Downmodulation of mitochondrial $F_0F_1$ ATP synthase by diazoxide in cardiac myoblasts: a dual effect of the drug

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Comelli M, Metelli G, Mavelli I. Downmodulation of mitochondrial $F_0F_1$ ATP synthase by diazoxide in cardiac myoblasts: a dual effect of the drug. Am J Physiol Heart Circ Physiol 292: H820–H829, 2007; doi:10.1152/ajpheart.00366.2006.—Similar to ischemic preconditioning, diazoxide was documented to elicit beneficial bioenergetic consequences linked to cardioprotection. Inhibition of ATPase activity of mitochondrial $F_0F_1$ ATP synthase may have a role in such effect and may involve the natural inhibitor protein IF1. We recently documented, using purified enzyme and isolated mitochondrial membranes from beef heart, that diazoxide interacts with the $F_1$ sector of $F_0F_1$ ATP synthase by promoting IF1 binding and reversibly inhibiting ATP hydrolysis. Here we investigated the effects of diazoxide on the enzyme in cultured myoblasts. Specifically, embryonic heart-derived H9c2 cells were exposed to diazoxide and mitochondrial ATPase was assayed in conditions maintaining steady-state $F_1$ binding (basal ATPase activity) or detaching bound IF1 at alkaline pH. Mitochondrial transmembrane potential and uncoupling were also investigated, as well as ATP synthesis flux and ATP content. Diazoxide at a cardioprotective concentration (40 $\mu$M cell-associated concentration) transiently downmodulated basal ATPase activity, concomitant with mild mitochondria uncoupling and depolarization, without affecting ATP synthesis and ATP content. Alkaline stripping of IF1 from $F_0F_1$ ATP synthase was less in diazoxide-treated than in untreated cells. Pretreatment with glibenclamide prevented, together with mitochondrial depolarization, inhibition of ATPase activity under basal but not under IF1-stripping conditions, indicating that diazoxide alters alkaline IF1 release. Diazoxide inhibition of ATPase activity in IF1-stripping conditions was observed even when mitochondrial transmembrane potential was reduced by FCCP. The results suggest that diazoxide in a model of normoxic intact cells directly promotes binding of inhibitor protein IF1 to $F_0F_1$ ATP synthase and enhances IF1 binding indirectly by mildly uncoupling and depolarizing mitochondria.

Pharmacological preconditioning is a cardioprotective state similar to IPC. Diazoxide, an antihypertensive and antihyperglycemic drug, is commonly used to induce preconditioning in animal models of ischemia-reperfusion (17, 20). This hydrophobic compound passively enters cells and organelles like mitochondria. Functionally, diazoxide is commonly considered as a selective opener of mitochondrial ATP-sensitive potassium (mitoKATP) channels (18, 19, 20), but at relatively high concentrations (>100 $\mu$M) the drug has other effects, including the inhibition of succinate dehydrogenase (22, 48). Interestingly, similar to IPC, diazoxide has beneficial bioenergetic consequences in perfused rat hearts and isolated rat heart mitochondria (2, 6, 33).

Activation of mitoKATP channels is considered to be a major trigger or end effector of IPC (19, 20, 40), even if some authors have provided renewed support for a role of sarcolemmal KATP channels in ischemic cardioprotection (47, 51). Evidence for the existence of mitoKATP channels and for their involvement in IPC derives from pharmacological studies, since KATP channel openers (e.g., diazoxide) mimic IPC and KATP channel blockers (e.g., glibenclamide) inhibit IPC (17, 19, 20, 23). However, the molecular structure of mitoKATP channels is not clear yet (12, 34), despite recent advances in understanding its structure (3, 30). Moreover, alternative mechanisms have been recently overviewed (4, 23).

Mitochondrial $F_0F_1$ ATP synthase is the major producer of ATP for contractile function and ionic homeostasis in cardiomyocytes (13). When oxygen deprivation collapses the mitochondrial electrochemical gradient, $F_0F_1$ ATP synthase switches from ATP synthesis to ATP hydrolysis and thus, during severe ischemia, is a major consumer of ATP (13). Therefore, inhibition of $F_0F_1$ ATP synthase during ischemia, which should conserve myocardial ATP, may play a key role in the energy-sparing effect elicited by ischemic and pharmacological preconditioning (2, 6, 24, 55). Numerous authors have suggested that inhibition of the ATPase activity of $F_0F_1$ ATP synthase is carried out by the enzyme’s natural inhibitor protein, IF1 (2, 7, 24, 55). IF1 is a noncompetitive inhibitor that reversibly binds with 1:1 stoichiometry to the $\alpha$-subunit of $F_1$, the catalytic sector of $F_0F_1$ ATP synthase; binding requires ATP hydrolysis associated with loss of proton motive force and is favored by a low mitochondrial electrochemical gradient (32). We recently documented (14, 43) that IF1 binding to $F_0F_1$ ATP synthase increases during IPC in goat heart in vivo. In addition, by studying both the purified enzyme and isolated mitochondrial membranes from beef heart, we demonstrated

