Direct Evidence for the Control of Mitochondrial Respiration by Mitochondrial Creatine Kinase in Oxidative Muscle Cells *in Situ**

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The efficiency of stimulation of mitochondrial respiration in permeabilized muscle cells by ADP produced at different intracellular sites, *e.g.* cytosolic or mitochondrial intermembrane space, was evaluated in wild-type and creatine kinase (CK)-deficient mice. To activate respiration by endogenous production of ADP in permeabilized cells, ATP was added either alone or together with creatine. In cardiac fibers, while ATP alone activated respiration to half of the maximal rate, creatine plus ATP increased the respiratory rate up to its maximum. To find out whether the stimulation by creatine is a consequence of extramitochondrial [ADP] increase, or whether it directly correlates with ADP generation by mitochondrial CK in the mitochondrial intermembrane space, an exogenous ADP-trap system was added to rephosphorylate all cytosolic ADP. Under these conditions, creatine plus ATP still increased the respiration rate by 2.5 times, compared with ATP alone, for the same extramitochondrial [ADP] of 14 μM. Moreover, this stimulatory effect of creatine, observed in wild-type cardiac fibers disappeared in mitochondrial CK deficient, but not in cytosolic CK-deficient muscle. It is concluded that respiration rates can be dissociated from cytosolic [ADP], and ADP generated by mitochondrial CK is an important regulator of oxidative phosphorylation.

Despite very significant efforts from many laboratories, the mechanism regulating the ATP synthesis resulting from oxidative phosphorylation in cells is not yet understood *in vivo*. The classical respiratory control phenomenon by ADP was discovered by Chance and Williams (1) for mitochondria *in vitro*. However, it failed to explain why in cardiac cells *in vivo*, a great increase in the rate of oxygen consumption can be observed under conditions of remarkable metabolic stability, notably at almost constant levels of phosphocreatine (PCr),[1] inorganic

phosphate (P$_i$), and ATP, and consequently ADP as calculated from the creatine kinase (CK) equilibrium (Refs. 2–4; see Refs. 5–7, for reviews). It was proposed that the rates of respiration could be modulated by different effectors, like reducing equivalents (NADH), calcium, proton motive force, and P$_i$ (4, 8). The presence of CK isoenzymes subcellularly compartmentalized has also been considered to play a primordial role in the control of the production and transport of energy into the cytoplasmic compartment. Indeed, cytosolic CK isoenzymes (muscle type MM-CK, brain type BB-CK, and MB-CK) are always co-expressed in a tissue-specific fashion together with a mitochondrial isoform (sarcomeric "ScCKmit" in skeletal muscle and heart or ubiquitous "UbCKmit" in non-muscle tissues). While in all adult skeletal muscle types, exclusively MM-CK and ScCKmit are co-expressed, small amounts of MB-CK, as well as BB-CK, are also found in cardiac muscle. The proportion of the ScCKmit *versus* total CK activity depends on the type of metabolism. This ratio is relatively high in oxidative muscle (heart and slow-twitch skeletal muscle), and it is low in glycolytic muscle (fast-twitch skeletal muscle). It has been proposed that in muscle cells, the different isoenzymes are functionally and/or structurally coupled either to sites of energy consumption (cellular ATPases, such as actomyosin ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase) or sites of energy production (glycolysis and mitochondria) to facilitate energy transport in the cytoplasmic compartment with creatine (Cr) and PCr as diffusible intermediates. According to this model, ADP produced by high free-energy demand in myofibrils is immediately rephosphorylated into ATP through the action of cytosolic CK, using the phosphoryl group from PCr, while Cr diffuses back to mitochondria to be rephosphorylated into PCr through the action of ScCKmit bound to the outer surface of the inner mitochondrial membrane. For an update on the PCr/CK system see Ref. 9, for review, and Ref. 10.

The ScCKmit, the structure of which is known now at an atomic resolution (11–13), is in a position to control the metabolic fluxes between mitochondria and cytoplasm, and to respond adequately to an increased energy demand in oxidative muscles. Considering the functional coupling of the ScCKmit with the ATP-ADP carrier and the restricted permeability of the outer mitochondrial membrane for adenine nucleotides (5, 14), the ADP produced in the intermembrane space by ScCKmit could be more efficient to stimulate the respiration than ADP produced directly in the cytoplasm (15). The increase of apparent affinity of oxidative phosphorylation for ADP in the presence of Cr in the respiratory medium surrounding permeabilized cardiac fibers or cardiomyocytes, as compared with affinity determined in the absence of Cr, supports this hypothesis (5, 7, 16). However, the relative role of the local (mitochondrial intermembrane space) ADP production by ScCKmit in the
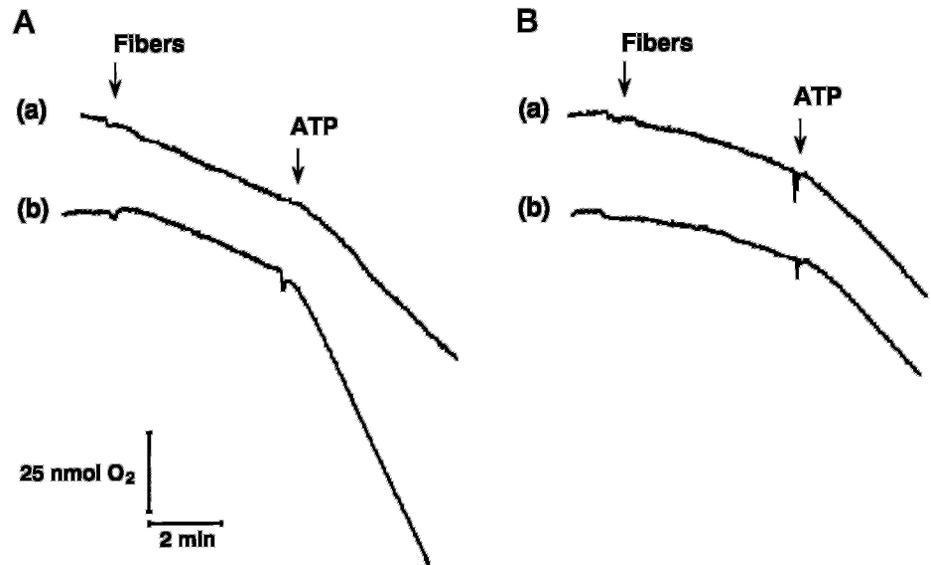
FIG. 1. **Oxygraph recordings: stimulation of respiration by 1 mM ATP in the absence (*a*) or presence (*b*) of Cr.** Oxygen consumption rates were evaluated in cardiac permeabilized fibers from wild-type (*panel A*) and ScCKmit-deficient (*panel B*) mice, in the presence of 5 mM glutamate and 2 mM malate as substrates. Respiration was stimulated by the addition of 1 mM ATP. In the *lower traces* (*b*), the respiratory medium was supplemented with 20 mM Cr.

