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Intramitochondrial adenylyl cyclase controls the turnover of nuclear-encoded subunits and activity of mammalian complex I of the respiratory chain

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ABSTRACT

In mammalian cells the nuclear-encoded subunits of complex I are imported into mitochondria, where they are assembled with mt-DNA encoded subunits in the complex, or exchanged with pre-existing copies in the complex. The present work shows that in fibroblast cultures inhibition by KH7 of cAMP production in the mitochondrial matrix by soluble adenylyl cyclase (sAC) results in decreased amounts of free non-incorporated nuclear-encoded NDUFS4, NDUFV2 and NDUFA9 subunits of the catalytic moiety and inhibition of the activity of complex I. Addition of permeant 8-Br-cAMP prevents this effect of KH7. KH7 inhibits accumulation in isolated rat-liver mitochondria and incorporation in complex I of "in vitro" produced, radiolabeled NDUFS4 and NDUFV2 subunits. 8-Br-cAMP prevents also this effect of KH7. Use of protease inhibitors shows that intramitochondrial cAMP exerts this positive effect on complex I by preventing digestion of nuclear-encoded subunits by mitochondrial protease(s), whose activity is promoted by KH7 and H89, an inhibitor of PKA.

1. Introduction

In mammals, complex I has 44 constituent subunits [1,2]. Fourteen are conserved from prokaryotes to humans; the others are termed supernumerary subunits. Seven of the conserved subunits are encoded by the mitochondrial DNA (mtDNA), all the others by nuclear genes [3,4]. Complex I biogenesis is thus a concerted process in which intramitochondrial expression of the mtDNA-encoded subunits is coordinated with extramitochondrial expression of the nuclear DNA-encoded subunits, their import and maturation in mitochondria, where, in a stepwise process, a copy of each subunit is assembled in the mature complex [5]. In addition to "de novo" biogenesis of the entire complex, nuclear encoded subunits, belonging to the matrix exposed catalytic moiety of the complex, undergo a relatively rapid turnover, with exchange of pre-existing with newly synthesized copies [6,7]. This exchange contributes to replace "aged", oxidatively damaged, subunits in the complex with new ones, thus preserving its functional activity [8].

The cAMP signal system, which functions at the crossroads of several cellular signal transduction networks, exerts a major role in the biogenesis and post-translational processing and activity of complex I (reviewed in [9]). cAMP produced in the cytosol primes, through phosphorylation by PKA of the cAMP response element protein (CREB) [10,11], the nuclear PGC-1α transcriptional cascade of respiratory chain complexes [12]. In addition, in mitochondria CREB phosphorylation by PKA activates the expression of mitochondrial genes of respiratory chain complexes, including seven subunits of complex I [13–15].

Previous work from our laboratory has shown that addition to cell cultures of the membrane permeant derivate of cAMP, 8-Br-cAMP (8-bromoadenosine 3′,5′-cyclic monophosphate), and, equally effective, activation of transmembrane adenylyl cyclase (tmAC) by cholera toxin [16] or the β-adrenoceptor agonist, isoproterenol [17], rescue the depression of complex I activity and the increase in cellular ROS level observed in murine and human fibroblast cultures in the G0 phase [18] and in oxidatively damaged cell cultures [8]. Other investigations showed that phosphorylation by extramitochondrial PKA of serine in the C-terminus RVS conserved site of the nuclear-encoded NDUFS4 subunit of complex I [19,20] promotes mitochondrial import and maturation of the precursor

Abbreviations: 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; CAI, carbonic anhydrase inhibitor; CREB, cAMP response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; tmAC, transmembrane adenylyl cyclase; mtDNA, mitochondrial DNA; NHDF-neo, neonatal normal human dermal fibroblasts; PBS, phosphate-buffered saline; PKA, cyclic AMP dependent protein kinase; PKI, protein kinase A inhibitor; RRL, rabbit reticulocyte lysate; RLM, rat liver mitochondria; ROS, reactive oxygen species; sAC, soluble adenylyl cyclase

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form of the protein and prevents retrograde back diffusion in the cytosol of the mature form of the protein [21].

In the mitochondrial matrix a soluble, bicarbonate-sensitive, adenylyl cyclase (sAC) [22] and all the members of the cAMP/PKA system are present [23–27]. It has been found that sAC-dependent cAMP production in mitochondria promotes cytochrome c oxidase activity, through phosphorylation of complex IV subunit IV-I [28], ATP production [29], and the mitochondrial pathway of apoptosis [30]. It has to be stressed that tmAC and sAC catalyze separate production of cAMP in the cytosol and in the mitochondrial-matrix respectively, being the mitochondrial membrane impermeable to the cyclic nucleotide.

In the present work we show that, in human fibroblast cultures KH7, which inhibits intramitohondrial cAMP production by sAC [31] and acetazolamide a carbonic anhydrase inhibitor (CAI) [32,33], depressed complex I activity, to the same extent as the inhibition of cytochrome *c* oxidase [34]. The KH7-induced depression of both enzymes was rescued by the addition of the membrane permeant 8-Br-cAMP, but not by the increase of cytosolic cAMP induced by isoproterenol. The depression of complex I activity caused by KH7 in fibroblast cultures was associated with decrease of the amount of nuclear-encoded NDUFS4, NDUFV2 and NDUFA9 subunits of complex I, which was also rescued by 8-Br-cAMP, but not by isoproterenol. It is also shown that KH7 depressed the accumulation in isolated rat-liver mitochondria (RLM) of "in vitro" expressed [^{35}S] labeled NDUFS4 and NDUFV2 subunits and their incorporation in complex I, this inhibition too being rescued by 8-Br-cAMP. Using protease inhibitors, evidence is obtained showing that intramitochondrial cAMP prevents the KH7-induced degradation of the non-incorporated pool of these complex I subunits by mitochondrial protease(s) and the inhibition of its activity. These findings showing an additional, critical role of cAMP in the regulation of complex I, provide new insight in mitochondrial pathophysiology.

2. Materials and methods

2.1. Cell cultures

Neonatal normal human dermal fibroblasts (NHDF-neo, Cambrex #CC-2509, East Rutherford, NJ, USA) were grown in the exponential phase in high glucose Dulbecco's modified Eagle's medium (DMEM) (EuroClone, Paignton, UK) supplemented with 10% fetal bovine serum (FBS), plus 2 mM glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin (Euroclone, Paignton, UK) at 37 °C, 5% CO_2. Further conditions are specified in the legends to figures.

2.2. Chemicals

[^{35}S] Met/Cys (1000 Ci/mmol) (Perkin Elmer, Milano, Italy), 8-Br-cAMP (Biolog Life Science Institute, Germany), Acetazolamide (Sigma-Aldrich, St. Louis, MO), catalytic subunit cAMP-dependent protein kinase (cPKA) (Promega, Mannheim, Germany), epoxomicin (Sigma-Aldrich, St. Louis, MO), KH7 (E-2-(1H-Benzo[d]imidazol-2-ylthio)-N′-(5-bromo-2-hydroxybenzylidene)propanehydrazide) (Sigma-Aldrich, St. Louis, MO), MG132 (Sigma-Aldrich, St. Louis, MO), Protein kinase A inhibitor (PKI) (Sigma-Aldrich, St. Louis, MO).

2.3. Spectrofluorimetric analysis

Intracellular H_2O_2 level was determined by the cell permeant probe 2′-7′dichlorodihydrofluorescin diacetate (H_2DCFDA) (Sigma-Aldrich, St. Louis, MO). Cells were incubated with 10 μM H_2DCFDA in DMEM. After 20 min incubation in the dark at 37 °C, the cells were collected by trypsinization, washed and resuspended in the assay buffer (100 mM potassium phosphate, pH 7.4, 2 mM $MgCl_2$). An aliquot was used for protein concentration determination. The probe fluorescence intensity (507 nm excitation and 530 nm emission wavelengths) was measured by a Jasco FP6200 spectrofluorimeter (see also ref. [17]).