ISCHEMIC PRECONDITIONING (IPC) induces a state of cardioprotection against subsequent prolonged ischemia-reperfusion injury. The cardioprotective mechanism of IPC is complex and involves a variety of triggers that activate multiple signaling pathways, most converging on mitochondria (37). The cellular survival program activated by IPC integrates several processes, including opening of ATP-sensitive potassium channels (20, 56), regulation of fatty acid metabolism (28), production of reactive oxygen species (41), and regulation of mitochondrial permeability transition (25). Moreover, IPC elicits protective effects on cellular bioenergetic status by downmodulating the ATPase activity of mitochondrial ATP synthase, thereby promoting cell survival after ischemic insult (2, 6, 14, 24, 43, 55).

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and is reported as units (micromoles of ATP per minute) per milligram of protein. Furthermore, the buffer composition (containing EGTA and <5 mM Na+ concentration) was chosen to minimize interference from Ca2+ and Na+-K+-ATPases (8); this was verified experimentally by performing the assay with 10 μM sodium orthovanadate and 2 mM ouabain, respectively. Oligomycin-sensitive ATPase activity, indicative of correct coupling of F0 and F1 moieties of the FoF1 ATP synthase complex, was assayed in the presence of 4 μM oligomycin (8). Oligomycin concentration was chosen on the basis of a dose-dependence study of the ATPase activity inhibitory effect, which permitted us to avoid using submaximal concentrations.

Cell-associated diazoxide concentration. H9c2 cells were incubated with 100 μM diazoxide in culture medium for 0–30 min and harvested by trypsinization. Cell pellets were extracted with 0.33 N perchloric acid (500 μl per 10-cm dish).

Cell-associated diazoxide concentration was determined by an HPLC method (54) using a LiChrosorb RP-8 column (10 cm × 4.6 mm internal diameter; particle size, 5 μm; Chrompack, Middelfd, The Netherlands). Calibration curves, obtained by adding known amounts of diazoxide to the culture medium immediately before trypsinization, were linear for the range 0.02–10 μg (r = 0.966); the sensitivity limit was 0.3 μg. The recovery of diazoxide from perchloric acid extracts was 85.2% (SD 0.9%) (n = 4), based on a standard curve. Diazoxide for standard solutions was dissolved in dimethyl sulfoxide and diluted in water or culture medium to a final concentration of 0.01–0.05%.

Mitochondrial ATPase assays. H9c2 cells, grown in 10-cm tissue culture plates, were incubated in culture medium for 0–30 min in the presence of 10–400 μM diazoxide or vehicle (0.05% dimethyl sulfoxide). In some experiments, cells were treated with carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) for 10 min at 25 μM or with vehicle (0.1% ethanol). In some experiments, cells were treated with 10 μM glibenclamide for 1 h. After treatments, cells were harvested by trypsinization (0.05% trypsin, 0.02% EDTA in phosphate-buffered saline), pelleted by centrifugation, resuspended in a diazoxide-free, ice-cold solution of phosphate-buffered saline (PBS), pelleted by centrifugation, resuspended in a diazoxide-free, ice-cold solution of phosphate-buffered saline (PBS), and homogenized by sonication (UP200s sonifier; Hielscher, Berlin, Germany). Conditions were controlled to expose catalytic sector F1 to solvent and to avoid uncoupling from the F0 domain (8).

Mitochondrial ATPase activity was monitored during ultrasonic treatment in the absence and in the presence of FCCP to evaluate breaking of mitochondria and formation of submitochondrial particles (SMP) and in the presence of oligomycin to ensure minimized damage to the enzyme coupling subunits during the treatment (7). Homogenization at pH 7.0 maintains the binding equilibrium between IF1 and FoF1 ATP synthase, permitting us to measure “basal” ATPase activity, while homogenization at pH 9.2 detaches 95% of bound IF1 (46), permitting us to measure “potential” (uninhibited) ATPase activity. Cell homogenates were assayed for ATPase immediately after preparation, and SMP were deproteinized by trypsinization. Cell homogenates were assayed for ATPase immediately after preparation, and SMP were deproteinized by trypsinization. Mitochondrial ATPase activity was assayed immediately after sonication. Total protein content was determined by Lowry et al. (56), with bovine serum albumin as standard.

