.21 ± 1.34	4.57 ± 1.35 (NS)
ascorbate + TMPD	13.09 ± 4.72	18.77 ± 7.08 (NS)

[a] Control mouse myoblasts and myoblasts incubated for 3 h with cholera toxin + IBMX were suspended in phosphate-buffered saline with phosphatase inhibitors and immediately respiration and enzymatic activities were determined as described under Materials and Methods. V_{max} and K_m were obtained from Lineweaver−Burk plots of NADH−UQ oxidoreductase activity measured on 5×10^5 sonicated cells suspended in 700 μL of 40 mM potassium phosphate buffer, pH 7.4, and 5 mM $MgCl_2$, containing 0.5 mM ATP, 1 μg/mL oligomycin, and 3 mM KCN; temperature 37 °C. Decylubiquinone (200 μM) was added and the reaction was started with various concentrations of NADH in the range 1.25−25 μM. Activities were corrected for the residual reaction in the presence of 1 mg of rotenone/mg of protein. Respiration was measured polarographically on a myoblast suspension [$(1.5-2) \times 10^6$ cells] in 650 μL of 75 mM sucrose, 30 mM Tris-HCl, 50 mM KCl, 0.5 mM EDTA, 0.5 mM $MgCl_2$, and 2 mM potassium phosphate buffer, pH 7.4, containing 1 μM carbonyl cyanide (*m*-chlorophenyl)hydrazone (CCCP). Cells were permeabilized with 0.002% digitonin. Substrate concentrations were 10 mM glutamate, 10 mM malate, 10 mM succinate, 2 mM ascorbate, and 0.2 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD). Values represent the means ± SE of three experiments. [b] V_{max} values are given in femtomoles per minute per cell. [c] K_m values are given as micromolar NADH. [d] Respiratory activity is given in femtoatoms of O per minute per cell.

Table 3: Effect of Dibutyryl cAMP and H89 on Complex I Activity in Mitoplasts Isolated from C2C12 Mouse Myoblasts[a]

control		H89		+dibutyryl-cAMP		+dibutyryl-cAMP + H89	
V_{max}[b]	K_m[c]	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
17.68 ± 7.35	6.14 ± 1.94	10.04 ± 3.84	4.89 ± 1.48	22.74 ± 6.24	7.42 ± 2.92	4.2	9

[a] Mitoplasts were prepared from myoblasts as described under Materials and Methods and immediately sonicated. NADH−UQ oxidoreductase activity was measured utilizing 100 μg of mitoplast proteins as described in the footnote to Table 2. The mitoplast suspension was preincubated for 5 min in the absence or in the presence of 5 μM dibutyryl-cAMP or 1 μM H89, an inhibitor of protein kinase A, or of 5 μM dibutyryl-cAMP + 1 μM H89; and then 200 μM decylubiquinone was added and the reaction was started with 1.25−25 μM NADH. The reaction was corrected for that measured in the presence of 1 μg/mL rotenone. The values represent the means of five experiments except for those carried out in the presence of dibutyryl-cAMP + H89 (means of two separate experiments). [b] V_{max} values are given in nanomoles per minute per milligram of protein. [c] K_m values are given as micromolar NADH.

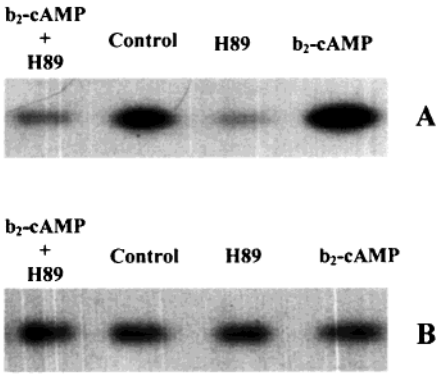

FIGURE 7: Immunodetection of the phosphorylated C-terminus and N-terminus of 18 kDa protein band of mitoplasts from myoblast cultures. Proteins of mitoplasts (30 μg) isolated from control myoblasts were incubated as described under Materials and Methods alone or in the presence of 5 μM dibutyryl-cAMP or in the presence of 1 μM H89. Complex I, separated by blue native PAGE, was resolved into its constituent subunits by SDS−PAGE. Proteins were then electrotransferred to nitrocellulose sheets for immunodetection of the phosphorylated C-terminus (A) and N-terminus (B) of the 18 kDa subunit with the specific antibodies. For other details see under Materials and Methods.

The activity analysis with histone as substrate shows that the PKA of the inner mitochondrial membrane exhibits affinities for ATP and cAMP and a pH optimum that are comparable to those reported for the cytosolic pool of PKA (*38*, *39*). The catalytic site of the mitochondrial PKA has been shown to be localized at the matrix side of the inner membrane, where proteins of 29, 18, and 6.5 kDa are exposed with the domains that are phosphorylated (*16*). Localization of the cAMP binding site in the R subunit remains to be determined.

The results of the experiments on myoblast cultures show that elevation in vivo of the intracellular level of cAMP induces serine phosphorylation in the 18 kDa subunit of complex I and activates the NADH−UQ oxidoreductase activity of this complex as well as the overall mitochondrial respiratory activity supported by NAD-linked substrates. Similar effects have also been observed in serum-starved mouse (*29*) and human fibroblasts (*44*). cAMP was effective in promoting phosphorylation of the 18 kDa subunit of complex I and activation of its NADH−UQ oxidoreductase also in isolated mitoplasts. Phosphorylation of the 18 kDa subunit by PKA, in addition to direct stimulation of the catalytic activity, could also influence the import in mitochondria of the protein and/or assembly of the complex.

Recently four different mutations in the NDUFS4 gene coding for the 18 kDa subunit of complex I have been found in children with complex I deficiency, exhibiting multisystem disorders. The first was a 5-bp base duplication of the cDNA of the 18 kDa protein, resulting in destruction of the phosphorylation consensus site in the C-terminus (*45*). Two other mutations resulted in truncated forms of the protein,

also with destruction of the phosphorylation site (*46*). A last mutation caused premature termination of the subunit (*47*). Biochemical investigations on the fibroblasts from the patients showed that the 5-bp duplication in the NDUFS4 gene abolished cAMP-dependent phosphorylation of this subunit and activation of mitochondrial respiration (*44*). In the fibroblasts from the patient with premature termination of the 18 kDa subunit, suppression of normal membrane assembly of complex I and of its activity was found (*47*).

All the above provides substantial evidence showing that cAMP-mediated intracellular signal transduction, through serine phosphorylation of the (NDUFS4) 18 kDa subunit of complex I, regulates the activity of complex I in mammalian tissues. Since complex I represents, at least under certain conditions, the rate-limiting step of mitochondrial respiratory chain, upregulation of complex I by cAMP-dependent phosphorylation of the 18 kDa subunit activates the overall cellular respiratory activity and aerobic ATP production, particularly in response to an increased energy demand. Considering the role that the cAMP cascade has in the cellular signal transduction of a variety of hormones and neurotransmitters, upregulation of mitochondrial respiration by cAMP emerges from our observations as one of the major control factor in the bioenergetic function of mammalian tissues.

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