IF$_1$ Function in Situ in Uncoupler-challenged Ischemic Rabbit, Rat, and Pigeon Hearts*

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Rabbit, rat, and pigeon are species representative of three cardiac muscle mitochondrial ATPase regulatory classes, a, b and c, respectively. Class a species contain a full complement of higher affinity ATPase inhibitor subunit, IF$_1$, in their cardiac muscle mitochondria and show marked IF$_1$-mediated mitochondrial ATPase inhibition during myocardial ischemia. Class b species contain low levels of higher affinity IF$_1$, and show very little IF$_1$-mediated ATPase inhibition during ischemia. Class c species contain a full complement of a lower affinity form of IF$_1$, and show a low-to-moderate level of IF$_1$-mediated ATPase inhibition during ischemia. In the present study we perfused hearts of a member of each regulatory class through the coronary arteries with the uncoupler, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone (FCCP), before making them ischemic. We then compared net rates of cell ATP depletion during ischemia in the FCCP-treated hearts to identically treated FCCP-free hearts. Thus, we tested the relative capacities of cardiac muscle mitochondria of the three species to avert a potentially greatly increased net rate of cell ATP depletion due to ATP hydrolysis by the fully uncoupled mitochondrial ATPase. We found that FCCP-uncoupling in situ had a relatively small effect on ATP depletion during ischemia in rabbit hearts, that it dramatically accelerated ATP depletion in ischemic rat hearts, and that it had an intermediate effect on ATP depletion in ischemic pigeon hearts. These results demonstrate for the first time the relative extents to which IF$_1$-mediated mitochondrial ATPase inhibition can slow cell ATP depletion due to the fully uncoupled mitochondrial ATPase in these three classes of hearts. They show that, in contrast to the situation in rabbit hearts, the low level of higher affinity IF$_1$, present in the cardiac muscle mitochondria of the rat, is, under these conditions, essentially nonfunctional, while the full complement of the lower affinity form of IF$_1$, present in the cardiac muscle mitochondria of the pigeon is partially functional in that it appeared to provide an intermediate level of protection against rapid cell ATP depletion.

A variety of studies suggest that the mitochondrial ATPase

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† This abbreviation is used: FCCP, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; RHMP, rabbit heart mitochondrial particles; SMP, submitochondrial particles.

inhibitor subunit, IF$_1$, is not required for ATP synthesis, but that it operates as a check on ATP hydrolysis by the mitochondrial ATPase under nonenergizing conditions. Thus, for example, Tagawa and co-workers (1, 2) have studied an IF$_1$-minus mutant of bakers’ yeast that can make ATP by oxidative phosphorylation just as well as wild-type cells, but, unlike the wild-type, it cannot survive exposure to mitochondrial uncoupling agents. Thus, while the wild-type can tolerate uncouplers because it possesses a means of slowing ATP hydrolysis by the unenergized mitochondrial ATP synthase, the mutant cannot overcome the effects of an uncoupler challenge on ATP depletion. Functional ATPase inhibitor subunit is also missing in Luft’s disease (3), a rare human mitochondrial myopathy described as non-thyroidal hypermetabolism (4). While the disease is characterized by normal phosphorylation capacity, there is “loose coupling” (5). Finally, work by ourselves has demonstrated that so-called fast heart rate mammalian species like the rat and pigeon have low levels of IF$_1$ in their cardiac muscle mitochondria (5, 6), yet these IF$_1$-poor mitochondria clearly make ATP normally both in situ and in vitro.

In the present study we examined for the first time the effects of the uncoupler, FCCP, introduced in situ through the coronary arteries, on net rates of ATP depletion during myocardial ischemia in three species representative of three different cardiac mitochondrial ATPase regulatory classes. FCCP uncoupling in situ had little effect on net rates of ATP depletion in ischemic rabbit hearts which possess a full complement of a higher affinity form of IF$_1$. However, it greatly accelerated net rates of ATP depletion in ischemic rat hearts, which possess only low levels of a higher affinity form of IF$_1$. Last, FCCP had an intermediate accelerating effect on ATP depletion in ischemic pigeon hearts which possess a full complement of a lower affinity form of IF$_1$. These studies demonstrate that, while the cardiac muscle mitochondria of the rat and pigeon lack an effective complement of IF$_1$, they suggest that mitochondria, which lack a functional complement of IF$_1$, may employ other mechanisms of inner mitochondrial membrane proton blockade and that these alternative mechanisms appear to be at least as effective as IF$_1$ in preventing ATP hydrolysis by the mitochondrial ATPase during myocardial ischemia in the absence of uncouplers.