control of respiration, in comparison to cytoplasmic ADP, has not been directly evaluated.

With different transgenic CK null mutant mice at hand (17, 18), M-CK and ScCKmit-deficient mice, as well as double knock-out mice, we were in a position to determine the relative roles of different CK isoenzymes and particularly that of ScCKmit in the regulation of mitochondrial respiration in different types of muscles. Therefore, in the current study, we investigated the characteristics of the regulation of mitochondrial respiration and its stimulation by Cr in heart, soleus, and white gastrocnemius taken from wild-type and transgenic mice, by using the permeabilized fiber technique (7).

These results show the control of the mitochondrial respiration rate by ADP generated via functionally coupled ScCKmit in close vicinity of the ATP-ADP carrier in the intermembrane space. It is proposed that this control of respiration by ScCKmit in oxidative fibers is due to both the functional coupling between ScCKmit and ATP-ADP carrier and the restricted permeability of the mitochondrial outer membrane for adenine nucleotides.

MATERIALS AND METHODS

Experimental Animals: CK-mutant Mice—In this study, we used wild-type C57BL/6 mice as controls and three transgenic mice models, derived by homologous recombination of stem cells, in which the genes of different CK isoforms have been ablated: (i) M-CK deficient mice that no longer express cytosolic muscle-type MM-CK (M-CK($-/-$) group) (17), (ii) mice deficient in sarcomeric mitochondrial CK (ScCKmit($-/-$) group) (18), as well as (iii) mice lacking both M-CK and ScCKmit isoforms (CK($-/-$) group). It should be noted that residual BB-CK, which is expressed in heart (1–5% of total CK in wild-type) is still present in these mutant types. There is no compensatory effect on the expression of any of the other CK isoforms in the different CK-mutants. However, some adaptational changes were described (17–19). While the mitochondrial design changes most prominently in the fast fibers of M-CK single and double-CK($-/-$) mutants, it seems to be unchanged either in the fast fibers of ScCKmit($-/-$) mutants or in cardiac fibers whatever the mutant types. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Preparation of Permeabilized Muscle Fibers: Oxygraphic Measurements—Permeabilized fibers were prepared according to the method described earlier (7, 20). The animals were anesthetized with Nembutal (100 mg/kg body weight, intraperitoneally). Then, the heart, still beating, and skeletal muscles (soleus and white gastrocnemius) were rapidly excised and placed into pre-cooled solution A containing in mM: CaK$_2$EGTA, 2.77; K$_2$EGTA, 7.23; MgCl$_2$, 6.56; dithiothreitol, 0.5; potassium methanesulfonate, 50; imidazole, 20; taurine, 20; Na$_2$ATP, 5.3; PCr, 15, pH 7.1, adjusted at 25 °C. All following procedures were carried

out at 4 °C. Muscle bundles were isolated (parallel to the main fiber direction) from left ventricles and both skeletal muscle types. After mechanical dilaceration of these bundles using needles to separate fibers from each other, samples were incubated with saponin (50 μg/ml solution A) at mild stirring for 30 min for complete solubilization of the sarcolemma. Permeabilized fibers were then washed (3 times to remove completely all saponin and metabolites) for 10 min in solution B containing in mM: CaK$_2$EGTA, 2.77; K$_2$EGTA, 7.23; MgCl$_2$, 1.38; dithiothreitol, 0.5; potassium methanesulfonate, 100; imidazole, 20; taurine, 20; K$_2$HPO$_4$, 3, pH 7.1, adjusted at 25 °C. The rates of oxygen consumption were recorded by using the two-channel high resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria) or Yellow Spring Clark oxygen electrode in 2 ml of solution B, supplemented with respiratory substrates (5 mM glutamate and 2 mM malate) and 2 mg/ml bovine serum albumin. The determinations were carried out at 25 °C. The solubility of oxygen was taken as 215 nmol of O$_2$/ml (20). After measurements, the fiber bundles were carefully removed, washed in distilled water, and the dry weight was determined (after 48 h at 80 °C) in order to express the absolute values of respiratory rates in nmol of O$_2$/min/mg dry weight.

