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Opening of Mitochondrial K^+ Channels Increases Ischemic ATP Levels by Preventing Hydrolysis

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Mitochondrial ATP-sensitive K^+ channels (mitoK_{ATP}) have been proposed to mediate protection against ischemic injury by increasing high-energy intermediate levels. This study was designed to verify if mitochondria are an important factor in the loss of cardiac ATP associated to ischemia, and determine the possible role of mitoK_{ATP} in the control of ischemic ATP loss. Langendorff-perfused rat hearts subjected to ischemia were found to have significantly higher ATP contents when pretreated with oligomycin or atractyloside, indicating that mitochondrial ATP hydrolysis contributes toward ischemic ATP depletion. MitoK_{ATP} opening induced by diazoxide promoted a similar protection against ATP loss. Diazoxide also inhibited ATP hydrolysis in isolated, nonrespiring mitochondria, an effect accompanied by a drop in the membrane potential and Ca^{2+} uptake. In hearts subjected to ischemia followed by reperfusion, myocardial injury was prevented by diazoxide, but not atractyloside or oligomycin, which, unlike diazoxide, decreased reperfusion ATP levels. Our results suggest that mitoK_{ATP}-mediated protection occurs due to selective inhibition of mitochondrial ATP hydrolysis during ischemia, without affecting ATP synthesis after reperfusion.

KEY WORDS: Heart mitochondria; K^+ channel; anoxia/reoxigenation; ATP synthase; mitochondrial permeability transition.

INTRODUCTION

Protection against ischemic myocardial damage can be achieved in many different manners, including treatment with opioids (Schultz *et al*., 1995; Schultz and Gross, 2001; Schwartz *et al*., 1997), acetylcholine (Yao *et al*., 1999; Yao and Gross, 1993), ATP-sensitive K^+ channel (K_{ATP}) agonists (Garlid *et al*., 1997; Grover and Garlid, 2000), mild oxidants (Baines *et al*., 1997; Tritto *et al*., 1997; Vanden Hoek *et al*., 1998), and ischemic preconditioning (Cohen and Downey, 1996; Murry *et al*., 1990; Van Winkle, 1996). Ischemic preconditioning, or the protective effect of a short, nondamaging, period of ischemia on subsequent long and normally harmful ischemia (Cohen and Downey, 1996; Murry *et al*., 1990; Van Winkle, 1996), promotes both the activation of protein kinase C (Ytrehus *et al*., 1994) and a temporary and mild increase in myocardial reactive oxygen species generation (Baines *et al*., 1997; Pain *et al*., 2000). This results in mitochondrial K_{ATP} (mitoK_{ATP}) opening, possibly though phosphorylation and critical thiol oxidation (Grover and Garlid, 2000). Indeed, preconditioning-like effects are obtained when hearts are treated with mild oxidants (Sharma and Singh, 2001; Valen *et al*., 1998; Yue *et al*., 2001), activators of protein kinase C (Tsuchida *et al*., 1994), or mitoK_{ATP} openers (Garlid *et al*., 1997), in a manner inhibited by mitoK_{ATP} antagonists (McCullough *et al*., 1991; Yao and Gross, 1993). Thus, all these cardioprotective schemes converge to a final cardioprotective result: the activation of mitoK_{ATP}.

Although mitoK_{ATP} opening may amplify cardioprotection by further increasing mitochondrial reactive oxygen generation (Forbes *et al*., 2001; Patel and Gross,

Key to abbreviations: 5-HD, 5-hydroxydecanoate; ATR, atractyloside; CK, creatine kinase; DMSO, dimethyl sulphoxide; DZX, diazoxide; FCCP, carbonyl cyanide *p*-trifluromethoxyphenylhydrazone; LDH, lactate dehydrogenase; mitoK_{ATP}, mitochondrial ATP-sensitive K^+ channels; oligo, oligomycin; RHM, rat heart mitochondria; $\Delta\Psi$, mitochondrial membrane potential.

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2001) and protein kinase C activity (Wang *et al*., 1999), a large amount of data also suggest that this channel is a coordinator of the end results of ischemic protection. As an example, ischemic preconditioning and K_{ATP} agonists improve high-energy phosphate levels in ischemia and early reperfusion (Fukuda *et al*., 2001; Grover *et al*., 1991; McPherson *et al*., 1993; Murry *et al*., 1990; Vuorinen *et al*., 1995), an effect certainly linked to changes in mitochondrial bioenergetics promoted by mitoK_{ATP}. Protection against ischemic damage has also been related to the recovery of higher mitochondrial membrane potentials (Xu *et al*., 2001), lower mitochondrial Ca^{2+} uptake (Ishida *et al*., 2001; Murata *et al*., 2001) and coupling between the adenine nucleotide translocase and creatine kinase (Laclau *et al*., 2001).

However, the exact effect of cardiac mitoK_{ATP} on mitochondrial function and how this effect promotes ischemic cardioprotection remains controversial. Initially, experiments suggested that mitoK_{ATP} opening was responsible for large drops in mitochondrial membrane potential due to K^+ cycling, resulting in a decreased capacity to accumulate mitochondrial Ca^{2+} and preventing Ca^{2+} overload-related mitochondrial dysfunction during ischemia (Holmuhamedov *et al*., 1998). However, later results showed that mitoK_{ATP} K^+ transport rates are too limited to promote a significant decline in the mitochondrial membrane potential of respiring mitochondria (Kowaltowski *et al*., 2001b). Indeed, in the absence of ischemia, cardioprotective concentrations of mitoK_{ATP} agonists do not measurably affect the membrane potential (Carrol *et al*., 2001; Kowaltowski *et al*., 2001b). In heart mitochondria incubated under physiological conditions, Ca^{2+} uptake and respiratory rates were also virtually unaffected by mitoK_{ATP} opening, and only matrix volumes were measurably increased by K^+ uptake via mitoK_{ATP} (Carrol *et al*., 2001; Kowaltowski *et al*., 2001b).

Mitochondrial volume can strongly influence the interaction between inner and outer membranes, resulting in changes in nucleotide transport into the mitochondrial matrix (Brdiczka *et al*., 1998; Gellerich *et al*., 1993). This finding lead to the hypothesis that mitoK_{ATP} could promote cardioprotection by decreasing ATP transport (and hydrolysis) into the mitochondrial matrix during ischemia (Dos Santos *et al*., 2002; Kowaltowski *et al*., 2001b). Thus, mitoK_{ATP} could decrease ATP-hydrolysis supported membrane potential generation and Ca^{2+} uptake during ischemia, without altering these parameters in respiring mitochondria. This manuscript concentrates on determining if ischemic mitochondrial ATP hydrolysis and Ca^{2+} uptake, in addition to postischemic ATP synthesis, are affected by mitoK_{ATP} opening.

MATERIALS AND METHODS

Rat Heart Perfusions

Hearts were rapidly removed from Male Sprague-Dawley rats weighing between 300 and 400 g, trimmed over ice (in order to inhibit preconditioning-induced infarct limitation), and Langendorff-perfused with 200 mL oxygenated Krebs-Hanseleit buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes, pH 7.0, at 37°C. Heart beat rates were left unpaced, and perfusion was maintained at a constant pressure of 70 mmHg. During the first 5 min of perfusion, no drugs were added to allow for heart stabilization, and a nonrecirculating mode was used to eliminate contaminating blood. After stabilization, a recirculating perfusion mode was initiated, and the DMSO-diluted drug or an equal amount of DMSO (1 μL/mL) was added directly to the perfusate. Under the conditions used, neither oligomycin nor atractyloside treatment promoted cardiac arrest prior to ischemia. In a set of preliminary experiments using 1 μg/mL oligomycin, two out of three hearts suffered cardiac arrest prior to ischemia, and the drug dose was reduced.