2.4. Mitoplast and inner mitochondrial membrane preparation

NHDF-neo cells were harvested from Petri dishes with 0.05% trypsin, 0.02% EDTA, pelleted by centrifugation at 500 ×*g* and then resuspended in phosphate-buffered saline, pH 7.4 (PBS). The cell suspension was exposed for 10 min on ice to 2 mg of digitonin/mg cellular protein and then centrifuged at 14,000 ×*g*. The pellet (mitoplast fraction) was resuspended in PBS. The inner mitochondrial membrane fraction was obtained by mitoplast exposure to ultrasounds and sedimentation by centrifugation at 100,000 ×*g* for 1 h. After centrifugation the pellet was resupended in PBS.

2.5. Rat liver mitochondria isolation

Mitochondria were isolated from rat liver as described in [21].

2.6. Enzymatic spectrophotometric assays

Isolated mitoplasts were exposed to ultrasound energy for 15 s at 0 °C to allow complete accessibility of substrates to enzymes.

The NADH-UQ oxidoreductase activity (complex I) was performed in 40 mM potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, in the presence of 3 mM KCN, 1 μg/ml antimycin, 200 μM decylubiquinone, using 30 μg of mitoplast proteins, by following the oxidation of 100 μM NADH at 340–425 nm ($\Delta\varepsilon = 6.81\ mM^{-1}\ cm^{-1}$). The activity was corrected for the residual activity measured in the presence of 1 μg/ml rotenone.

Cytochrome c oxidase (complex IV) activity was measured by following the oxidation of 10 μM ferrocytochrome c at 550–540 nm ($\Delta\varepsilon = 19.1\ mM^{-1}cm^{-1}$). Enzymatic activity was measured in 10 mM phosphate buffer, pH 7.4, using 20 μg of mitoplast proteins. This rate was inhibited over 95% by KCN (2 mM).

Succinate-cytochrome *c* oxidoreductase (complex II + III) activity was measured at 550–540 nm ($\Delta\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$) as the initial rate of antimycin-sensitive cytochrome c reduction.

2.7. cAMP assay

Cells were harvested from petri dishes with 0.05% trypsin, 0.02% EDTA and pelleted by centrifugation at 500 ×*g*. The cell pellet resuspended in PBS was utilized for homogenate preparation, by potterization, and for mitoplast preparation as described above. The samples of the homogenate and mitoplast were checked for lactate dehydrogenase activity. cAMP level was measured in the homogenate and mitoplast samples using a direct immunoassay kit (Assay Designs, Ann Arbor, Michigan, USA) as described by the manufacturer. Total protein concentration was determined by Bio Rad protein assay. The cAMP level measured in mitoplast fraction was corrected for contamination by the homogenate, evaluated by lactic dehydrogenase activity.

2.8. Electrophoretic procedures and western blotting

Proteins from fibroblast cell lysate, mitoplast fraction or their inner membrane fraction were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% fatty acid free dry milk in 500 mM NaCl, 20 mM Tris, 0.05% Tween 20 pH 7.4 (TTBS) for 3 h at 4 °C and probed with antibodies against NDUFS4 (Thermo scientific, Pierce Antibodies, Lausanne Switzerland), NDUFA9, NDUFB6, COX IV, β-ATPase, Core II (Invitrogen, Paisley, UK), NDUFV2 (Cell Signalling, Danvers, MA, USA), NDUFB11 (Prim SRL, Milan, Italy) and actin (Sigma-Aldrich, St. Louis, MO). After being washed in TTBS, the membrane was incubated for 60 min with antirabbit or antimouse IgG peroxidase-conjugated

antibody (diluted 1:5000). Immunodetection was then performed, after further TTBS washes, with the enhanced chemiluminescence (ECL) (Euroclone, Paignton, UK).

Separation of oxidative phosphorylation complexes was performed by two-dimensional blue-native electrophoresis (BNE)/SDS-PAGE. Mitoplast fraction or RLM (50 μg) were suspended in 750 mM aminocaproic acid, 50 mM Bis–Tris, 0.5 mM EDTA plus 1% or 0.4% (*w*/*v*) lauryl maltoside, respectively. Protein were loaded on 5%–12% gradient native-gel followed by a second dimension in 12% SDS-PAGE. After the electrophoretic separation the proteins were transferred to nitrocellulose and immunoblotted with the NDUFA9, NDUFB6, COX IV, β-ATPase and Core II antibodies described above. Densitometric analysis was performed by VersaDoc imaging system (BioRad, Milan, Italy).

2.9. cDNA constructs, in vitro translation and import assay

Full-length human NDUFS4 and NDUFB11 cDNAs were generated by reverse transcriptase-PCR, using RNA extracted from primary fibroblasts from skin biopsies of control subjects. The cDNAs were cloned in pCDNA3.1(+) vector with the T7 promoter as described in [21]. Plasmid constructions were confirmed by DNA sequencing. The full-length human NDUFV2 cDNA was purchased from OriGene Technologies (Rockville, MD, USA).

In vitro transcription/translation of cDNAs was performed in rabbit reticulocyte lysate (RRL) system (Promega Biotech, Madison, WI) as reported in [21]. Essentially, 1 μg of construct was added to 50 μl Promega standard mixture, containing T7 RNA polymerase and a standard amino acid mixture with [^{35}S]Methionine/Cysteine (20 μCi). Incubation was done at 30 °C for 90 min.

5 μl of radioactive RRL translation mixture was added to the import mixture containing: rat liver mitochondria (500 μg proteins), 210 mM mannitol, 7 mM Hepes, pH 7.4, 0.35 mM $MgCl_2$, 2.5 mg/ml BSA, 3 mM ATP, 3 mM GTP, 15 mM malate, 30 mM pyruvate and 1 mg/ml chloramphenicol; final volume 200 μl. Incubation was done at 30 °C. After incubation, aliquots of the mixture were transferred to ice-cooled tubes and supplemented with protease inhibitors (1 μl per 250 μg mitochondrial proteins). Mitochondria were spun down from the import mixture at 4000 ×*g* and 50 μg mitochondrial proteins of each sample were separated by SDS-PAGE and/or BNE (see also [8]). After electrophoresis the gels were dried. Radioactive protein bands were detected by Personal FX at "phosphorus imager" (BioRad, Milan, Italy) and quantified by VersaDoc (BioRad, Milan, Italy).

2.10. Proteolytic activity assay

90 μg rat liver mitochondria were incubated in the absence or in the presence of KH7, 8-Br-cAMP, MG132 or epoxomicin at 30 °C in 210 mM mannitol, 7 mM Hepes, pH 7.4, 0.35 mM $MgCl_2$. After 15 min incubation, 0.2% *w*/*v* Triton X-100, 0.25 μM protein kinase A inhibitor (PKI), 5 μM okadaic acid, and 2 mM ATP (Sigma-Aldrich, St. Louis, MO) were added, before adding 12 μg casein. The incubation was, then, prolonged and aliquots of mitochondrial proteins were taken at the times specified in Fig. 7 and resuspended in SDS-lysis buffer. Proteins were separated by SDS-PAGE and stained with comassie blue.

2.11. Animals

All experiments were performed in accordance with national and institutional guidelines for animal welfare, adhering to protocols approved by the institutional committee on research animal care. A written confirmation of authorization was obtained from local authority (n. 60895-X10, Comitato Etico per la Sperimentazione Animale, Area Sicurezza sul Lavoro, Dipartimento Amministrativo per la Sanità, University of Bari).