Determination of ADP Concentration—The oxygraphic samples (1 ml), immediately frozen in liquid nitrogen, were stored at −80 °C until [ADP] was determined. All proteins (bovine serum albumin) present in the respiratory medium were precipitated by 60% perchloric acid (100 μl). Then, the samples were centrifuged at 17,000 × g for 10 min (4 °C). The supernatants (600 μl) were neutralized by a solution 2 M KHCO$_3$ (420–470 μl depending on the samples). The samples were spun once more at 17,000 × g for 10 min (4 °C) and the supernatants loaded directly onto the HPLC column. The adequate separation of adenine nucleotides was performed by reversed phase chromatography on a HPLC system using a PepRPC HR 5/5 column and a gradient system, as described previously (21). The injection volume was 40 μl for standards or neutralized extracts. Eluent A contained 50 mM KH$_2$PO$_4$, 5 mM tetrabutylammonium hydrogen sulfate, pH 6.0, and Eluent B contained 50 mM KH$_2$PO$_4$, 5 mM tetrabutylammonium hydrogen sulfate, 40% methanol, pH 6.0. The flow rate was 1 ml/min. After 3 min with Eluent A alone, an increasing gradient from 0 to 70% of Eluent B was performed in 14 min. Then, the gradient was increased to 100% Eluent B (40% methanol) in 1 min and maintained during 3 min to clean the column as much as possible. To return to Eluent A, a decreasing gradient from 100% to 0% Eluent B within 2 min was used. Using this procedure, the elution of ADP occurred at 12.3 min. For the calculation of [ADP] in oxygraphic samples, the calibration was made by injecting known quantities of ADP standard (previously determined by spectrophotometric method: absorbance of a standard ADP solution was measured at neutral pH and λ = 259 nm, and [ADP] calculated using an extinction coefficient equal to 15.4×10^{-3} M^{-1}).

All reagents were purchased from Sigma, or Roche Molecular Biochemicals (Germany) for ATP and ADP. The results are expressed by mean ± S.E. Statistical comparisons were made using the Anova test (variance analysis and Fisher test), and $p < 0.05$ was taken as the level of statistical significance.

RESULTS

Due to the compartmentation of the ADP production within muscle cells *in vivo*, *e.g.* in the cytosol or within the mitochondrial intermembrane space (22), it is important to know what the relative efficiencies of stimulation of mitochondrial respiration by ADP from these different intracellular sources are. To address this question, investigations were carried out on permeabilized fibers in which there was a powerful ADP-regenerating system. Indeed, while soluble cytoplasmic constituents including BB-, MB-CK, and unbound MM-CK (around 50, and 80% of total CK activity, respectively, for heart and skeletal muscle) were lost during the permeabilization procedures of sarcolemma, mitochondria, and other intracellular structures such as myofilaments and sarcoplasmic reticulum were intact. Moreover, proteins connected to these structures such as ATPase and bound MM-CK were still present. Thus, it is possible to stimulate mitochondrial respiration by activating the endogenous production of ADP.

In this study, we stimulated mitochondrial respiration by the addition of either 1.0 mM ATP or 1.0 mM ATP together with 20 mM Cr. Depending on these conditions, three different pathways led to ADP generation: 1) high myofibrillar ATPase activity occurred under saturating [ATP] of 1 mM; 2) compartmentalized adenylate kinase (AK) activity participated also to ADP production from ATP and AMP; 3) compartmentalized CK, not only ScCKmit in the mitochondrial intermembrane space (corresponding to around 60 and 20% of total CK activity remaining in the permeabilized fibers of heart and skeletal muscle, respectively) but also bound MM-CK to myofibrils and sarcoplasmic reticulum (corresponding to around 40 and 80% of total CK activity remaining in the permeabilized fibers of heart and skeletal muscle, respectively). These relative contents of ScCKmit and MM-CK in permeabilized fiber extracts were estimated, after cellulose polyacetate electrophoresis, by staining for CK activity (not shown). By activating these different pathways for ADP generation, our protocol reproduced most closely what occurs in the intracellular medium inside the cell.

To assay the extramitochondrial steady state [ADP], aliquots of the medium surrounding the permeabilized fibers were taken in each oxygraphic experiment. As there is a rapid diffusional equilibrium of metabolites between the intracellular bulk phase and the surrounding medium (23), the [ADP] measured in samples is supposed to reflect the cytosolic [ADP]. In order to determine the relative contribution of ScCKmit and bound MM-CK in the control of mitochondrial respiration observed in permeabilized wild-type fibers, we performed all the experiments in muscles from the three different types of CK-deficient mice.

Determination of Extramitochondrial ADP Concentration after Induction of Mitochondrial Respiration by ADP Produced by Different Metabolic Pathways—As the control of mitochondrial respiration *in vivo* depends on the specific metabolic properties of the muscle (16), our experiments were carried out on three different muscles: heart and soleus (oxidative type), as well as white gastrocnemius (glycolytic type). While a high apparent K_m for exogenous ADP (around 150–200 μM for control mouse) was observed in oxidative slow-twitch permeabilized fibers, a very low apparent K_m for exogenous ADP (around 5–10 μM) characterized the white fast-twitch permeabilized fibers. These data are not shown, but appeared similar to those previously published (24, 25). We observed a significant decrease of the apparent K_m for ADP in heart fibers from M-CK-deficient mice (102 $\pm$ 11 *versus* 157 $\pm$ 6 μM in wild-type fibers), and a significant increase of the apparent K_m for ADP in gastrocnemius fibers from M-CK-deficient mice (8.7 $\pm$ 0.8 *versus* 4.6 $\pm$ 1.0 μM in wild-type fibers). This last observation was also made in
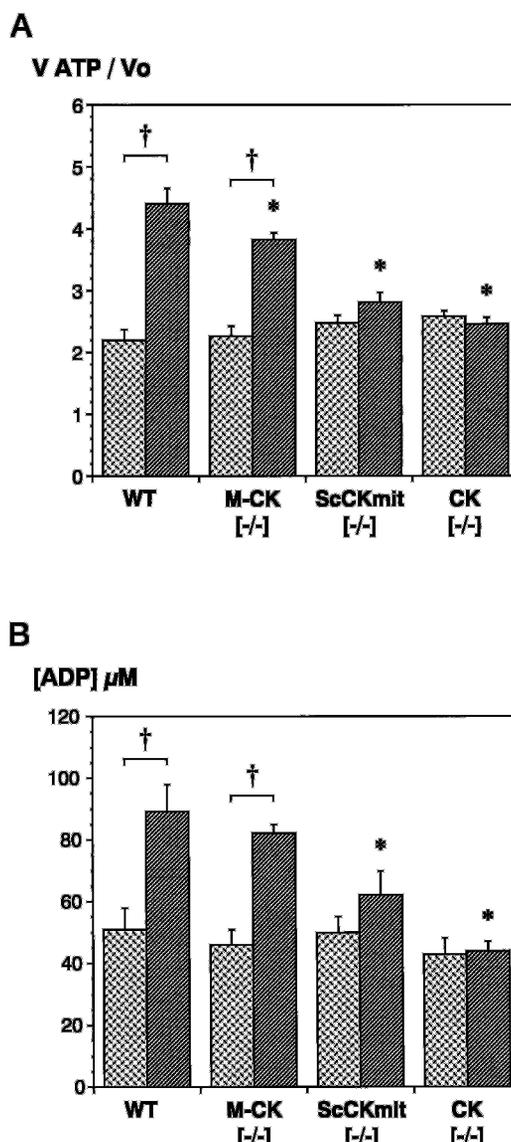

