Biochimica et Biophysica Acta 1847 (2015) 143–152

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Membrane potential regulates mitochondrial ATP-diphosphohydrolase activity but is not involved in progesterone biosynthesis in human syncytiotrophoblast cells

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ARTICLE INFO

Article history:
Received 16 May 2014
Received in revised form 17 September 2014
Accepted 7 October 2014
Available online 14 October 2014

Keywords:
ATP-diphosphohydrolase
Placental mitochondria
Mitochondrial bioenergetics
ATP hydrolysis
Progesterone synthesis

ABSTRACT

ATP-diphosphohydrolase is associated with human syncytiotrophoblast mitochondria. The activity of this enzyme is implicated in the stimulation of oxygen uptake and progesterone synthesis. We reported previously that: (1) the detergent-solubilized ATP-diphosphohydrolase has low substrate specificity, and (2) purine and pyrimidine nucleosides, tri- or diphosphates, are fully dephosphorylated in the presence of calcium or magnesium (Flores-Herrera 1999, 2002). In this study we show that ATP-diphosphohydrolase hydrolyzes first the nucleoside triphosphate to nucleoside diphosphate, and then to nucleotide monophosphate, in the case of all tested nucleotides. The activation energies (E_a) for ATP, GTP, UTP, and CTP were 6.06, 4.10, 6.25, and 5.26 kcal/mol, respectively; for ADP, GDP, UDP, and CDP, they were 4.67, 5.42, 5.43, and 6.22 kcal/mol, respectively. The corresponding Arrhenius plots indicated a single rate-limiting step for each hydrolyzed nucleoside, either tri- or diphosphate. In intact mitochondria, the ADP produced by ATP-diphosphohydrolase activity depolarized the membrane potential ($\Delta\Psi_m$) and stimulated oxygen uptake. Mitochondrial respiration showed the state-3/state-4 transition when ATP was added, suggesting that ATP-diphosphohydrolase and the F_1F_0-ATP synthase work in conjunction to avoid a futile cycle. Substrate selectivity of the ATP-diphosphohydrolase was modified by $\Delta\Psi_m$ (*i.e.* ATP was preferred over GTP when the inner mitochondrial membrane was energized). In contrast, dissipation of $\Delta\Psi_m$ by CCCP produced a loss of substrate specificity and so the ATP-diphosphohydrolase was able to hydrolyze ATP and GTP at the same rate. In intact mitochondria, ATP hydrolysis increased progesterone synthesis as compared with GTP. Although dissipation of $\Delta\Psi_m$ by CCCP decreased progesterone synthesis, NADPH production restores steroidogenesis. Overall, our results suggest a novel physiological role for $\Delta\Psi_m$ in steroidogenesis.

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1. Introduction

One of the main functions of the placenta is the synthesis of progesterone (P4) to maintain pregnancy. Mitochondria from human syncytiotrophoblast cells contain the machinery for steroid synthesis. It consists of an electron transport chain (ETC-P450scc) composed by the cytochrome P450scc (CYP11A1; EC 1.14.15.6) that receives electrons from NADPH + H^+ through two proteins: adrenodoxine and adrenodoxine reductase. These proteins are located in the inner mitochondrial membrane and transform cholesterol into pregnenolone (P5) [3–5]. An additional enzyme, type II 3β-hydroxysteroid-dehydrogenase-Δ4-5 isomerase (3βHSD) also embedded in the inner mitochondrial membrane of syncytiotrophoblast cells, transforms pregnenolone into progesterone [4,5].

Mitochondria are best known as the major source of ATP in aerobic cells. Oxidative phosphorylation provides the main source of ATP. This metabolic pathway relies on the activity of two components: the oxidative and the phosphorylation systems. The oxidative system (*i.e.* respiratory chain) couples redox reactions to the production of a proton electrochemical gradient that drives the synthesis of ATP by the phosphorylation system (*i.e.* F_0F_1-ATP synthase and the ADP/ATP and phosphate carriers). Studies conducted in primary and MA-10 tumor Leydig cells suggest an interrelation between steroidogenesis and oxidative phosphorylation. Steroidogenic mitochondria perform a double role: synthesize ATP and produce hormones. Steroidogenesis is affected when the classic mitochondrial electron-transport chain (ETC), membrane potential ($\Delta\Psi_m$), or ATP synthesis is disrupted [6,7], suggesting a close relationship between both metabolic pathways.

The relative amount of ETC-P450scc and classic ETC components present in cells depends on the type of steroidogenic tissue. In acute-

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http://dx.doi.org/10.1016/j.bbabio.2014.10.002
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regulated steroidogenic tissues (*i.e.* the adrenal gland and gonads) the content of ETC-P450scc is several times higher than in the classic ETC. In the syncytiotrophoblast, a constitutive steroidogenic tissue, the amounts of both electron transfer chain components is similar [8]. This suggests that the activity of both pathways will generate enough ATP for the cell to function properly and enough P4 to maintain pregnancy. Both processes are equally important for the physiological role of human placenta, and their activity must be tightly regulated.

Human syncytiotrophoblast mitochondria contain accessory enzymes, like the ATP-diphosphohydrolase. This enzyme is tightly bound to mitochondrial membranes and is involved in progesterone synthesis, mainly in cholesterol transport [1]. Studies have related cholesterol transport across the membranes of syncytiotrophoblast mitochondria with the activity of mitochondrial ATP-diphosphohydrolase [1,2]. Probably, ATP-diphosphohydrolase provides the required energy to drive cholesterol transport between mitochondrial membranes in an analogous way to that of the mitochondrial GDPase in the adrenal gland [9]. The underlying molecular mechanism involved remains unknown.

Simultaneously, in the intact and energized mitochondria, the ATP-diphosphohydrolase hydrolyzes ATP to ADP. The latter promotes oxygen uptake and ATP synthesis by the F_1F_0-ATP synthase [10]. The activities of the ATP-diphosphohydrolase (ATP hydrolysis) and the F_1F_0-ATP synthase (ATP synthesis) must be coordinated to avoid a futile cycle and energy dissipation.

Nevertheless, the physiological role of the ATP-diphosphohydrolase must be examined in intact syncytiotrophoblast mitochondria. Since ATP-diphosphohydrolase activity is involved in progesterone synthesis [2] and mitochondrial bioenergetics [10], regulatory mechanisms must be involved to keep trophoblast cells alive and functional. In the present work we evaluated the relationship between $\Delta\Psi_m$, ATP-diphosphohydrolase activity, and progesterone synthesis in syncytiotrophoblast mitochondria. Results suggest that ATP-diphosphohydrolase activity is modified by $\Delta\Psi_m$, but an increase in NADPH content and ATP hydrolysis supports progesterone synthesis when $\Delta\Psi_m$ decreases. This study puts forward a novel physiological role for the $\Delta\Psi_m$ in human placenta steroidogenesis.

2. Experimental procedures

2.1. Isolation of human syncytiotrophoblast mitochondria

Full term human placenta was collected immediately after normal delivery at the IMSS Hospital No. 4, approval under the Ethical Committee regulations. Mitochondria were prepared as previously described [3]. Briefly, placental cotyledons were placed in ice-cold 250 mM sucrose and 1 mM EDTA, 10 mM Tris, pH 7.4. The suspension was homogenized by means of a Polytron (Brinkmann Instruments, Westbury, NY, USA), at 3000 rev/min for 1 min for two cycles separated by an interval of 1 min. The whole process was carried out at 4 °C. The pH of the homogenate was adjusted to pH 7.4 with Tris and centrifuged at 1500 *g* for 15 min. The supernatant was recovered and centrifuged at 4000 *g* to pellet the cytotrophoblast mitochondria (*i.e.* heavy mitochondria). The supernatant was centrifuged again at 16,000 *g* for 15 min and the pellet containing the syncytiotrophoblast mitochondria (*i.e.* light mitochondria) was resuspended in the same solution and then centrifuged at 1500 *g* for 10 min to remove any remaining erythrocytes. Mitochondria were pelleted by centrifugation at 12,000 *g* for 10 min. The resulting syncytiotrophoblast mitochondria were loaded on a 35% sucrose solution (25 ml) and centrifuged at 15,000 *g* for 45 min at 4 °C. The mitochondrial fraction was collected, suspended in 250 mM of sucrose, 1 mM of EDTA, and 10 mM of Tris (pH 7.4) and centrifuged at 16,000 *g* for 15 min at 4 °C; the mitochondrial pellet was suspended in this buffer and stored at 4 °C. Protein concentration was measured as reported by [11,12].