3. Results

3.1. Effect of intramitochondrial cAMP on the activity of respiratory chain complexes and cellular H_2O_2 level in fibroblast cultures

Analysis of the cellular and mitochondrial cAMP levels in human fibroblast cell cultures shows (Fig. 1) that, under the experimental conditions used, the mitochondrial contribution to the total cellular cAMP content amounted to about 30%. β-agonist stimulation (isoproterenol 1 μM, 35 min) increased the cellular level of cAMP, without any effect on its amount in the mitochondrial fraction. Treatment of fibroblast cultures with KH7 (25 μM, 35 min), which reduced by approximately 30% the overall cellular cAMP content, suppressed almost completely the mitochondrial cAMP content.

The enzymatic activities of the respiratory chain complexes were then analyzed (Fig. 2). KH7 treatment of fibroblast cultures depressed the activities of complex I and complex IV, both activities being rescued by 8-Br-cAMP, but not by isoproterenol. No effect was observed on the activities of complex II/III. KH7 treatment of the fibroblast cultures, in the exponential growth-phase, resulted also in a large increase of the cellular H_2O_2 level, which was, in this case, prevented by 100 μM 8-Br-cAMP, but not by isoproterenol (Fig. 2D).

Treatment of the fibroblast culture with the carbonic anhydrase inhibitor (CAI) [33], caused, like KH7, inhibition of complex I and complex IV activities and promoted ROS production. These effects of CAI were rescued, as observed with KH7, by 8-Br-cAMP (Fig. 2, panels E, F, G).

3.2. KH7 affects the level of specific nuclear-encoded complex I subunits in fibroblast cultures

The amounts in the fibroblast lysate of the NDUFA9, NDUFS4 and NDUFV2 nuclear-encoded subunits of complex I, were decreased upon 35 min treatment of the fibroblast cultures with KH7 (Fig. 3A). It has to be noted that, according to the molecular weight-exhibited in the SDS-PAGE run, the immunodetected spots represent the mature forms of these subunits, produced in the cytosol, after their import in mitochondria and cleavage of the presequences by mitochondrial maturase [21]. The KH7-induced decrease of the amount of these subunits was prevented by the concomitant presence in the fibroblast cultures of 8-Br-cAMP, but not by isoproterenol. Treatment of the fibroblasts with CAI caused, like KH7, a decrease of NDUFA9, NDUFV2 and NDUFS4 subunits of complex I, which was rescued, as observed with KH7, by 8-Br-cAMP (Supplementary Fig. 1). KH7 and CAI treatments of the fibroblast cultures, alone and in the presence of 8-Br-cAMP or

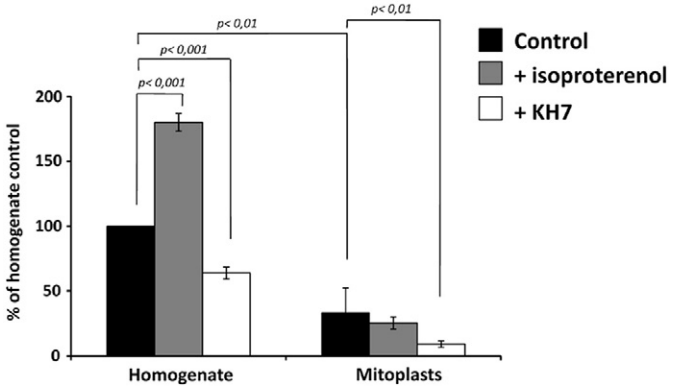

Fig. 1. Effect of KH7 and isoproterenol on the overall cellular and mitoplast level of cAMP in human fibroblast cultures. cAMP level in the cell homogenate and mitoplast fraction. Experimental conditions: ■ fibroblasts grown in DMEM with 10% fetal bovine serum; ▩ fibroblasts treated for 35 min with 1 μM isoproterenol; □ fibroblasts treated for 35 min with 25 μM KH7. The histograms represent the mean values of three or more cAMP determinations expressed as percentage with respect to the control homogenate. Error bars indicate standard deviations. For other experimental details see under Materials and methods.

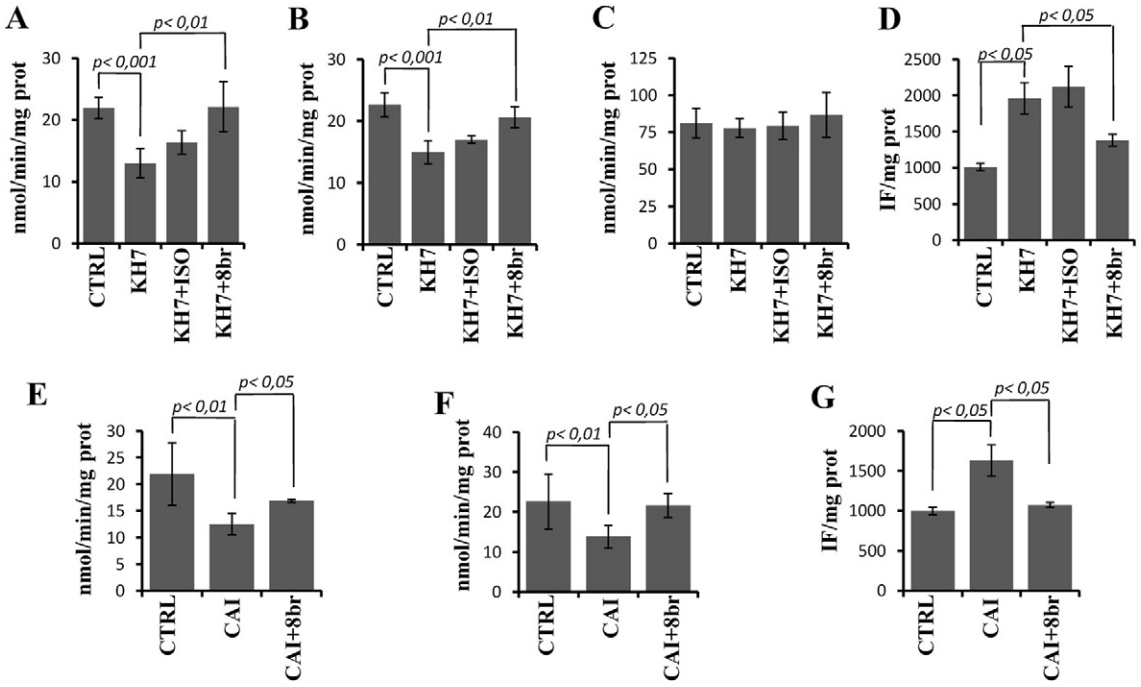

Fig. 2. Effects of KH7, CAI, isoproterenol and 8-Br-cAMP treatment on the activity respiratory chain complexes and H_2O_2 level in fibroblast cultures. The NADH:ubiquinone oxidoreductase (Panels A and E), cytochrome c oxidase (Panels B and F) and succinate-cytochrome *c* oxidoreductase (Panel C) activities were determined in the fibroblast mitoplast fraction, isolated as described under Materials and methods. Panels D and G, H_2O_2 level in fibroblasts, detected fluorimetrically by the H_2DCFDA probe and expressed as relative fluorescence intensity (IF). Experimental conditions: (CTRL) fibroblasts grown in DMEM with 10% fetal bovine serum; (KH7) fibroblasts treated for 35 min with 25 μM KH7; (KH7 + ISO) fibroblasts treated for 35 min with KH7 plus 1 μM isoproterenol; (KH7 + 8br) fibroblasts treated for 35 min with KH7 plus 100 μM 8-Br-cAMP; (CAI) fibroblasts treated for 35 min with 1 μM CAI; (CAI + 8br) fibroblasts treated for 35 min with CAI plus 8-Br-cAMP. The histograms represent the mean values of three or more determinations. Errors bars indicate standard deviations. For experimental details see under Materials and methods.