FIG. 2. **Determination of mitochondrial respiratory stimulation and extramitochondrial [ADP] when ADP is produced by different ways: study in permeabilized cardiac fibers.** The *graph* in *panel A* shows statistical data of the experiments presented in Fig. 1 for the wild-type (*WT*) and the three different types of transgenic CK mice (see "Materials and Methods"). The stimulation of respiration by 1 mM ATP was evaluated by the ratio of the respiration rate in the presence of 1 mM ATP on the respiration rate with substrates only, before ATP addition, ($V_{ATP\ 1\ mM}/V_0$). V_0 values were close to 6 nmol of O_2/min/mg dry weight for all groups. The same evaluations were made in respiratory medium supplemented with 20 mM Cr (*hatched bars*). In *panel B*, the steady-state extramitochondrial [ADP] after stimulation of respiration by 1 mM ATP alone or in the presence of Cr was determined in the medium surrounding the fibers, by HPLC method. The values are expressed in micromolar. The values are mean $\pm$ S.E. from four to eight different mice, $p < 0.05$ was taken as the level of significance. * different from wild-type value; † difference between the Cr and non-Cr groups.

gastrocnemius fibers from ScCKmit and double knock-out mice. But we did not observe any modification of the K_m for ADP in heart and soleus fibers from ScCKmit and double knock-out mice.

Fig. 1 shows representative oxygraph readings for permeabilized cardiac fibers from wild-type (*A*) and ScCKmit-deficient mice (*B*), when mitochondrial respiration was stimulated by 1 mM ATP in the presence or absence of Cr. In wild-type fibers, addition of ATP alone activated the oxygen consumption (VO_2) to half its maximal value (15.78 $\pm$ 0.83 nmol of O_2/min/mg dry

FIG. 3. **Determination of mitochondrial respiratory stimulation and extramitochondrial [ADP] when ADP is produced by different ways: study in permeabilized skeletal fibers.** As for cardiac fibers in Fig. 2, these graphs show the oxygraphic data (*A*) and the extramitochondrial [ADP] (*B*) obtained for soleus muscle (*I*) and gastrocnemius muscle (*II*). For experimental conditions see the legend of Fig. 2. In these types of muscles, V_0 values were close to 3 and 1 nmol of O_2/min/mg dry weight, respectively, for soleus and gastrocnemius. The values of ($V_{ATP\ 1\ mM}$/V_0) ratio and [ADP] are mean $\pm$ S.E. from four to eight different mice, $p < 0.05$ was taken as the level of significance. * different from wild-type value; † difference between the Cr and non-Cr groups.
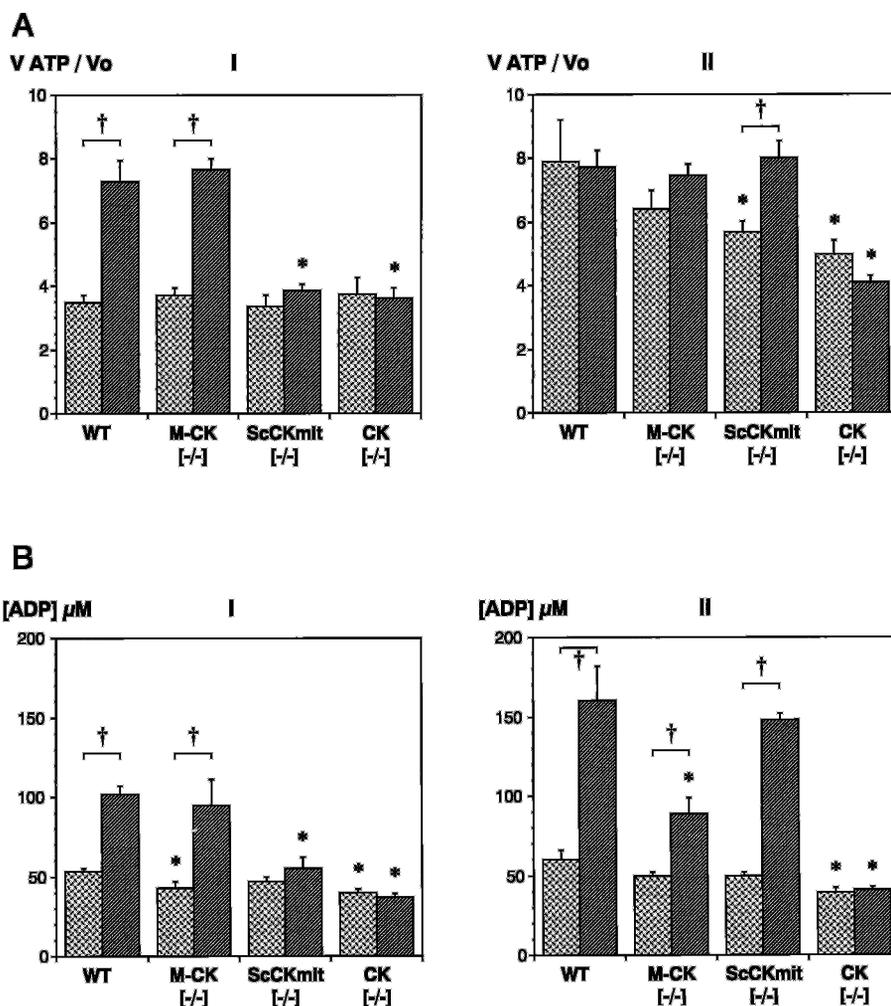
weight). In the presence of 20 mM Cr in the respiratory medium, a similar addition of ATP increased the VO_2 up to the maximal value (29.06 ± 1.18 nmol of O_2/min/mg dry weight), as it was observed at a saturating [ADP] of 1 mM (not shown). This stimulating effect of Cr was not observed in fibers from ScCKmit knock-out mice (Fig. 1*B*). In order to facilitate the comparison between groups in each type of muscle, the stimulation of respiration by 1 mM ATP was expressed by the ratio V_{ATP}/V_0, where V_0 is the VO_2 before ATP addition. Fig. 2*A* presents the mean values of this ratio obtained in cardiac fibers for each group of mice, and Fig. 2*B* those of the extramitochondrial [ADP] corresponding to the steady state, after 1 mM ATP addition alone or in the presence of Cr. While we observed an [ADP] of 51 ± 7 μM when respiration (around 50% of maximal activation) was induced by 1 mM ATP alone, an [ADP] of 89 ± 9 μM was measured under maximal stimulation of the respiration by Cr together with ATP. In cardiac fibers from M-CK-deficient mice, we observed qualitatively the same results as those obtained in cardiac wild-type fibers (Fig. 2, *A* and *B*), although the activation of respiration by Cr was slightly lower. By contrast, both the stimulation of respiration and the increase of [ADP], caused by the presence of Cr, were drastically decreased and completely suppressed in cardiac fibers from ScCKmit and double-CK knock-out mice, respectively (Fig. 2).