Maximal ATPase activity (V_max) was determined by coupling the production of ADP to the oxidation of NADH via pyruvate kinase and lactic dehydrogenase reactions with a diazoxide-free assay buffer containing an ATP-regenerating system (Mn2+: 50 Tris-HCl pH 7.4, 30 sucrose, 50 KCl, 4 MgCl2, 2 NaATP, 2 EGTA, 2 phosphoenolpyruvate, and 300 NADH with 3 IU lactate dehydrogenase and 4 IU pyruvate kinase). Activity was measured by following NADH oxidation at 340 nm on a Perkin-Elmer Vis-UV Spectrometer Lambda 14

METHODS

Chemicals and reagents. All materials were purchased from Sigma (St. Louis, MO), unless otherwise stated.

Cell cultures. H9c2, a clonal line of rat embryonic heart-derived myoblasts, was obtained from American Type Culture Collection (CRL-1446; Rockville, MD). The cells were maintained in culture medium consisting of Dulbecco’s modified Eagle’s medium (Euroclone, Devon, United Kingdom), 10% fetal bovine serum (Biochrom, Berlin, Germany), penicillin (100 U/ml), streptomycin (100 μg/ml), and glucose (4 mM) to prevent loss of differentiation potential.

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For confocal microscopy, labeled cells were washed three times with culture medium and examined under a laser scanning confocal microscope (TCS NT; Leica, San Jose, CA). Fluorescence was excited by the 488-nm line of an argon laser, and emission was recorded at 525 and 590 nm. For quantitative analyses, pixel intensities were analyzed with MetaMorph software (Crisel Instruments, Rome, Italy).

For flow cytometry, labeled cells were harvested by trypsinization, washed three times in culture medium, resuspended at 10^6 cells/ml, and analyzed (10,000 cells/sample) with a FACScan cytometer (Becton Dickinson, Mannheim, Germany). The excitation wavelength was 488 nm, and emission was monitored at 582 nm. Data were analyzed with Cell Quest Software (Becton Dickinson, New York, NY).

Statistical analysis. Data are reported as means (SD) unless otherwise indicated. Intergroup comparisons were made with Student's t-test for two groups and by one-way ANOVA followed by post hoc Tukey's test for multiple groups. A value of \( P < 0.05 \) was considered to be statistically significant.

RESULTS

We first examined the effects elicited on the ATPase activity of mitochondrial F₀F₁ ATP synthase by the exposure of cultured H9c2 myoblasts to diazoxide. When subconfluent cultures were homogenized at pH 7.0, ATPase activity was 0.126 U/mg (SD 0.006) \((n = 20)\). The presence of 10 \( \mu \)M Na orthovanadate and 2 mM ouabain reduced ATPase activity by 2% and 7%, respectively, confirming that the assay conditions were specific for mitochondrial ATPase. Nevertheless, the oligomycin-sensitive ATPase activity, indicative of correctly assembled enzyme complex, was low [0.063 U/mg (SD 0.002)], although still in the range reported for cell lines assayed under experimental conditions avoiding mitochondrial isolation (5). This may suggest that disturbed assembly/stability of F₀F₁ ATP synthase complex occurred in H9c2 cells, considering that the homogenization conditions were carefully controlled to minimize damage to the coupling subunits of the enzyme.

When H9c2 cells were treated with 0–400 \( \mu \)M diazoxide for 10 min before homogenization at pH 7.0, a mild but significant dose-dependent decrease in both oligomycin-sensitive and insensitive ATPase activity was observed at concentrations known to have cardioprotective effects (50–200 \( \mu \)M) (Fig. 1). The maximum inhibitory effect (26% and 27% in the two cases) was achieved at 100 \( \mu \)M. Diazoxide had no effect on cell viability (data not shown).

The time course analysis of the inhibitory effects of 100 \( \mu \)M diazoxide revealed maximal reduction in ATPase activity over 10 min (Fig. 2A). Thereafter, activity remained relatively constant until it returned to the control value at 30 min. The profile of cell-associated diazoxide during the time course paralleled that of the inhibitory effects, with a maximum concentration at 10 min (Fig. 2B). Concentrations of cell-associated diazoxide at all time points were less than the 100 \( \mu \)M added to the medium and below those at which the drug exerts pharmacological effects other than mitoKATP activation (e.g., inhibition of succinate dehydrogenase).

The fact that diazoxide inhibition of ATPase was transient pointed to a reversible downmodulation of F₀F₁ ATP synthase, probably by naturally occurring IF₁, similar to that we previously observed on IPC (14, 43). To test this hypothesis, taking into account that IF₁ binding to F₀F₁ ATP synthase is optimal at pH 6.7 and that sonication at pH 9.2 releases 95% of bound IF₁, binding to F₀F₁ ATP synthase was optimal at pH 6.7 and that sonication at pH 9.2 releases 95% of bound...

