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Diazoxide affects the IF_1 inhibitor protein binding to F_1 sector of beef heart F_0F_1ATPsynthase

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Abstract

Diazoxide, a selective opener of the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}), has been reported to enhance F_0F_1ATP-synthase inhibition during ischemia, but the underlying mechanisms are still unclear. Here, we demonstrate that diazoxide directly interacts with the F_1 sector of beef heart F_0F_1ATPsynthase markedly promoting the binding of the inhibitor protein (IF_1) to β subunit. More specifically, the treatment of soluble F_1 with one equivalent of diazoxide was sufficient to decrease the K_d of IF_1–F_1 complex at low pH. Such effect was revealed only on the cycling enzyme, while no effect was observed in the absence of Mg-ATP. However, diazoxide binding occurred independently from the catalysis, as shown by the structural changes induced by the drug in not catalytically active F_1 and revealed by CD spectra. In addition, kinetic analysis of ATP hydrolysis demonstrated that diazoxide exerts a stabilising role on Mg-ADP bound in the catalytic site of the β subunit adopting the tight conformation (β_{DP}). In accordance, a stabilising effect of Mg-ADP at the nucleotide binding domain (NBD) has been reported also for K_{ATP} channel. These results suggest that diazoxide binds to β subunit at NBD, which is highly conserved in the ATP-binding cassette protein family, thus inducing nucleotide stabilisation and favouring F_1 conformation suitable for IF_1 binding. Finally, diazoxide also increased IF_1 binding to membrane bound F_1, while it did not influence the energisation-dependent IF_1 release. As IF_1 binding mediates the F_0F_1ATPsynthase inhibition, we suggest that such mechanism may contribute to cardioprotection during ischemia.

Keywords: Diazoxide; Mitochondria; F_0F_1ATPsynthase; Inhibitor protein IF_1; Nucleotide binding domain; Preconditioning

1. Introductions

F_0F_1ATPsynthase, a member of the ATP-binding cassette protein family [1], is the enzyme responsible for the synthesis of the majority of cellular ATP from ADP and Pi using energy derived from the transmembrane proton motive force [2]. Catalysis takes place in the extrinsic membrane domain, known as F_1, which is composed of five polypeptides, α, β, γ, δ and ε, assembled with the stoichiometry 3:3:1:1:1. Both α and β subunits contain nucleotide binding domains (NBDs) and the catalytic sites are in the β subunits at the interface with the adjoining α subunits.

The activity of mitochondrial ATPsynthase is regulated by ADP, a proton motive force ($\Delta\mu H^+$) and by a natural protein inhibitor, IF_1 [3], a basic protein of 84 amino acids in length [4]. IF_1 is a non-competitive inhibitor, which binds to F_1 sector of ATPsynthase in a 1:1 stoichiometry and fully inhibits the enzyme. Low pH and $\Delta\mu H^+$ [5], as well as the hydrolysis of Mg-ATP [6], promote the formation of the inhibited IF_1–F_1 complex. On the contrary, the restoration of a proton motive force favouring ATP synthesis displaces IF_1 from its inhibitory site [7].

IF_1-mediated inhibition is widely accepted to be essential during myocardial ischemia, when ATPsynthase switches from ATP synthesis to ATP hydrolysis as a consequence of membrane potential decrease [3,6]. In addition, inhibition of myocardial ATPsynthase, most probably by IF_1 binding, has been observed in ischemic preconditioning, which consists of short, non-damaging periods of ischemia

Abbreviations: mitoK_{ATP}, mitochondrial ATP-sensitive K^+ channel; sK_{ATP}, sarcolemmal ATP-sensitive K^+ channel; NBDs, nucleotide binding domains; F_1, soluble isolated F_1 domain; IF_1, inhibitor protein; ndF_1, F_1 depleted of Mg^{2+} and nucleotides; SMPs, submitochondrial particles; DMSO, dimethylsulfoxide

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promoting protective effects on subsequent prolonged ischemia [7]. However, contradicting observations have been also reported [4].

Diazoxide is a cardioprotective drug, which mimics ischemic preconditioning, but its underlying mechanisms are still unclear. Most of evidences point to the involvement of the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}), which is more sensitive to diazoxide opening compared to the sarcolemmal K_{ATP} channels (sK_{ATP}) [8]. However, it is debated if mitoK_{ATP} is a trigger or an end-effector. In addition, the structural differences responsible for the variation in selectivity between mito-K_{ATP} and sK_{ATP} are not known [9], also because the subunit composition of mitoK_{ATP} has not yet completely defined [10]. Nevertheless, in the K_{ATP} channel complexes nucleotide binding to NBDs is essential for diazoxide effect to be revealed [11].

Very recently, different authors [12–14] showed in isolated and perfused rat heart that diazoxide causes potentiation of ATPsynthase inhibition during ischemia along with cardioprotection. However, the mechanistic link between opening of mitoK_{ATP} and inhibition of ATPsynthase mediated by diazoxide remains obscure [12,14].

Considering the crucial role of NBDs for diazoxide effects on K_{ATP} channel, as well as the high homology between the NBDs in the F_1 sector of ATPsynthase and the regulatory subunits of K_{ATP} channel, the sulfonylurea receptor subunits (SUR), we have investigated if diazoxide directly affects the F_1 catalytic activity and/or the inhibitory IF_1–F_1 complex formation. With this aim, the myocardial enzyme isoform has been studied and incubations with IF_1 have been performed at low pH mimicking ischemia conditions. Soluble isolated F_1 domain (F_1) has been used as the favourite model, since IF_1–F_1 complex formation is so energetically favoured with respect to the inverse reaction that it can be considered irreversible [15]. In addition, the effect of diazoxide on IF_1 binding/release to/from membrane bound ATPsynthase was assayed.