2.2. Mitochondrial oxygen consumption

Oxygen uptake was estimated polarographically using a Clark type electrode in a mixture containing 250 mM of sucrose, 10 mM of HEPES pH 7.4, 1 mM of EGTA, 1 mM of EDTA, 10 mM of succinate, 10 mM of KH_2PO_4, 5 mM of $MgCl_2$, 0.2% bovine serum albumin and 1 mg/ml of syncytiotrophoblast mitochondrial protein [2]. Temperature was set at 37 °C and oxygen consumption was stimulated by the addition of 300–500 nmol of ATP or ADP (state 3 of respiration). Respiratory control was defined as oxygen uptake rate of state 3/oxygen uptake rate of state 4 (state 4 of respiration started when all ADP was converted to ATP and respiration slowed down) [13]. Where indicated, mitochondria were incubated with 5 μM carboxyatractyloside (CAT) to inhibit the translocation of adenine nucleotides by blocking the ADP/ATP carrier. Simultaneously, 10 μM of carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) was added to depolarize the inner membrane and stimulate maximal oxygen uptake (*vide infra*). At the indicated times (see Figs. 3–5) an aliquot was withdrawn and used to determine the nucleotide concentration by HPLC (*vide infra*).

2.3. Mitochondrial membrane potential ($\Delta\Psi_m$)

The following media was used to determine the $\Delta\Psi_m$ of syncytiotrophoblast mitochondria: 125 mM of KCl; 5 mM of $MgCl_2$; 10 mM of acetate–Tris, pH 7.4; 10 mM of Tris–HCl, pH 7.4; 1 μM of rotenone; 3.3 mM of H_3PO_4, pH 7.4; 9.6 mM of Safranine O, and 1 mg of mitochondrial protein/ml. Generation of the membrane potential was initiated by adding 10 mM of succinate–Tris, pH 7.4 [14] to the solution containing syncytiotrophoblast mitochondria. Where indicated, mitochondria were incubated with 5 μM CAT to inhibit the ADP/ATP carrier, and 10 μM of CCCP was added to abolish $\Delta\Psi_m$. The membrane potential was evaluated in a double beam spectrophotometer by using the difference of wavelengths between 533 and 511 nm. The final volume was 1.5 ml and was kept at 25 °C.

2.4. Activity determinations of mitochondrial enzymes

Activities of complex I (NADH:DCPIP oxidoreductase) and complex II (succinate:DCPIP oxidoreductase) were determined spectrophotometrically at 600 nm by following the reduction of the artificial electron acceptor 2,6-dichlorophenol-indophenol (DCPIP; 50 μM; $\varepsilon_{DCPIP} = 21\ mM^{-1}\ cm^{-1}$). Mitochondria were permeabilized with 0.01% Triton X100, incubated in 120 mM of KCl, 5 mM of $MgCl_2$, 1 mM of EGTA, 30 mM of KH_2PO_4, pH 7.4, and either 500 μM of NADH (complex I) or 2 mM of succinate (complex II). Complex II was activated by preincubation in the presence of 0.2 mM of phenazine methosulfonate (PMS) for 10 min at 25 °C [15,16]. Protein concentration of syncytiotrophoblast mitochondria was 50 μg/ml and the reaction was started by the addition of NADH or succinate.

Mitochondrial ATP-diphosphohydrolase activity was determined either by nucleotide separation by HPLC (*vide infra*) or by measuring the release of inorganic phosphate (Pi) as described by Flores-Herrera et al. [1], using an ATP-diphosphohydrolase enriched fraction [1], or isolated syncytiotrophoblast mitochondria. Briefly, proteins (50 μg) were incubated in a final volume of 0.5 ml at 30 °C in 30 mM Tris–HCl (pH 8.5), and the ATP-diphosphohydrolase reaction was started by the addition of the substrate (Mg-nucleotide complex) plus 1 mM of free Mg^{2+}. Aliquots were withdrawn at one minute intervals and used for nucleotide separation by HPLC (*vide infra*), or mixed with the malachite–molybdate–Triton X-100 mixture for Pi determination, as described by Lanzetta et al. [17]. The nucleotides used were ATP, ADP, GTP, GDP, UTP, UDP, CTP, CDP or TTP. The experiments were performed at least four times in duplicate.

2.5. Nucleotide separation by HPLC

Quantification of nucleotides was performed from either mitochondrial oxygen consumption experiments or a fraction enriched with a detergent-solubilized ATP-diphosphohydrolase. At the indicated times aliquots from ATP-diphosphohydrolase nucleotide hydrolysis activity or oxygen uptake determination were withdrawn and mixed with trichloroacetic acid (6% final) to stop the reaction. Nucleotides were separated by anion exchange HPLC on a Hypersil SAX column (120 Å, 5 μm, 250 × 4.6 mm) from Alltech International. The low concentration buffer (A) was 5 mM $NH_4H_2PO_4$ (pH 2.8) and the high concentration buffer (B) was 750 mM $NH_4H_2PO_4$ (pH 3.7). The sample was loaded on the column equilibrated with buffer (A). Then, a gradient of buffer (B) (30 min, 0–100%) was used for elution. The flow rate was 1 ml/min and detection was performed at 254 nm [18].

2.6. Mitochondrial progesterone synthesis

Progesterone synthesis was determined at 37 °C as reported previously [2] in 120 mM of KCl, 10 mM of MOPS, 0.5 mM of EGTA, 10 mM of isocitrate, 4 μg of aprotinin/ml, 1 μM of leupeptin, and 5 mM of KH_2PO_4, pH 7.4, in a final volume of 500 μl with 1 mg/ml of syncytiotrophoblast mitochondrial protein. Where indicated, 25 μM 22-(*R*)-hydroxy-cholesterol was added to verify cytochrome P450scc, adrenedoxin, adrenedoxin reductase, and 3β-hydroxysteroid dehydrogenase activities [19]. After 20 min of incubation the reaction was arrested with 75 μl methanol and progesterone concentration was determined using a radioimmunoassay kit (Diagnostic Systems Laboratories, Inc. Webster, Texas, USA), according to the manufacturer's instructions. The concentration of progesterone at time zero was subtracted from the amount of progesterone quantified at 20 min and the resulting net progesterone synthesis was reported.

2.7. Sample preparation for native electrophoresis

The ATP-diphosphohydrolase from syncytiotrophoblast mitochondria was resolved by native PAGE following the general procedures reported previously [20,21], with minor modifications [22]. Briefly, syncytiotrophoblast (2 mg) mitochondria were suspended in 50 mM Bis-Tris and 500 mM 6-aminocaproic acid, pH 7.0, and solubilized by adding digitonin, at a detergent/protein ratio of 2 (g/g) in a final volume of 200 μl. The mixture was incubated for 30 min at 4 °C and centrifuged at 100,000 *g* for 30 min at 4 °C. The supernatants were recovered and immediately loaded on a linear polyacrylamide gradient gel (5–10%) for Blue Native PAGE (BN-PAGE) or Clear Native PAGE (CN-PAGE) [21]. The molecular weight of ATP-diphosphohydrolase activity was estimated by using the digitonin-solubilized bovine mitochondrial complexes as standard.

2.8. In-gel catalytic activity assays

The in-gel activity assays were performed as described by [18]. Briefly, gel strips were preincubated in 30 mM Tris–HCl, pH 8.5, 5% glycerol, 15 mM $CaCl_2$ for 30 min at 37 °C in the presence or absence of the complex V inhibitor oligomycin (5 μg/ml), or 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA, 1 mM) the ATP-diphosphohydrolase inhibitor. The equilibration solution was discarded and the gel strips were then added to the assay buffer containing 30 mM of Tris–HCl, pH 8.5, 5% glycerol, 15 mM of $CaCl_2$ and 5 mM of ATP, ADP, GTP or GDP, with or without oligomycin (5 μg/ml) or FSBA (1 mM). After incubation at 37 °C for approximately 2 h, nucleotide hydrolysis correlated with the development of white calcium phosphate precipitates. The reaction was stopped using 50% methanol, and subsequently, the gel was transferred to water and scanned against a dark background as described previously [22].