Global heart ischemia was initiated 10 min after drug administration by stopping the perfusion and immersing hearts in 37°C perfusate. After 30-min ischemia, hearts were either homogenized for ischemic ATP content determination or reperfused with recirculating drug-free Krebs-Hanseleit buffer for 30 min. Aliquots (1 mL) of the perfusate were collected for creatine kinase and lactate dehydrogenase activity determination after 30 min reperfusion.

All studies were conducted in accordance with institutional guidelines for animal care. Six hearts were studied under each experimental condition, for a total of 72 perfused hearts included in this study. Hearts were eliminated from the study if the time between heart removal and the beginning of perfusion was >3 min or if air bubbles were present in the system ($n = 4$).

Heart ATP Contents

Hearts were rapidly removed from the perfusion apparatus. Their ventricles were excised, weighed and placed in 6 mL of 0.8 M $HClO_4$, a procedure that took less than 20 s. The tissue was then homogenized, the pH adjusted between 5 and 8 with KOH, and volumes completed to 10 mL. Samples were stored frozen at −80°C. Prior to ATP determination, the thawed samples

were centrifuged, and the supenatants were diluted 1:10 in 1 M phosphate buffer, pH 7.0. ATP concentrations were determined by light emission at 560 nm on a Hitachi F4500 spectrofluorometer using 40 mg/mL of a commercial luciferin–luciferase kit (Sigma® L0633 or Promega® FF2021), as described by Ellis and Gardner (1980). Light emission during the first 120 s following the addition of luciferin–luciferase was integrated, and data were calibrated using known concentrations of ATP. Data were expressed as total ventricular ATP content, which equals the concentration of ATP measured in the undiluted sample multiplied by 0.01 and correlates well with ATP levels/dry weight (Ellis and Gardner, 1980).

Creatine Kinase Activity

Creatine kinase activity was determined in frozen perfusate aliquots using commercial kits (Doles® 1070 or Sigma® 49A), accompanying the time-dependent formation of NAD(P)H at Ex = 352 nm and Em = 464 nm on a Hitachi F4500 spectrofluorometer between 5 and 10 min after the reaction was started, when traces showed maximum linearity. Curves were calibrated using lyophilized bovine heart creatine kinase.

Lactate Dehydrogenase Activity

Lactate dehydrogenase activity was determined in refrigerated perfusate aliquots exactly as described by Sigma's LD-500 kit. Final absorbencies were determined at 525 nm on an Ultrospec 1000 spectrophotometer.

Mitochondrial Isolation

Rat heart mitochondria were isolated exactly as described previously (Kowaltowski *et al*., 2001b). Briefly, rat hearts were quickly excised and washed in ice-cold buffer containing 300 mM sucrose, 10 mM K^+-HEPES buffer, pH 7.2, and 1 mM K^+ EGTA. The tissue was finely minced and incubated in the presence of 1 mg nagarse during 10 min. Excess nagarse was removed by washing the heart fragments in the same buffer containing 1 mg/mL BSA, and the samples were homogenized manually. The resulting suspension was centrifuged at 600*g* for 4 min, and the supernatant recentrifuged at 9000*g* for 8 min. The mitochondrial pellet was then washed once or twice until a blood-free, compact pellet was obtained. This pellet was suspended in a 200–300 μL of BSA-containing buffer and kept over ice. Figure 1

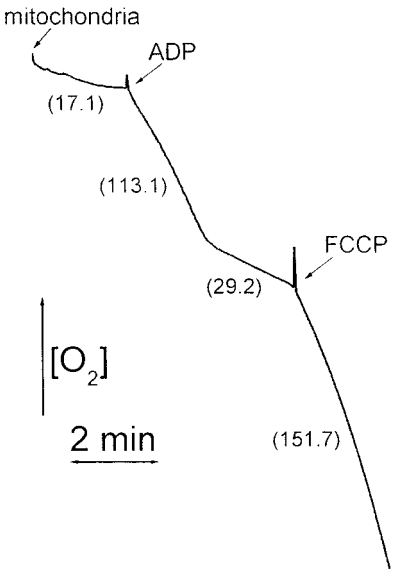

Fig. 1. Respiratory control of isolated rat heart mitochondria. Isolated rat heart mitochondria (RHM) were incubated at 37°C in 125 mM sucrose, 65 mM KCl, 10 mM Hepes, pH 7.2, 2 mM K^+ phosphate, 1 mM Mg^{2+}, 2 mM succinate, and 1 μM rotenone. ADP (100 μM) and FCCP (1 μM) were added where indicated. Respiratory rates (ng atom O · min^{-1} · mg^{-1}) are indicated in parenthesis.

shows a typical respiratory control trace of these isolated mitochondria, measured using a Clark-type electrode in an air-tight, temperature controlled chamber. Oxygen consumption was coupled to oxidative phosphorylation, since respiratory rates increased when ADP was added. Respiration spontaneously decreased after the added ADP was phosphorylated, although the respiratory rate now (in parenthesis) was slightly higher than that observed in the absence of added ADP. This effect is typical of heart mitochondria, which are rich in ATPase activity, maintaining a low level of ADP production. Under our conditions, ATPase activity was low enough to allow mitochondria to spontaneously resume a slower respiratory rate, but was sufficient to decrease ATP concentrations from 200 μM to approximately 150 μM after 5 min incubation. This effect was taken into account when ATP hydrolysis rates were determined in isolated mitochondria (see Fig. 3(D)).

Mitochondrial Membrane Potential Estimation

Mitochondrial membrane potentials were estimated by following safranin fluorescence (Akerman and Wikstrom, 1976) at Ex = 495 nm and Em = 586 nm on a Hitachi F4500 spectrofluorometer, under the conditions described in the figure legend.

Mitochondria Ca^{2+} Uptake

Mitochondrial Ca^{2+} uptake was estimated from fluorescence changes of 0.1 μM Ca^{2+} green 5N (hexapotassium salt), using a Hitachi F4500 spectrofluorometer at excitation and emission wavelengths of 506 and 531 nm, respectively (Kowaltowski *et al*., 2001b).

Anoxic Incubations

The reaction medium was extensively purged with N_2 (10–20 min), a condition in which oxygen was undetectable by a Clark-type electrode (Hansatech Instruments). Oxygenated media contained 210–230 nmol oxygen/mL.

ATP Hydrolysis by Isolated Mitochondria

Isolated mitochondria (0.5 mg/mL) were incubated during 5 min in anoxic media containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes, pH 7.2, 1 mM Mg^{2+}, 2 mM succinate, and 1 μM rotenone, in the presence of 0.2 mM ATP. Other additions are indicated in the figure legend. After 5 min, the mitochondrial suspensions were treated with 1 μg/mL oligomycin, frozen in liquid nitrogen, thawed to 4°C and quickly centrifuged to remove mitochondrial membrane fragments. ATP concentrations in the supenatant were estimated by integrating light emission promoted by the addition of 40 mg/mL luciferin/luciferase (Ellis and Gardner, 1980). An experiment in which the incubation was conducted in the presence of oligomycin indicated the ATP concentration decrease unrelated to the mitochondria ATP synthase under our conditions (see Fig. 3(D)).

Data Analysis

Data analysis was conducted using SigmaStat® multiple pairwise Tukey tests for comparisons between experimental groups or Spearman rank order correlations for determining the significance of correlations. Data shown represent averages ± SEM of six repetitions, except data in Figs. 1, 3(A) and (B), and 4(A), which are representative traces of similar experiments conducted using at least three different mitochondrial preparations.