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Fig. 1. Dose-dependent effects of diazoxide on mitochondrial ATPase activity (open bars) and oligomycin-sensitive ATPase activity (gray bars) in H9c2 myoblasts. Cells were exposed to vehicle or 10–400 \( \mu \)M diazoxide for 10 min before homogenization at pH 7.0, in the presence or in the absence of oligomycin (see METHODS). Data are means (SD) of 3 experiments made in duplicate. \(* P < 0.001 vs. control (Student’s t-test).\)

Fig. 2. Time courses of diazoxide inhibition of mitochondrial ATPase and cell-associated diazoxide concentration. Cells were treated with 100 \( \mu \)M diazoxide for 0–30 min. A: ATPase activity in homogenates prepared at pH 7.0. B: cell-associated diazoxide concentration. Data are means (SD) from at least 4 experiments performed in duplicate. \(* P < 0.001 vs. time 0 control (Student’s t-test); data marked with the same superscript letters are significantly different \((P < 0.05, Tukey’s test).\)
IF1 (46), we compared mitochondrial ATPase activity in homogenates prepared at pH 7.0 and 9.2 (Fig. 3A). In control cells (treated with vehicle), ATPase activity was only 20% higher (P<0.05) in homogenates made at pH 9.2 (potential ATPase activity) than at pH 7.0 (basal ATPase activity), indicating a low level of bound IF1 under physiological conditions. In cells treated with 100 μM diazoxide for 10 min and homogenized at physiological pH, ATPase activity was 26% less than in nontreated cells, as shown earlier; homogenization at pH 9.2 increased ATPase activity by only 21%, similarly to nontreated cells and therefore was unable to overcome the inhibitory effects of the drug. Treatment with 25 μM FCCP (to increase IF1 binding through the loss of ΔΨm) also inhibited ATPase by 25%, showing that free IF1 is available in cells but in a low amount. FCCP concentrations lower than 25 μM had little effect in this cell system (data not shown). Homogenization at pH 9.2 fully overcame the protonophore’s inhibitory effects, returning ATPase activity to that of control cells and confirming that the inhibitory action of FCCP is due to increased IF1 binding. FCCP treatment had no effect on cell viability, and its vehicle had no effect on ATPase activity (data not shown). Control experiments using SMP deprived of IF1 showed that ATPase activity was not affected by diazoxide at both pH 7.0 and 9.2 (Fig. 3B), suggesting that the drug did not interfere per se with the catalytic activity of the enzyme and confirming the role of IF1 in the ATPase activity inhibition afforded by diazoxide.

The time course of diazoxide inhibition of mitochondrial ATPase assayed in homogenates prepared at pH 9.2 was similar to that observed in homogenates prepared at physiological pH, with a rapid and significant decrease between 0 and 10 min and recovery at 30 min (Fig. 4A). At all time points, ATPase activity in alkaline extracts was greater than that in pH 7.0 extracts. With the consideration that alkaline treatment removes >95% of bound IF1 (46), the ATPase activity observed in control cells at pH 9.2 was taken as the activity of IF1-free enzyme. On this basis, we calculated the amount of IF1-inhibited enzyme as the ratio of the enzyme activity measured in alkaline extracts to the activity of IF1-free enzyme. Fig. 4. Time course of diazoxide inhibition of mitochondrial ATPase in homogenates prepared at pH 7.0 and 9.2 (Fig. 3A). In control cells (treated with vehicle), ATPase activity was only 20% higher (P<0.05) in homogenates made at pH 9.2 (potential ATPase activity) than at pH 7.0 (basal ATPase activity), indicating a low level of bound IF1 under physiological conditions. In cells treated with 100 μM diazoxide for 10 min and homogenized at physiological pH, ATPase activity was 26% less than in nontreated cells, as shown earlier; homogenization at pH 9.2 increased ATPase activity by only 21%, similarly to nontreated cells and therefore was unable to overcome the inhibitory effects of the drug. Treatment with 25 μM FCCP (to increase IF1 binding through the loss of ΔΨm) also inhibited ATPase by 25%, showing that free IF1 is available in cells but in a low amount. FCCP concentrations lower than 25 μM had little effect in this cell system (data not shown). Homogenization at pH 9.2 fully overcame the protonophore’s inhibitory effects, returning ATPase activity to that of control cells and confirming that the inhibitory action of FCCP is due to increased IF1 binding. FCCP treatment had no effect on cell viability, and its vehicle had no effect on ATPase activity (data not shown). Control experiments using SMP deprived of IF1 showed that ATPase activity was not affected by diazoxide at both pH 7.0 and 9.2 (Fig. 3B), suggesting that the drug did not interfere per se with the catalytic activity of the enzyme and confirming the role of IF1 in the ATPase activity inhibition afforded by diazoxide.