isoproterenol, did not produce, on the other hand, any significant change in the amount of the membrane embedded nuclear-encoded NDUFB6 and NDUFB11-subunits of complex I (Fig. 3A and Supplementary Fig. 1) neither in the amount of core II subunit of complex III, subunit IV of complex IV and the β subunit of complex V (F_oF_1 ATP synthase) (Fig. 3A). The addition of valinomycin or CCCP to the fibroblast cultures had, on the other hand, no effect on the amounts of the NDUFS4 and NDUFA9 subunits of complex I in the fibroblast lysate (Fig. 3, inset). KH7 treatment of the fibroblast cultures resulted in a significant decrease of the amount of the NDUFS4 subunit in the total mitoplast fraction (Fig. 3B), but had no effect on the amount of this subunit, as well as of the NDUFA9 and NDUFB6 subunits, in the inner-membrane mitochondrial fraction of the mitoplasts (Fig. 3C). Two-dimensional, blue-native/SDS-PAGE and western blotting of respiratory chain complexes show that the KH7 and 8-Br-cAMP treatment of the fibroblast cultures did not have, in the short term incubation used (35 min), any effect on the amount of the nuclear encoded subunits in the assembled complex I, as well as in complex III, IV and V (Fig. 3D).

3.3. KH7 affects the accumulation of complex I subunits in isolated rat liver mitochondria

The effect of sAC inhibition by KH7 on the mitochondrial import/accumulation of newly synthesized complex I subunits was examined in a hybrid experimental model in which the import into isolated rat liver mitochondria (RLM) of [^{35}S]Met/Cys radiolabeled subunits of the complex, produced "in vitro" by expression in the rabbit reticulocyte lysate (RRL) of their full-length human cDNA was followed. The autoradiography patterns presented in Fig. 4A show that the [^{35}S] labeled NDUFS4 protein was imported into RLM, where it underwent cleavage of the presequence, generating the mature form (with lower molecular weight). The precursor, bound to the surface of mitochondria, was completely digested by trypsin treatment (see also [21]). The amount of the mitochondria imported NDUFS4, which was increased by added purified catalytic subunit of PKA (see also [21]), was depressed by KH7. The KH7-dependent decrease of the imported NDUFS4 protein was, however, not prevented by the addition of PKA. RLM, used in the import experiments, were also processed by BNE to separate the assembled respiratory chain complexes. The autoradiography shows that the imported [^{35}S] labeled NDUFS4 was incorporated in the RLM complex I. The incorporation was enhanced by PKA (Fig. 4B), but depressed by KH7. This effect of KH7 was prevented by 8-Br-cAMP, but not by added PKA. The import into RLM of two other "in vitro" expressed [^{35}S] labeled subunits of complex I, namely NDUFV2 and NDUFB11, was also followed (Fig. 4C and D). KH7 depressed, as observed for NDUFS4, the mitochondrial accumulation of the NDUFV2 subunit and its incorporation in the RLM complex I. The depressing effect exerted by KH7 on NDUFS4 and NDUFV2 was rescued by 8-Br-cAMP. The mitochondrial import of the [^{35}S] labeled NDUFB11 subunit was, on the other hand, unaffected by KH7 and 8-Br-cAMP. It can also be noted that, in the short incubation time used (35 min), the newly imported [^{35}S] labeled NDUFB11 did not assemble in the RLM complex I (Fig. 4D). In these experiments the immunoblot spot of the NDUFB6 subunit was used as a marker of the location of complex I in the native gel, and the immunoblot density of COX-IV subunit as a marker for protein loading in the gel (see Fig. 4B and D).

3.4. Effect in fibroblast cultures of protease inhibitors on the level of subunits and activity of complex I

The level of the mature, nuclear-encoded subunits of complex I is likely to be determined by the balance between their expression, mitochondrial import/maturation, and proteolytic digestion by mitochondrial proteases, as well as by the proteasome, for those copies of the subunits which undergo retrograde back diffusion in the cytosol (see [21,35]). Treatment of the fibroblast cultures with MG132, an inhibitor of proteasome, as well as of mitochondrial Lon protease [35,36], prevented the KH7-induced decrease of the level of NDUFA9, NDUFS4 and NDUFV2 in the fibroblast lysate (Fig. 5A). The level of the membrane