RESULTS

Mitochondrial ATP Hydrolysis Occurs During Cardiac Ischemia

The mitochondrial ATP synthase, normally responsible for ADP phosphorylation, is reversible in the absence of a proton gradient, hydrolyzing ATP and pumping protons into the intermembrane space. During ischemia, the lack of oxygen and substrates may lead to mitochondrial ATP hydrolysis, energetic depletion and cell death.

In order to determine the importance of ATP hydrolysis during ischemia and the effect of mitoK_{ATP} on this hydrolysis, we measured ventricular ATP contents in Langendorff-perfused rat hearts after 30 min of global ischemia conducted in the presence of ATP synthase inhibitors and mitoK_{ATP} regulators. As observed previously (Garlid *et al*., 1997), considerable ischemic contracture occurred in untreated hearts during this period, with an average onset 9 ± 1.5 min after perfusion was halted, but not in hearts pretreated (10 min) with 30 μM diazoxide (a mitochondrially-specific K_{ATP} opener, Garlid *et al*., 1996). In addition, we found ATP to be very severely depleted in ischemic hearts (Fig. 2) in a manner prevented by diazoxide and reversed by 200 μM 5-hydroxydecanoate, an inhibitor of mitoK_{ATP} (Jaburek *et al*., 1998). These results are in full agreement with previous data showing that non-mitochondrially selective K_{ATP} openers increase ATP levels in ischemic hearts (Fukuda *et al*., 2001; Grover *et al*., 1991; McPherson *et al*., 1993; Tanonaka *et al*., 1999).

Interestingly, pretreating hearts with 0.5 μg/mL oligomycin, an inhibitor of the ATP synthase, or 10 μM atractyloside, which prevents nucleotide transport into the mitochondrial matrix, also increased ventricular ATP levels, to an extent very similar to that promoted by diazoxide.

MitoK_{ATP} Opening Prevents Mitochondrial ATP Hydrolysis

Since ischemic ATP levels were similar when hearts were pretreated with ATP synthase inhibitors and diazoxide, we evaluated if the higher ATP levels found in ischemic hearts treated with diazoxide were related to an inhibition of ATP hydrolysis. Isolated heart mitochondria were used to measure the membrane potential sustained by proton pumping both at the respiratory chain and by the ATP synthase (Fig. 3(A)). In oxygenated media, this membrane potential was unaffected by diazoxide, both in the absence (results not shown, see Kowaltowski *et al*., 2001b) and presence of added ATP (compare lines a and b). This confirms previous results showing that heart mitoK_{ATP}

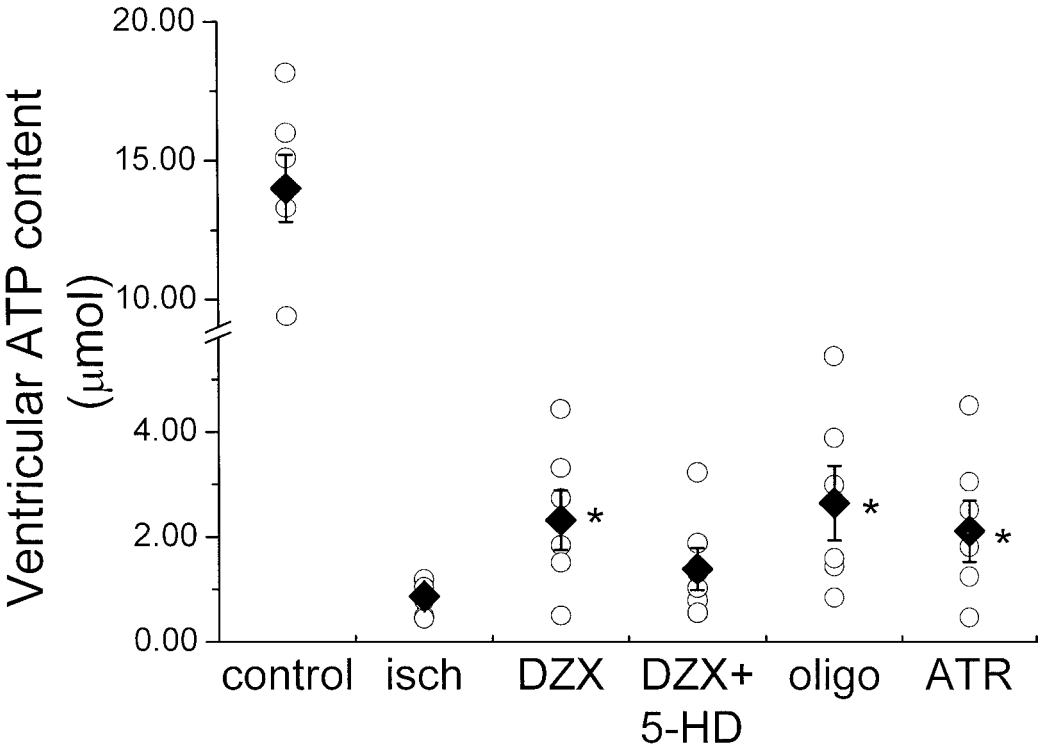

Fig. 2. Ventricular ATP levels in ischemic hearts are increased by mitoK$_{ATP}$ opening and ATP synthase inhibition. Rat hearts were subjected to 30 min global normothermic ischemia (as described in Materials and Methods) after 10 min perfusion in the presence of no further additions (isch), 30 μM diazoxide (DZX), 30 μM diazoxide and 200 μM 5-hydroxydecanoate (DZX + 5-HD), 0.5 μg/mL oligomycin (oligo), or 10 μM atractyloside (ATR). Control (nonischemic) hearts were maintained perfused during the extent of the complete experiment. Estimated total ventricular ATP levels are represented for six individual hearts (○) under each condition, in addition to the averages ± SEM (♦). $^*p < 0.05$, when compared to untreated ischemic hearts.

K^+ transport rates are too limited to measurably affect respiration-supported membrane potentials (Kowaltowski *et al*., 2001b). In media in which O_2 was eliminated to undetectable levels by extensive N_2 purging, no membrane potential was observed unless exogenous ATP was added (lines c and d). The addition of ATP resulted in the build-up of a hydrolysis-supported membrane potential which, as observed previously (St-Pierre *et al*., 2000), was lower than the membrane potential supported by respiration. Diazoxide prevented ATP-sustained membrane potential generation (compare lines c and d, Fig. 3(A)), suggesting that it inhibits mitochondrial ATP hydrolysis. Indeed, in a series of six experiments conducted using three different mitochondrial preparations, diazoxide significantly reduced the fluorescence response measured after the addition of ATP (Fig. 3(C)). When the same experiment was conducted in media containing only Na^+ salts (Fig. 3(B) and (C)), diazoxide did not alter the ATP-supported membrane potential, linking this membrane potential effect to mitoK$_{ATP}$, which does not transport Na^+.

Parallel measurements of ATP levels in the supernatants of these anoxic mitochondrial suspensions indicated that, indeed, ATP hydrolysis was inhibited by diazoxide (Fig. 3(D)). Here, mitochondria were incubated under anoxic conditions similar to those in Fig. 3(A), in the presence of 200 μM ATP. After 5 min, mitochondria were frozen, ruptured, the membrane fragments removed, and the remaining ATP concentrations measured. We found that control mitochondria, in which mitoK$_{ATP}$ was closed, hydrolyzed more ATP than mitochondria in which mitoK$_{ATP}$ was opened by diazoxide or in which the ATP synthase was inhibited by oligomycin.