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sured after sonication at pH 7.0 to the value of the activity of IF1-free enzyme. With respect to the 20% inhibition of the ATPase activity of F0F1 ATP synthase observed in nontreated cells during diazoxide treatment, we calculated 41% of IF1-inhibited enzyme (at 10 and 20 min), which then returned to 20% at 30 min (Fig. 4B).

To determine whether the effects of diazoxide involved uncoupling of mitochondrial respiration, we assayed the complex. With respect to the 20% inhibition of the ATP synthesis flux, an overall low rate [2.1 nmol O2·min⁻¹·mg protein⁻¹ (SD 0.2)] was observed in control cells. This suggests that the proton gradient driving ATP formation was not perfectly tight to oxidative phosphorylation and that the ATP synthesis control over respiration was quite low. The low coupling might depend on both basal proton leak and disturbed assembly/stability of F0F1 ATP synthase complex, which we suggested above to occur in control cells on the basis of the observed low value of oligomycin-sensitive ATPase activity. When cells were incubated with 100 μM diazoxide for 10 min, ATP synthesis flux increased but not significantly so. As expected, 10 min of 25 μM FCCP blunted ATP synthesis flux. Finally, in cells treated with 100 μM diazoxide for 10 min, cellular ATP content showed a negligible, nonsignificant increase (Fig. 5B), while in cells treated with 25 μM FCCP for 10 min ATP content was significantly reduced. Thus, in normoxic cells exposed to diazoxide, mitochondrial F0F1 ATP synthase normally synthesized ATP despite a significantly reduced Vmax of ATP hydrolysis.

The fact that diazoxide did not reduce ATP levels agrees with its small effect on ATP synthesis flux and suggests that the transient uncoupling is too moderate to significantly alter mitochondrial energetics. If this is the case, membrane depolarization should also be moderate. Therefore, we investigated the effects of diazoxide on Δψm by monitoring the fluorescence intensity of the lipophilic cation JC-1. At confocal microscopy, control cells had punctuate red staining indicative of normal mitochondrial uptake of JC-1 driven by high Δψm (Fig. 6A). After treatment with diazoxide (10 min, 100 μM), cells maintained punctuate staining but were predominantly green with some yellow, indicative of lower Δψm. Quantification of red fluorescence intensity (590 nm) indicated a 23% (SD 2) decrease in Δψm compared with control cells. Cells treated with FCCP had blotchy green-yellow fluorescence, indicating lower Δψm but also undefined mitochondrial edges and leakage of dye. Similar results were observed at flow cytometry: diazoxide treatment for 10 min altered the peak of the red fluorescence intensity distribution (Fig. 6B). Mean fluorescence intensity was 26% (SD 2) less than in control cells (P < 0.001), in agreement with confocal microscopy results. The intensity profile for a 30-min treatment with diazoxide was similar to that of control cells. FCCP treatment resulted in a 54% (SD 3) reduction in mean red fluorescence intensity (P < 0.001 vs. control cells). These results are in agreement with the effects of diazoxide on mitochondrial ATPase, and they suggest that diazoxide treatment of H9c2 cells moderately and transiently decreases Δψm, similar to its inhibition of F0F1 ATP synthase.

Because of its hydrophobic nature, diazoxide passes through membranes and may interact with F0F1 ATP synthase irrespectively of its effects on mitoKATP channels or mitochondrial depolarization. To test this hypothesis, H9c2 cells were preexposed for 1 h to glibenclamide before treatment with 100 μM diazoxide. When compared with no preexposure, glibenclamide pretreatment also blocked diazoxide’s time-dependent inhibition of mitochondrial ATPase activity, assessed in homogenates made at pH 7.0