This study demonstrates that diazoxide can interact with the F_1 sector, thus enhancing IF_1 binding and inhibiting the enzyme. This suggests that different diazoxide actions may be due to drug binding to conserved structures in proteins belonging to the family of the ATP-binding cassette.

2. Materials and methods

2.1. Materials

Diazoxide was purchased from Aldrich and dissolved in alkaline solution (water solution of NaOH at pH 11.3), addition of which did not alter the pH of the samples. When indicated diazoxide was dissolved in DMSO. Glybenclamide was purchased from Aldrich and dissolved in DMSO.

All the other chemicals were commercial samples of the purest quality.

2.2. Preparation of purified proteins and submitochondrial particles

The soluble isolated F_1 domain (F_1) was prepared from beef heart mitochondria as in [16]. Before each treatment, an F_1 suspension in ammonium sulphate was pelletted and resuspended in 20 mM Tris–HCl, pH 6.7.

F_1 depleted of Mg^{2+} and nucleotides (ndF$_1$) was obtained according to [17]. The enzyme fractions with $A_{280}/A_{260} > 1.9$, which contained less than 0.2 mol of nucleotides per mol of F_1, were collected and stored at −20 °C in the presence of 50% (v/v) glycerol. Aliquots of ndF$_1$ were diluted in 20 mM Tris–HCl, pH 6.7, in the presence of 10–20% (v/v) glycerol as indicated in the figure legends. The inhibitor protein IF_1 was prepared as reported in [18].

AS particles and Mg-ATP particles were prepared from heavy beef heart mitochondria as in [19,20], respectively. IF_1 content of AS and Mg-ATP particles, determined as in [21], were 0.07 ± 0.05 mol IF_1/mol and 0.88 ± 0.05 mol IF_1/mol F_0F_1ATPsynthase, respectively.

2.3. Formation of F_1–IF_1, ndF$_1$–IF_1 and F_0F_1–IF_1 complexes and kinetic analyses

2.2 μM F_1 or ndF$_1$ were suspended in 20 mM Tris–HCl, pH 6.7 or in 20 mM Tris–HCl, 10–20% (v/v) glycerol, pH 6.7, respectively, and treated with 2.2 or 22 μM diazoxide for 10 min at 37 °C. Then, 0.52 μM aliquots of F_1 were prepared by dilution in the same buffer, while 0.52 μM aliquots of ndF$_1$ were added to an ATP regenerating buffer (20 mM Tris–HCl, 10% glycerol, 1.5 mM phosphoenolpyruvate, 1 mM KCl, 4 UI pyruvate kinase, pH 6.7). Finally, the samples were treated with 0.52–3.12 μM of IF_1 and incubated at 37 °C with 10 μM–1 mM Mg-ATP for different times, as indicated in the figure legends [22].

AS particles (2 mg/ml), which are deprived of IF_1 (see above), were incubated for 10 min at 37 °C in 250 mM sucrose, 10 mM MOPS, pH 6.7, plus 100 μM diazoxide in the absence or presence of 75 mM K_2SO_4. In some experiments, AS particles were treated for 10 min with 10 μM glybenclamide before or after incubation with diazoxide. Then, 1.72 μM of IF_1 and 1 mM Mg-ATP were added and incubated again for 30 s–10 min.

As the quantity of IF_1 bound to the F_1 sector is strictly correlated with the degree of the inhibition of the ATPase activity determined at saturating ATP [23], the binding of IF_1 to F_1, ndF$_1$ and AS particles was followed by measuring the residual ATPase activity of aliquots removed from the samples at different incubation times (see below).

The dissociation constant (K_d) of isolated and membrane bound F_1 with IF_1 was determined from the slope of the plot of $[IF_1]_0/\alpha$ against $1/(1-\alpha)$, where $[IF_1]_0$ is the initial IF_1 concentration and α is the fraction of the enzyme complexed with IF_1 at equilibrium. In fact, as reported in [18], it can be assumed that every molecule of bound IF_1

completely inhibits the enzyme, so that α is given by $1 - \alpha = V/V_0$, where V_0 is ATPase activity in the absence of IF_1 and V is ATPase activity after equilibration with IF_1. The relationship between $[IF_1]_0/\alpha$ and $1/(1 - \alpha)$ was calculated by linear regression using Prism 4.0 (GraphPad Software, USA).

2.4. Release of IF_1 from Mg-ATP particles

Mg-ATP particles were washed twice by centrifugation, suspended in 21 mM Tris–H_3PO_4, 5 mM magnesium acetate, pH 7.3 and incubated at 37 °C for 2 h with 2.5 mM sodium malonate [23]. Mg-ATP particles (2 mg/ml) were then treated for 2 min with 0.1% (v/v) DMSO or 100 μM diazoxide dissolved in DMSO. After that, 50 mM succinate was added for 4 and 30 min.

2.5. ATPase activity assay

F_1 and ndF_1 aliquots were removed and transferred to a spectrophotometric cuvette containing saturating ATP (3 mM) and the ATP-regenerating pool (20 mM Tris–HCl, 1.5 mM phosphoenolpyruvate, 1 mM KCl, 4 mM $MgCl_2$, 200 μM NADH, 2 UI pyruvate kinase and 3 UI lactic dehydrogenase) to assay the residual ATPase activity at 37 °C [24]. The final F_1 and ndF_1 concentration was 5.2 nM and the specific activity at 37 °C in the pool was 31 ± 0.3 U/mg and 35 ± 0.4 U/mg, respectively.

