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Regulation of oxidative phosphorylation in mitochondria of epididymal bull spermatozoa

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The regulation of oxidative phosphorylation was studied with digitonin-treated epididymal bull spermatozoa in which mitochondria are directly accessible to low molecular compounds in the extracellular medium. Due to the high extramitochondrial ATPase activity in this cell preparation, it was possible to stimulate respiration to a small extent only by added hexokinase in the presence of glucose and adenine nucleotides. Added pyruvate kinase plus phospho*enol*pyruvate, however, strongly suppressed the respiration. Under these conditions, the respiration was found to depend on the extramitochondrial [ATP]/[ADP] ratio in the range of 1–100. The contribution of the adenine nucleotide translocator to this dependence was determined by titration with the irreversible inhibitor carboxyatractyloside in the presence of ADP. Using lactate plus malate as substrate, the active state respiration was controlled to about 30% by the translocator, whereas 12 and 4% were determined in the presence of L-glycerol-3-phosphate and malate alone, respectively. In order to compare the results with those for intact cells, the adenine nucleotide patterns were determined in intact and digitonin-treated spermatozoa under conditions of controlled respiration in the presence of vanadate and carboxyatractyloside, respectively. About 21% of total cellular adenine nucleotides were found in digitonin-treated cells representing the mitochondrial compartment. While allowing for the intramitochondrial amount of adenine nucleotides, the cytosolic [ATP]/[ADP] ratio was estimated to be 6-times higher than the mitochondrial ratio in intact cells. It is concluded from the data presented that the principal mechanism by which oxidative phosphorylation in sperm mitochondria is regulated via the extramitochondrial [ATP]/[ADP] ratio is the same as that demonstrated for other isolated mitochondria.

Introduction

Due to the simplicity of their cellular energy metabolism, intact spermatozoa represent an appropriate object for investigating into the regulation of the oxidative energy metabolism. It was found for these cells that variations in motility as induced by vanadate, the inhibitor of dynein ATPase, led to shifts in the cellular [ATP]/[ADP] ratio, which, in turn, caused changes in the respiratory rate [1,2]. However, the range of response of mitochondrial respiration to this ratio markedly differed from that observed with isolated mitochondria of different sources [3]. The obvious difference might be caused by compartmentation of adenine nucleotides in the cytosolic and mitochondrial compartments, leading to lower

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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total [ATP]/[ADP] ratios than the cytosolic ones. On the other hand, sperm mitochondria may differ in their response to the extramitochondrial [ATP]/[ADP] ratio from that of other mitochondria. For spermatozoa the intracellular distribution of adenine nucleotides as well as the mitochondrial response to the extramitochondrial [ATP]/[ADP] ratio are unknown. In the present study, spermatozoa with digitonin-permeabilised plasma membrane were used to investigate into both problems. In particular, the dependence of respiration on the extramitochondrial [ATP]/[ADP] ratio and the role of the adenine nucleotide translocator were studied, since the latter is known to be an important reaction step for the control of respiration coupled to ADP phosphorylation [3–7]. The adenine nucleotide patterns of the mitochondrial and the cytosolic compartments were determined to compare the results with those for intact cells.

Materials and Methods

Spermatozoa were prepared from the caudae epididymidis of bulls within two hours of slaughter. The cells were collected by retrograde flushing with a medium of 120 mM NaCl and 50 mM Hepes (pH 7.4). Spermatozoa were washed once by centrifugation for 6 min at $600 \times g$. For experiments with intact epididymal spermatozoa the cells were incubated in a medium containing 140 mM NaCl, 6 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris and 10 mM Hepes (pH 7.4). Spermatozoa for permeabilization were resuspended in a KCl medium described by Keyhani and Storey [8] and containing 113 mM KCl, 3 mM $MgCl_2$, 5 mM KH_2PO_4 and 20 mM Tris (pH 7.4). Digitonin treatment was performed by dropwise addition of the digitonin stock solution (5 mg/ml) under rapid stirring up to a final concentration of 5 μg digitonin per μl of packed cells (about $14 \cdot 10^6$ cells) [9]. The suspension was centrifuged for 10 min at $800 \times g$, resuspended in KCl medium and stored at 0–4 °C until use. The basic incubation medium for digitonin-treated spermatozoa contained 110 mM sucrose, 60 mM KCl, 15 mM glucose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM EDTA and 60 mM Tris (pH 7.4).