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Fig. 5. Diazoxide increases mitochondrial respiration in H9c2 cells without affecting ATP synthesis flux or cellular ATP content. Subconfluent cells were exposed to diazoxide or FCCP, used as a positive control to uncouple mitochondrial respiration from ATP synthesis. A: mitochondrial respiration (open bars) and ATP synthesis flux (gray bars). Mitochondrial O2 consumption was registered in nonpermeabilized cells suspended in culture medium in the absence and presence of the 2 agents (100 μM diazoxide, 5 μM FCCP). ATP synthesis flux was determined as the difference between respiration rate detected in absence of oligomycin and that detected in presence of 10 μM oligomycin. B: cellular ATP content. HPLC determination of ATP concentration was performed in acidic extracts of trypsinized cells (100 μM diazoxide, 25 μM FCCP). Data are means (SD) of at least 5 experiments performed in duplicate. *P < 0.005 vs. untreated controls; †P < 0.005 vs. diazoxide-treated cells (Student’s t-test).
Thus, when mitoKATP channels are blocked, diazoxide is unable to transiently induce mitochondrial depolarization and inhibit ATPase. We further compared mitochondrial ATPase activity in homogenates prepared at pH 7.0 and pH 9.2 from cells subjected to various pharmacological treatments (Fig. 8). Figure 8A demonstrates that, as already shown, ATPase activity from control and diazoxide-treated cells was ~20% higher ($P < 0.05$) in homogenates made at pH 9.2 than at pH 7.0, because of alkaline stripping of IF$_1$. Pretreatment with glibenclamide blocked the inhibitory effects of diazoxide in homogenates prepared at physiological pH and did not alter the limited increase in ATPase activity observed at pH 9.2. This result suggests that diazoxide treatment altered IF$_1$ release from ATP synthase at alkaline pH, irrespective of the effects elicited on $\Delta\psi_m$. When the experiments were repeated in presence of 25 $\mu$M FCCP (Fig. 8B), we again observed FCCP inhibition of ATPase in pH 7.0 homogenates but full recovery of ATPase activity in pH 9.2 homogenates; these results were not altered by pretreatment with glibenclamide, indicating that FCCP uncoupling stimulated IF$_1$-mediated ATPase inhibition independently of the state of mitoKATP channels. When cells were treated with both diazoxide and FCCP, there was no additive inhibitory effect on ATPase assessed in pH 7.0 homogenates, likely because of limited IF$_1$ availability, and the recovery of ATPase activity in alkaline homogenates was only partial (as observed with diazoxide alone). Finally, when cells were treated with both diazoxide and FCCP after preexposure to glibenclamide, ATPase activity in pH 7.0 homogenates was reduced to the same extent as without glibenclamide, indicating that the inhibition was due to FCCP uncoupling rather than to diazoxide. However, when these triply treated cells were homogenized at pH 9.2 to strip bound IF$_1$, there was only modest recovery of ATPase, attributable to the inhibitory effects of diazoxide. Notably, in all four diazoxide-treated samples, ATPase activity measured after alkaline stripping of IF$_1$ was significantly lower than that in the...
respective nondiazoxide controls, while for all four FCCP-treated samples, there were no significant differences in such activity compared with the respective non-F CCP controls (Fig. 8). These results show that, even in presence of a K_{ATP} channel blocker, diazoxide interfered with alkaline release of IF1 from F_{0}F_{1} ATP synthase. Furthermore, diazoxide had this effect even when IF1 binding was increased by the depolarizing effects of FCCP.

**DISCUSSION**

Because an improved mitochondrial energy state is recognized to play an important role in the biochemical mechanisms of IPC and pharmacological preconditioning (2, 24, 43, 55), we used cultured rat H9c2 myoblasts to characterize the effects elicited by diazoxide on mitochondrial F_{0}F_{1} ATP synthase in intact cells. In this model system, under normoxic conditions, diazoxide inhibited the ATPase activity of F_{0}F_{1} ATP synthase in a transient manner, with a maximal effect of 26% at 100 μM. This effect of diazoxide coincided with a transient and mild mitochondria uncoupling and membrane depolarization but was achieved without reducing ATP synthesis flux or cellular ATP content. Concerning ATP synthesis flux, the limitation of the experimental setup consisting of the fact that oligomycin increases proton motive force by inhibiting its dissipation by ATP synthesis as well as proton leak proportionally should be considered. Nevertheless, because diazoxide had no effect on oligomycin sensitivity of basal respiration, we can expect that the limitation was common to both control and diazoxide-treated cells, thus not altering the finding that ATP synthesis rate was not affected by diazoxide. Our finding are consistent with those of Fryer et al. (17), who found that the rate of ATP synthesis in isolated mitochondria of diazoxide-treated heart was not affected by the drug. Furthermore, the unaltered ATP levels were in accordance. The mitochondrial effects of diazoxide were blocked by pretreating cells with glibenclamide, a K_{ATP} channel blocker. However, even in the presence of glibenclamide or FCCP (which strongly uncouples and depolarizes mitochondria), the effect of diazoxide was observed as a lower ATPase activity in homogenates prepared by sonication at pH 9.2 (i.e., by an effective way of releasing bound IF1). These results suggest that diazoxide elicits its effects through interference with IF1 binding and/or release, although other pH-dependent pharmacological effects of the drug cannot be ruled out. Moreover, these results are consistent with a dual effect of diazoxide leading to ATPase inhibition: by mildly uncoupling and depolarizing mitochondria, diazoxide indirectly favors binding of IF1 to F_{0}F_{1} ATP synthase and thereby inhibits ATPase activity; in addition, diazoxide directly alters IF1 binding/release to/from F_{0}F_{1} ATP synthase, as IF1 alkaline stripping is less effective. This is in accordance with our previous report (9), in which we described a lower K_{d} value of IF1 binding to purified F_{1} in the presence of diazoxide,
although it did not influence the energization-dependent IF\textsubscript{1} release. The selective action of diazoxide, inhibiting the hydrolase but not the synthase activity of F\textsubscript{0}F\textsubscript{1} ATP synthase, may be responsible for the beneficial effects of pharmacological preconditioning on bioenergetics and cell survival during ischemia-reperfusion. A similar action has been reported by Grover et al. (21) for a series of selective ATPase inhibitors.