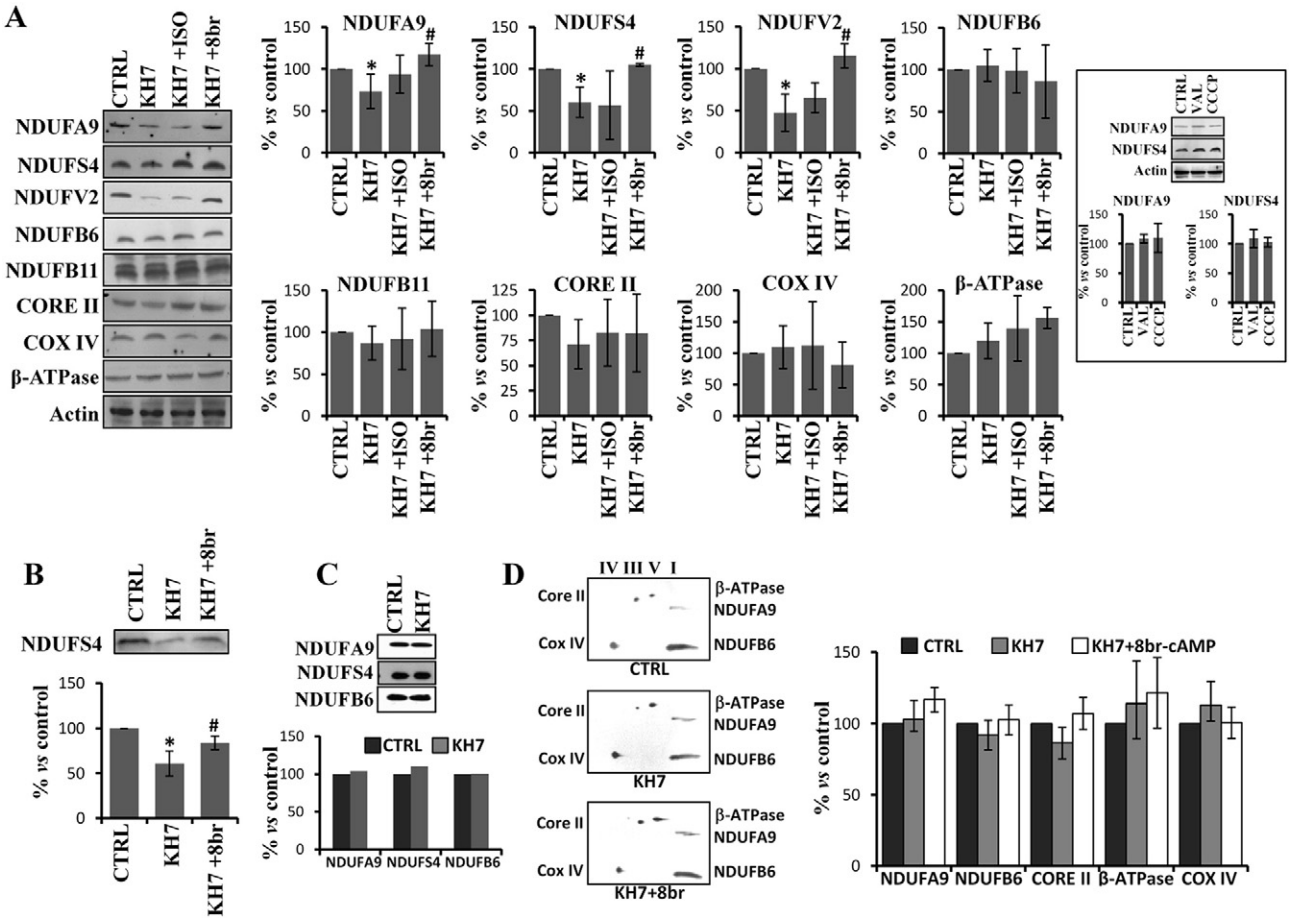

Fig. 3. Effect of KH7 treatment on the levels of mitochondrial respiratory chain subunits in fibroblast cultures. Fibroblasts were cultivated in standard culture conditions (CTRL), treated 35 min with KH7 (KH7), with KH7 plus isoproterenol (KH7 + ISO), or KH7 plus 8-Br-cAMP (KH7 + 8br). Proteins of cellular lysate (Panel A) and mitoplast fraction (Panel B) were loaded on 12% SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted with the antibodies against the subunits specified in the figure. Protein loading was assessed by reprobing the blots with the β-actin antibody. Panel C, the pellet of the inner mitochondrial membrane, obtained by sonication of the mitoplast fraction was resuspended in 30 μl of PBS, proteins separated by SDS/PAGE and subjected to immuno-detection with the antibodies described in the figure. Panel D, mitoplast proteins were processed by 2D, BNE/SDS-PAGE, followed by transfer to nitrocellulose membranes for immuno-detection with antibodies against complex I (NDUFA9 and NDUFB6), complex III (core II), complex IV (COX IV), and complex V (β-ATPase) subunits. The histograms represent the percentage changes with respect to the ADU (arbitrary densitometric units) of control untreated fibroblasts. The values are means ± standard deviation of three separate experiments. * $p < 0.05$, KH7 vs CTRL; # $p < 0.05$ KH7 + 8br vs KH7; Student's *t* test. For other details see legend to Fig. 2 and under Materials and methods. The inset shows the effects of valinomycin and CCCP treatment on the levels of complex I subunits in fibroblast cultures. Experimental conditions: (CTRL) fibroblasts grown in DMEM with 10% fetal bovine serum; (Val) fibroblasts treated for 35 min with 5 μM valinomycin; (CCCP) fibroblasts treated for 35 min with 2 μM CCCP. Proteins from fibroblast cell lysate were loaded on 12% SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted with the antibodies against the subunits specified in the figure. Protein loading was assessed by reprobing the blots with the β-actin antibody. The histograms represent the percentage changes with respect to the ADU of control untreated fibroblasts. The values are means ± standard deviation of three separate experiments.

integral, KH7 insensitive, NDUFB11 was unaffected by MG132. Epoxomicin, which is a specific proteasome inhibitor [37], exerted a smaller sparing effect on the KH7-induced decrease of the level of the NDUFA9, NDUFS4 and NDUFV2 subunits (Fig. 5A). Direct immunoassay showed that MG132 had no effect on the overall amount of the nuclear-encoded subunits in the assembled complex I separated by 2D (blue native/SDS-PAGE) from the KH7-treated fibroblasts (Fig. 5, inset).

The sparing effect exerted by MG132 on the KH7-induced decrease of the subunits prevented the KH7-induced depression of the enzymatic activity of complex I (Fig. 5B). The smaller sparing effect exerted by epoxomicin on the level of the subunits had, on the other hand, no effect on the KH7-induced inhibition of complex I activity. It can also be noted that MG132 or epoxomicin treatment of the fibroblast cultures had no effect on the KH7-induced depression of the enzymatic activity of complex IV (Fig. 5C).

3.5. Effect of MG132 and epoxomicin on the import/accumulation in RLM of [^{35}S] labeled complex I subunits

The sparing effect of protease inhibitors on the mitochondrial accumulation of matrix-exposed subunits of the complex was also verified in the RLM import model. The results presented in Fig. 6 show that MG132, but not epoxomicin, prevented the KH7-induced decrease of the accumulation in RLM of the [^{35}S] labeled NDUFS4 and NDUFV2 subunits (Fig. 6A). MG132 but not epoxomicin prevented the KH7-induced decrease of the amount of the [^{35}S] labeled NDUFS4 subunit incorporated in the RLM complex I (Fig. 6B).

3.6. Modulation of RLM protease activity by effectors of the cAMP system

The mitochondrial protease activity of RLM was measured by following the digestion of casein by Triton X-100 permeabilized organelles. After incubation of RLM with KH7, KH7 plus MG132 or KH7 plus epoxomicin, a protein kinase inhibitors (PKI) and okadaic acid, phosphatase inhibitor, were added to the incubation mixture, before adding casein, to prevent any possible change in the intrinsic phosphorylation state of casein used in the measurements. The results (Fig. 7) show that RLM treatment with KH7 promoted the digestion of casein, which was completely blocked by MG132, but unaffected by epoxomicin. The KH7 induced digestion of casein by RLM was largely prevented by the addition to the incubation mixture of 8-Br-cAMP (Fig. 7C). On the other hand, the addition of the PKA inhibitor H89, promoted casein degradation (Fig. 7C).