In the experiments adapted to study the dependence of respiration on the extramitochondrial [ATP]/[ADP] ratio, the incubation medium was complemented by 2.5 mM lactate, 2.5 mM malate, and 2 mM phospho*enol*pyruvate. The extramitochondrial [ATP]/[ADP] ratio was varied by addition of various amounts of yeast hexokinase (EC 2.7.1.1) or rabbit muscle pyruvate kinase (EC 2.7.1.40).

Concentrations of spermatozoa were assayed by using a spermatocrit procedure and calculated as μl packed cells [10]. In previous work, it was checked to ensure that the number of spermatozoa per volume of packed cells did not significantly differ for intact and digitonin-treated spermatozoa [9]. Respiration was determined polarographically at 37 °C while adopting an oxygen solubility as described by Reynafarje et al. [11]. The rate of oxygen uptake was recorded simultaneously in the carboxyatractyloside titration experiments. Adenine nucleotides were assayed spectrophotometrically by standard enzymic procedures [12] from samples quenched by perchloric acid (final concentration, 0.55 M).

Chemicals and enzymes. Lactic acid was a product of Sigma (St. Louis). Sodium monovanadate was obtained from Merck (Darmstadt) and prepared as stock solution as described previously [1]. The enzymes, malate, phospho*enol*pyruvate (cyclohexylammonium salt), carboxyatractyloside, and FCCP were purchased from Boehringer (Mannheim). All other chemicals were of analytical grade.

Results

Investigation into mitochondrial functions of spermatozoa requires the permeability barrier of the plasma membrane for low-molecular compounds to be removed without impairing the mitochondrial intactness. Fig. 1 illustrates the effect on succinate oxidation of the treatment of spermatozoa with various amounts of digitonin. Trace a shows that the rate was low in untreated spermatozoa. The full capacity for succinate oxidation could be measured after addition into the polarographic chamber, of an appropriate amount of digitonin which eliminates the restricted permeability of the plasma membrane for externally added succinate [10]. Spermatozoa in

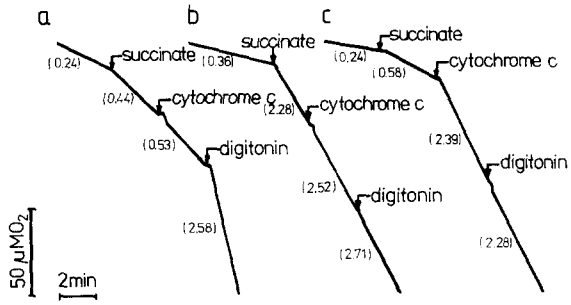

Fig. 1. Succinate oxidation by epididymal spermatozoa before and after digitonin treatment. Untreated cells (trace a) and cells permeabilised with 5 µg (trace b) or 35 µg digitonin per µl cells (trace c) were used. The additions were 5 mM succinate, 53 µM cytochrome *c* and 156 µM digitonin (trace a) or 64 µM digitonin (traces b and c), and 8.3 µM rotenone was present at all times. Sperm concentrations (µl cells per ml) were: 21 (a), 7.5 (b) and 9.3 (c). Numbers in parentheses indicate nmol O_2/min per µl cells.

trace b were pretreated with 5 µg digitonin per µl cells as described in Material and Methods; this lead to a nearly total permeability for succinate. The missing effect of added cytochrome *c* and the comparable final rates of respiration indicated that mitochondria had an intact outer membrane. On the other hand, the use of five times the amount of digitonin (trace c) was followed by a low-rate succinate oxidation which could be considerably stimulated by cytochrome *c*. This along with the decreased final rate after digitonin addition was suggestive of an injury of mitochondria.