Fig. 3 presents the results of similar experiments carried out on two types of skeletal muscle, *i.e.* soleus (Fig. 3*I, A* and *B*) and gastrocnemius (Fig. 3*II, A* and *B*). Concerning the data obtained in soleus (oxidative) fibers, the very same conclusions can be drawn as for cardiac fibers. In both cases, the comparison between wild-type and transgenic groups showed that the

increase of both respiration and cytosolic [ADP] observed in the presence of Cr was related to the ScCKmit reaction.

In glycolytic type fibers from wild-type mice (Fig. 3*II*), the addition of 1 mM ATP induced a VO_2 close to the maximal value, as it was reached with saturating [ADP], being around 0.1 mM [ADP] in this type of fibers (not shown). While the V_{ATP}/V_0 ratio was not changed by the presence of Cr, cytosolic [ADP] was increased from 60 ± 6 μM in the absence of Cr to 160 ± 22 μM in its presence. In gastrocnemius fibers from double-CK knock-out mice, as observed before with oxidative fibers, the [ADP] was not changed in the presence of Cr, the values being even significantly lower than the control value. However, by contrast to what was found in oxidative fibers, the elevation of [ADP] in the presence of Cr was as high in glycolytic fibers from ScCKmit-deficient mice as that in wild-type fibers, and it was of smaller magnitude in glycolytic fibers from M-CK knock-out mice. Therefore, while the elevation of [ADP] in the presence of Cr was related to the ScCKmit activity in oxidative permeabilized fibers (heart and soleus), it seemed that the M-CK rather than the ScCKmit was mainly implicated in the accumulation of ADP into the cytosol of glycolytic fibers (Figs. 2*B* and 3*B*). This observation can be related to the presence of a larger amount of M-CK (lower amount of ScCKmit) in glycolytic than in oxidative fibers. Furthermore, it could be noticed that the stimulation of respiration by ATP alone tended to be lower in the transgenic groups than in the wild-type group. In gastrocnemius fibers, considering that the level of ADP produced from 1 mM ATP added was close to the saturating [ADP], and the apparent K_m for ADP was significantly higher in the three mutant type groups than in wild-type

group, the decreased V_{ATP} could be explained by a cytosolic [ADP] below the saturating concentration. When the [ADP] was maintained to saturating concentration by M-CK or ScCKmit activity in the presence of Cr, V_{ATP} was maximal. In gastrocnemius fibers from double-CK knock-out mice, the significant increase of V_0 was also partly responsible for decreasing the ratio of V_{ATP}/V_0.

Suppression of the Influence of Extramitochondrial ADP on Respiration by Use of a Competitive ADP-Trap System—Although in oxidative fibers, we observed parallel tendencies for elevation of VO_2 and [ADP] in the cytosol, this increase of the extramitochondrial [ADP] could not alone explain the stimulation of respiration at maximal rate. Indeed, Cr increased the [ADP] to a value close to 90 μM, which, however, was still much lower than needed for maximal VO_2 (1 mM ADP). Thus, to find out whether the stimulation of respiration by Cr in oxidative fibers was only a consequence of extramitochondrial [ADP] increase, or, as we supposed it, directly correlated with ADP generation by ScCKmit in the intermembrane space, we repeated the first experiment (stimulation of respiration by ATP ± Cr), followed by the addition of pyruvate kinase (PK) and phosphoenolpyruvate (PEP), at saturating levels of 10 units/ml and 5 mM, respectively, in order to rephosphorylate and thus eliminate cytosolic ADP. Finally, Ap$_5$A was used at a saturating concentration of 200 μM to eliminate an interference of AK. Under these conditions, there was a competition for ADP between oxidative phosphorylation and the ADP-trap system. After respiratory rates were stationary, samples were taken for the determination of [ADP] by HPLC. This experiment was carried out only on cardiac fibers.