2.9. Isolation of steroidogenic contact sites

Steroidogenic contact sites were isolated as reported by Uribe et al. [23]. Briefly, 20–25 mg of mitochondrial protein was incubated at 4 °C with 10 mM H_3PO_4, adjusted to pH 7.3 with Tris base in the presence of 10 μg aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride, and 10 μg leupeptin/ml. After incubation, sucrose was added to attain a concentration of 0.38 M. The resulting mixture was incubated for another 20 min at 4 °C and then centrifuged at 12,500 *g* for 10 min. The pellet containing mitoplasts was recovered and incubated in 1 mM H_3PO_4, adjusted to pH 7.3 with Tris base, for 20 min at 4 °C. Sucrose was added to reach a concentration of 0.31 M and the mixture was incubated for another 20 min at 4 °C and centrifuged at 102,000 *g* for 1 h. The pellet containing the inner membrane fraction was sonicated four times in an ice bath for five seconds in a MSE Soniprep 150 at maximal output. The fraction containing the inner membranes was layered over a discontinuous sucrose gradient (densities of 1.06 to 1.29 g/ml) and centrifuged at 96,000 *g* for 20 h at 4 °C. The steroidogenic contact sites were recovered at sucrose densities of 1.20–1.22 g/ml and washed three times with 0.25 M sucrose, 1 mM EDTA, with pH adjusted with Tris base to 7.4, and recovered by centrifugation at 137,000 *g* for 30 min at 4 °C. Protein content was determined as described above and the obtained samples were stored at −70 °C.

2.10. Tandem mass spectrometry (LC/ESI–MS/MS)

The mitochondrial inner membrane obtained during the isolation of steroidogenic contact sites (see previous section) was incubated in 100 mM ammonium bicarbonate (pH = 7.8) for 30 min and centrifuged at 100 000 ×*g* at 4 °C. The pellet containing the mitochondrial inner membranes was sent to the Proteomics Core Facility at the University of Arizona, USA.

2.11. Statistical analyses

Statistical analyses (one- and two-way analysis of variance, ANOVA) of the data were performed using Sigma Stat software, version 3.5. When necessary, nonlinear regression of the data to a single exponential decay equation was performed in Sigma Plot software, version 10.0.

2.12. Materials

Analytical grade reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), E. Merck (Darmstadt, Germany), and BioRad (Hercules, CA, USA).

3. Results

3.1. Functional state of syncytiotrophoblast mitochondria

We calculated respiratory controls from oxygen uptake traces, using succinate as a substrate, to determine the functional integrity of isolated syncytiotrophoblast mitochondria (Table 1). Oxygen uptakes in state 3 and state 4 were 110 ± 18 ng atom of oxygen/min·mg protein, and 19 ± 6 ng atom of oxygen/min·mg protein, respectively. The value of the respiratory control was 5.5 ± 1.2. Adding CCCP to energized mitochondria increased the permeability of the membrane to protons and induced maximum respiration rate (200 ± 35 n atom g of oxygen/min·mg protein) and dissipation of $\Delta\Psi_m$ (Fig. 4 later in the paper), inhibiting oligomycin-sensitive-ATP synthesis. These data indicated functional coupling of respiration and ATP synthesis in syncytiotrophoblast mitochondria. In addition, activities of 110 ± 27 μmol/min·mg protein for the NADH:DCPIP oxidoreductase (complex I), and 7 ± 1.5 μmol/min·mg protein for the succinate: DCPIP oxidereductase (complex II) were obtained (Table 1). These results indicated the presence of functional mitochondria that retained

Table 1
Bioenergetics and steroidogenic parameters of syncytiotrophoblast mitochondria.

Oxygen uptake	
State 3[a]	110 ± 18 ng atom of oxygen/min·mg protein
State 4[b]	19 ± 6 ng atom of oxygen/min·mg protein
Respiratory control[c]	5.5 ± 1.2
Complexes activities[d]	
Complex I	110 ± 27 μmol/min·mg
Complex II	7 ± 1.5 μmol/min·mg
Progesterone synthesis[e]	
Control	143 ± 1.5 ng progesterone/min·mg
+22(*R*)-hydroxy-cholesterol	606 ± 52 ng progesterone/min·mg

[a] Defined as oxygen consumption stimulated by ADP added in presence of succinate as substrate. Values are the mean ± S.D. (n = 20 independent determinations from different placental tissue).

[b] Defined as oxygen consumption reduction because all ADP added was converted to ATP. Values are the mean ± S.D. (n = 20 independent determinations from different placental tissue).

[c] Respiratory control = oxygen uptake rate of state 3/oxygen uptake rate of state 4. Values are the mean ± S.D. (n = 20 independent determinations from different placental tissue).

[d] Specific activities from complexes I and II were measured spectrophotometrically in sonicated mitochondria: complex I, NADH:DCPIP oxide reductase; and complex II, succinate:DCPIP oxide reductase. Complex II activity was stimulated as described in the Experimental procedures section. Values shows are the mean ± S.D. (n = 9 independent determinations from different placental tissue).

[e] Progesterone synthesis was determined as described in the Experimental procedures section. The 22(*R*)-hydroxy-cholesterol was used to verify cytochrome P450scc, adrenedoxin, adrenedoxin reductase and 3β-hydroxysteroid dehydrogenase activities [19]. Values here are the mean ± S.D. from four determinations, from four different placental tissues.

the ability to increase the consumption of oxygen and the synthesis of ATP upon the addition of ADP.

Human placental mitochondria are steroidogenic organelles that synthesize progesterone, due to the presence of the type II 3-β-hydroxy steroid dehydrogenase in their inner membrane [3–5]. We determined steroidogenic activity of syncytiotrophoblast mitochondria to verify their physiological function. As observed in Table 1 synthesis of progesterone by syncytiotrophoblast mitochondria was 143 ± 12 ng progesterone/min·mg protein (Fig. 6 later in the paper), reaching a maximum of 606 ± 52 ng progesterone/min·mg protein in the presence of 22-(*R*)-hydroxy-cholesterol, which is a soluble substrate used to verify cytochrome P450scc, adrenedoxin, adrenedoxin reductase and 3β-hydroxysteroid dehydrogenase activities [19]. These results agree with the specialized role of syncytiotrophoblast tissue [3] and support the functional integrity of the isolated syncytiotrophoblast mitochondria used in this work.

3.2. Nucleotide hydrolysis by mitochondrial ATP-diphosphohydrolase

We designed the experiments in the present work to investigate the possible involvement of $\Delta\Psi_m$ in mitochondrial ATP-diphosphohydrolase activity and progesterone synthesis in human syncytiotrophoblast cells. We first obtained a detergent-solubilized ATP-diphosphohydrolase fraction to determine their nucleotide hydrolysis activity [1]. We monitored the time course of nucleotide hydrolysis by HPLC (Figs. 1 and 1S). Hydrolysis of nucleoside diphosphates (NDP) by mitochondrial ATP-diphosphohydrolase was associated with the accumulation of the corresponding nucleoside monophosphate (NMP), whether a purine or pyrimidine nucleotide was involved (Figs. 1A and 2S). A transient accumulation of NDP was observed when a nucleoside triphosphate (NTP) was hydrolyzed. NDP were dephosphorylated to NMP (Figs. 1B and 2S). Since this hydrolyzing activity is exerted by the mitochondrial ATP-diphosphohydrolase, it can be inhibited by 5′-*p*-fluorosulfonyl benzoyl adenosine [1]. The kinetics of ATP-diphosphohydrolase was similar regardless of the substrate of choice (Figs. 1 and 2S), confirming its low substrate specificity [1]. Additionally, we determined the activation energy (E_a) for the