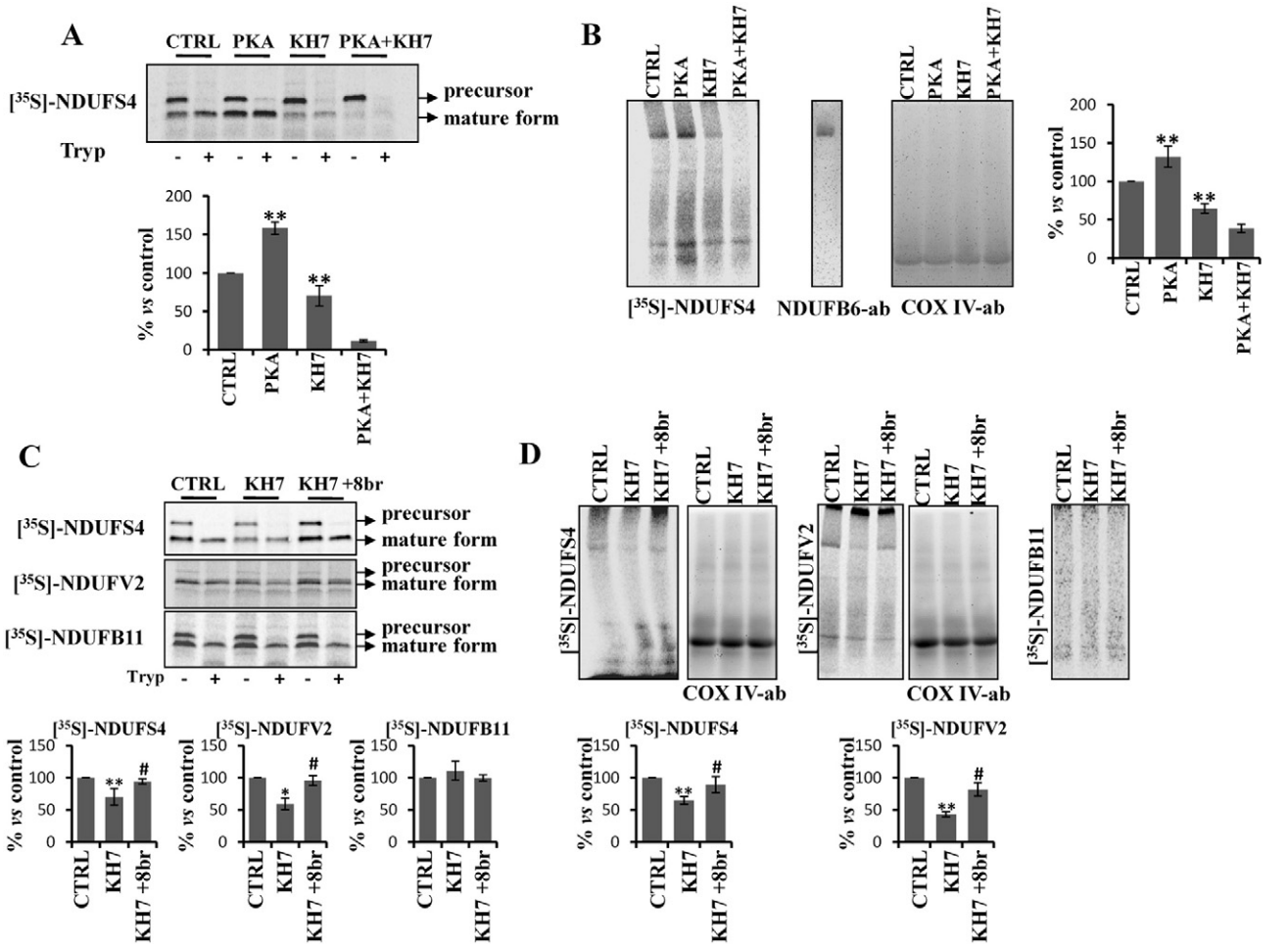

Fig. 4. Effect of KH7 on the accumulation of "in vitro" produced complex I subunits in isolated rat-liver mitochondria. [^{35}S]Met-labeled NDUFS4, NDUFV2 and NDUFB11 proteins were produced by "in vitro" RRL transcription/translation of full length human cDNAs of the subunits. 5-μl aliquots of the RRL translation mixture were added to the RLM import mixture. Panel A, mitochondrial import of [^{35}S] labeled NDUFS4. Experimental conditions: (CTRL) no addition; (PKA) import incubation in the presence of cPKA (1 unit per 10 μg mitochondrial proteins) plus 7.5 mM sodium fluoride; (KH7) import incubation in the presence of 50 μM KH7; (PKA + KH7) import incubation in the presence of KH7 plus cPKA. After 60 min incubation at 30 °C, mitochondria were spun down from the import mixture, before or after trypsin treatment (1 μg per 50 μg mitochondrial proteins, 35 min on ice) as specified in the figure. The solubilized pellets, containing 50 μg of mitochondrial proteins, were analyzed by SDS–PAGE and autoradiography. Panel B, Mitochondrial proteins of trypsinized samples in Panel A were spun down from the import mixture and analyzed by BNE and autoradiography. The location of assembled complex I was assessed by immunoblotting with an antibody against the NDUFB6 subunit of complex I. Protein loading was assessed by probing the membrane with the COX IV antibody. Panel C, mitochondrial import experiments of [^{35}S] labeled NDUFS4, NDUFV2 and NDUFB11. Experimental conditions: (CTRL) no addition; (KH7) import incubation in the presence of KH7; (KH7 + 8br) import incubation in the presence of KH7 plus 100 μM 8-Br-cAMP. Panel D, Mitochondrial proteins of trypsinized samples in Panel C were spun down from the import mixture and analyzed by blue native electrophoresis and autoradiography. The histograms show the mean ADU (as percentage of control) of radioactive bands corresponding to the trypsin-resistant mature form of radiolabeled proteins (panels A and C) and of the radiolabeled bands incorporated in complex I (panels B and D). The values are means ± standard deviation of three separate experiments. * $p < 0.05$, ** $p < 0.01$: PKA vs CTRL, KH7 vs CTRL; # $p < 0.05$, KH7 + 8br vs KH7; Student's *t* test. For other details see under Materials and methods.

4. Discussion

In mammalian cells the nuclear-encoded subunits of complex I are continuously imported into mitochondria, where they are integrated, in sequential steps, with the mitochondrial-encoded subunits to form the mature functional complex, or separately exchanged with their pre-existing copies in the complex [5,7,8]. The results from previous [8,9,21] and present work show that both cytosolic and mitochondrial pools of cAMP regulate the turnover of specific nuclear encoded subunits of complex I. Phosphorylation of the NDUFS4 subunit by extramitochondrial PKA promotes the mitochondrial import of the precursor of the protein [21]. The results of the present work show that the intramitochondrial cAMP, produced by sAC, modulates the concentration of this and other specific nuclear-encoded subunits of the catalytic moiety of complex I and its NADH-ubiquinone oxidoreductase activity.

Treatment of fibroblast cultures with KH7, which inhibits intramitochondrial production of cAMP (Fig. 1), causes a marked (35–50%) decrease of the overall amount of the mature forms of the nuclear-encoded NDUFS4, NDUFV2 and NDUFA9 subunits of complex I, but left unaffected their total amount in the assembled complex in the inner mitochondrial membrane. Of these subunits, which are the last to be assembled in the complex, NDUFS4 and NDUFV2 belong to the NADH dehydrogenase (N) module, NDUFA9 to the electron output (Q) module [5]. No effect of KH7 was, on the other hand, exerted on the level of the nuclear-encoded NDUFB6 and NDUFB11 subunits, which belong to the membrane embedded moiety of complex I [5]. The KH7-induced decrease of the nuclear-encoded subunits of complex I, which are essential for the NADH-ubiquinone oxidoreductase of the enzyme, was associated with depression of the activity of complex I. This was accompanied as already observed in other cases [9] by enhanced level of cellular H_2O_2. Both KH7 effects were counteracted by the membrane permeant 8-Br-cAMP, but not by isoproterenol-induced production of cAMP in the cytosol. Effects similar to those of KH7, were reproduced by treatment of the fibroblast cultures with acetazolamide, a carbonic anhydrase inhibitor (CAI), which also causes a decrease in the mitochondrial level of cAMP [33]. The observed marked decrease of the overall amount of the NDUFS4, NDUFV2 and NDUFA9 subunits, caused by KH7 and CAI in the fibroblast