When indicated, ndF_1 was diluted in 20 mM Tris–HCl, 10–20% (v/v) glycerol, pH 6.7, and incubated with 2.5 mM $MgSO_4$ plus 0.52 mM ADP to induce the Mg-ADP inhibited form of the enzyme [25]. ATPase activity of ndF_1 or Mg-ADP inhibited F_1 was also measured in the ATP-regenerating pool containing 100 μM ATP and 2 μM diazoxide. The final protein concentrations were 0.2 μM and 15 nM, respectively. AS and Mg-ATP particles ATPase activity was assayed at different times (see figures) by transferring aliquots to a spectrophotometric cuvette containing saturating ATP (3 mM) and the ATP-regenerating pool (30 mM sucrose, 50 mM Tris–HCl, 50 mM KCl, 4 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 2 mM EGTA, 1 mg/ml rotenone, 0.3 mM NADH, 4 UI pyruvate kinase and 3 UI lactic dehydrogenase) at 37 °C [26]. The final particle concentration was 0.01 mg/ml.

The activity assays were run using a Perkin-Elmer Vis-UV Spectrometer Lambda 14.

2.6. F_1 fluorescence analysis

0.52 μM F_1 was suspended in 20 mM Tris–HCl, pH 6.7, and incubated for 10 min at 37 °C in the absence or presence of 5.2 μM diazoxide. Then, 0.52 μM aurovertin B was added to the sample and the fluorescence analysis was carried out at 37 °C [27]. When the fluorescence signal was stable (after 5 min), Mg-ATP was added to a final concentration of 2.5 mM.

The spectra were run using a Perkin-Elmer Luminescence Spectrometer LS50.

2.7. Circular dichroism spectra

0.52 μM ndF_1 was suspended in 20 mM Tris–HCl, 20% (v/v) glycerol, pH 6.7 and incubated for 10 min at 37 °C in the absence or presence of 0.52 or 5.2 μM diazoxide. CD spectra were measured in far UV region using a Spectropolarimeter Jasco J-600 with a quartz cell of 1 mm path length at 37 °C. α-Helix content was calculated from the θ_{222} value according to Chen et al. [28].

2.8. Protein assay

Mg-ATP and AS particle protein concentration was estimated by Lowry et al. [29], while F_1 and ndF_1 concentration was determined by bicinchoninic acid method as in [30].

3. Results

The formation of the inhibited IF_1–F_1 complex has been obtained initially by incubating F_1 at pH 6.7 with IF_1 under conditions which ensured the enzyme catalytic cycle, supporting the formation of a productive orientation for IF_1 binding. The degree of IF_1 association has been followed by measuring the residual ATPase activity, as specified in Section 2. Fig. 1A shows the results obtained by incubating F_1 with different excesses of IF_1 for 10 min in the presence of millimolar concentrations of Mg-ATP. In the absence of diazoxide, about 50% of the ATPase inhibition was obtained with three excesses of IF_1, in accordance with [15]. When F_1 was treated with one equivalent of diazoxide before IF_1 addition, 50% inhibition was obtained at a lower excess of IF_1 per F_1, indicating that diazoxide markedly favoured the IF_1 binding at the different molar excesses of IF_1 per F_1. In addition, Fig. 1A shows that such effect was not significantly different when the enzyme was incubated with 10 eq. of diazoxide per F_1, suggesting that one equivalent gave the maximal effect.

K_d value for the combination of F_1 with IF_1 was calculated from the slope of the plot of $[IF_1]_0/\alpha$ against $1/(1 - \alpha)$, as specified in Section 2. Fig. 1B clearly shows that K_d changed upon the diazoxide treatment of F_1. In the absence of diazoxide K_d was 7.6×10^{-7} M, in accordance with data reported by Harris and Das [31]. This value significantly decreased to 2.5×10^{-7} M in the presence of one diazoxide equivalent per F_1 ($P < 0.001$), while no further decrease was observed when 10 eq. diazoxide were added.

In order to test if the F_1 catalytic cycle was essential for diazoxide effect, F_1 depleted of Mg^{2+} and nucleotides (ndF_1) was pre-treated with diazoxide and then incubated