Functional criteria of mitochondria in cells treated with the optimum amount of digitonin are demonstrated in Fig. 2. High ratios of ADP-stimulated to carboxyatractyloside-inhibited rates of respiration indicate the high degree of coupled mitochondria (trace a). Respiration was also stimulated to a substantial extent by addition of AMP (trace b). This requires the presence of adenylate kinase and demonstrates the intactness of the mitochondrial outer membrane.

The adenine nucleotides remaining in the digitonin-treated spermatozoa after washing were supposed to represent the mitochondrial pool. The adenine nucleotide patterns of intact and digitonin-treated sperm suspensions were compared to estimate their distribution between the mitochondrial and the cytosolic compartments. Listed in Table I are the measured adenine nucleotides of digitonin-treated spermatozoa along with data for the intact cells of the same cell preparations. In order to establish comparable conditions, controlled states of respiration were adjusted in intact and digitonin-treated spermatozoa with vanadate and carboxyatractyloside, respectively. The respective rates of respiration found for the cells under these conditions were 0.62 and 0.60 nmol O_2/min per µl cells. The data given in Table I were adopted to calculate cumulative adenine nucleotides and [ATP]/[ADP] ratios which are presented in Table II. Considerable differences were noted in the adenine nucleotide patterns of the sperm preparations. They were much greater than the standard deviations obtained for the individual preparations. Obviously, they were caused by a different equipment of the individual sperm populations, a finding also yielded with intact ejaculated spermatozoa [1,10]. On an average, about 21% of the total celular adenine nucleotides were found in digitonin-treated spermatozoa. The total cellular [ATP]/[ADP] ratio of epididymal spermatozoa was identical with that measured after vanadate inhibition in ejaculated spermatozoa [1,2]. The cytosolic patterns were calculated from the differences in the contents of the separate adenine nucleotide species

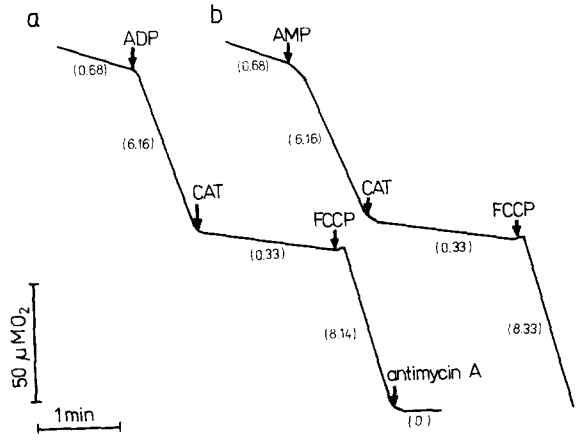

Fig. 2. Response of permeabilised spermatozoa to ADP or AMP and effectors of oxidative phosphorylation. Spermatozoa (17 µl cells per ml) permeabilised with 5 µg digitonin per µl cells were incubated with 2.5 mM lactate/2.5 mM malate. Additions were: 1 mM ADP or AMP, 40 µM carboxyatractyloside (CAT), 0.4 µM antimycin A or 0.2 µM FCCP. Numbers in parentheses indicate nmol O_2/min per µl cells.

TABLE I

ADENINE NUCLEOTIDE PATTERNS OF INTACT AND DIGITONIN-TREATED SPERMATOZOA AT INHIBITED EXTRAMITOCHONDRIAL ATP TURNOVER

Intact spermatozoa (92–138 μl cells/ml) and digitonin-treated spermatozoa (75–144 μl cells/ml) obtained from the same sperm preparation were incubated in the presence of 5 mM lactate/5 mM malate plus 0.25 mM vanadate (for the former) and 33 μM carboxyatractyloside (for the latter) for 10 min at 37 °C. After the preincubation, three samples were taken for the adenine nucleotide assay. Data represent mean ± S.D. for duplicate measurements of the three samples.