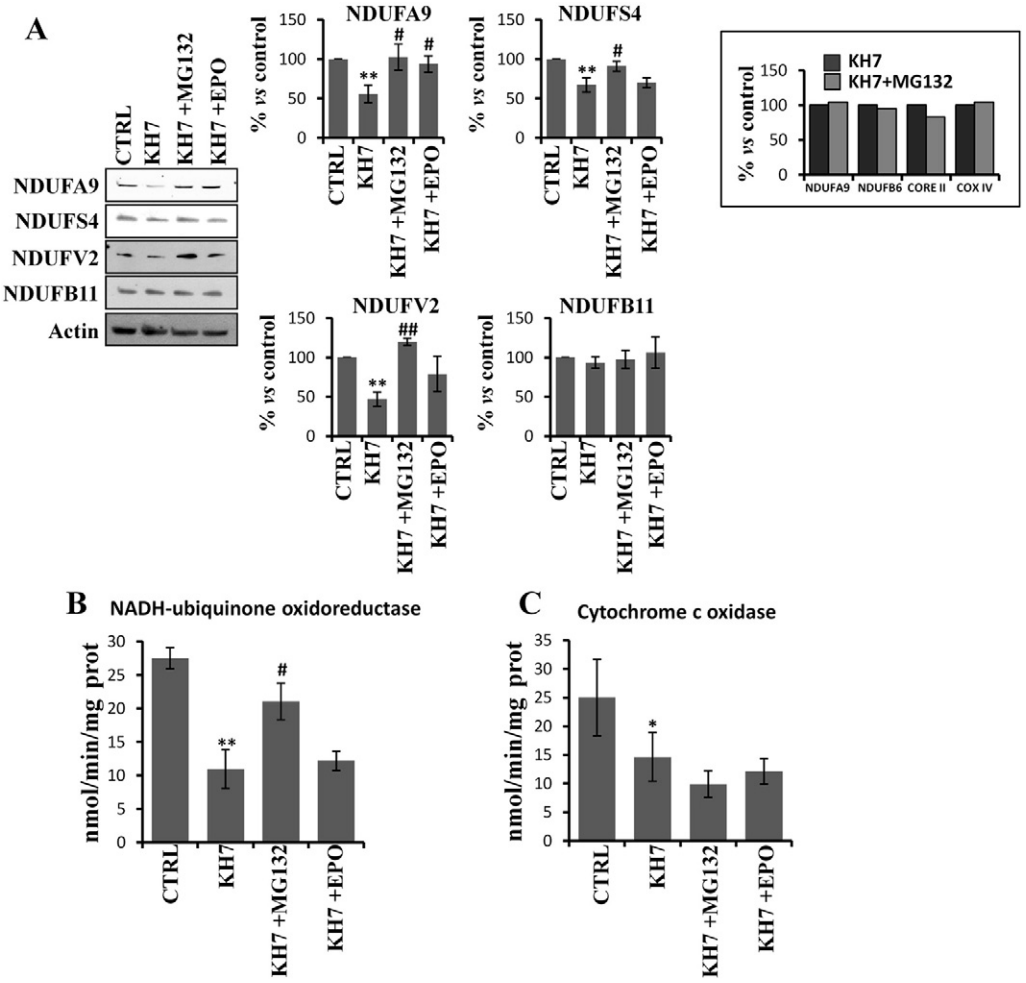

Fig. 5. Effect of MG132 and epoxomicin on KH7-dependent induced decrease of subunit levels and activity of complex I in fibroblast cultures. Panel A, Immuno-detection of subunit levels in the fibroblast lysate. Panel B, NADH:ubiquinone oxidoreductase activity determined in the fibroblast mitoplast fraction. Panel C, cytochrome *c* oxidase activity determined in the fibroblast mitoplast fraction. Fibroblasts were cultivated in standard culture conditions (CTRL); treated 35 min with 25 μM KH7 (KH7); KH7 plus 10 μM MG132 (KH7 + MG); KH7 plus 5 μM epoxomicin (KH7 + EPO). The histograms represent the percentage changes with respect to the ADU of control untreated fibroblats. The values are means ± standard deviation of three independent experiments. * $p < 0.05$, ** $p < 0.01$: KH7 vs CTRL; # $p < 0.05$, ## $p < 0.01$: KH7 + MG132 vs KH7, KH7 + EPO vs KH7. Student's *t* test. For other details see legend to Fig. 3 and under Materials and methods. The histograms in the inset represent the densitometric semiquantitative analysis of the assembled subunits of the respiratory chain complexes. Fibroblast cultures were treated with KH7 and KH7 plus MG132. After 35 min incubation, the mitoplasts were isolated, proteins separated by 2D, BNE/SDS-PAGE, and transferred to nitrocellulose membranes for immuno-detection with the specified antibodies.

lysate, reveals a free non-incorporated pool of these subunits with accounts for about one half of their total cellular amounts.

The depressing effect exerted by KH7 on the mitochondrial amount of mature form of the subunits of complex I was verified in the experimental model in which the import/accumulation in RLM of "in vitro" expressed [^{35}S] labeled human NDUFS4 and NDUFV2 subunits was followed. Previous work from our laboratory has shown that phosphorylation of the C-terminal RVS conserved site of the nuclear-encoded NDUFS4 subunit of complex I [20] by extramitochondrial PKA promotes the membrane potential driven mitochondrial import of the precursor form of the protein [21]. The imported precursor, processed by mitochondrial maturase with cleavage of the positively charged leader sequence, generates the mature form which is then incorporated in the complex (see Fig. 4). It can be noted the positive outcome of this hybrid import system confirms the functional inter-species equivalence of these subunits, whose human and rat aminoacid sequences present more than 90% identity ([20], sequences obtained and aligned with UniProt website). The accumulation of [^{35}S] labeled human NDUFS4 and NDUFV2 proteins in RLM and their incorporation in the RLM complex I were depressed by KH7. This inhibitory action of KH7 too was completely prevented by 8-Br-cAMP. The mitochondrial accumulation of the membrane integral [^{35}S] labeled NDUFB11 subunit was, on the other hand, unaffected by KH7 and 8-Br-cAMP.

Insight into the mechanism of this role of intramitochondrial cAMP is provided by the experiments in which the effect of protease inhibitors on the KH7-induced decrease of the amount of these subunits was tested. Treatment of fibroblast cultures with MG132, an inhibitor of the proteasome as well as of Lon, completely prevented the KH7-induced decrease of the non-incorporated amounts of the NDUFA9, NDUFS4 and NDUFV2 subunits. Epoxomicin, which inhibits only the proteasome, exerted a smaller protective effect against the KH7-induced decrease of these subunits. MG132, but not epoxomicin, prevented the KH7-induced depression of complex I activity. It can also be noted that MG132 had no effect on the KH7-induced inhibition of cytochrome c oxidase.

The sparing effect of MG132 was also observed in the RLM import model, in which it prevented the depressing action of KH7 on the accumulation in mitochondria of [^{35}S] labeled NDUFS4 and NDUFV2 subunits and their incorporation in the RLM complex I. In this system epoxomicin did not prevent the KH7 depression of subunit accumulation in RLM and incorporation in the RLM complex I. These findings show that the intramitochondrial critical amount of specific nuclear-encoded subunits, of the catalytic moiety of the complex, is controlled