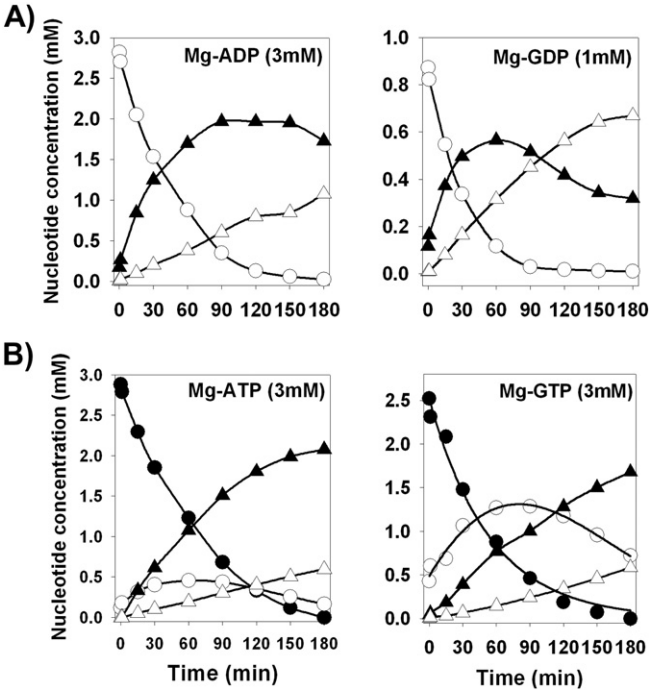

Fig. 1. Nucleotide hydrolysis of isolated syncytiotrophoblast mitochondrial ATP-diphosphohydrolase. ATP-diphosphohydrolase from syncytiotrophoblast mitochondria was isolated and its hydrolytic nucleotide activity was determined as described in the Experimental procedures section. Nucleotide concentration was quantified at different times by HPLC. A) Hydrolysis of Mg-ADP (3 mM) or Mg-GDP (1 mM) by mitochondrial ATP-diphosphohydrolase: (○) = nucleoside diphosphate (NDP); (▲) = nucleoside monophosphate (NMP); (Δ) = nucleoside. B) Hydrolysis of Mg-ATP (3 mM) or Mg-GTP (3 mM) by mitochondrial ATP-diphosphohydrolase: (•) = nucleoside triphosphate (NTP); (○) = NDP; (▲) = NMP; (Δ) = nucleoside. The figure shows representative experiments.

solubilized ATP-diphosphohydrolase activity by measuring the reaction rate constant (V_{max}) at different temperatures and by plotting $\ln(V_{max})$ *versus* 1/T (Fig. 3S). Data were adjusted to the integrated form of the Arrhenius equation: $E_a = ((RT_2T_1)/(T_2 - T_1))\cdot\ln(V_{max})$. The Arrhenius plot was linear in the temperature range spanning 10–55 °C (Fig. 3S), suggesting a single rate-limiting step. A sudden drop in the Arrhenius plot at low 1/T (high temperature, 60–70 °C) indicated protein denaturation (Fig. 3S). The E_a values for nucleoside triphosphates such as ATP, GTP, UTP, and CTP were 6.06, 4.10, 6.25, and 5.26 kcal/mol, respectively. For ADP, GDP, UDP, and CDP the E_a values were 4.67, 5.42, 5.43, and 6.22 kcal/mol, respectively. These results suggest that solubilized ATP-diphosphohydrolase had a similar rate-limiting step for either tri- or diphosphates nucleoside hydrolysis.

3.3. BN-PAGE analysis

To support the hypothesis that a single enzyme hydrolyzes ATP, ADP, GTP, or GDP, we conducted blue native PAGE of syncytiotrophoblast mitochondria (Fig. 2). Since the calcium–nucleotide complex can be used as a substrate by the mitochondrial ATP-diphosphohydrolase, but not by the F_1F_0-ATP synthase, the in-gel activity was determined in the presence of $CaCl_2$ with ATP, ADP, GTP or GDP as substrate, in the absence or presence of oligomycin (not shown). Phosphohydrolytic activity produced a single band of calcium phosphate precipitate (Fig. 2), which displayed no oligomycin inhibition but identical electrophoretic mobility with an apparent molecular weight of 167 kDa. FSBA inhibited ATP-diphosphohydrolase activity with any of the tested substrates (Fig. 2). Results indicated that solubilized ATP-diphosphohydrolase from syncytiotrophoblast mitochondria was the only enzyme capable of hydrolyzing calcium–nucleotide complexes. These results confirmed the wide spectrum of substrates

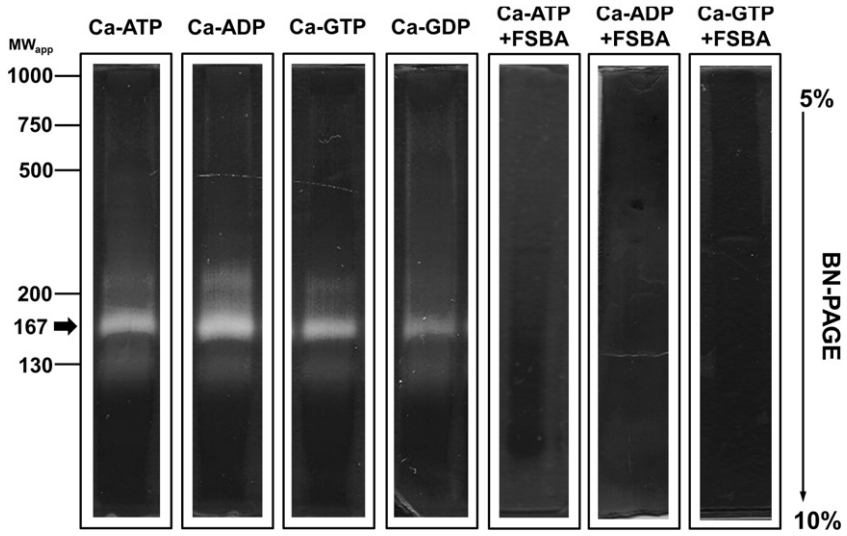

Fig. 2. In-gel activity of digitonin-solubilized mitochondrial ATP-diphosphohydrolase from syncytiotrophoblast in native gels. Mitochondria were solubilized using digitonin (2 g/g of protein), and ATP-diphosphohydrolase was separated by BN-PAGE. Native-PAGE was performed in linear polyacrylamide gradient gels from 5 to 10% as described in the Experimental procedures section. Electrophoresis was conducted at 30 V for 12 h at 4 °C. In the spots containing ATP-diphosphohydrolase activity, white precipitates of calcium phosphate appeared within 2 h after the addition of Ca–ATP, Ca–ADP, Ca–GTP or Ca–GDP complex. The presence of 1 mM of FSBA inhibited ATP hydrolysis while oligomycin (5 μg/mg) did not modify the hydrolytic activity (data not shown). The molecular weight of ATP-diphosphohydrolase activity was estimated by using the digitonin-solubilized bovine mitochondrial complexes as standard. The figure shows representative experiments from four different mitochondrial preparations.

that ATP-diphosphohydrolase exhibits. In the second approach, we analyzed the ATP-diphosphohydrolase activity associated to intact mitochondria undergoing oxygen uptake or progesterone synthesis. The tandem mass spectrometry (LC/ESI–MS/MS) analysis showed the isoform 2 of the ATP-diphosphoydrolase (P49961-2|ENTP1_HUMAN), which belong to the ectonucleoside triphosphate diphosphohydrolase group, with a molecular weight of 59 kDa. The hydropathy analysis displayed four transmembrane segments (see Fig. 4S). However, no mitochondrial targeting presequence was observed.

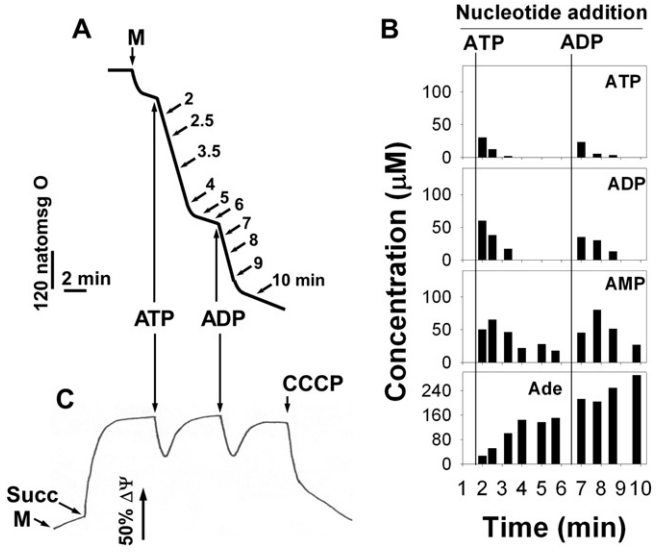

Fig. 3. Mitochondrial ATP-diphosphohydrolase induces mitochondrial oxygen consumption and $\Delta\Psi_m$ depolarization. Syncytiotrophoblast mitochondria were isolated as described in the Experimental procedures section. A) Mitochondria were incubated in oxygen uptake medium at 37 °C and mitochondrial respiration was stimulated by ATP or ADP addition. Arrows indicate sequential additions of (M) mitochondria (1 mg/ml); ATP (130 μM); or ADP (130 μM). Simultaneous to mitochondrial oxygen uptake recording, an aliquot was withdrawn at the indicated time (bold arrows) and used to quantify nucleotide concentration by HPLC (B). Concentrations of ATP, ADP, AMP or Adenosine (Ade) at different times after addition of ATP or ADP during the time course of oxygen uptake showed in (A). C) $\Delta\Psi_m$ measurement with Safranine O as described in the Experimental procedures section. Arrows indicate sequential additions of mitochondria (M); 10 mM succinate (Succ); ATP (130 μM); ADP (130 μM); CCCP (10 μM). In all cases curves show representative experiments of at least four different mitochondrial preparations.