We have previously shown that mitoK$_{ATP}$ does not alter Ca^{2+} uptake in respiring mitochondria (Kowaltowski *et al*., 2001b), because the K^+ transport rate through the channel is insufficient to decrease the membrane potential (and driving force for Ca^{2+} uptake). Since in anoxic mitochondria we found that mitoK$_{ATP}$ opening decreases the membrane potential, we decided to evaluate if Ca^{2+} uptake could be affected under these anoxic conditions.

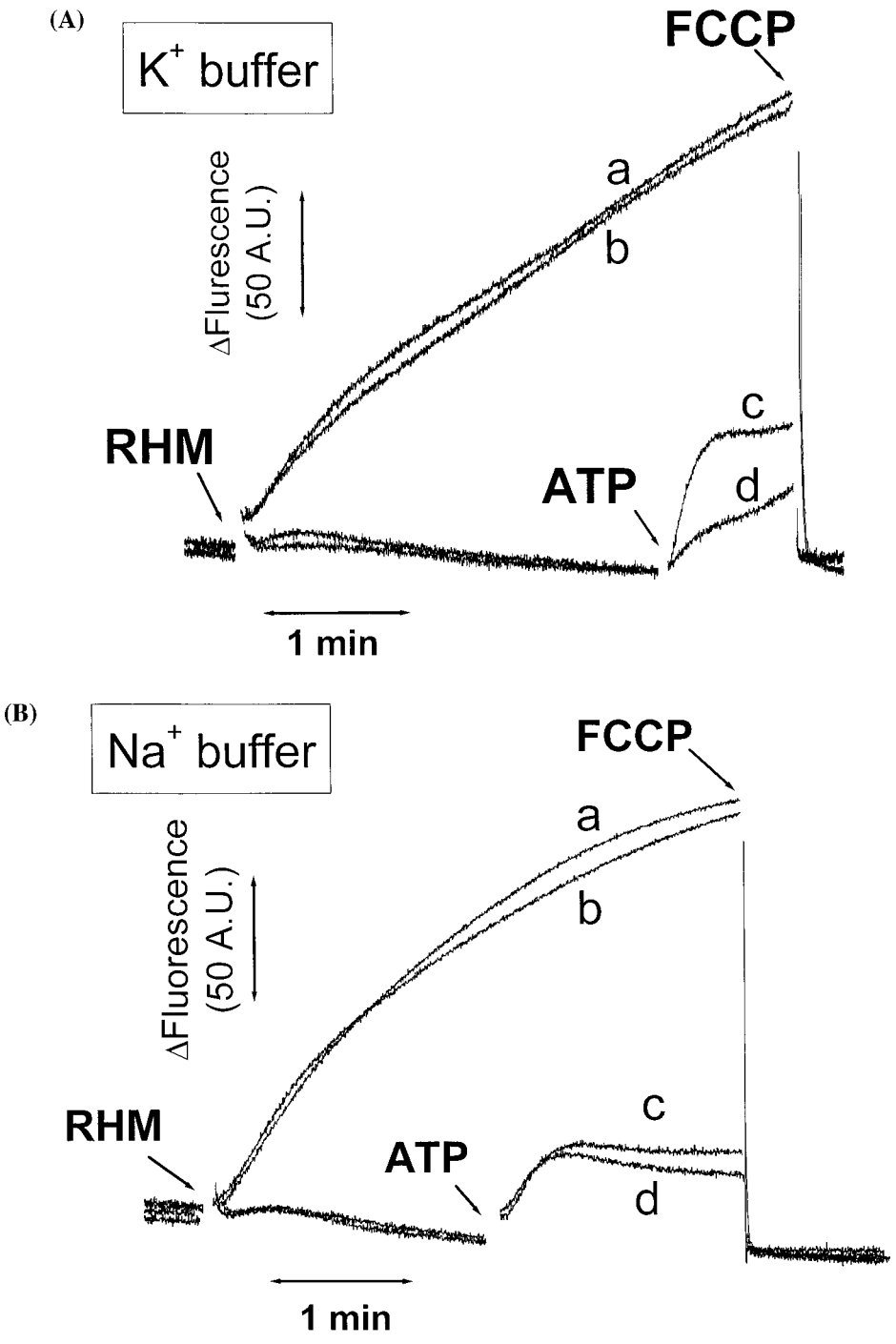

Fig. 3. MitoK_{ATP} prevents ATP hydrolysis in anoxic mitochondria. RHM (0.5 mg/mL) were incubated at 37°C in 125 mM sucrose, 65 mM KCl (Panels A and D) or NaCl (Panel B), 10 mM Hepes, pH 7.2, 1 mM Mg^{2+}, 2 mM succinate, 1 μM rotenone, 5 μM safranin (Panels A–C), 0.2 mM ATP (lines a and b), and 30 μM diazoxide (lines b and d) at 37°C, and their membrane potential was estimated as described in Materials and Methods. ATP (0.2 mM, lines c and d) and 1 μM FCCP (all traces) were added where indicated. The experiments shown in lines c and d were conducted in reaction media purged with N_2 to achieve anoxia. In Panel C, data from six experiments such as those in Panels A and B were pooled, and safranin fluorescence measured 1 min after the addition of ATP was subtracted from the fluorescence in the presence of FCCP to determine ΔFluorescence. In Panel D, RHM (0.5 mg/mL) were incubated in anoxic media in the presence of 0.2 mM ATP (control), 0.2 mM ATP and 30 μM diazoxide (DZX) or 0.2 mM ATP, and 1 μg/mL oligomycin (oligo). ATP contents ($n = 6$) were determined after 5 min as described in Materials and Methods. $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared to controls.