In the cell system investigated here, the maximal effect of diazoxide on F\textsubscript{0}F\textsubscript{1} ATP synthase was observed at 100 \(\mu\text{M}\), a relatively high concentration but nonetheless within the pharmacological range. Although some effects have been observed at lower concentrations (18, 39), other authors have also used 100 \(\mu\text{M}\) (1, 22). To our knowledge, no authors have measured cell-associated concentrations of diazoxide, which may vary in different cells depending on the efficiency of drug-extruding machinery. When H9c2 cells were exposed to 100 \(\mu\text{M}\) diazoxide, the cell-associated drug concentrations were 30–40 \(\mu\text{M}\) over 30 min. Therefore, in the experiments reported here, low intracellular concentrations of diazoxide should have helped avoid undesired effects such as succinate dehydrogenase inhibition (22, 48).

Our results regarding the ability of IF\textsubscript{1} in inhibiting ATPase activity in rat cells in response to stimuli (i.e., diazoxide exposure) are in agreement with those of Das (13) and Hassanen et al. (24), who observed in hearts of small rodents IF\textsubscript{1}-dependent inhibition of ATPase during ischemia and IPC; conversely, Rouslin and Broge (46) reported no significant inhibition. The low level (20\% of bound IF\textsubscript{1}) observed in H9c2 cells under normoxic, physiological conditions is in accordance with Schwerzmann and Pedersen (49) and Rouslin (45), who both reported a 0.2 ratio of bound IF\textsubscript{1} to catalytic sector F\textsubscript{1} in rat. Finally, the modest mitochondrial uncoupling achieved with diazoxide in H9c2 cells is similar to that reported by Minners et al. (38, 39), who indicated properly in a modest uncoupling the cellular response of preconditioning-mediated cardioprotection. The mechanism by which uncoupling of oxidative phosphorylation in preconditioning results in a beneficial adaptive mitochondrial response to cellular stress has not been established yet. It may include reduction of excessive reactive oxygen species generation in the context of ischemia (41), uncoupling-induced augmentation of glucose uptake (31), and preservation of mitochondrial calcium overload (26). Inhibition of ATPase activity of F\textsubscript{0}F\textsubscript{1} ATP synthase, due to the dual effect of diazoxide on IF\textsubscript{1} binding documented here, may be central to the adaptive response by attenuating ATP depletion. It should be emphasized that diazoxide elicited decrease of \(\Delta\psi\text{m}\) and enhancement of respiration, but not decrease of mitochondrial ATP synthesis and enhancement of ATP hydrolysis. Therefore, diazoxide cannot be considered to act as a “conventional protonophore” under the experimental conditions we used, although we cannot exclude such an effect on a different timescale. We can speculate that the expected increase of ATP hydrolysis may be counteracted by the effect altering the steady state of IF\textsubscript{1} binding/release to/from the F\textsubscript{0}F\textsubscript{1} ATP synthase that diazoxide afforded concomitantly, resulting in promotion of IF\textsubscript{1} binding and inhibition of ATPase activity. Conversely, at present we do not have a clear explanation for the finding that the expected decrease in ATP synthesis flux was undetectable.