3.4. ATP hydrolysis by ATP-diphosphohydrolase stimulates mitochondrial respiration and depolarizes the inner membrane

To evaluate the ATP-diphosphohydrolase activity during mitochondrial oxygen uptake, the time course of the hydrolysis of nucleotides was analyzed by HPLC (Fig. 3). Syncytiotrophoblast mitochondria were energized by succinate, and oxygen uptake was stimulated by ATP (Fig. 3A). Results show that ATP-diphosphohydrolase hydrolyzed ATP and produced ADP (Fig. 3B), which in turn was translocated into the mitochondrial matrix, where it was transformed to ATP by the F_1F_0-ATP synthase at the expense of the proton electrochemical gradient ($\Delta\mu_{H+}$). Simultaneously, the inner membrane was depolarized (Fig. 3C). This series of events is defined as state 3 of mitochondrial respiration [13], and continues until the ATP-diphosphohydrolase hydrolyses ATP to ADP and ADP to AMP. In this situation $\Delta\Psi_m$ increased (Fig. 3C) and mitochondrial respiration decreased to a minimum, a condition that is known as state 4 of mitochondrial respiration [13] (Fig. 3). A new cycle of mitochondrial oxygen uptake stimulation, depolarization of $\Delta\Psi_m$, ATP synthesis and ATP-diphosphohydrolase activity was observed when ADP was added (Fig. 3). It is crucial to highlight that ATP-diphosphohydrolase activity is closely related to an increase in mitochondrial respiration and inner membrane depolarization in the presence of ATP. Additionally, syncytiotrophoblast mitochondria contain a phosphatase [1] that is responsible for adenosine production from AMP. However, inhibiting it with phenylalanine or sodium molibdate [1] did not modify the results described (data no shown).

3.5. Substrate selectivity and catalytic rate by ATP-diphosphohydrolase during mitochondrial respiration

We added a different nucleoside triphosphate to support the notion that the ATP-diphosphohydrolase activity could be modified by mitochondrial bioenergetics during oxygen uptake (Fig. 4). After the transition from state 3 to 4 of mitochondrial respiration, addition of GTP did not stimulate oxygen uptake (Fig. 4A), nor depolarized the inner membrane (Fig. 4B). Although GTP was hydrolyzed by ATP-

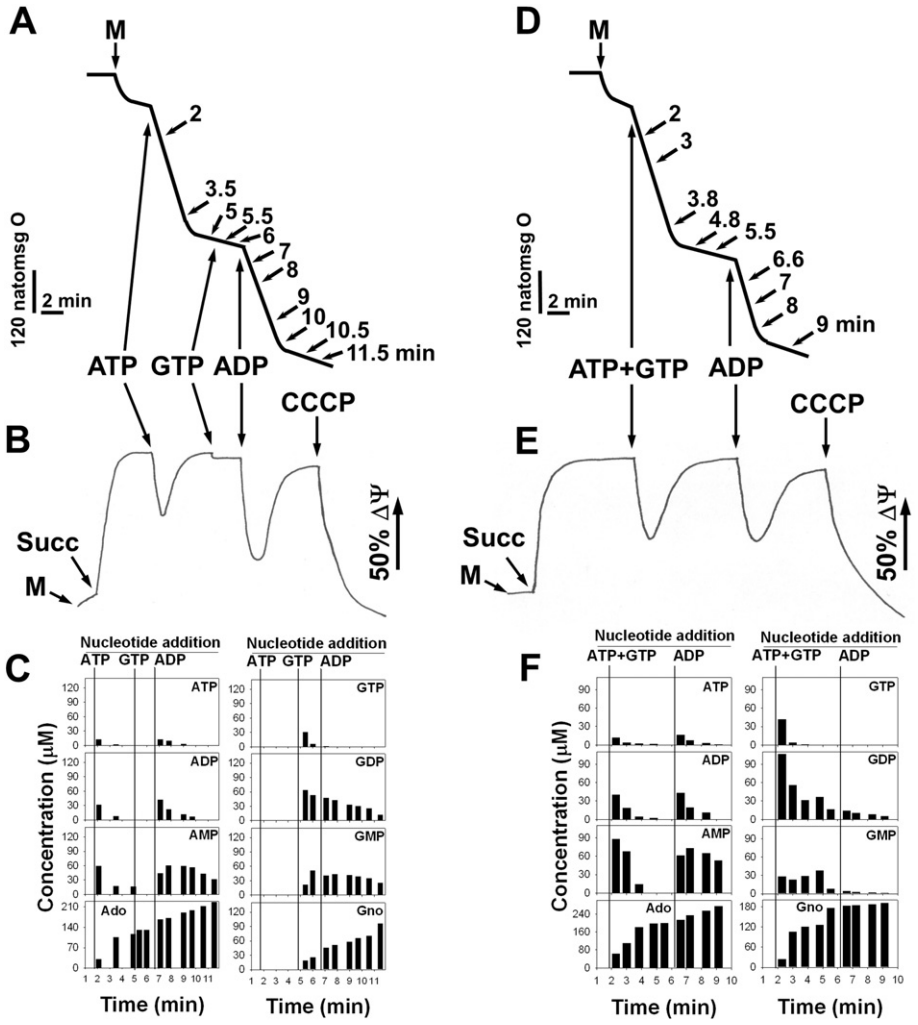

Fig. 4. Mitochondrial ATP-diphosphohydrolase selectively hydrolyzes ATP in energized and coupled mitochondria. A) Mitochondria were incubated in oxygen uptake medium at 37 °C and mitochondrial respiration was stimulated by ATP or ADP addition. The arrows indicate sequential additions of (M) mitochondria (1 mg/ml); ATP (130 μM); GTP (130 μM); or ADP (130 μM). During mitochondrial oxygen uptake an aliquot was withdrawn at the indicated time (bold arrows) and used to quantify nucleotide concentration by HPLC. B) $\Delta\Psi_m$ measurement with Safranine O as described in Fig. 3. Arrows indicate the sequential additions of mitochondria (M); 10 mM succinate (Succ); ATP (130 μM); GTP (130 μM); ADP (130 μM); CCCP (10 μM). C) Concentrations of ATP, ADP, AMP, Ade, GTP, GDP, GMP, or guanosine (Gno) after addition of ATP, GTP or ADP during oxygen uptake. D) Mitochondria were incubated in oxygen uptake medium and a mixture of ATP (130 μM) and GTP (130 μM) was added to stimulate respiration. Oxygen uptake (D), $\Delta\Psi_m$ measurement (E) and nucleotide concentration (F) were determined as described in the Experimental procedures section. Arrows indicate sequential additions of mitochondria (M); 10 mM succinate (Succ); a mixture of ATP + GTP (130 μM each one); ADP (130 μM); CCCP (10 μM). In all cases curves show representative experiments of at least four different and independent mitochondrial preparations.

diphosphohydrolase (855 ± 137 μmol/mg·min), its catalytic rate was lower than that observed with ATP (1910 ± 265 μmol/mg·min) (Figs. 4C and 6B). Indeed, addition of ADP, in the presence of GDP produced from GTP hydrolysis, induced oxygen consumption and decreased $\Delta\Psi_m$, while the rate of GDP hydrolysis was very small (Fig. 4C). This result contrasts with the one of the detergent-solubilized ATP-diphosphohydrolase, which displays a similar hydrolysis rate for ATP, ADP, GTP, and GDP (Fig. 1).

To examine substrate selectivity of the mitochondrial ATP-diphosphohydrolase, we added simultaneously ATP and GTP (Fig. 4D). The enzyme consistently hydrolyzed ATP instead of GTP (Fig. 4F), and the produced ADP induced oxygen consumption and membrane depolarization (Fig. 4D and E, respectively).