Sperm preparation	Adenine nucleotides (nmol per μl cells)					
	intact cells			digitonin-treated cells		
	ATP	ADP	AMP	ATP	ADP	AMP
1	3.81 ± 0.06	0.48 ± 0.02	0.25 ± 0.03	0.57 ± 0.02	0.24 ± 0.02	0.38 ± 0.01
2	4.81 ± 0.17	0.53 ± 0.03	0.37 ± 0.01	0.69 ± 0.16	0.26 ± 0	0.26 ± 0.09
3	4.07 ± 0.17	0.63 ± 0.01	0.29 ± 0	0.37 ± 0.03	0.25 ± 0.03	0.11 ± 0.02
4	4.27 ± 0.18	0.58 ± 0.05	0.49 ± 0.03	0.87 ± 0.05	0.32 ± 0.05	0.38 ± 0.02
5	4.77 ± 0	0.34 ± 0.02	0.27 ± 0.02	0.48 ± 0.04	0.14 ± 0	0.14 ± 0.03

in intact and digitonin-treated spermatozoa. The cytosolic [ATP]/[ADP] ratios estimated in this way were about 6-times greater than the mitochondrial ones. Additionally, we calculated the mass action ratio of adenylate kinase in the cytosolic compartment. Although this ratio was somewhat lower than the equilibrium constant of adenylate kinase as assumed for cytosolic conditions [18], it was comparatively fixed in four of the sperm suspensions studied.

As can be calculated from the data in Table II cytosolic [ATP]/[ADP] ratios from 10 to 20 were built up by spermatozoal mitochondria in the absence of any extramitochondrial ATP utilization. On the other hand it is known for isolated mitochondria of other sources that [ATP]/[ADP] ratios of about 100 result in the controlled state of respiration [3–5]. The question arose whether controlled respiration in digitonin-treated spermatozoa alike would occur at high extramitochondrial [ATP]/[ADP] ratios. Therefore, these permeabilised cells were incubated with adenine nucleotides and various amounts of hexokinase as successfully used to vary the extramitochondrial ATP consumption [3,5]. However, as illustrated on the left in Fig. 3, the rate of ATP-stimulated respiration in the absence of hexokinase nearly reached the active state respiration in the presence

TABLE II

CELLULAR, MITOCHONDRIAL AND CALCULATED CYTOSOLIC CONTENTS OF ADENINE NUCLEOTIDES AND [ATP]/[ADP] RATIOS

Data were calculated from Table I; values for mitochondria were taken from digitonin-treated cells, values for cytosol from the difference between intact cells and digitonin-treated cells. Means and standard deviations refer to five different sperm preparations.

Sperm preparation	Σ(ATP + ADP + AMP)			[ATP]/[ADP]			$[ATP]_c/[ADP]_c$	$[ADP]_c^2$
	cells (nmol per μl cells)	mitochondria (nmol per μl cells)	(%)	cells	mitochondria	cytosol	$[ATP]_m/[ADP]_m$	$[ATP]_c \cdot [AMP]_c$
1	4.54 ± 0.11	1.19 ± 0.05	26.2	8.0 ± 0.3	2.4 ± 0.1	13.0	5.4	–
2	5.71 ± 0.18	1.21 ± 0.25	21.2	9.2 ± 0.2	2.7 ± 0.6	15.3	5.7	0.15
3	4.99 ± 0.17	0.73 ± 0.05	14.6	6.5 ± 0.3	1.5 ± 0.2	9.7	6.5	0.22
4	5.34 ± 0.17	1.58 ± 0.04	29.6	7.4 ± 0.8	2.7 ± 0.6	13.1	4.9	0.18
5	5.37 ± 0	0.76 ± 0.01	14.2	14.3 ± 0.9	3.4 ± 0.3	21.5	6.3	0.07
$\bar{x}$	5.19	1.09	21.2	9.1	2.5	14.5	5.8	0.16
± S.D.	± 0.44	± 0.35	± 6.9	± 3.1	± 0.7	± 4.4	± 0.7	± 0.06