MATERIALS AND METHODS

One and one-half kg male New Zealand White rabbits, 300-g male Sprague-Dawley rats, and 800-g American Show Racer pigeons of either sex were anesthetized with sodium pentobarbital intravenously to effect for rabbits or intraperitoneally to effect for rats and pigeons) and killed by the rapid removal of their hearts while the animals were fully anesthetized. Upon removal, the hearts were placed immediately into either ice-cold 180 mM KCl, 10 mM EGTA* (KE solution) for hearts used for the immediate preparation of mitochondria or IF$_1$, or cold Krebs-bicarbonate buffer for hearts to be cannulated and perfused. For the preparation of mitochondria, the hearts were minced finely in ice-cold 180 mM KCl, 10 mM EGTA, 0.5% bovine serum albumin, 10 mM MOPS-KOH, pH 7.2 (KEAM solution) and mitochondria prepared from the cardiac muscle minces by Polytron homogenization as described earlier (7, 8). For experiments utilizing control-energized mitochondria (Table I), the mitochondria were energized by shaking them vigorously for 10 min at 37 °C in 0.25 M sucrose, 1 mM EGTA, 20 mM MOPS-KOH, pH 7.2

The abbreviations used are: FCCP, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; RHMP, rabbit heart mitochondrial particles; SMP, submitochondrial particles.
are, respectively, (6, 19, 20), the rabbit, rat, and pigeon are representative of mitochondrial ATPase activities, and the ratios of the two in rabbit, pigeon, and rat heart mitochondria. As reported by us earlier (9–12). The in vitro incubations of inhibitor-containing extracts with IF1-depleted RHMP for the experiments presented in Fig. 1 were carried out for 20 min at 37 °C at pH 6.2 in a final volume of 2.0 ml. The incubation medium contained 0.25 m sucrose, 0.1 m MgATP, and 20 mE MES-KOH, pH 6.2, in the presence of the KCl concentrations indicated. Aliquots of RHMP containing the equivalent of 0.5 mg of rabbit heart mitochondria were added from a pooled suspension of RHMP made from a known amount of mitochondria. Aliquots of mitochondrial extracts from each species that contained the amount of IF1 present in 0.5 mg of mitochondria were added to the particles. Thus, the naturally occurring or species-endogenous ratios of IF1 to ATPase were employed in these experiments. The rat heart SMP titration procedure described by us previously (5, 11–13) was used for the normalization of the IF1-containing extracts for the experiments presented in Fig. 1. Mitochondrial ATPase activity was measured sonicated mitochondria (Tables I) or in RHMP (Fig. 1) using a modification of the method of Tzagoloff (14) as described by us previously (5, 7, 8, 11). The IF1 content determinations presented in Table I were carried out as described previously using our rat heart SMP titration procedure (5, 11, 12). Mitochondrial respiration and degrees of respiratory coupling remaining in mitochondria from FCCP-free and treated hearts (Fig. 2) were measured polarographically at 30 °C using a Gilson model K-IC Oxygraph equipped with a Clark oxygen electrode as described earlier (15). Briefly, approximately 1.5 mg of mitochondria were used per assay, and the assay medium contained 0.25 m sucrose, 10 mE MOPS-KOH, pH 7.4, 2.5 mE P, 6.25 mglucamate, and 6.25 mglucose. Respiration was initiated by the addition of approximately 500 nmo1 of ADP.

For the ATP depletion time course experiments (Fig. 3), the hearts were cannulated through the aorta and perfused retrogradely at 37 °C with Krebs-bicarbonate buffer, pH 7.4, bubbled continuously with 95% O2, 5% CO2. The perfusion buffer was pumped at 20 ml/min for rabbit and pigeon hearts or at 10 ml/min for rat hearts. Once cannulated, the hearts were perfused for 10 min (control hearts) followed by 30 s at the same rate of flow with the same buffer containing 20 μM FCCP (FCCP-treated hearts). After perfusion, the hearts were rapidly removed from the cannula and quickly sectioned into ventricular myocardial samples of roughly equal size. The tissue samples were then made ischemic by placing them into sealed Ziploc plastic bags immersed in a circulating water bath at 37 °C as described earlier (5–8). At the times indicated, they were removed from the bags and immediately frozen in liquid nitrogen. The frozen samples were lyophilized overnight and the lyophilized tissue samples finely powdered and extracted with trichloroacetic acid. The acid extracts were then deproteinized by centrifugation and known amounts of the total removed for the enzymatic assay of ATPase and were oxidized to acid. The acid extracts were then deproteinated by centrifugation and known amounts of the total removed for the enzymatic assay of ATPase and were oxidized to acid.