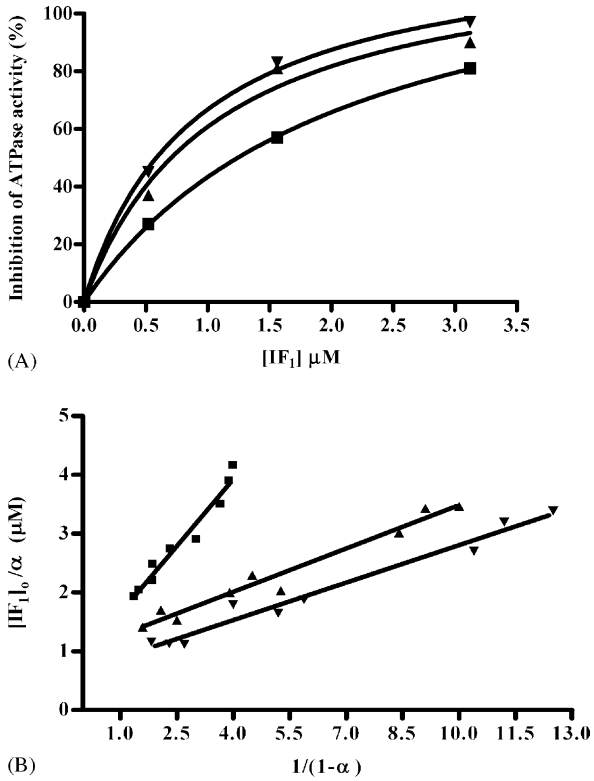

Fig. 1. Effect of diazoxide on K_d for the combination of F_1 with IF_1. (A) 0.52 μM F_1 suspended in 20 mM Tris–HCl, pH 6.7, was incubated for 10 min at 37 °C in the absence (■) or in the presence of 0.52 μM (▲) or 5.2 μM (▼) diazoxide. Subsequently, 0.52–3.12 μM of IF_1 plus 1 mM Mg-ATP were added and the samples were further incubated for 10 min at 37 °C. Aliquots were then removed and transferred to a spectrophotometric cuvette containing saturating ATP (3 mM) and the ATP-regenerating pool as indicated in Section 2 to assay the residual ATPase activity at 37 °C. The final F_1 concentration was 5.2 nM. The figure shows one experiment representative of three. (B) Plot of results using the equation: $[IF_1]_0/\alpha = K_d/(1-\alpha) + E_0$, where $[IF_1]_0$ is the inhibitor concentration at the beginning of the reaction, α is the enzyme fraction engaged in the inhibitor complex at equilibrium and E_0 is the initial enzyme concentration. K_d were calculated as the slopes of the lines generated by linear regression. r^2 value was 0.9599 for data obtained in the absence of diazoxide and 0.9637 or 0.9713 for those obtained in the presence of 0.52 or 5.2 μM diazoxide, respectively.

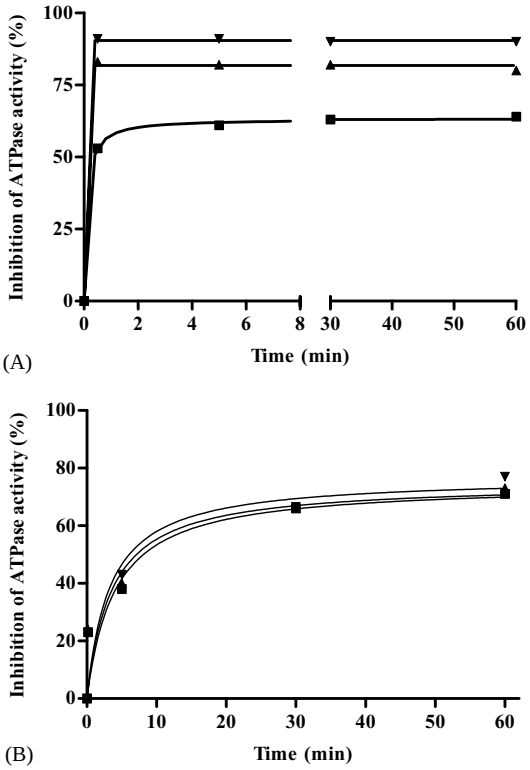

Fig. 2. Diazoxide affects IF_1 binding only to catalytically active F_1. (A) Diazoxide effect on cycling F_1. 2.2 μM of ndF_1 in 20 mM Tris–HCl, 10% glycerol, pH 6.7, was incubated for 10 min at 37 °C in the absence (■) or presence of 2.2 μM (▲) or 22 μM (▼) diazoxide. Thereafter, the ndF_1 samples were diluted to 0.52 μM in an ATP-regenerating buffer as reported in Section 2 and mixed with 1 μM IF_1 and 10 μM Mg-ATP, pH 6.7. At the times indicated, aliquots were transferred to a spectrophotometric cuvette containing saturating ATP (3 mM) and the ATP-regenerating pool to assay the residual ATPase activity at 37 °C. The final F_1 concentration was 5.2 nM. The figure shows one experiment representative of four. (B) Diazoxide effect on not cycling F_1. 2.2 μM of ndF_1 in 20 mM Tris–HCl, 20% glycerol, pH 6.7, was incubated for 10 min at 37 °C in the absence (■) or presence of 2.2 μM (▲) or 22 μM (▼) diazoxide. F_1 samples were diluted to 0.52 μM in the same buffer and mixed with 1 μM IF_1. At the time indicated aliquots were transferred to a spectrophotometric cuvette containing saturating ATP (3 mM) and the ATP-regenerating pool (see Section 2) to assay the residual ATPase activity at 37 °C. The final F_1 concentration was 5.2 nM. The figure shows one experiment representative of five.

with IF_1 either in the presence or in the absence of Mg-ATP. In this experiment ndF_1 was added together with low Mg-ATP concentration (10 μM) and an ATP-regenerating system in order to ensure a slow and continuous enzyme cycling and to overcome the Mg-ADP enzyme inhibition. Although the rate of ATP hydrolysis was low, 30 s were sufficient to reach the plateau value of IF_1–F_1 binding (see Fig. 2A), which was similar to that obtained in the presence of millimolar Mg-ATP and using the same IF_1 excesses per F_1 (see Fig. 1A). In addition, according to Fig. 1A, one diazoxide equivalent increased the degree of inhibition by 20% and no further increase in IF_1 binding was observed when the enzyme was pre-incubated with 10 molar excess of diazoxide. Considering the slow rate of ATP hydrolysis during the rapid combination of F_1 and IF_1, it can be estimated that the number of ATP molecules needed to be hydrolysed for formation of an IF_1–F_1 complex was lower than 200 under all conditions. This is the value previously found upon incubation with millimolar Mg-ATP in the absence of an ATP regenerating system [18]. The reduction of the turnover value we observed in comparison with [18] was probably due to the fact that IF_1 was bound to F_1 in continuous cycling.