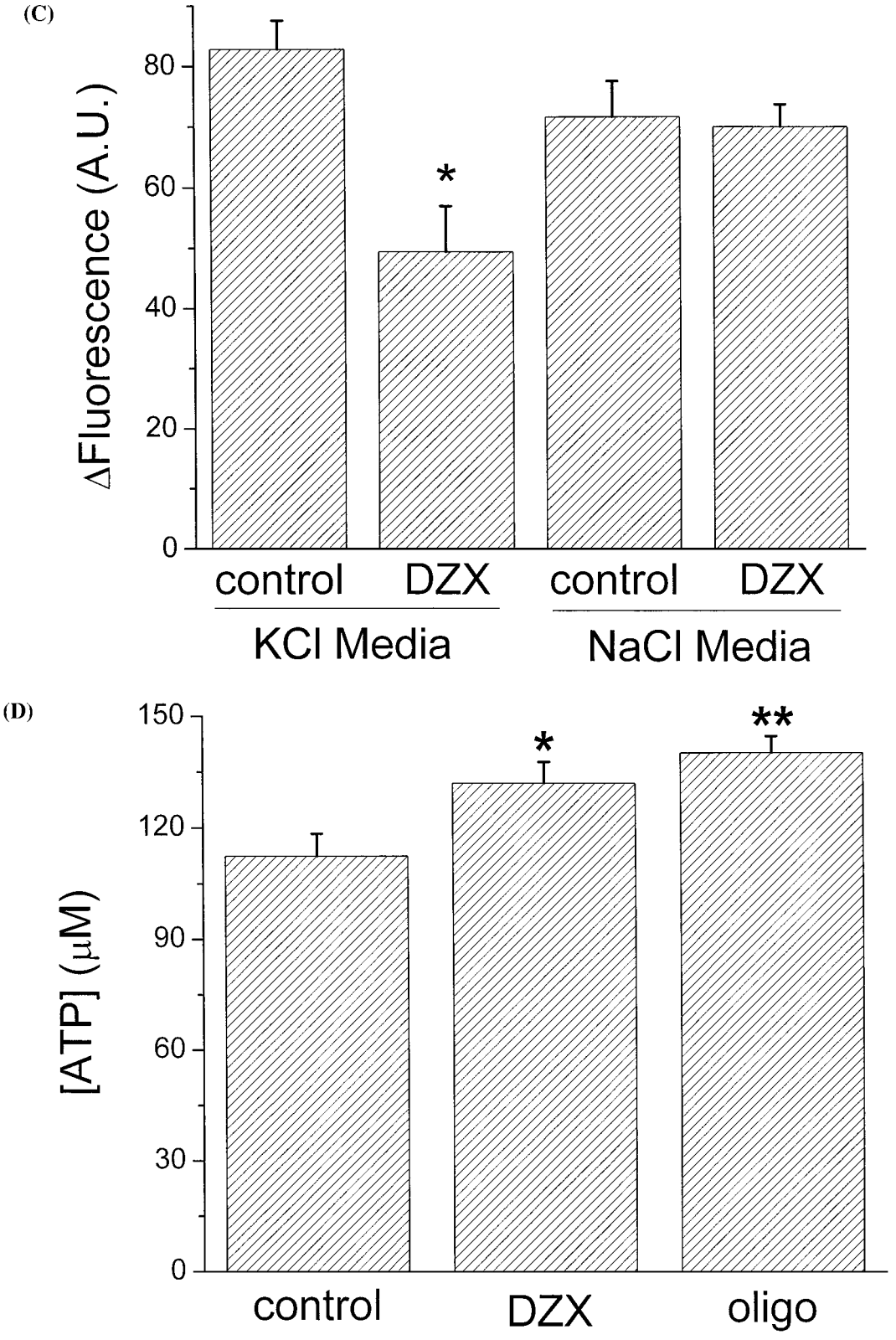

Fig. 3. (*Continued*)

Figure 4 shows that Ca^{2+} uptake in anoxic mitochondria treated with diazoxide is significantly reduced in comparison to control mitochondria. The strong inhibitory effect of oligomycin on this uptake indicates that it is dependent on the membrane potential generated by ATP hydrolysis. Thus, although mitoK_{ATP} does not significantly affect the membrane potential and Ca^{2+} uptake supported by respiration (Kowaltowski *et al*., 2001b), it does decrease these parameters in anoxic preparations, in which ATP hydrolysis generates the membrane potential.

Ischemic ATP Levels Determine Reperfusion Myocardial Injury

In order to correlate our previous findings to ischemic heart damage, we subjected a second group of hearts to 30 min ischemia followed by 30 min reperfusion. Creatine kinase (CK) activity in the perfusate was measured to evaluate myocardial injury. As expected, hearts subjected to ischemia/reperfusion showed higher CK activity in the perfusate (Fig. 5(A)), an effect prevented by diazoxide and reversed by 5-hydroxydecanoate. Oligomycin and atractyloside promoted very variable effects on reperfusion CK release. While some hearts treated with these drugs exhibited low CK release, some hearts released CK in levels much higher than untreated hearts. Lactate dehydrogenase (LDH) levels in the perfusion liquid, determined in parallel, closely matched relative CK release levels in the individual hearts (Fig. 5(B), Spearman rank order correlation $= 0.93$, $p < 0.01$). This result suggests that CK activity determination correctly reflects tissue damage, and that a difference in sensitivity of individual hearts to oligomycin and atractyloside occurs.

Although neither oligomycin nor atractylate were present in the perfusate during reperfusion, we found that reperfusion ATP levels in hearts pretreated with these drugs were significantly decreased in relation to control hearts (Fig. 6). Thus, these drugs were not completely washed out during reperfusion and impaired ATP synthesis. In contrast, ATP levels in diazoxide-treated hearts did not differ significantly from control hearts. When analyzed together with the results in Figs. 2–5, these data suggest that diazoxide protects against damage by decreasing ischemic ATP hydrolysis without hampering ATP synthesis after reperfusion.

DISCUSSION

Since the initial observation that opening ATP-sensitive K^+ channels results in reduced ischemic injury (Grover *et al*., 1989), many studies have concentrated on understanding the mechanism through which drugs such as nicorandil or pinacidil promote cardioprotection. Although sarcolemal K_{ATP} channels were first assumed to mediate this cardioprotection, later evidence suggested that protection could be achieved without any alteration in plasma membrane K^+ transport (Grover and Garlid, 2000).

The description of ATP-sensitive K^+ channels in the inner mitochondrial membrane (Inoue *et al*., 1991; Paucek *et al*., 1992) suggested a possible intracellular target for these cardioprotective drugs. Indeed, by studying relative sensitivity to pharmacological regulators, Garlid's group located a selective opener of mitoK_{ATP} (diazoxide, which required concentrations 2000 times higher to open sarcolemal K_{ATP}, Garlid *et al*., 1996), and demonstrated that this drug was an effective cardioprotective agent at concentrations that only affected mitoK_{ATP} (Garlid *et al*., 1997). The role of mitoK_{ATP} in ischemic cardioprotection was later confirmed by a number of independent groups (Baines *et al*., 1997; Sato *et al*., 2002; Wang *et al*., 1999).

Subsequent work demonstrated that mitoK_{ATP} channels were also involved in the mechanism through which a variety of conditions promoted ischemic protection, including ischemic preconditioning (Baines *et al*., 1997) and treatment with adenosine and opioids (Huh *et al*., 2001; Zhao *et al*., 2001). The fact that all these cardioprotective schemes converge to mitoK_{ATP} opening suggests that this channel regulates key conditions for protection of the ischemic heart.

MitoK_{ATP} opening is most effective when added prior to or during early ischemia (Das *et al*., 2001). Thus, we investigated if mitoK_{ATP} could play a role in ATP hydrolysis promoted by the mitochondrial ATP synthase under ischemic conditions (see Fig. 7). ATP hydrolysis by the ATP synthase is limited by the presence of the inhibitor subunit (for review, see Green and Grover, 2000) which is activated during early ischemia (Vander Heide *et al*., 1996). Nevertheless, mitochondrial ATP hydrolysis is still quite substantial in rat heart tissue, since inhibitor subunits are not necessarily present in all ATP synthase complexes (Green and Grover, 2000; Rouslin, 1991) and the inhibitory effect of this protein is not total, ranging from 50 to 80% (Rouslin *et al*., 1997). We have confirmed that ATP hydrolysis occurs during ischemia using a whole rat heart model by showing that the ATP synthase inhibitor oligomycin and the adenine nucleotide translocase inhibitor atractyloside increase the levels of ATP at 30 min global ischemia (Fig. 2). This result is in agreement with previous results (Green *et al*., 1998; Vuorinen *et al*., 1995) showing a similar effect of oligomycin, but is in contrast with the results from Rouslin's group, who found