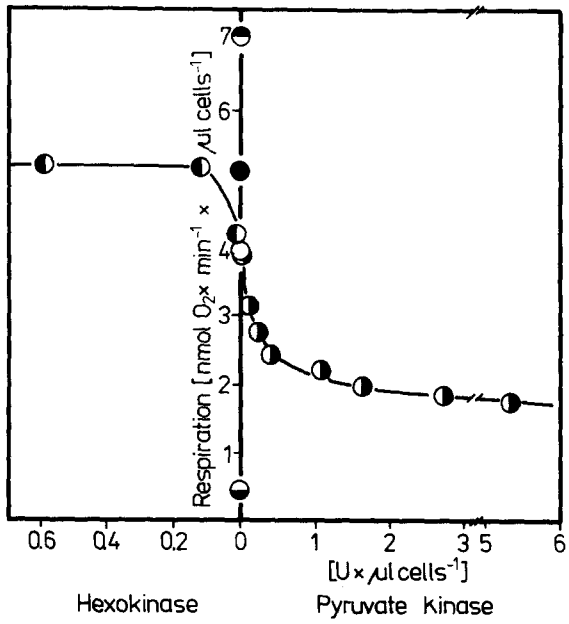

Fig. 3. Variation of respiratory rates of digitonin-treated epididymal spermatozoa by changes in the extramitochondrial ATP turnover. Digitonin-treated spermatozoa (12 µl cells/2.5 ml sample) were incubated as described in Materials and Methods. Pyruvate kinase (◑) or hexokinase (◐) were added as indicated. Reactions were started by addition of 1.7 mM ATP (○); the resulting stationary respiratory rates are given. The rates in the presence of 2 mM ADP (●), 0.12 µM FCCP (◓) or 40 µM carboxyatractyloside (◒) are presented for comparison.

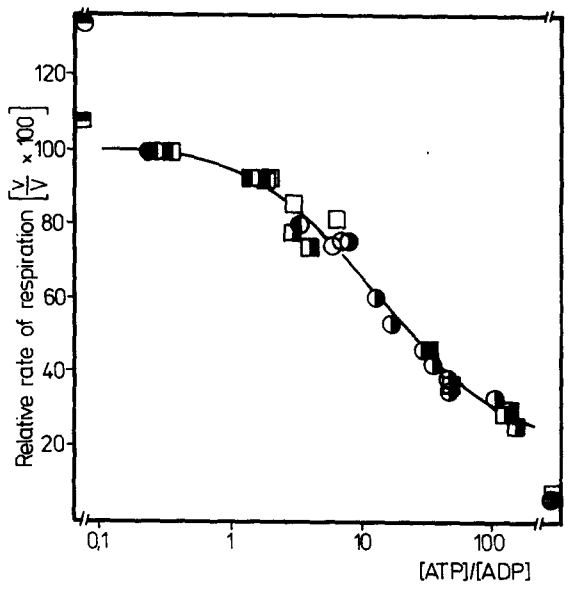

Fig. 4. Mitochondrial respiration vs. extramitochondrial [ATP]/[ADP] ratio. The data were taken from the experiment depicted in Fig. 3 and an additional experiment (squares). Samples for the adenine nucleotide determination were withdrawn from the incubations mixture after 2.5 min and quenched immediately by $HClO_4$. Due to the different maximal rates of respiration measured with excess of hexokinase (5.18 and 4.41 nmol O_2/min per µl cells), the rates were expressed in percent of these values. The curve was obtained by a least-square fit [2] of Eqn. 1 to data resulting in the following parameter values: $V_{resp} = 101.5\%$, $V_0 = 20.1\%$, $K_{resp} = 13.6$ and $n = 0.944$. Additionally, the relative rates of uncoupled (◓, ◨) and carboxyatractyloside-inhibited (◒, ◧) respiration are given.