When not catalytically active ndF_1 was incubated with IF_1 in the absence of any Mg-ATP, IF_1 binding occurred, but much more slowly. After 60 min the same degree of inhibition observed in the catalytically active F_1 was obtained (Fig. 2B), indicating that the same equilibrium position of IF_1–F_1 complex was reached. Under this condition, the treatment with either 1 or 10 eq. of diazoxide did not alter IF_1 binding to F_1. These results clearly indicate that the drug favours the formation of the inhibited

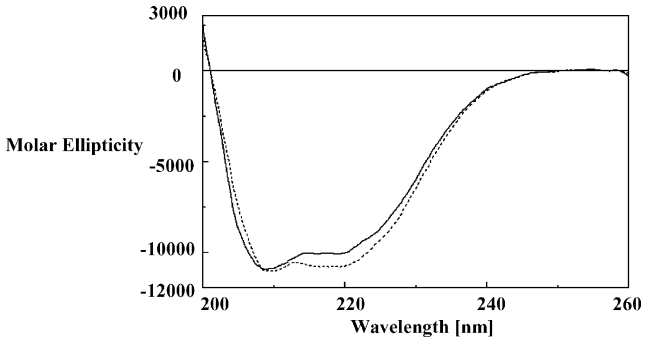

Fig. 3. CD spectra of ndF_1 pretreated or not with diazoxide. 0.52 μM ndF_1 was incubated or not with 0.52 μm diazoxide (see Section 2). Then, CD spectra were run at 37 °C with a quartz cell of 1 mm path length between 200 and 260 nm at a rate of 20 nm/min. ndF_1: solid line; ndF_1 pretreated with diazoxide: dotted line. The figure shows one experiment representative of three.

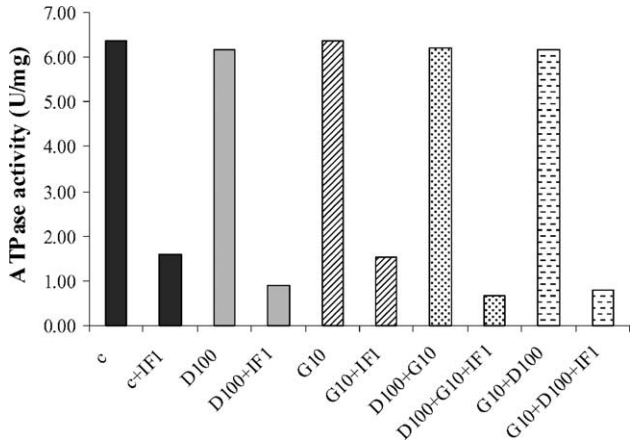

Fig. 4. Effect of diazoxide on IF_1 binding to AS particles in the absence or presence of glybenclamide. AS particles were suspended in 250 mM sucrose, 75 mM K_2SO_4 and 10 mM MOPS, pH 6.7 at 2 mg/ml (c). The particles were treated for 10 min with 100 μM diazoxide and then for 10 min with 10 μM glybenclamide (D100 + G10) or vice versa (G10 + D100) or for 20 min with 100 μM diazoxide (D100) or 10 μM glybenclamide (G10) separately. After taking a sample for basal ATPase activity measurement, 2 μM IF_1 and 1 mM Mg-ATP were added and after 10 min the residual ATPase activity was similarly assayed in the presence of 3 mM ATP and 0.01 mg/ml of AS particles. The figure shows one experiment representative of three.

IF_1–F_1 complex selectively when F_1 is catalytically active (cycling).

Although the catalytic F_1 cycle is essential for diazoxide effect revelation, its binding might occur independently. CD spectra of ndF_1 have been run to obtain direct evidence of diazoxide binding to the catalytically inactive enzyme. Diazoxide significantly changed the ndF_1 molar ellipticity, consequent to alterations of the enzyme secondary structure (Fig. 3). Specifically, diazoxide stabilised the protein structure by increasing its α-helix content from 32.3 to 35.2%. Moreover, the effect caused by one equivalent of drug was similar to that caused by 10 eq. (spectrum not shown), suggesting that the main structural effect was due to the binding of one equivalent.

To test diazoxide effects on membrane bound ATPsynthase, submitochondrial particles deprived of IF_1 (AS particles—see Section 2) were either pre-treated or not with diazoxide and either incubated or not for 10 min with different excesses of IF_1 and millimolar Mg-ATP. Diazoxide per se slightly inhibited the ATPase activity ($5.4 \pm 1.4\%$ decrease). When IF_1 was subsequently added, in accordance with the results obtained with isolated F_1, diazoxide favoured the formation of the inhibited IF_1–F_0F_1 complex. In particular, the dissociation constant K_d, 1.36×10^{-8} M in the absence of diazoxide according to [18], significantly decreased to 0.17×10^{-8} M ($P < 0.001$) in the presence of 100 μM diazoxide (data not shown). Assuming 0.35 nmol of F_1/mg of particle protein in AS particles [19], this drug concentration corresponds to about one hundred equivalents of diazoxide per F_1. However, such molar ratio is speculative, because the actual drug concentration is unknown. In fact, diazoxide is hydrophobic and could be easily trapped by the lipid bilayer [9].