RESULTS AND DISCUSSION

Table I lists the IF1 contents, the maximal energized mitochondrial ATPase activities, and the ratios of the two in rabbit, rat, and pigeon heart mitochondria. As reported by us earlier (6, 19, 20), the rabbit, rat, and pigeon are representative of three distinct mitochondrial ATPase regulatory classes. These are, respectively, (a) species that contain a full complement of higher affinity ATPase inhibitor subunit, IF1, in their cardiac muscle mitochondria and show marked IF1-mediated mitochondrial ATPase inhibition during myocardial ischemia, (b) species that contain low levels of higher affinity IF1, and show very little IF1-mediated ATPase inhibition during ischemia, and (c) species that contain a full complement of a lower affinity form of IF1, and show a low-to-moderate level of ATPase inhibition during ischemia. Fig. 1 shows the effect of varying ionic strength on the inhibition of the ATPase in rabbit submitochondrial particles by species-endogenous levels of IF1 from rabbit, rat, and pigeon heart mitochondria. As is evident from Fig. 1, physiological ionic strength interferes significantly more with ATPase inhibition by the ATPase inhibitor than by rabbit IF1. At species-endogenous levels, the rat inhibitor has very little effect on ATPase activity at any ionic strength. However, as we have shown earlier, when the concentration of the rat inhibitor is increased to the level present in rabbit heart mitochondria, it behaves much like the rabbit inhibitor in the face of increasing ionic strength (6, 20). The data presented in Table I are consistent with the presence of species-specific differences in IF1-ATPase interaction that might prove useful for distinguishing between possible species-related differences in IF1-ATPase interaction was based largely on earlier studies, which showed that increasing ionic strength...
interfered with IF1-mediated ATPase inhibition in bovine heart SMP (21).

While pigeon was the lower affinity IF1-possessing species chosen for the present study, it may be noted that this ATPase regulatory class also includes guinea pig, turtle, and frog (6). Thus while three of the four members of this ATPase regulatory class that have been studied by us are either a bird, a reptile, or an amphibian, of the 15 vertebrate species examined by us thus far, the guinea pig is the only mammalian species found to contain a lower affinity ATPase inhibitor (6). In that the guinea pig system resembles that present in birds, reptiles and amphibians (6, 19, 20), it may be useful to regard it as an evolutionarily less modern system than that present in the other mammals that have been investigated. Indeed, work by others on molecular phylogenetic relationships between guinea pigs and rodents and between guinea pigs and other mammalian orders suggests that the old classification of guinea pigs as rodents should be abandoned (22). The guinea pig would appear to be a member of a separate order of mammals that branched off from the vertebrate evolutionary tree earlier than rodents but somewhat later than marsupials, birds, and reptiles (22).

Fig. 2 shows representative polarographic tracings for mitochondria from FCCP-free and FCCP-perfused rabbit, rat, and pigeon hearts. Mitochondrial respiration and coupling were measured using a Gilson Oxygraph equipped with a Clark oxygen electrode at 30 °C using glutamate and malate as substrates.

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Fig. 2 shows representative polarographic tracings for mitochondria isolated from FCCP-free (control) and FCCP-perfused rabbit, rat, and pigeon hearts. While mitochondria from control hearts exhibited good respiratory control and coupling, those isolated from hearts perfused for 30 s with 20 μM FCCP were completely uncoupled. It may be mentioned that the FCCP-perfused hearts stopped beating prior to the conclusion of FCCP perfusion.

Fig. 3 presents representative ATP depletion time courses for control and FCCP-treated rabbit, rat, and pigeon hearts during total ischemia. The hearts were perfused retrogradely with Krebs-bicarbonate for 10 min minus and plus an additional 30 s with 20 μM FCCP. They were then quickly sectioned and the tissue samples made totally ischemic at 37 °C for the times shown. ATP was then determined as described in the text.