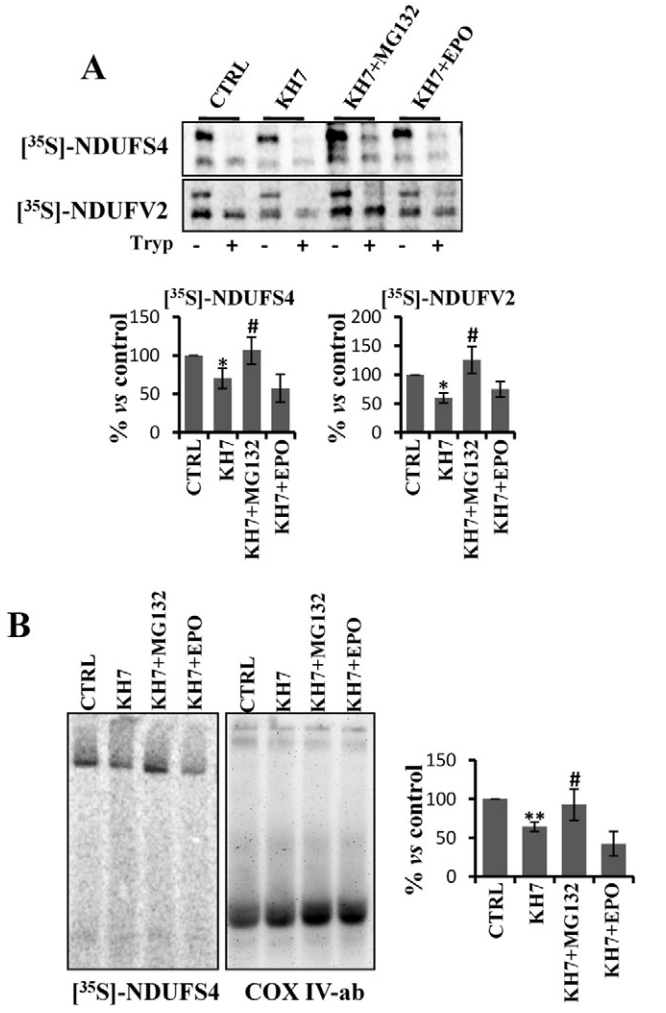

Fig. 6. KH7-induced decrease of accumulation of complex I subunits in rat-liver mitochondria is prevented by MG132 but not by epoxomicin. Panel A, mitochondrial import experiments of [^{35}S] labeled NDUFS4 and NDUFV2. (CTRL), no addition; (KH7), import incubation in the presence of 50 μM KH7; (KH7 + MG), import incubation in the presence of KH7 plus 10 μM MG132; (KH7 + EPO) import incubation in the presence of KH7 plus 5 μM epoxomicin. After 60 min incubation at 30 °C, mitochondria were spun down from the import mixture before or after trypsin treatment. The solubilized pellets, containing 50 μg of mitochondrial proteins, were analyzed by SDS–PAGE and autoradiography. Panel B, Mitochondrial proteins of trypsinized samples in Panel A were spun down from the import mixture and analyzed by BNE and autoradiography. Protein loading was assessed by probing the membrane with the COX IV antibody. The histograms show the mean ADU (as percentage of control) of radioactive bands corresponding to the trypsin-resistant mature form of radiolabeled proteins (panel A) and of the radio-labeled bands incorporated in complex I (panel B). The values are means ± standard deviation of three separate experiments. * $p < 0.05$, ** $p < 0.01$: KH7 vs CTRL; # $p < 0.05$: KH7 + MG132 vs KH7; Student's t test. For other details see legend to Fig. 4 and under Materials and methods.

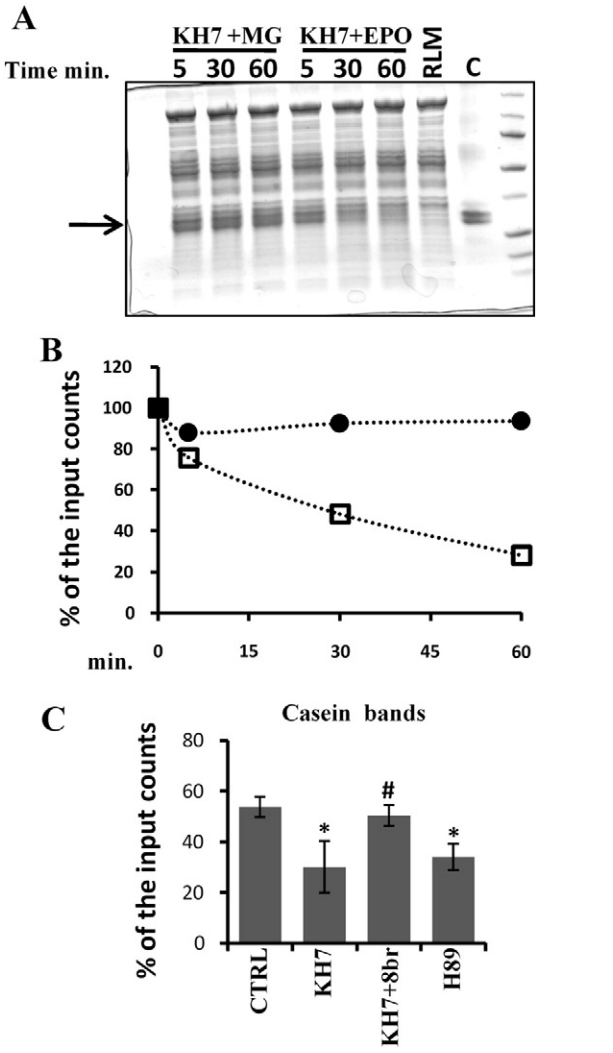

Fig. 7. Effect of KH7, 8-Br-cAMP and H89 on proteolysis of casein in rat liver mitochondria. Panel A, casein digestion was followed in triton X-100 solubilized RLM incubated in the presence of 50 μM KH7 plus 10 μM MG132 (KH7 + MG, filled circles of the plot) or in the presence of KH7 plus 5 μM epoxomicin (KH7 + EPO, open squares of the plot) at 30 °C, for the time intervals indicated in the figure. After incubation, proteins were separated by SDS-PAGE and stained with comassie blue. The lane (RLM) represents the SDS-PAGE of RLM proteins incubated in the absence of added casein. Lane (C) represents the SDS-PAGE of the purified casein sample. The ADU of stained purified casein (in lane C) represents the 100% of the input counts. Panel B, the plots represent the ADU of the stained casein, expressed as percentage of input counts. Panel C, casein digestion was followed in RLM incubated for 60 min at 30 °C in the absence (CTRL), in the presence of 50 μM KH7 (KH7), in the presence of KH7 plus 100 μM 8-Br-cAMP (KH7 + 8br), in the presence of 100 nM H89 (H89). The histograms represent the ADU of the stained casein, expressed as percentage of input counts. The values are means of three determinations. * $p < 0.05$: KH7 vs CTRL and H89 vs CTRL; # $p < 0.05$: KH7 + 8br vs KH7. Student's t test. For other details see under Materials and methods.

by the balance between import/maturation in mitochondria and their proteolytic degradation [38,39].

Mitochondrial proteases include two AAA proteases, the Lon protease, the metallopeptidase OMA1 and the ClpXP protease [39]. MG132, which is reported to inhibit in addition to the proteasome also the Lon protease [35,36], completely prevented the KH7 induced digestion of purified casein by RLM extract, as, indeed, observed for the KH7 induced digestion of nuclear encoded NDUFA9, NDUFS4 and NDUFV2 subunits of complex I in fibroblast cultures (Fig. 5) as well as the digestion of the radiolabeled NDUFS4 and NDUFV2 subunits incorporated in vitro in RLM (Fig. 6). These observations favor the Lon protease as responsible for the KH7 promoted proteolysis of the nuclear-encoded subunits of complex I. This doesn't, however, exclude the possibility that the other mitochondrial protease(s) are also involved.

The KH7-promoted digestion of specific nuclear-encoded subunits of complex I was completely prevented by the concomitant addition of 8-Br-cAMP. Also the KH7-induced digestion of casein by RLM extract was prevented by 8-Br-cAMP, and promoted by the PKA inhibitor H89. This shows that the KH7-induced proteolysis involves the cAMP system. Collapse of mitochondrial membrane potential by valinomycin, or the uncoupler CCCP, in fibroblast cultures did not induce, on the other hand, degradation of the NDUFA9 and NDUFS4 subunits of complex I (inset in Fig. 3). It can also be noted that KH7 did not promote degradation of the NDUFB11 subunit in fibroblast cultures (Fig. 3), neither its import in RLM in vitro, a process which, like the import of NDUFS4, is $\Delta\psi$ dependent (see also [21]). This seems to exclude the possibility that, at least under the prevailing experimental conditions used, the

KH7 effects on complex I subunits and activity, in addition to the depression of the cAMP system, involve also changes in membrane potential. The increase in ROS level induced by KH7 treatment of fibroblast cultures, which was prevented by 8-Br-cAMP, could also be involved in the KH7-induced degradation of the NDUFS4, NDUFV2 and NDUFA9 subunits (see ref. [40]). The minor sparing effect on the KH7-induced decrease of complex I subunits, produced by epoxomicin inhibition of the proteasome in fibroblast cultures, is likely due to inhibition of degradation in the cytosol of the mature forms of complex I subunits released from mitochondria by retrograde back diffusion [cf. 21]. Obviously the sparing effect of this fraction of subunits has no effect on the catalytic activity of complex I in mitochondria.

In conclusion regulation by cytosolic and mitochondrial cAMP of the import and proteolytic processing respectively controls the intramitochondrial level of free nuclear encoded NDUFS4, NDUFV2 and NDUFA9 subunits of complex I. These subunits, belonging to the catalytic moiety of the complex, which protrudes in the matrix space, are exposed to oxidative damage by ROS produced in this space [6–8]. Maintenance of a substantial intramitochondrial free pool of these subunits contributes to preserve the functional capacity of complex I by replacing ROS damaged subunits in the assembled complex with newly produced copies [8].

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