Furthermore, the ability of glybenclamide, a blocker of K_{ATP} channel, to prevent/reverse diazoxide effect on membrane bound ATPsynthase was investigated. AS particles were incubated with 10 μM glybenclamide [32] and then with 100 μM diazoxide or vice versa before evaluating IF_1 binding to F_0F_1. The experiments were run both in the absence (data not shown) and in the presence (see Fig. 4) of 75 mM K_2SO_4, which mimics the mitochondrial environment [8]. Under this latter condition a lower basal IF_1 binding to AS particles was observed, as reported by [18]. In all cases diazoxide effect on IF_1–F_0F_1 complex formation was observed, suggesting that the conventional dose of glybenclamide neither prevents nor reverts diazoxide effect.

To verify if diazoxide also affects IF_1 release induced by $\Delta\mu H^+$ generation, Mg-ATP particles rich in IF_1 (see Section 2) were treated or not with diazoxide (100 μM) and the ATPase activity was assayed before and after energisation by succinate. To minimise IF_1 rebinding, the particles were washed by centrifugation to eliminate any nucleotides before diazoxide addition and the experiments were carried out at neutral pH in the absence of added nucleotides. As shown in Fig. 5, no effect on the energisation-dependent release of IF_1 from membrane bound F_1 occurred. This is not surprising, considering that the unidirectional rate of IF_1 release is controlled by $\Delta\mu H^+$, whilst it is not dependent on Mg-ATP hydrolysis catalysed by membrane bound F_1 [5]. This result then allows the proposal that K_d decrease due to diazoxide was only consequent to increase of IF_1 binding to F_1.

Diazoxide enhances K_{ATP} channel opening by stabilising the Mg-ADP complex at the NBD able to hydrolyse Mg-ATP at high rate (NBD2) [33]. In order to test if diazoxide also stabilises Mg-ADP in the tight catalytic site of F_1 (β_{DP}), ndF_1 was treated with a very low ADP concentration in the presence of saturating Mg^{2+}, which selectively induces Mg-ADP binding to such site [25].

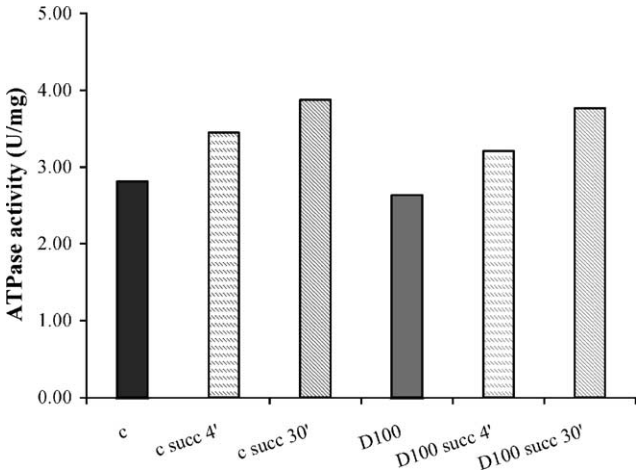

Fig. 5. Lack of diazoxide effect on IF_1 release from membrane bound F_1. Mg-ATP submitochondrial particles were suspended at 2 mg/ml (see Section 2). The particles were treated for 2 min with 100 μM diazoxide dissolved in DMSO and the basal ATPase activity assayed. Thereafter 50 mM succinate was added and the activity at 37 °C assayed after 4 and 30 min in the ATP-regenerating pool (3 mM ATP); c, control; D100 in 100 μM diazoxide. The final protein concentration of Mg-ATP SMPs in cuvette was 0.01 mg/ml. The figure shows one experiment representative of five.