Fig. 4*A* illustrates the principle of the method used. As AK was inhibited by Ap$_5$A, only CK isoenzymes are represented in the scheme. In the absence of PK + PEP system, supposedly cytosolic ADP contributes to the control of mitochondrial respiration. In the presence of an ADP-trap system, the role played by cytosolic ADP in the control of the respiration should be reduced to a minimum. This gives the possibility to evaluate the importance of ADP production, in the intermembrane space, by ScCKmit for the stimulation of respiration. *Panel B* of Fig. 4 shows the profile of adenine nucleotide contents present in the medium surrounding permeabilized fibers when respiration was stimulated by 1 mM ATP in the absence (*a*) and presence (*b*) of PK, PEP, and Ap$_5$A. Under this latter condition (*b*), we observed only a very small quantity of ADP as compared with profile *a*. An increase of the AMP content, probably due to the inhibition of AK activity, was also detected. Representative oxygraph readings obtained in this experiment for cardiac fibers from wild-type are depicted in Fig. 5*A*. Here, the traces show that the respiration rate observed after PK, PEP, and Ap$_5$A additions was still significantly higher in the presence of Cr (*b*) than in its absence (*a*), while the initial rates were identical. The oxygraph reading in Fig. 5*B* shows that the ADP-trap system used here was efficient, since it was able to suppress maximal stimulation induced by the addition of a saturating [ADP] (1 mM). Importantly, subsequent addition of Cr again stimulated respiration. In order to quantitatively interpret the data obtained from the experiments such as presented in Fig. 5*A*, the ATP-induced increase of VO_2, which persisted after the "PK + PEP and Ap$_5$A" system had been added to the respiratory chamber, was calculated from the difference between VO_2 measured under these conditions (after Ap$_5$A addition) and V_0.

Fig. 6 shows the mean values of the ATP-induced increase of VO_2 (*A*) and extramitochondrial [ADP] (*B*) in the presence or absence of Cr for wild-type and all three transgenic animal groups. With the ADP-trap system, the extramitochondrial
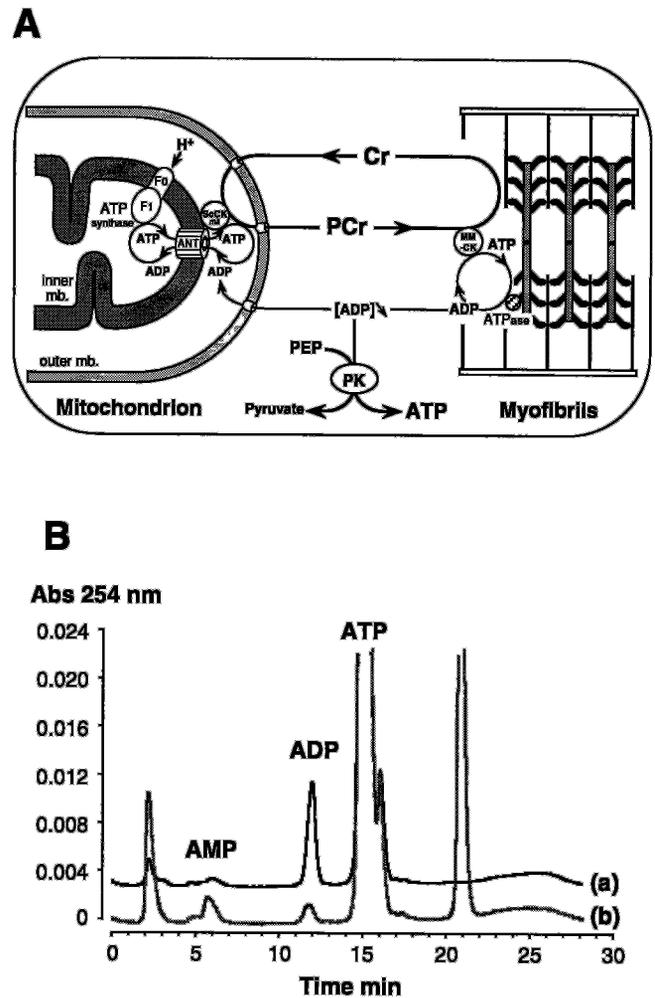
FIG. 4. **System used to investigate the control of respiration by mitochondrial intermembrane ADP produced by ScCKmit.** *Panel A* illustrates the role of the Cr-PCr system for intracellular energy transport in muscle cells. This schematic representation shows the compartmentation of CK isoenzymes and adenines nucleotides. The respiration can be controlled by different sources of ADP, that is cytosolic ADP or ADP produced in the intermembrane space by ScCKmit. By the use of a competitive ADP-trap system (5 mM PEP and 10 UI/ml PK) the cytosolic ADP is continuously rephosphorylated and thus its influence on the respiration rate is reduced to a minimum (see the text). *Panel B* shows HPLC chromatographs of adenines nucleotides from oxygraphic samples before (*a*) and after (*b*) the ADP-trap system addition and AK inhibition by Ap$_5$A. The different peaks of AMP, ADP, and ATP are indicated by *arrows*. The other peaks are related to compounds of respiratory buffer and accumulated metabolites.

[ADP] was decreased to a minimal value of around 12–14 μM, independently of the presence of Cr in the respiratory buffer, and in all experimental groups. The results with wild-type fibers showed that while the stimulation of respiration by ATP alone was very significantly decreased under very low extramitochondrial [ADP], as compared with what is observed in the absence of an ADP-trap system, the increase of respiration was still ×2.5 higher in the presence of Cr than in its absence, without any measurable changes in the extramitochondrial [ADP]. This was also true in fibers from M-CK-deficient hearts. But when ScCKmit was absent, the increase of the ATP-induced respiration in the presence of Cr disappeared. These results are direct evidence that the higher stimulation of respiration in the presence of Cr is due to local ADP generation in the intermembrane space by ScCKmit activity and that ScCKmit-produced ADP is preferentially used by oxidative phosphorylation rather than by the ADP-trap system.