of ADP. This stimulation is caused by cellular ATPases, mainly dynein ATPase, present in the preparation [9]. Still, as shown it was possible to suppress the respiration by increasing amounts of pyruvate kinase in the presence of phospho*enol*-pyruvate. This procedure was successfully adopted by Gellerich et al. [13] with liver mitochondria to counteract the ATP-splitting activity of externally added hexokinase. Even though the respiration decreased strongly due to ATP production by pyruvate kinase, high activities of this enzyme were needed. Nevertheless, the lowest respiratory rate obtained was still considerably higher than in the carboxyatractyloside-inhibited state. On the other hand, addition of small amounts of hexokinase in the presence of glucose led to an identical rate of respiration as that for ADP. Samples were withdrawn for the assay of adenine nucleotides to determine the dependence of respiration on the extramitochondrial [ATP]/[ADP] ratio at each stationary state of respiration as established by addition of hexokinase or pyruvate kinase. The results of two experiments are summarized in Fig. 4. In the light of the different maximal rates of respiration, the respiration was expressed as relative rates. As shown, it was then possible to describe the relation between respiration and the [ATP]/[ADP] ratio by a uniform curve. The latter was obtained by a least-square fit to a simple kinetic equation similar to that used in studies with intact spermatozoa [2]:

$$v_{resp} = \frac{V_{resp}}{1 + (X/K_{resp})^n} + V_0 \qquad (1)$$

where X is the extramitochondrial [ATP]/[ADP] ratio, V_{resp} the maximum rate of [ATP]/[ADP]-de-

pendent respiration, K_{resp} the [ATP]/[ADP] ratio for the half maximum of this rate, n an apparent cooperativity coefficient, and V_0 the rate remaining at infinitely large [ATP]/[ADP] ratios.

Although [ATP]/[ADP] ratios of up to 150 were measured, the minimal rate of respiration V_0 was considerably higher than the measured carboxyatractyloside-inhibited respiration found in both experiments (8.4 and 9.5%, respectively).

The general dependence of mitochondrial respiration on the extramitochondrial [ATP]/[ADP] ratio was mainly attributed to the kinetic properties of the adenine nucleotide translocator [3,5,14]. But the amount of control exerted by the adenine nucleotide translocator was found to depend on the respiratory rate and, with the exception of the fully active state of respiration, also on the kinetic properties of the extramitochondrial ATP-utilizing enzymes [13,15]. Therefore, to study the role of the adenine nucleotide translocator in the control of sperm respiration, its flux control coefficient was determined in the active state respiration by titration with the irreversible inhibitor, carboxyatractyloside [6]. Two typical titrations in the presence of different substrates obtained from the same preparation of digitonin-treated spermatozoa are shown in Fig. 5. The active state respiration was much more sensitive to inhibition by carboxyatractyloside with lactate plus malate as substrates than in the presence of malate only, while yielding a substantially lower rate of active respiration. To calculate the flux control coefficient according to Groen et al. [6] the initial slope dJ/dI and the carboxyatractyloside amount I_{max} for maximal inhibition must be derived from the titration curve. The flux control coefficient C^J then results as:

$$C^J = \frac{dJ}{dI} \cdot \frac{I_{max}}{J} \qquad (2)$$

where J is the respiratory rate in the absence of the inhibitor. The results of such titration experiments in the presence of various substrates are summarized in Table III. As already shown qualitatively in Fig. 5, the flux control coefficient was seen to depend markedly on the kind of hydrogen donor. Comparison of the data for lactate plus malate and malate only revealed that a diminished supply of the respiratory chain with reducing equivalents led to a drastic decrease in flux control coefficient of the adenine nucleotide translocator. It is reasonable to conclude that under such conditions the flux control coefficient of the substrate-supplying reactions is elevated. A similar effect was observed with isolated liver mitochondria by Böhme et al. [16]. Yet, the highest respiratory rates were measured with L-glycerol-3-phosphate, although maximum control coefficients of the adenine nucleotide translocator were found with lactate plus malate. In this context, it must be taken into account that L-glycerol-3-phosphate is oxidized via the flavoprotein-dependent L-glycerol-3-phosphate dehydrogenase. Due to the lowered ADP/O ratio, the flux via the adenine nucleotide tranlocator at the same rate of respiration with L-glycerol-3-posphate as substrate is smaller than during oxidation of lactate plus malate.

The higher flux control coefficient of the adenine nucleotide translocator with lactate plus malate is consistent with observations indicating that in this case the active state respiration could

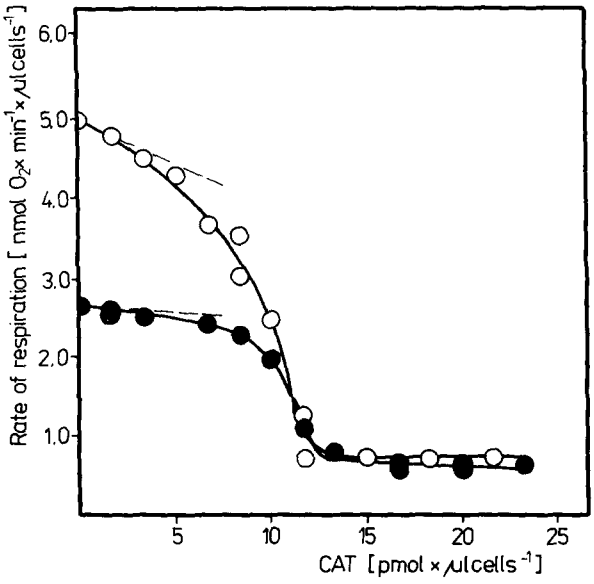

Fig. 5. Titration of active state respiration by carboxyatractyloside (CAT). Digitonin-treated spermatozoa (30 µl cells/2.9 ml sample) were titrated with carboxyatractyloside in the presence of 1 mM ADP and 2.5 mM lactate plus 2.5 mM malate (○) or 5 mM malate alone (●). The stationary rates of respiration after addition of carboxyatractyloside are given. Both titration curves include data from two titration experiments.

TABLE III

FLUX CONTROL COEFFICIENTS OF THE MITOCHONDRIAL ADENINE NUCLEOTIDE TRANSLOCATOR IN THE ACTIVE STATE RESPIRATION OF DIGITONIN-TREATED SPERMATOZOA

Titrations with carboxyatractyloside (CAT) were performed as described in Fig. 5. The flux control coefficients (C^J) were calculated according to Eqn. 2. Data represent mean ± S.D. for the given number of titrations performed with three individual sperm preparations.

Substrate	Number of titrations	J (nmol O_2/min per μl cells)	I_{max} (pmol CAT per μl cells)	C^J
2.5 mM lactate + 2.5 mM malate	7	4.91 ± 0.19	11.3 ± 0.9	0.29 ± 0.05
5 mM malate	5	2.50 ± 0.33	10.9 ± 1.4	0.04 ± 0.02
5 mM L-glycerol-3-phosphate	4	5.30 ± 0.73	11.3 ± 1.2	0.12 ± 0.03

be stimulated considerably by uncoupling, but not with malate alone or with L-glycerol-3-phosphate [9]. The I_{max} values represent a measure of the carboxyatractyloside-binding capacity of mitochondria. Expectedly, these values were independent of both the substrate used and the various sperm preparations studied.