3.6. $\Delta\Psi_m$ determines ATP-diphosphohydrolase substrate selectivity and catalytic rate

$\Delta\Psi_m$ is a central component of mitochondrial metabolism that provides the driving force for oxidative phosphorylation, for the import of proteins and metabolites, and for regulating the activity of membrane proteins like the adenine nucleotide translocase (ANT) [24–26]. We examined the effect of the mitochondrial protonophore and respiration uncoupler CCCP on $\Delta\Psi_m$ to assess whether the ATP-diphosphohydrolase substrate selectivity was mediated by $\Delta\Psi_m$ (Fig. 5). Syncytiotrophoblast mitochondria were incubated with CAT to inhibit the adenine nucleotide translocase and avoid ATP internalization into the mitochondrial matrix in the presence of CCCP (see the Experimental procedures section). When CCCP was added to mitochondria, oxygen uptake was stimulated and $\Delta\Psi_m$ collapsed (Fig. 5A and B, respectively). Further addition of ATP did not modify oxygen consumption nor $\Delta\Psi_m$. Importantly, ATP-diphosphohydrolase activity was lower (812 ± 30 μmol/mg·min) when compared to control conditions, (*i.e.* in the absence of CCCP). Also, a transient accumulation of ADP was observed (Figs. 5C and 6B).

In identical experimental conditions, GTP addition to CCCP-uncoupled mitochondria rendered a lower rate of GTP hydrolysis (642 ± 37 μmol/mg·min), and a transient accumulation of GDP (Figs. 5D–F and 6B), similar to the results obtained with ATP. To compare ATP and GTP hydrolysis in CCCP-uncoupled mitochondria, both nucleotides were added at the same time (Fig. 5G–I). The ATP-diphosphohydrolase catalyzed simultaneously the hydrolysis of both nucleotides and displayed similar velocities (Fig. 5I), without any

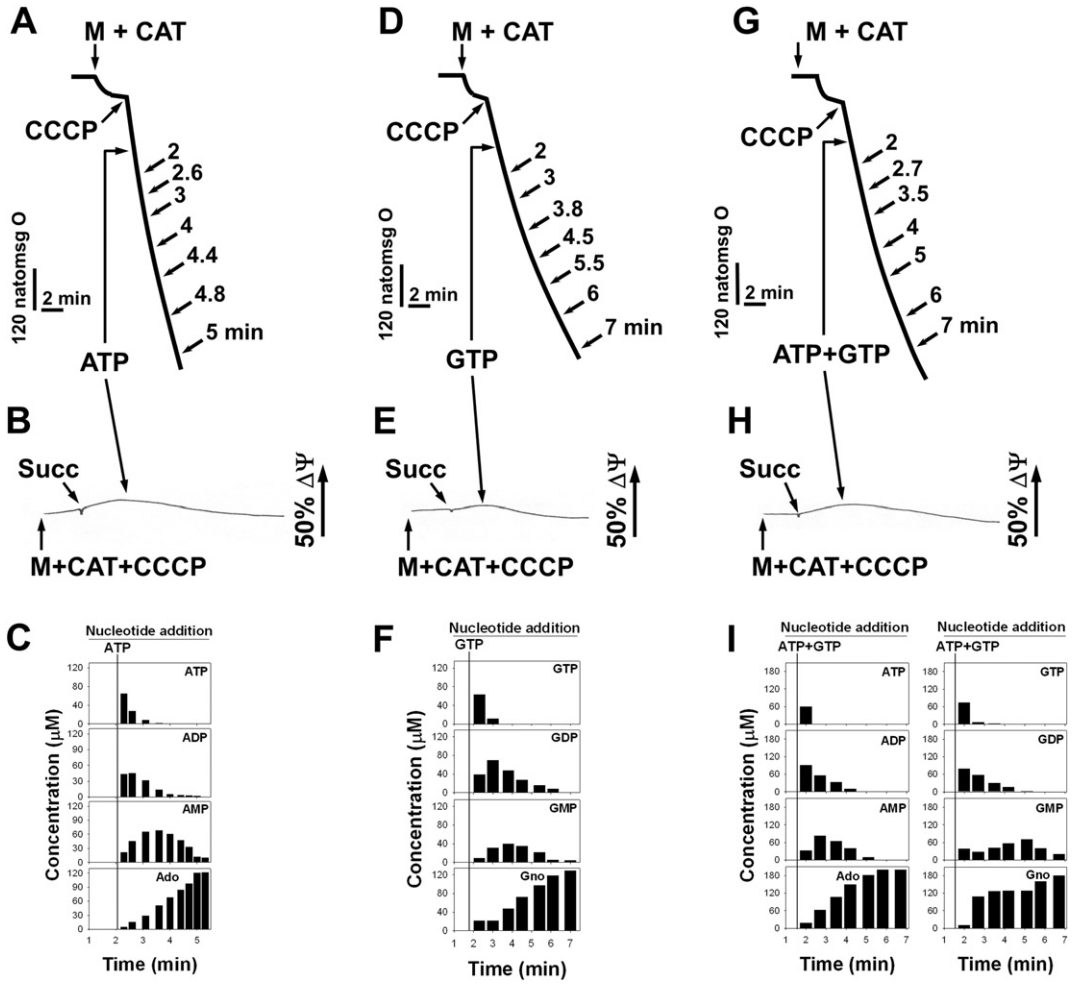

Fig. 5. $\Delta\Psi_m$ regulates syncytiotrophoblast mitochondrial ATP-diphosphohydrolase substrate selectively. Mitochondria were incubated in oxygen uptake medium (described in the Experimental procedures section) plus 5 μM carboxyatractyloside (CAT) to inhibit the translocation of adenine nucleotides by blocking the ADP/ATP carrier. CCCP (10 μM) was added to collapse $\Delta\Psi_m$ and obtain the maximum oxygen uptake rate. After CCCP, the addition of 130 μM ATP (A), 130 μM GTP (D) or an ATP + GTP mixture (130 μM each one, G) was performed. Mitochondria were incubated with CAT (5 μM), CCCP (10 μM), and 10 mM of succinate (Succ), and then 130 μM of ATP (B), 130 μM of GTP (E), or an ATP + GTP mixture (130 μM of each, H) was added. At the times indicated in the oxygen uptake recording, an aliquot was withdrawn to quantify the nucleotide concentration by HPLC when ATP (C), GTP (F), or ATP + GTP (I) were added. The figure shows representative experiments of at least four different and independent mitochondrial preparations.

selectivity for ATP as observed in Fig. 4. This observation suggests that substrate selectivity of ATP-diphosphohydrolase (*i.e.* ATP *versus* GTP preference) is regulated by $\Delta\Psi_m$.

3.7. Progesterone synthesis by syncytiotrophoblast mitochondria

In intact syncytiotrophoblast mitochondria, ATP, ADP, GTP, and GDP hydrolysis by the ATP-diphosphohydrolase has been associated with progesterone synthesis, particularly with cholesterol flux between mitochondrial membranes [2]. As ATP-diphosphohydrolase inhibition with FSBA decreased progesterone production [2] we explored the control of $\Delta\Psi_m$ on progesterone synthesis (Fig. 6A). Syncytiotrophoblast mitochondria were incubated as described in the Experimental procedures section in the presence of isocitrate to maintain a high NADPH/$NADP^+$ ratio to supply energy to P450scc [27]. Under these conditions mitochondrial progesterone (P4) synthesis was 143 ± 12 ng P4/min·mg protein; it increased to 391 ± 10 ng P4/min·mg protein when ATP was added, and to 606 ± 52 ng P4/min·mg protein if 22-(*R*)-hydroxy cholesterol was present (Fig. 6A). The concomitant addition of ATP and 22-(*R*)-hydroxy cholesterol augmented progesterone production to 594 ± 82 ng P4/min·mg protein. This increase in progesterone synthesis was observed even in the presence of CCCP (427 ± 15 and 536 ± 12 ng/mg·min respectively) (Fig. 6A). The addition of ADP, GTP, or GDP slightly increased progesterone synthesis (340 ± 82; 206 ± 17, and 173 ± 19 ng P4/min·mg protein, respectively). However, when mitochondria were incubated without isocitrate, with the consequent suppression of NADPH synthesis, progesterone production decreased even in the presence of ATP, ADP, GTP or GDP (100 ± 10; 75 ± 12; 53 ± 9, and 44 ± 11 ng/mg·min, respectively) (Fig. 6A). ATP-diphosphohydrolase activity was evaluated simultaneously for progesterone synthesis (Fig. 6B). In the presence of $\Delta\Psi_m$ adenine nucleosides, tri- and diphosphates were preferentially hydrolyzed over guanosine nucleotides, *i.e.* GTP or GDP (Fig. 6B, black bars). In the presence of CCCP (which collapsed the $\Delta\Psi_m$), ATP-diphosphohydrolase activity was similar with all nucleotides tested (ATP, ADP, GTP, and GDP) (Fig. 6B, white bars). Although CAT was added prior to $\Delta\Psi_m$ dissipation with CCCP, it did not have any significant effect on ATP-diphosphohydrolase activity (Fig. 6B, gray bars).