FIG. 5. **Oxygraph recordings: effect of the ADP-trap system on respiration induced by ADP produced from different sources.** Respiratory rate was evaluated in cardiac permeabilized fibers from wild-type mice, in the presence of 5 mM glutamate and 2 mM malate. In *panel A*, as in Fig. 1, the respiration was stimulated by the addition of 1 mM ATP. Then, 5 mM PEP plus 10 UI/ml PK and 200 μM Ap5A were successively added. In the *lower trace* (*b*), the respiratory medium was supplemented with 20 mM Cr. In *panel B*, respiration was stimulated by the addition of 1 mM ADP, then inhibited (like in *panel A*) by the ADP-trap system, and finally reactivated by the addition of 20 mM Cr.
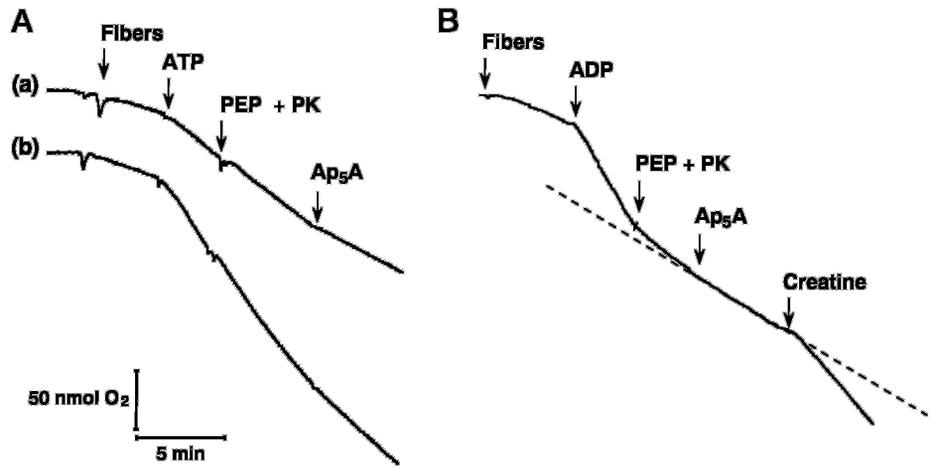

DISCUSSION

The results of this study confirm that the regulation of mitochondrial respiration in oxidative muscle differs from that in glycolytic muscle (24, 26). While oxidative phosphorylation in glycolytic fibers (from gastrocnemius) showed a very high sensitivity for extramitochondrial ADP, we found that the sensitivity of oxidative phosphorylation for extramitochondrial ADP was very low in oxidative fibers (from heart and soleus). Moreover, from the comparison between the results obtained in permeabilized fibers from wild-type and transgenic mice, it appears obviously that the ADP produced by ScCKmit reaction plays a more important role in the control of the mitochondrial respiration in oxidative than in glycolytic fibers. Furthermore, in the experiments on cardiac fibers, we demonstrate clearly that, interestingly, there is no direct and simple relationship between the rate of mitochondrial respiration and the [ADP] in the extramitochondrial medium, which would correspond to the cytoplasmic space in the cells *in vivo*. Instead, the respiration rate depends on the generation of ADP by multiple reactions in different intracellular compartments, in particular by ScCKmit. Due to its "strategic" localization inside the mitochondrial intermembrane space, ScCKmit, activated by the presence of Cr, can effectively stimulate respiration, while the [ADP] in the cytosol is maintained unchanged. So, the ADP generated by ScCKmit in the intermembrane space was preferentially used by the ATP-ADP carrier in the inner membrane for oxidative phosphorylation, rather than by the PK + PEP system.

This can be explained by the close functional coupling between ScCKmit and ATP-ADP carrier (15, 27, 28). Furthermore, the ScCKmit, present *in vivo* in an octameric form (29), is able to interact simultaneously with both inner and outer membranes (30, 31) and to form complexes with outer mitochondrial membrane porin pores ("voltage-dependent anion channels") and ATP-ADP carriers (32) in the so-called mitochondrial contact sites (33). These contact sites may play an important role for efficient energy export into the cytosol by a direct channeling of substrates and products between the interaction partners (13, 34).

The inaccessibility of the intermembrane space ADP to PK + PEP system can also be due to the restricted permeability of the mitochondrial outer membrane for adenine nucleotides (6, 14). Previously, the restricted free exchange of ADP through the outer membrane (14, 15) was not observed while studying the control of the mitochondrial respiration on isolated organelles. Under standard *in vitro* conditions, the porin pores are usually opened and permit essentially free passage of adenine nucleotides. The apparent K_m of oxidative phosphorylation for ADP is close to the K_m of the ATP-ADP carrier for ADP, which is in the range of 5–10 μM (35). Recently, the role of the outer membrane in the control of affinity for ADP was evidenced *in vivo* by the permeabilized fiber (or cell) technique and *in vitro* by studying the isolated mitochondria in surrounding medium containing macromolecules, like dextran, which induce an ultrastructural configuration representing better the *in vivo* status (14). At the colloid osmotic pressure increased, the outer membrane of isolated mitochondria appeared to be less permeable to adenine nucleotides, like what was observed in permeabilized fibers. This has primarily consequences for the ADP that is present at low levels and thus may build up diffusion gradients across the outer membrane (14). Actually, the nature of the factors restricting the permeability of the outer mitochondrial membrane is not clear yet, and is still a matter of scientific debate. A possibility, which could explain the effect of macromolecules (14) is a decreased permeability of the porin pores caused by oncotic pressure reducing the diameter of the pore (36, 37). However, there is increasing evidence showing specific interaction of mitochondria with cytoplasmic structural elements, presumably cytoskeleton, which may strongly influence the intracellular mechanisms of regulation of mitochondrial functions (6, 38). According to this last hypothesis, a specific factor possibly related to cytoskeleton would control the pore opening. Whatever the causes for its restricted permeability, the mitochondrial outer membrane is generally considered as a diffusion barrier diminishing both the transport of extramitochondrial formed ADP into the intermembrane space, and the export of intermembrane space produced ADP into the cytoplasm (14, 15).