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Fig. 3 presents representative ATP depletion time courses for control and FCCP-treated rabbit, rat, and pigeon hearts during total ischemia. It is evident from the data that total uncoupling of the mitochondrial ATPase in situ had relatively little effect on the net rate of ATP depletion in the ischemic rabbit heart. In contrast, FCCP uncoupling in situ had a marked accelerating effect on the rate of ATP depletion in ischemic rat hearts. Thus, while total ATP depletion took approximately 30 min in FCCP-free rat hearts, it took only approximately 2 min in the FCCP-perfused rat hearts. The re-
results with pigeon hearts were intermediate in that the effect of uncoupling in situ was marked, but not nearly as extreme as in the rat. These results demonstrate directly for the first time the relative extents to which IF1-mediated mitochondrial ATPase inhibition can slow cell ATP depletion due to the fully uncoupled mitochondrial ATPase in these three classes of hearts. They show that, in contrast to the situation in rabbit hearts, the low level of higher affinity IF1 present in the cardiac muscle mitochondria of the rat is, under the conditions used, essentially nonfunctional, while the full complement of the lower affinity form of IF1 present in the cardiac muscle mitochondria of the pigeon is partially functional in that it appeared to provide an intermediate level of protection against rapid cell ATP depletion.

In an earlier study we demonstrated that oligomycin inhibition of the mitochondrial ATPase in situ in canine hearts significantly slowed net rates of ATP depletion during a subsequent ischemic interval (17). These same studies showed that oligomycin inhibition in situ had little ATP-sparing effect in ischemic rat hearts due to a variety of factors discussed in that study (17) and in a later report (23). In the present study, rat hearts were perfused with buffer for 5 min followed by 10 μM oligomycin for 5 min followed by 20 μM FCCP for 30 s as a control for the “FCCP alone” protocol. While FCCP alone caused a cessation of rat heart contraction in approximately 20 s and a concomitant very rapid ATP depletion already evident in the zero time ischemic samples (Fig. 3), in contrast, oligomycin perfusion of rat hearts caused the heart rate to decrease approximately 5-fold from 251 ± 8 beats/min (n = 4) to a sustained rate of 58 ± 10 beats/min (n = 4), but, interestingly, during the subsequent 30 s FCCP perfusion, the heart rate actually increased by approximately 30% to 74 ± 6 beats/min (n = 4). This important and interesting observation may be explained by an increased glycolytic rate supported by the resumption of mitochondrial electron flow due to FCCP uncoupling of oligomycin inhibited, but still coupled electron transport. Thus, lactate accumulation increased from 20.5 μmol/g wet weight after 10 min of ischemia in hearts treated with oligomycin alone to 37.5 μmol/g wet weight after 10 min in hearts treated with oligomycin followed by FCCP. The resumption of mitochondrial electron flow in the oligomycin-inhibited mitochondria in situ presumably facilitated the reoxidation of cytosolic NADH to NAD⁺ (via the α-glycerophosphate and/or malate-oxaloacetate shuttle) required for the glyceraldehyde-3-P dehydrogenase reaction thereby increasing glycolytic ATP generation. Thus, not only did an initial oligomycin inhibition in situ preemp the cessation of contractile activity that would have occurred upon subsequent FCCP perfusion by blocking an uncoupler stimulation of ATP hydrolysis by the mitochondrial ATPase, but it also made possible an increased rate of contractile activity upon the introduction of FCCP through the effect of the uncoupler on glycolytic ATP production.

As interesting as these results are in themselves, they raise additional questions about possible fundamental interspecies differences in mechanisms of mitochondrial inner membrane proton blockade. Thus, in the absence of uncoupler, the two so-called fast heart rate species studies here, the rat and pigeon, appeared to resist ATP depletion during ischemia some-what better than the rabbit, the slow heart rate species studied (Fig. 3). In earlier reports we showed that, while phosphate carrier-mediated P/H⁺ symport is the primary channel for equilibrating protons across the mitochondrial inner membrane of cardiac muscle mitochondria of slow heart rate species including rabbit (19, 24) and dog (23), these same studies demonstrated that rat and pigeon heart mitochondria did not require added P, to equilibrate protons across the inner membrane (19, 24). While both slow and fast heart rate species presumably possess an active phosphate carrier in their cardiac muscle mitochondria, fast heart rate species may also employ some additional means of trans-membrane proton equilibration. Thus, there appears to be something fundamentally different between these two classes of species with respect to the mechanisms they employ both for equilibrating protons and for maintaining proton gradients across the inner membrane. In a similar vein, Jones and co-workers (25, 26) have suggested that, in anoxic rat hepatocytes, mechanisms exist to preserve the mitochondrial transmembrane proton distribution so that the pH gradient across the mitochondrial membrane is preserved under anaerobic conditions. Thus, species like the rat that lack a functional complement of IF1 in many of their tissues (6), appear to possess alternative mechanisms for mitochondrial inner membrane proton blockade that may be at least as effective as IF1 under nonenergizing conditions in situ. Why species employ one mechanism of proton blockade (or equilibration) as opposed to another in their cardiac muscle mitochondria remains to be elucidated.

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