This suggested that $\Delta\Psi_m$ might be involved in cholesterol flow by regulating ATP-diphosphohydrolase activity, but for progesterone synthesis the NADPH/$NADP^+$ ratio is important. To verify this possibility, we used the mitochondrial steroidogenic contact sites [23] from human placenta as an alternative experimental approach.

These contact sites can synthesize progesterone [23] since they contain the whole steroidogenic machinery, including the cytochrome

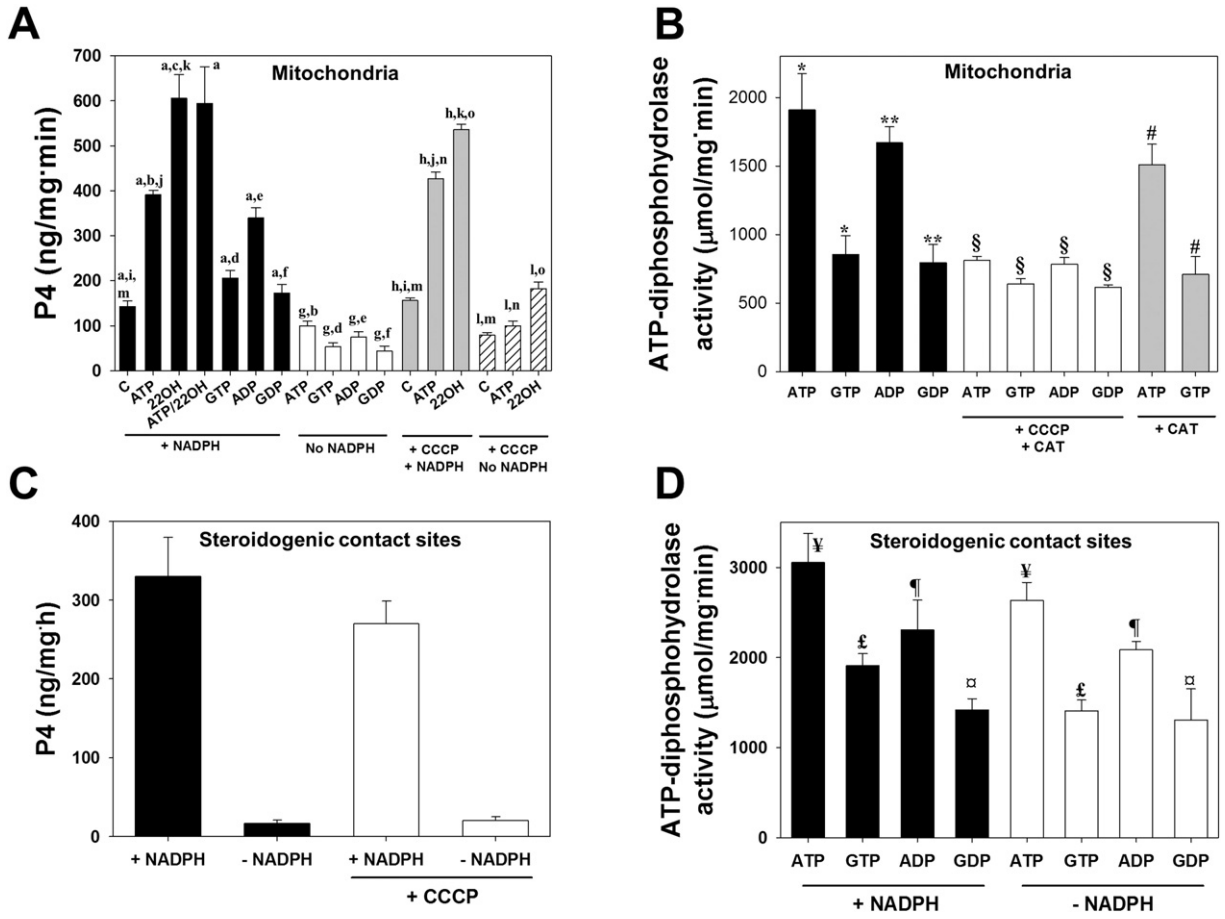

Fig. 6. Steroidogenesis and nucleotide hydrolysis in syncytiotrophoblast mitochondria or steroidogenic contact sites. A) Progesterone synthesis by intact mitochondria. Mitochondria were incubated in the presence (black bars) or absence (white bars) of isocitrate to maintain NADPH production (see text for details). ATP (1 mM), GTP (1 mM), ADP (1 mM), GDP (1 mM) or 22-hydroxy cholesterol (22OH, 25 μM) was added to stimulate progesterone synthesis. Alternatively, CCCP (10 μM) was added in the presence (gray bars) or absence (dashed bars) of isocitrate. The results are the mean ± S.D. of n = 4 different and independent mitochondrial preparations. B) ATP-diphosphohydrolase activity in intact mitochondria incubated in the presence (white bars) or absence (black bars) of CCCP. Results are the mean ± S.D. with n = 4. C) Progesterone production by steroidogenic contact sites in the presence (+NADPH) or absence (−NADPH) of 2 mM of NADPH. CCCP was added (white bars) to test its effect on progesterone synthesis. Results are the mean ± S.D. with n = 3. D) ATP-diphosphohydrolase activity by steroidogenic contact sites in the presence (+NADPH, black bars) or absence (−NADPH, white bars) of 2 mM NADPH. Results are the mean ± S.D. with n = 3. The one-way ANOVA analysis showed a statistical significant difference ($p \leq 0.001$) between different groups of data marked with: a, g, h, l, *, and **. The difference is greater than it would be expected by chance (all pairwise multiple comparison procedures were performed with the Tukey test). The comparison marked with § shows no significant difference ($p \leq 0.001$). The two way ANOVA analysis showed a statistically significant difference ($p \leq 0.005$) between groups marked with: b, c, d, e, f, l, j, k, m, n, o, ¥, £, ¶, and ¤ (all pairwise multiple comparison procedures were performed with the Holm–Sidak method).

P450scc, the 3-β-hydroxy-steroid dehydrogenase, the adrenodoxin and adrenodoxin reductase, the ATP-diphosphohydrolase [23], and a NADP-dependent isocitrate dehydrogenase [27]. It is important to mention that these contact sites do not generate $\Delta\Psi_m$, so that the participation of this parameter was excluded. Contact sites were isolated as described in the Experimental procedures section, incubated in either the absence or presence of isocitrate to stimulate NADPH production, with or without CCCP, and the amount of progesterone synthesis was determined (Fig. 6C). NADPH is the substrate of the adrenodoxin reductase that supports cytochrome P450scc activity, and isocitrate dehydrogenase activity regenerates NADPH [27]. Progesterone synthesis reached a value of 330 ± 50 ng/mg·h in the presence of NADPH production, while in the absence of NADPH there was no progesterone synthesis (Fig. 6C, black bars). CCCP had no effect on progesterone synthesis in the presence of NADPH (Fig. 6C, white bars). ATP-diphosphohydrolase activity associated with the contact sites was unaffected by the presence or absence of NADPH (Fig. 6D).

4. Discussion

Human placenta is essential to maintain pregnancy. Mitochondria of syncytiotrophoblast cells, besides generating ATP, synthetize progesterone using cholesterol as a substrate [3]. Therefore, syncytiotrophoblast mitochondria must reconcile ATP synthesis with hormone production.

Isolated syncytiotrophoblast mitochondria retain their ability to couple oxygen uptake to ATP synthesis as well as their capacity to synthesize progesterone (Table 1). The specific hormone(s) or substance(s) that modulate P4 synthesis and ATP production during pregnancy are currently unknown [3,28]. The most striking observation of this work shows that the ATP-diphosphohydrolase, an accessory enzyme involved in cholesterol flux between outer and inner syncytiotrophoblast mitochondrial membranes [1,2], might be regulated by $\Delta\Psi_m$.

Kinetic characterization of the detergent-solubilized ATP-diphosphohydrolase from mitochondria showed that this enzyme had low substrate selectivity (Figs. 1 and 2). It was capable of hydrolyzing purine or pyrimidine, tri or diphosphate nucleotides with similar affinities [1]. Its activation energy showed values between 4 and 6 kcal/mol with a single rate-limiting step during catalysis.