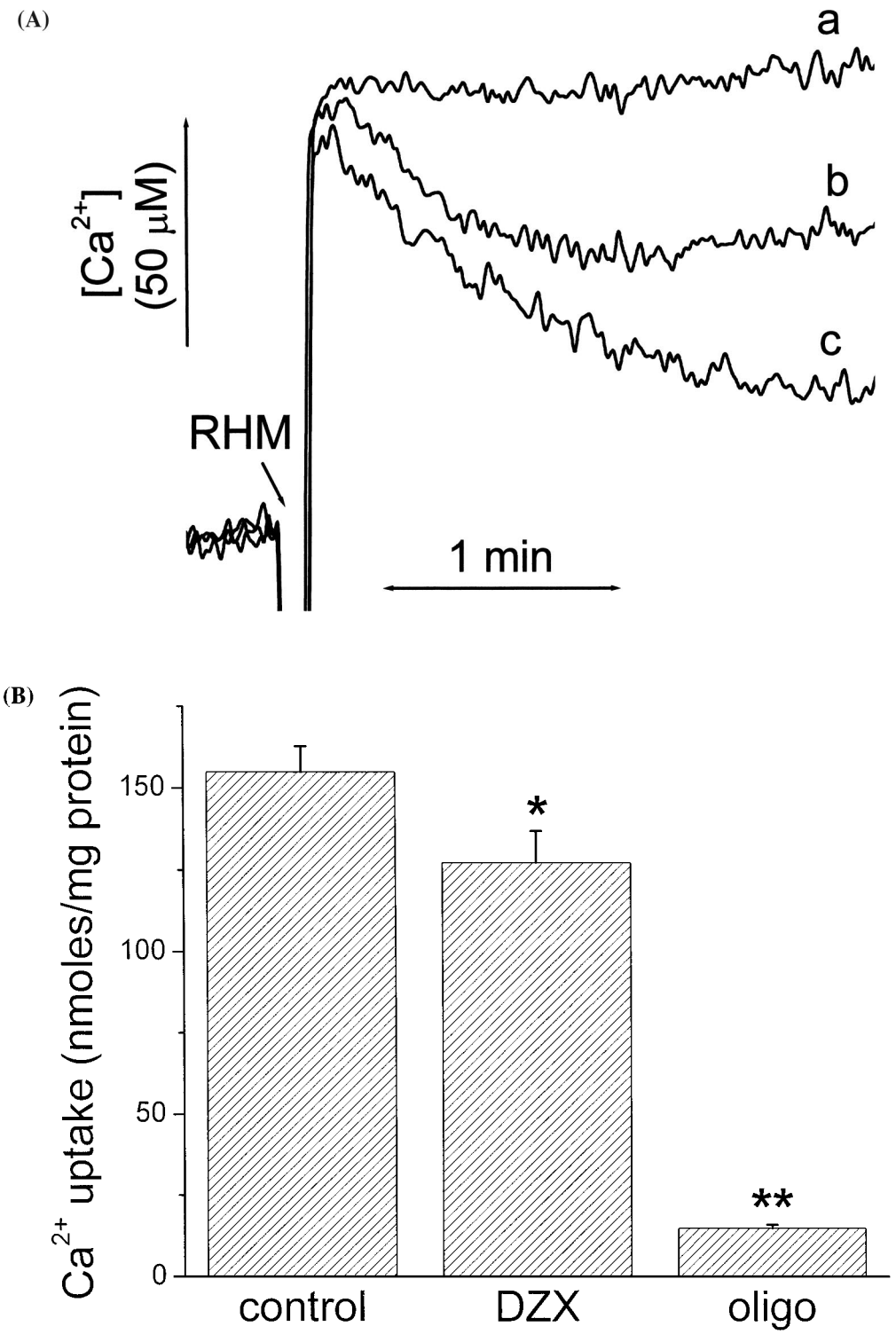

Fig. 4. MitoK_{ATP} prevents Ca^{2+} uptake in anoxic mitochondria. In Panel A, RHM (0.5 mg/mL) were incubated at 37°C in anoxic KCl media under conditions similar to Fig. 2, in the presence of 0.2 mM Ca^{2+}, 0.2 mM ATP (all lines) and 1 μg/mL oligomycin (line a), 30 μM diazoxide (line b), or no further additions (line c). Extramitochondrial Ca^{2+} levels were monitored in the presence of 0.1 μM Ca^{2+} Green, as described in Materials and Methods. In Panel B, Ca^{2+} uptake (per mg mitochondrial protein) was determined 2 min after the addition of RHM in six experiments such as those shown in Panel A. $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared to controls.

that oligomycin promoted an increase in ischemic rat ventricular ATP levels only at 20 min ischemia (Rouslin *et al*., 1990).

Diazoxide treatment promoted a similar increase in heart ATP levels, a finding which, added to the knowledge that the drug is mitochondrially active, suggests that it may also prevent mitochondrial ATP hydrolysis. The data shown in Fig. 2 confirm this idea by demonstrating that, in isolated anoxic heart mitochondrial suspensions, diazoxide decreases the membrane potential sustained by ATP hydrolysis and maintains higher ATP contents. The effects of mitoK$_{ATP}$ opening on ATP hydrolysis in ischemic mitochondria are in line with previous data showing that preconditioned hearts (in which mitoK$_{ATP}$ is activated by brief periods of ischemia) present slower ATP depletion rates (Murry *et al*., 1990). It should be noted that ATP hydrolysis activity in submitochondrial particles of preconditioned hearts has been measured previously, and found to be equal to that of control hearts (Vander Heide *et al*., 1996). However, mitoK$_{ATP}$ regulation cannot change ATP hydrolysis rates under these conditions, since the channel does not regulate matrix K^+ levels in inside-out submitochondrial particles.

Although the exact mechanism through which diazoxide decreases ATP hydrolysis is unknown, it may involve changes in mitochondrial permeability to nucleotides (Dos Santos *et al*., 2002) when mitochondrial volume is increased by K^+ transport through this channel (Carroll *et al*., 2001; Kowaltowski *et al*., 2001b). In respiring isolated heart mitochondria, mitoK$_{ATP}$ promotes K^+ ion transport into the matrix, stimulated by the membrane potential (Jaburek *et al*., 1998; Kowaltowski *et al*., 2001b). Because the K^+ transport rate is extremely limited, this K^+ entry does not