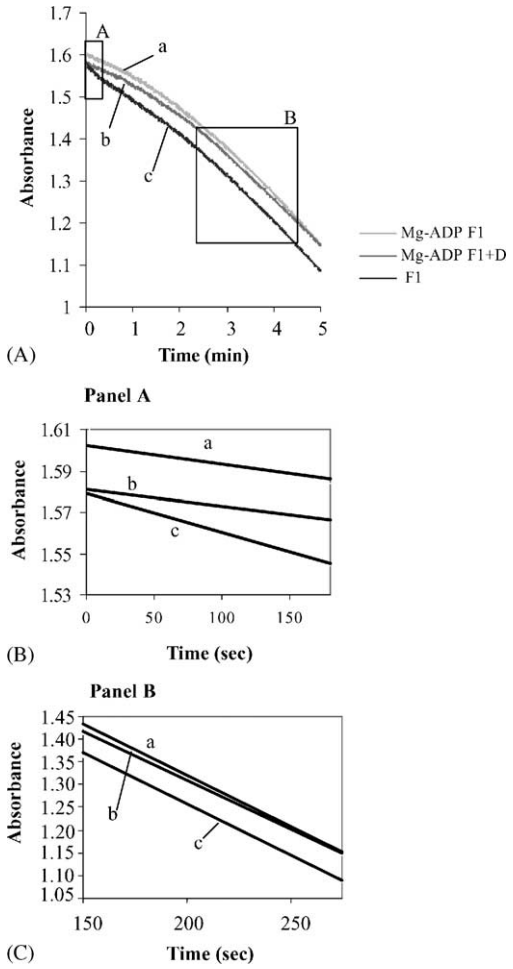

Fig. 6. Effect of diazoxide on the reactivation of the Mg-ADP inhibited F_1. 2.2 μM of ndF_1 in 20 mM Tris–HCl, 20% glycerol, pH 6.7, was incubated for 10 min at 37 °C in the absence (curves a and c) or presence (curve b) of 22 μM diazoxide. Then aliquots were incubated for 15 min at 37 °C with 2.5 mM $MgSO_4$ plus 2.2 μM ADP to induce the enzyme in the Mg-ADP inhibited form (curves a and b). The ATPase activity was assayed spectrophotometrically at 37 °C with 100 μM ATP in the ATP-regenerating pool in the absence or presence of diazoxide. The steady-state ATPase activity was 0.61 ± 0.053 U/mg in ndF_1 inhibited (curve a) or not (curve c) by Mg-ADP and significantly decreased to 0.58 ± 0.054 U/mg ($P < 0.001$) after diazoxide treatment (curve b). The final F_1 concentration was 15 nM. The figure shows one experiment representative of five. *Panel A*: The trendline equations of the curves in the first 200 s are the following. Curve (a): $y = -9E{-}05x + 1.6019$; curve (b): $y = -8E{-}05x + 1.5811$; curve (c): $y = -0.0002x + 1.579$. *Panel B*: The trendline equations of the curves between 2.5 and 4.5 min are the following. Curve (a): $y = -0.1122x + 1.715$; curve (b): $y = -0.1068x + 1.6827$; curve (c): $y = -0.1119x + 1.6492$.

The enzyme was then treated or not with diazoxide and the kinetics of ATP hydrolysis were followed. As reported in Fig. 6 the enzyme treated with Mg-ADP showed a strongly inhibited initial rate that was reversed only by continuous ATP hydrolysis, according to [25]. When this inhibited form of F_1 was treated with diazoxide, the reactivation during the catalytic cycle was slower. More specifically, in the first 200 s, during which the Mg-ADP inhibition was evident, the curve slope in the presence of diazoxide decreased of around 11% (see panel A). However, it must be considered that, because of the delay between starting the reaction and initiating the recording, the rapid-phase process completed within 5 s would not have been detected. Between 2.5 and 5 min (see panel B), when the steady-state ATP hydrolysis was reached, the difference between the two curves was only of 5%. The same low degree of inhibition ($P < 0.001$) was observed when diazoxide effect was tested on the steady-state ATP hydrolysis of ndF_1 (data not shown). Then, diazoxide appeared to prevent the release of the inhibitory Mg-ADP from a catalytic site and to slightly inhibit the catalytic activity. Moreover, both in ndF_1 and in Mg-ADP inhibited F_1 the inhibitory effect disappeared upon F_1 dilution, namely when diazoxide was not present in the ATP hydrolysis assay pool, indicating that the drug binding was reversible.

When the enzyme was complexed with aurovertin, a different fluorescence response to Mg-ATP was observed in diazoxide-treated F_1 in the presence of Mg-ADP excess, which converts the enzyme to an inhibited form (see Section 2). The drug did not affect the binding of aurovertin, as it did not alter the fluorescence of aurovertin–F_1 complex. On the other hand, diazoxide induced a lower response of the complex to Mg-ATP addition (Fig. 7). This result suggests that diazoxide induces a conformational change in F_1, which alters the catalytic cycle, thereby slightly inhibiting the enzyme, as shown in Fig. 6. However, as demonstrated in Figs. 1 and 2, the inhibition was much more marked when F_1 treated with diazoxide was incubated with IF_1. Then, diazoxide poorly inhibits the enzyme per se, but markedly promotes IF_1 binding to F_1 significantly increasing the degree of inhibition.

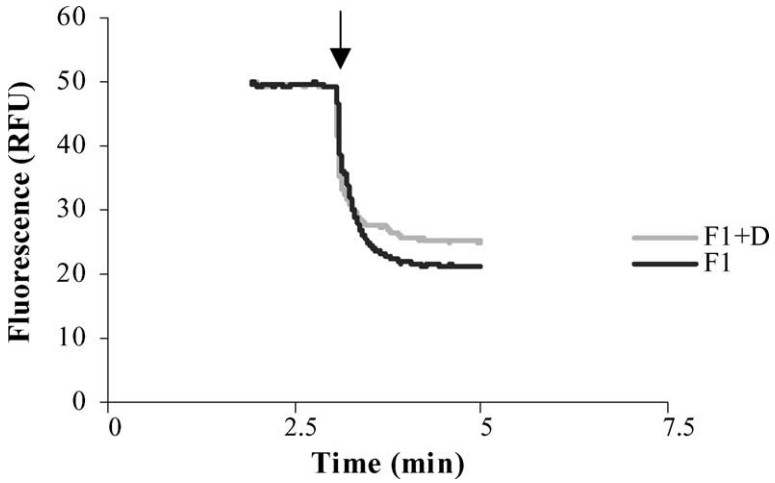

Fig. 7. Effect of diazoxide on Mg-ATP induced aurovertin fluorescence change in ADP-inhibited F_1. 0.52 μM of the Mg-ADP inhibited F_1 (see Section 2) was suspended in 20 mM Tris–HCl, pH 6.7, and incubated for 10 min at 37 °C in the absence or presence of 5.2 μM diazoxide. After that, 0.52 μM aurovertin B was added to the samples and the fluorescence analysis was carried out at 37 °C. When indicated by the arrow, Mg-ATP was added to a final concentration of 2.5 mM. The figure shows one experiment representative of four.