In sharp contrast, ATP-diphosphohydrolase is regulated by $\Delta\Psi_m$ in energized mitochondria. If mitochondria were energized (*i.e.* with succinate), the ATP-diphosphohydrolase preferentially hydrolyzed ATP, even if other substrates such as GTP, were present (Fig. 4), and

promoted mitochondrial oxygen uptake coupled to ATP synthesis (Fig. 3). In energized mitochondria from all organisms tested, ADP but not ATP increased the respiration rate. This is controlled by the adenine nucleotide translocase (ANT), which does not recognize outer mitochondrial ATP if $\Delta\Psi_m$ is generated by ETC. This is a key control mechanism that prevents a futile-cycle and energy waste as heat.

In syncytiotrophoblast mitochondria energized with succinate, ATP addition induced oxygen uptake through the ATP-diphosphohydrolase activity, which hydrolyzed ATP to ADP and inorganic phosphate (Fig. 3 and [1,10]). The activities of the ATP-diphosphohydrolase (ATP hydrolysis) and F_1F_0-ATP synthase (ATP synthesis) do not produce permanent oxygen uptake stimulation ([10] and the present study) as has been observed with the brain hexokinase associated with the outer mitochondrial membrane [29].

This observation is consistent with the proposed role for this enzyme [2]; it was suggested that the ATP-diphosphohydrolase could perform sequential hydrolysis of ATP to ADP, and ADP to AMP and simultaneously stimulate cholesterol transport between mitochondrial membranes for progesterone synthesis [2] (Fig. 7). Although cholesterol transfer during steroidogenesis in the adrenals glands occurs through a macromolecular complex that consists of outer membrane proteins such as the mitochondrial membrane translocator protein (TSPO), and the TSPO-associated protein PAP7 that bind and lead the regulatory subunit RI-α of the cAMP-dependent protein kinase (PKARIα) towards mitochondria [30], the TSPO protein in human syncytiotrophoblast mitochondria remains unidentified [31]. A potential candidate exists. A multiprotein complex, the steroidogenic contact sites, has been associated with cholesterol transport and steroidogenesis in human syncytiotrophoblast mitochondria [23]. These contact sites contain proteins such as HSP60 [32], ANT, VDAC, cytochrome P450scc, adrenodoxin reductase, adrenodoxin, NADP-dependent isocitrate dehydrogenase, 3-β-hydroxysteroid dehydrogenase, STARD3 protein and ATP-diphosphohydrolase [3,23].

The molecular weight of the ATP-diphosphohydrolase calculated from native-gel was 163 kDa, in contrast with the 59 kDa determined from tandem mass spectrometry. The reported molecular weight from SDS-PAGE, radiation-inactivation or gel filtration goes from 64 to 70 kDa [33–35]. The molecular weights in native conditions can be explained by one of the following hypotheses: A) that the native state of mitochondrial ATP-diphosphohydrolase is a homo-oligomer (*i.e.* a dimer or trimer), as has been reported for other organisms [36,37], or B) that ATP-diphosphohydrolase interacts with other mitochondrial membrane protein(s) (*i.e.* steroidogenic contact site proteins). We can hypothesize that $\Delta\Psi_m$ regulates ATP-diphosphohydrolase activity through the close interactions between proteins from contact sites. Although ATP-diphosphohydrolase lacks a classic mitochondrial targeting presequence, it has been reported that many mitochondrial hydrophobic membrane proteins are synthesized without cleavable extensions [38]. These proteins typically contain several targeting signals that are distributed over the entire length of the protein [39]. However, this hypothesis needs to be elucidated.

Even though $\Delta\Psi_m$ reflects efficient mitochondrial oxygen consumption and ATP synthesis, it is not crucial for progesterone synthesis in syncytiotrophoblast cells. When intact mitochondria were incubated in the presence of CCCP to collapse $\Delta\Psi_m$, and isocitrate to maintain NADPH, cholesterol was efficiently transformed into progesterone (Fig. 6A). Although in the presence of CCCP ATP-diphosphohydrolase did not show substrate selectivity and its activity was decreased, ATP hydrolysis in the presence or absence of CCCP increased cholesterol transport and its transformation into progesterone, in the presence of NADPH. This suggested that the remaining activity of the ATP-diphosphohydrolase (around 50%) fully supports the synthesis of progesterone.

As $\Delta\Psi_m$, respiration and ATP synthesis are solid indicators of functional mitochondria, they are believed to be crucial for Leydig cell steroidogenesis [6,7]. However, using steroidogenic contact sites from syncytiotrophoblast mitochondria in this study allowed us to focus on the role of ATP (ATP-diphosphohydrolase activity) and NADPH (NADP-dependent isocitrate dehydrogenase activity) in the synthesis of progesterone; it is important to mention that steroidogenic contact sites do not generate $\Delta\Psi_m$, but transform cholesterol into progesterone (Fig. 6C). With this experimental approach, we were able to establish that progesterone synthesis is sensitive to the presence of NADPH and nucleotide hydrolysis but insensitive to $\Delta\Psi_m$ (the present study and [23]).

In syncytiotrophoblast cells $\Delta\Psi_m$ regulates the specificity of ATP-diphosphohydrolase allowing most of the ATP available to be used for cholesterol transport during progesterone synthesis (Fig. 7), leaving GTP, GDP and other nucleotides available for other important metabolic reactions (*i.e.* proteins synthesis). However, in some pathological events such as preeclampsia, calcium accumulation in mitochondria of trophoblast cells may collapse $\Delta\Psi_m$ and interfere with ATP synthesis. In an attempt to sustain progesterone synthesis, ATP-diphosphohydrolase may use any nucleotide available (*i.e.* GTP), to maintain the required cholesterol flux for progesterone synthesis.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.10.002.

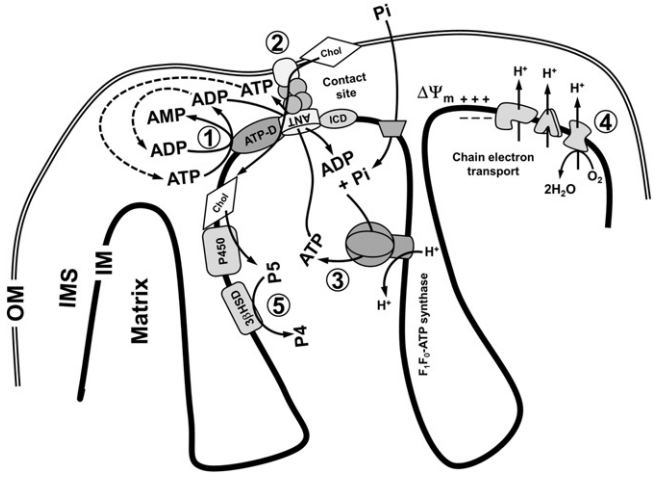

Fig. 7. Model for the regulation of mitochondrial ATP-diphosphohydrolase by $\Delta\Psi_m$ in syncytiotrophoblast cells. $\Delta\Psi_m$ induces ATP hydrolysis by ATP-diphosphohydrolase (1) during cholesterol transport (2) at the contact sites. ADP can be hydrolyzed to AMP by ATP-diphosphohydrolase (1) or can enter the mitochondrial matrix for ATP synthesis by F_1F_0-ATP synthase (3), stimulating oxygen uptake and the proton pump by CTE (4). Cholesterol is transformed into pregnenolone (P5) by cytochrome P450scc and then into progesterone (P4) by 3β-hydroxysteroid dehydrogenase (5). Steroidogenic contact sites are constituted by different proteins including adenine nucleotide translocase (ANT), ATP-diphosphohydrolase (ATP-D), NADP-dependent isocitrate dehydrogenase (ICD), cytochrome P450scc (P450) and 3β-hydroxysteroid dehydrogenase (3βHSD). OM = outer mitochondrial membrane; IM = inner mitochondrial membrane; IMS = inter membrane space; Chol = cholesterol.

Acknowledgements

We dedicate this work as a memorial to Dr. Guillermo Mendoza-Hernandez, an exceptional friend and colleague, and coauthor of this paper, who passed away suddenly on July 13, 2012. This work was partially supported by research grants IN214914 (OFH), IN209614 (JPP) and IN211912 (FM) from Dirección General de Asuntos del Personal Académico (DGAPA) from Universidad Nacional Autónoma de México (UNAM), as well as Grant 168025 from CONACYT (FM). Mercedes Esparza-Perusquía is a Ph.D. student of the Biological Science Program of Universidad Nacional Autónoma de México (511021118) and fellow of CONACYT (254400).

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