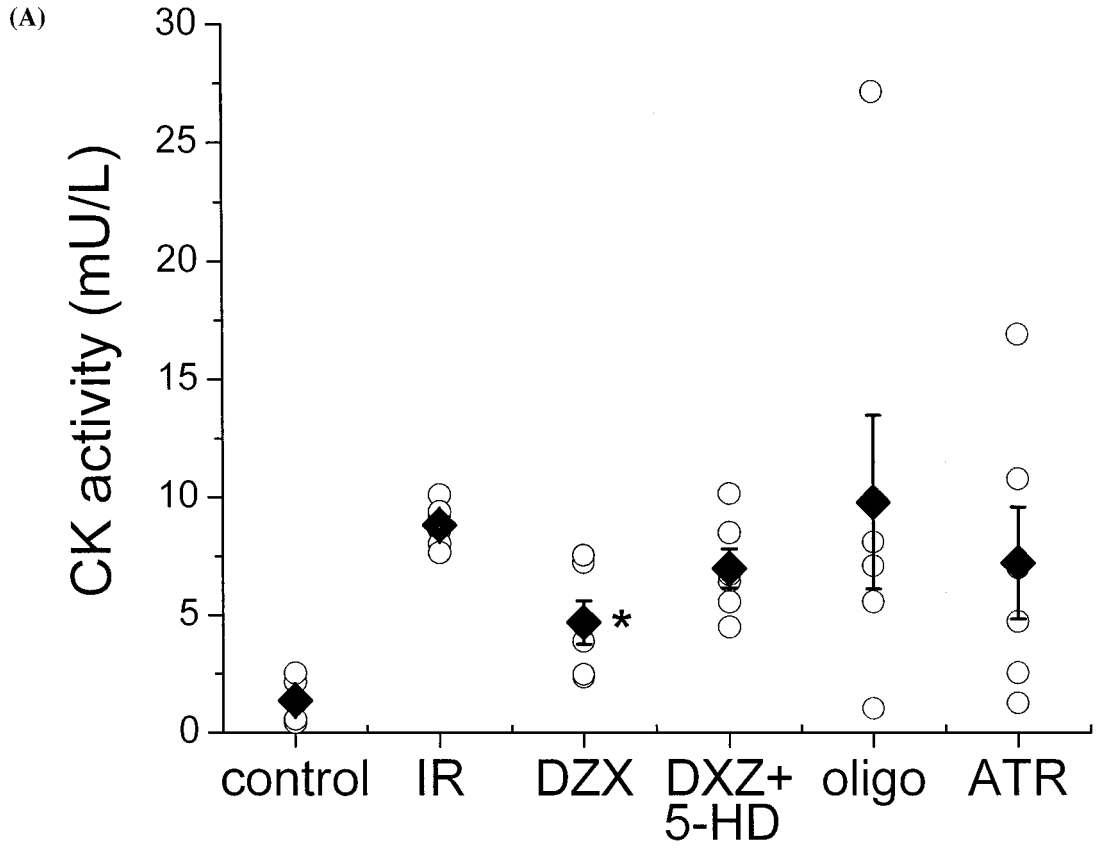

Fig. 5. CK and LDH activity in perfusion liquids from hearts subjected to ischemia/reperfusion. Rat hearts were subjected to 30 min global normothermic ischemia and 30 min reperfusion, after 10 min pretreatment with no drugs (IR), 30 μM diazoxide (DZX), 30 μM diazoxide and 200 μM 5-hydroxydecanoate (DZX + 5-HD), 0.5 μg/mL oligomycin (oligo), or 10 μM atractyloside (ATR). Control (nonischemic) hearts were maintained perfused during the extent of the complete experiment. Creatine kinase (CK) and lactate dehydrogenase (LDH) activity in the perfusion liquid were measured as described in Materials and Methods. $^*p < 0.05$, when compared to IR.

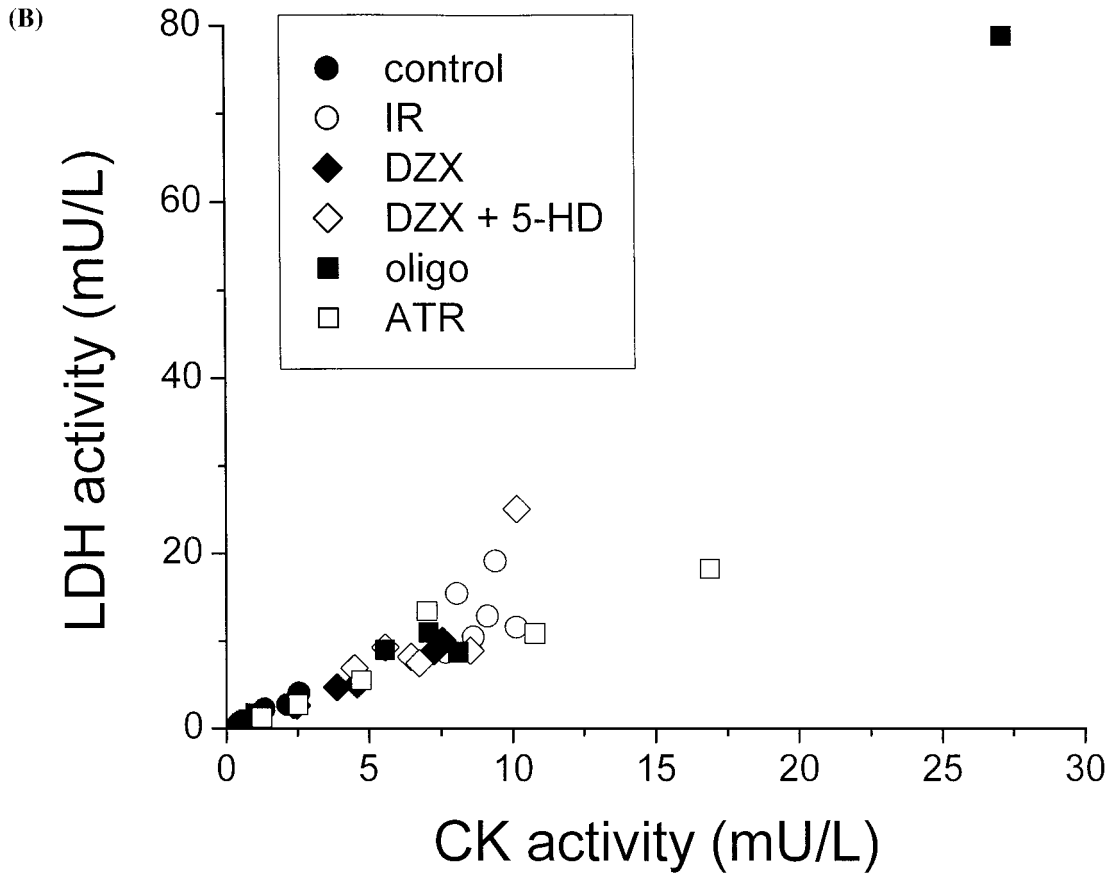

Fig. 5. (*Continued*)

cause any measurable decrease in the membrane potential or prevent mitochondrial Ca^{2+} uptake, but does result in a ∼20% increase in matrix volume (Carroll *et al*., 2001; Jaburek *et al*., 1998; Kowaltowski *et al*., 2001b). In nonrespiring mitochondria, the drop in membrane potential due to the lack of proton pumping by the respiratory chain decreases matrix volume, an effect less dramatic when diazoxide is present (Kowaltowski *et al*., 2001b). Matrix volume modifies the interactions between the inner and outer mitochondrial membranes, resulting in changes in the transport properties of ADP and ATP across these membranes (Brdiczka *et al*., 1998; Dos Santos *et al*., 2002; Gellerich *et al*., 1993). Indeed, hearts subjected to ischemia present large changes in ADP transport properties across their membranes, while ischemic hearts in which mitoK_{ATP} was opened by preconditioning preserve their ADP transport properties (Laclau *et al*., 2001). These changes in ADP and ATP transport properties may be responsible for the decrease in ATP-supported membrane potential and ATP hydrolysis in nonrespiring mitochondria observed in this paper (Fig. 3), improving ischemic ATP levels, as represented in Fig. 7.

Although the increase in ventricular ATP levels promoted by diazoxide during ischemia is not large, it may be sufficient, in these nonbeating hearts, to maintain vital activities such as membrane ion pumps. In fact, earlier studies measuring high-energy phosphate levels in ischemia show quite clearly that hearts must loose over 90% of their ATP to suffer significant damage (Jennings *et al*., 1978). We also found that lower ATP hydrolysis (Figs. 2 and 3(D)) during ischemia results in a reduced membrane potential (Fig. 3(A) and (C)) and mitochondrial Ca^{2+} uptake (Fig. 4). Our results agree with literature data showing lower membrane potentials and mitochondrial Ca^{2+} accumulation in ischemic cells treated with mitoK_{ATP} openers (Di Lisa *et al*., 1995; Ishida *et al*., 2001; Murata *et al*., 2001). Excessive Ca^{2+} uptake has deleterious effects in mitochondria such as nonselective inner membrane permeabilization (the mitochondrial permeability