4. Discussion

The results so far presented indicate that diazoxide strongly affects the equilibrium position of IF_1–F_1 complex formation, thus promoting the enzyme inhibition, both in soluble and membrane bound complex. The experiments with soluble F_1 clearly show that the diazoxide functional effect depends on the enzyme catalytic cycle. In fact, no diazoxide effect has been observed when IF_1 has been incubated with F_1 not catalytically active. This observation suggests that the drug favours F_1 conformation suitable for IF_1 binding during the catalytic cycle. A scheme of IF_1 action based on the crystal structure of IF_1–F_1 complex has been very recently proposed [34]. In that structure, IF_1 is bound at the interface between the β subunit adopting the tight conformation (β_{DP}) and the adjoining α subunit (α_{DP}) in the C-terminal region of both. According to this structure, it has been proposed that IF_1 binding to its site at β_{DP}/α_{DP} interface of F_1 prevents the conformational changes in the catalytic sites required for rotary catalysis, thus supporting that ''cross-talk'' through the catalytic site and IF_1 binding domain is essential for the inhibitory action.

Our data show that diazoxide exerts a stabilising role on Mg-ADP bound in the catalytic site of β_{DP}. Similarly, in sK_{ATP}, diazoxide stabilises Mg-ADP in the NBD2 of its SUR subunit, which sustains ATP hydrolysis at high rate [33]. These considerations suggest that the diazoxide binding target region may be located in NBDs both in SUR subunits of K_{ATP} channel and in β subunits of ATPsynthase. In addition, the finding that one equivalent of diazoxide was sufficient to give the F_1 maximal functional effects suggests that only one of the three NBDs located in β subunits contains the high affinity site for the drug. Thus, it is tempting to propose that diazoxide binds to the catalytic domain of β_{DP}, thus inducing the nucleotide stabilisation and facilitating IF_1 binding in the C-terminal domain of β_{DP}.

In K_{ATP} channel diazoxide action at NBD2 requires ATP binding at NBD1, which has very low ATPase activity, providing evidence that co-operative interactions of the NBDs are critical for the drug effect [35]. Similarly, in F_1 the catalytic cycle is known to be accompanied by nucleotide-dependent structural interactions through the NBDs contained in the non-catalytic and catalytic subunits of the enzyme [36]. Thus, since diazoxide effect is elicited selectively on cycling F_1, ''cross-talk'' through NBDs appears to mediate the drug effect in ATPsynthase as in K_{ATP} channel.

Although diazoxide effects were evident only in catalytically active F_1, the CD spectra of the enzyme depleted of Mg^{2+} and nucleotides indicated that one equivalent of diazoxide binds even in the absence of the catalytic cycle. As in K_{ATP} channel diazoxide binding, unlike its functional effects, is independent from ATP hydrolysis [9], our data further support the common drug behaviour versus F_1 and K^{ATP} channel. It cannot be excluded that diazoxide binds to a different region in catalytically active F_1 and in F_1 devoid of Mg^{2+} and nucleotides. However, it is noteworthy to remember that either exogenous inhibitors or metal ions [37,38] bind to β subunits with the same stoichiometry and at the same sites both in the presence and in the absence of nucleotides, thus supporting the concept of the inherently asymmetric assembly of F_1 [39].

In summary, our data suggest that the binding of one equivalent of diazoxide to NBD of one of the three β subunits, i.e. β_{DP}, is sufficient to induce the conformational changes able to increase IF_1 binding. However, this does not exclude that other NBDs in β/α subunits can bind diazoxide with lower affinity.

Diazoxide enhancement of ATPsynthase inhibition in isolated and perfused rat heart has been already reported [12–14]. It has been proposed that such inhibition could

contribute to cardioprotection by reducing the ATP depletion [12,13], as well as by accelerating the mitochondrial depolarisation and preventing mitochondrial Ca^{2+} accumulation [14]. However, the regulatory messengers linking the opening of mitoK_{ATP} and ATPsynthase inhibition are still debated [14]. Most specifically, it has been hypothesised that diazoxide action on the mitochondrial ATP hydrolysis could be partially mediated by changes in the sizes of the mitochondrial intermembrane space [40], but also by IF_1 binding to ATPsynthase, this latter being not influenced by the too small inner membrane depolarisation caused by mitoK_{ATP} opening [14]. The data reported in this study show that a direct interaction between diazoxide and F_1 occurs, resulting in enhancement of IF_1 binding at low pH. This enhancement also occurs at physiological K^+ ion concentration, which is regulated by mitoK_{ATP} and H^+–K^+ exchangers [8]. Considering the hydrophobic nature of diazoxide, which allows it to reach its cellular targets via the lipid bilayer [9], the direct interaction with the enzyme might represent a mechanism to attenuate in vivo mitochondrial nucleotide hydrolysis, leading to cardioprotection during ischemia.

The finding that in our model glybenclamide does not prevent/revert diazoxide effect towards IF_1 binding to membrane bound ATPsynthase excludes its direct interaction with the enzyme. Nevertheless, in mitochondria glybenclamide may indirectly counteract the enhancement of IF_1 binding to F_1 mediated by diazoxide. In fact, K_{ATP} channel closure prevents pH decrease and allows Ca^{2+} accumulation in mitochondria [14], both known as factors favouring free with respect to bound IF_1 [3]. Moreover, the finding that in the submitochondrial particles diazoxide appears not to affect the energisation-dependent IF_1 release is in accordance with the observation that the mitochondrial ATP synthesis during the reperfusion phase after prolonged ischemia is not affected by the drug [12].

In conclusion, the structural similarities of the adenine nucleotide binding proteins may explain, at least partially, the metabolic actions of diazoxide [13]. Therefore, we suggest that F_1 might be a good model for diazoxide binding sequence localisation and for a further understanding of the molecular mechanisms involved in mitoK_{ATP} channel activation.

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