Diazoxide uncoupling may be due to \(\text{K}^+\) influx induced by the opening of mitoK\textsubscript{ATP} channels and/or to the “protonophoric” effect of the drug (23, 27, 38, 39). Unfortunately, we do not know whether or not \(\text{K}^+\) cycling contributes to the uncoupling and mitochondrial depolarization elicited by diazoxide in our conditions. We did not perform experiments addressing this question, considering that a number of side effects, possibly occurring by culturing cells under conditions of low \(\text{K}^+\) concentration, may challenge data reliability in intact cells. On the other hand, with regard to the effect afforded by diazoxide on F\textsubscript{0}F\textsubscript{1} ATP synthase and promoting IF\textsubscript{1} binding to the enzyme, in our previous paper (9) we reported experiments run with isolated membrane-bound enzyme and documented that IF\textsubscript{1} binding to submitochondrial particles was similarly affected by diazoxide in both the absence and the presence of 75 mM K\textsubscript{2}SO\textsubscript{4}. Thus we may suggest that in cells the effect altering the steady state of IF\textsubscript{1} binding/release to/from the enzyme was also afforded by diazoxide independently of \(\text{K}^+\) cycling. Conversely, the effect inducing IF\textsubscript{1} binding and mediated by mitochondrial depolarization possibly depended on energy-diverting \(\text{K}^+/\text{H}^+\) cycling. In the resting state of our cells, in which \(\text{O}_2\) consumption is low and \(\Delta\psi\text{m}\) is high, concomitant with the effects observed by us (i.e., mild loss of \(\Delta\psi\text{m}\) and inhibition of ATPase activity mediated by IF\textsubscript{1} binding), a modest increase of mitochondrial \(\text{K}^+\) influx and matrix expansion may be caused by diazoxide, as suggested by Garlid and coworkers (11, 15, 19, 20). In this hypothesis, the induction of IF\textsubscript{1} binding to ATP synthase consequent to mild loss of \(\Delta\psi\text{m}\) afforded by diazoxide may be an additional molecular consequence of mitoK\textsubscript{ATP} channel opening, besides preservation of mitochondrial intermembrane architecture and of the normal low outer membrane permeability to ADP/ATP (15). Our data concerning the protective effect of glibenclamide may prompt us to conclude, within a pharmacological approach, that this was the case. Nevertheless, metabolic effects of glibenclamide not related to the activity of specific K\textsubscript{ATP} channels have been reported (23) and must be considered, together with the finding that the sensitivity of mitochondrial Na\textsuperscript{+} and \(\text{K}^+\) channels to glibenclamide critically depends on factors such as Mg\textsuperscript{2+}, ATP, and GTP concentrations (29, 50).

Although mitoK\textsubscript{ATP} channels have been implicated in most studies regarding IPC and pharmacological preconditioning, there is no conclusive molecular evidence for their existence and no definitive information regarding structural properties. Studies in which mitoK\textsubscript{ATP} channels are pharmacologically manipulated, including the present study, must be interpreted with caution because of the multiple metabolic effects of drugs active at these potassium channels. As already mentioned, besides the actions on ATPase reported here and by others (2, 6, 55) and those on succinate dehydrogenase (22, 48), diazoxide also acts as a protonophoric uncoupler (26, 27, 33) and inhibits other nucleotide-requiring cellular ATPases (16). Furthermore, glibenclamide reduces fatty acid oxidation by inhibiting carnitine palmitoyltransferase (35) and blocks ATP-binding cassette transporters (42) and chloride channels (56); whether these actions, other than K\textsubscript{ATP} channel inhibitors, play a role in its ability to block diazoxide-induced preconditioning remains to be tested. Drug potency and targets may be critically dependent on the metabolic state of the cells and on the experimental conditions chosen (29).

As reported by many authors (2, 6, 24, 55), we believe that reducing ATP hydrolysis rate in ischemia is a fundamental
aspect of cardioprotection. In our opinion, both the mechanism documented by Garlid and coworkers and that suggested by us and involving a dual effect on ATP synthase may contribute to diazoxide-mediated reduction of energy waste. We therefore speculate that the modest dissipation of Δψm caused by diazoxide in the resting state of our cells, whether mediated by mitoKATP channels or by other mechanisms, could be sufficient to induce IF1 binding to ATP synthase, thereby inhibiting ATP hydrolysis catalyzed by the enzyme but not ATP synthesis. Therefore, mild uncoupling and the dual effect on ATP synthase documented by us during normoxia may represent important effects helping in the search for the mechanisms, still unknown, by which diazoxide confers cardioprotection during ischemia.

In conclusion, we observed that in normoxic intact cells, diazoxide has a dual effect on mitochondrial ATP synthase that can be summarized as follows. Diazoxide-triggered mitochondrial uncoupling and depolarization inhibit ATPase activity by promoting binding with the membrane potential-sensitive inhibitor IF1. Concomitantly, diazoxide interferes with the binding/release of IF1 to/from the enzyme, further enhancing the inhibition. The transient nature of diazoxide effects we observed remains to be explained. Nevertheless, the finding that cell-associated drug concentration significantly varies over the same timescale and parallels such effects may suggest a threshold effect of the drug concentration, which appeared to diminish to the initial value at 30 min as a likely consequence of high efficiency of drug-extruding machinery. Furthermore, it should be emphasized that in fully energized mitochondria diazoxide-induced reduction of Δψm likely has a relatively small and transient effect, whereas under conditions of metabolic stress relatively small decreases in Δψm can translate into large decreases of matrix Ca2+ and large changes in the functional state of mitochondria and specifically of ATPase/synthase. Under such conditions diazoxide effects may be thus amplified and made lasting. It is also possible that a deeper investigation of the actual relevance of energy-diverting K+/H+ cycling as a mechanism of the effects documented here (as discussed above) may provide a contribution to the explanation of their transient nature. The biological relevance of these results versus cardioprotection needs further in vivo investigation.

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DUAL EFFECT OF DIAZOXIDE ON MITOCOCHONDRIAL F0F1 ATP SYNTHASE

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