The ADP-trap system has been used earlier by Gellerich and collaborators (22) on rat heart mitochondria to evidence the maintaining of a substantial CK-stimulated respiration in the presence of excess PK. More recently, a study with isolated rat liver mitochondria showed that a significant fraction of the ADP generated either by intermembrane space AK or by outer membrane bound hexokinase isoenzyme I, was not accessible to extramitochondrial PK (39). This competitive system was also used in isolated cardiac myofibrils to evidence the role of myofibrillar-CK in the relaxation of rigor tension (40) and to show that the Ca^{2+} uptake by the cardiac sarcoplasmic reticulum ATPase *in situ* strongly depends on bound CK (41). However, it was not clear how the ScCKmit, in its position between oxidative phosphorylation and cytosolic processes of energy utilization, influenced the relationships between mitochondrial respiration and cytoplasmic ADP. The use of permeabilized fibers from wild-type and CK-mutant transgenic mice answered this question, since in this technique mitochondria are not isolated but studied in their intracellular surroundings *in situ*. By using the ADP-trap system, in permeabilized cardiac

A

ATP-induced increase of VO₂

nmol O₂ / min / mg d.w.

B

[ADP] μM
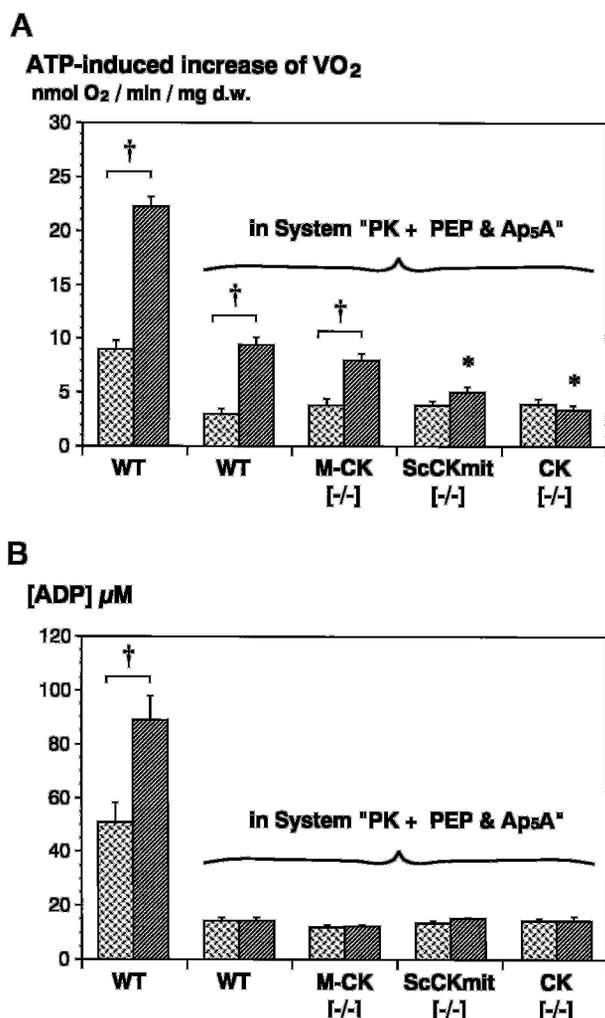
FIG. 6. **Direct evidence for the dissociation between the respiration rate and the extramitochondrial [ADP].** The *graph* in *panel A* shows the statistical data of the experiments presented on Fig. 5 for the wild-type (WT) and the three different types of CK transgenic mice. The ATP-induced increase of the respiration, which persists after ADP-trap system addition and inhibition of AK, is expressed, in nmol of O₂/min/mg dry weight, by the difference between the respiration rate after Ap₅A addition and the rate before ATP addition. The same evaluations were made in respiratory medium supplemented with 20 mM Cr (*hatched bars*). In *panel B*, the steady-state extramitochondrial [ADP], after addition of the ADP-trap system and inhibition of AK, was determined from the oxygraphic sample, as described in Fig. 2, and expressed in micromolar. For comparison, the ATP-induced increase of the respiration ($V_{\text{ATP 1 mM}} - V_0$) and steady-state extramitochondrial [ADP] obtained in wild-type fibers, in the absence of ADP-trap system and AK inhibition, are also plotted on the *left* of graphics. The values are mean ± S.E. from four to 10 different mice, $p < 0.05$ was taken as the level of significance. * different from wild-type value; † difference between the Cr and non-Cr groups.

fibers, we directly demonstrate that the respiration rate can be dissociated from extramitochondrial [ADP], when the ScCKmit reaction is activated. However, it is interesting to note that in the presence of PK and PEP, the ScCKmit activated by physiological [Cr] of 20 mM was not able to maximally activate respiration (Fig. 6*A*). Obviously, some contribution from extramitochondrial ADP is necessary to achieve the maximal rate of respiration, or some leak of ADP produced in the intermembrane space through the outer membrane into the cytosol, which is exacerbated by the presence of the ADP-trap system, limits the respiration to a submaximal value. In M-CK(−/−) cardiac fibers, as the outer membrane permeability has been found higher (lower K_m for ADP) than in wild-type fibers, the leak of ADP could be increased. Changes of the outer mem-

brane permeability may be in line with the ability of M-CK(−/−) muscles to import PCr into mitochondria and produce ATP, as the reverse flux observed in ³¹P NMR studies showed (17).

To summarize, the results of this work emphasize the importance of compartmentation and local production of ADP for the regulation of the oxidative phosphorylation in mitochondria *in vivo*. However, these results are not sufficient to solve the problem of the regulation of mitochondrial respiration in working heart cells, where both the energy producing and energy utilizing systems are interconnected by the whole network of energy transfer systems, such as the entire CK and AK systems, diffusional exchange of metabolites, and glycolytic system (42). Under *in vivo* conditions, a great variety of steady state levels of metabolites may exist depending on the workload, the state of enzyme systems, the metabolite levels, and the pathology. In all these cases different intracellular factors may play different roles in the regulation of respiration. An interesting possibility is a redistribution of the control of respiration among the parallel pathways of feedback regulation, such as Cr and Pᵢ fluxes from myofibrils to mitochondria. These complex problems will still require further quantitative studies before being solved completely.

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