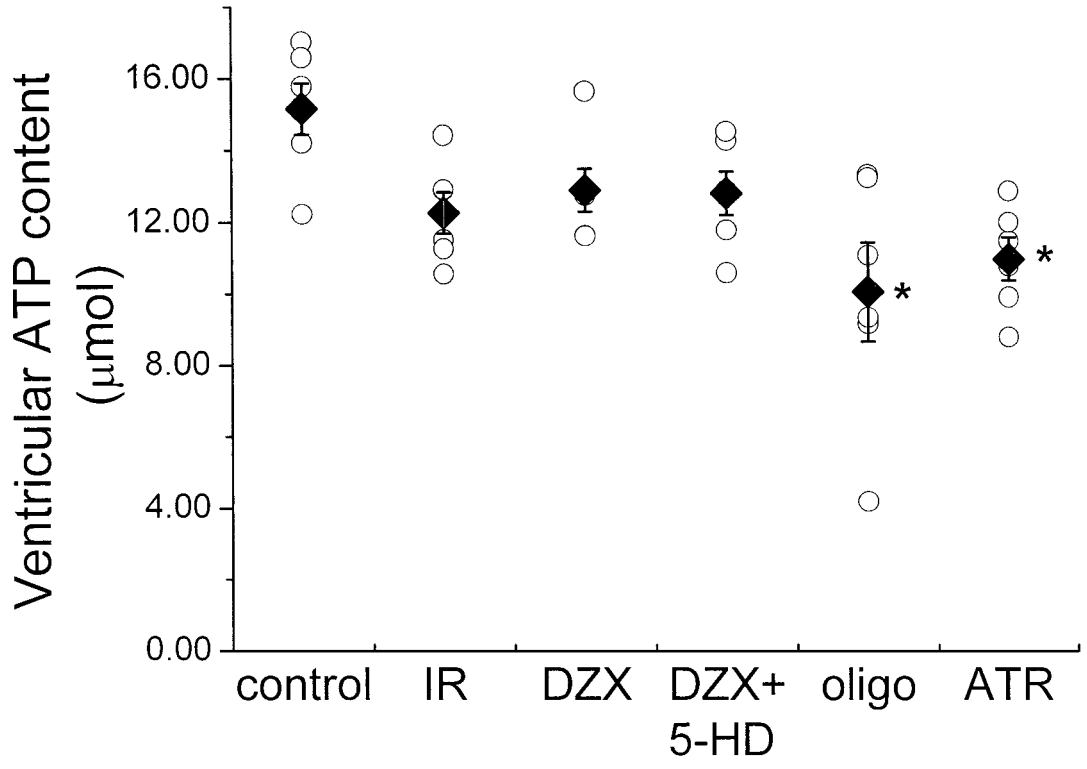

Fig. 6. Reperfusion ventricular ATP contents. Rat hearts were subjected to ischemia and reperfusion as described in Fig. 4, after 10 min pretreatment with no drugs (IR), 30 μM diazoxide (DZX), 30 μM diazoxide and 200 μM 5-hydroxydecanoate (DZX + 5-HD), 0.5 μg/mL oligomycin (oligo), or 10 μM atractyloside (ATR). Control (nonischemic) hearts were maintained perfused during the extent of the complete experiment. Estimated total ventricular ATP levels are represented for six individual hearts (○) under each condition, in addition to the averages ± SEM (♦). $^*p < 0.05$, when compared to untreated (IR) hearts.

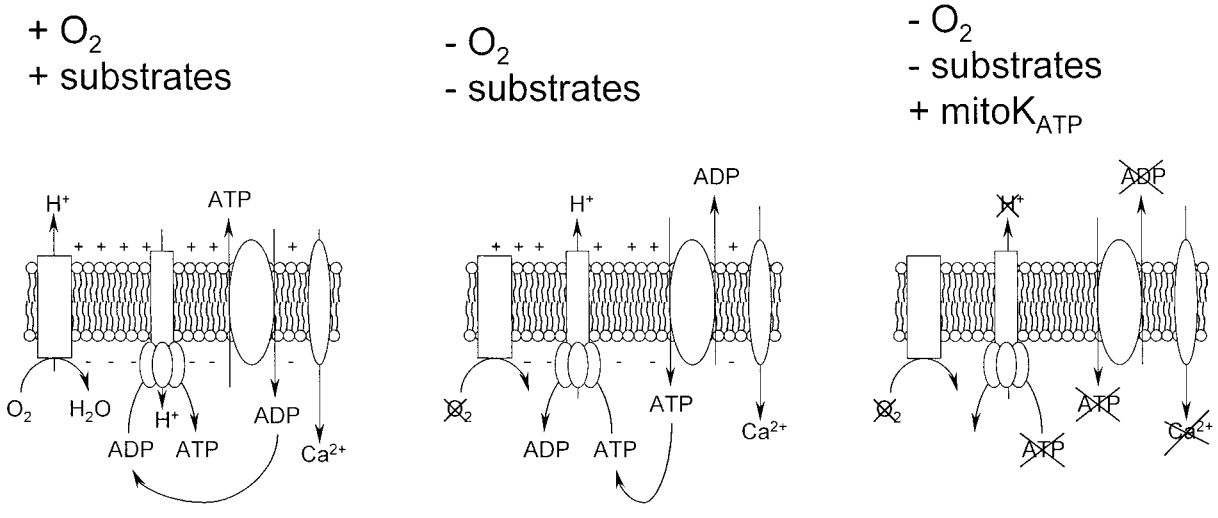

Fig. 7. MitoK_{ATP} opening decreases anoxic mitochondrial membrane potential, ATP hydrolysis and Ca^{2+} uptake. In the presence of O_2 and substrates, the respiratory chain generates a transmembrane H^+ gradient, which is used by the ATP synthase to phosphorylate ADP transported into mitochondria through the adenine nucleotide translocator. The H^+ gradient also provides the driving force for mitochondrial Ca^{2+} uptake through the Ca^{2+} uniporter. Under ischemic conditions, neither O_2 nor substrates are present, and no H^+ transport occurs through the respiratory chain. The lack of a respiratory-chain supported membrane potential promotes the reversal of ATP synthase activity, with the production of ADP and H^+ pumping. Cytosolic ATP levels decrease, and Ca^{2+} uptake is present, due to the ATP hydrolysis-supported H^+ gradient. Under ischemic conditions in which mitoK_{ATP} is open, ATP hydrolysis is inhibited, probably due to limited transport into the mitochondrial matrix by the adenine nucleotide translocator (Dos Santos *et al*., 2002), maintaining cytosolic ATP levels higher. The lack of an ATP hydrolysis-supported H^+ gradient also results in a reduction of Ca^{2+} uptake into mitochondria.

transition), and a proven role in the pathogenesis of injury following ischemia/reperfusion (see Halestrap, 1999 and Kowaltowski *et al*., 2001a for reviews). We believe the reduction in Ca^{2+} uptake ischemic mitochondria exhibit when treated with diazoxide may prevent the permeability transition, contributing to postischemic recovery. At the moment, we are conducting experiments to evaluate the effect of diazoxide on the induction of mitochondrial permeability transition in hearts submitted to ischemia/reperfusion.

When we measured tissue damage in hearts subjected to ischemia followed by reperfusion in the presence of diazoxide, oligomycin and atractyloside, we found that diazoxide prevented CK and LDH release from the reperfused hearts, but not oligomycin and atractyloside (Fig. 5), which presented very variable responses. The lack of protection in these hearts can be explained by the impaired ability to recover ATP levels during reperfusion (Fig. 6), an effect not observed in diazoxide-treated hearts. Thus, we found that diazoxide can directly modulate the ability of nonrespiring mitochondria to consume ATP without decreasing ATP production in respiring mitochondria. The decrease in ATP hydrolysis promoted by diazoxide results in a conservation of ventricular ATP levels during ischemia, reduced mitochondrial Ca^{2+} uptake and improved tissue recovery after reperfusion. Based on our findings, we suggest that the cardioprotective effect of mitoK_{ATP} opening is directly linked to the inhibition of mitochondrial ATP consumption in nonrespiring mitochondria.

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