Discussion

Measuring the adenine nucleotide contents of intact and digitonin-treated cells of the same suspension of epididymal spermatozoa enabled us to determine the distribution of these metabolites between the mitochondrial and the cytosolic compartments. This approach requires that mitochondria in digitonin-treated preparations be intact. The intactness of the mitochondrial outer membrane is indicated by the missing effect of external cytochrome *c* on succinate oxidation as well as by the stimulating action exerted by AMP on respiration. Furthermore, there was no loss of mitochondria during the digitonin treatment as indicated by comparable final rates of succinate oxidation in untreated and in digitonin-treated cells (cf. Fig. 1). The maximal rate of oxidation observed with lactate plus malate in the presence of uncoupler (cf. Fig. 2) corresponds to the uncoupled rate of lactate oxidation by intact spermatozoa [1,2]. Since lactate oxidation is coupled to the intramitochondrial NAD^+ pool, a remarkable leakage of intramitochondrial nucleotides during the digitonin treatment is not likely to occur. The intactness of mitochondria in digitonin-treated spermatozoa was demonstrated by their functional criteria, too. The mitochondria were well coupled as indicated by high ratios of the active to the carboxyatractyloside-inhibited rates of respiration in the order of 10 (cf. Figs. 2, 3 and 5). The difference between these two rates lends itself to the calculation of a capacity of sperm mitochondria for ADP phosphorylation which is about twice the rate of the ATP turnover found in intact ejaculated spermatozoa [1]. On the other hand, it cannot be ruled out that part of the remaining adenine nucleotides are bound to extramitochondrial proteins. This, however, would require a high-affinity binding, since, otherwise, the bound nucleotides would be removed by the washing procedure.

For the range of the extramitochondrial [ATP]/[ADP] ratio from 1 to 100 in which the respiration of spermatozoal mitochondria was found to respond to this ratio, there were two remarkable differences seen in comparison to the range of other mitochondria. The half-maximum rate of respiration in digitonin-treated spermatozoa was measured at [ATP]/[ADP] ratios of about 14 (cf. Fig. 2). In rat liver mitochondria, this figure ranged from 50 to 100 as a function of the substrate oxidized [17]. The shift towards smaller ratios may have been caused by particular properties of the sperm mitochondria. In this connection it is noteworthy that a relatively large apparent K_m for ADP was observed for the respiration in digitonin-treated spermatozoa [9]. In part, it may also be produced by phospho*enol*pyruvate present in the incubation mixture since such an effect of phospho*enol*pyruvate was noted with liver mitochondria, too (Gellerich, F.N., personal communication). The second difference when compared to liver mitochondria was the obvious failure to suppress the respiration by added pyruvate

kinase to the same extent as by carboxyatractyloside, a feature which may be caused by the tight binding of spermatozoal mitochondria to the axoneme [18] such that at least part of the ADP produced by dynein ATPase in the axoneme reaches the mitochondria rather than the extracellular pyruvate kinase. Similar effects were observed with heart mitochondria if ADP was generated by creatine kinase in the mitochondrial intermembrane space [19]. Also in this case, extramitochondrial pyruvate kinase was unable to suppress the respiration to the same extent as did carboxyatractyloside.

The 30% contribution by the adenine nucleotide translocator to the control of respiration as seen in digitonin-treated spermatozoa with lactate plus malate as substrates (Table III) is not directly comparable to the situation in intact cells. The respiratory rate of the cells was comparable to about 50% of active state respiration of digitonin-treated cells. In the range of half-maximum rates of respiration, the contribution of the translocator to the control is known to be reduced by reactions of extramitochondrial ATP utilization [13,15]. These reactions were found to control the ATP flux in intact spermatozoa to about 80%, whereas merely the remaining 20% were exerted by mitochondrial reaction steps [2]. Therefore, this latter value is supposed to be an upper limit of respiratory control by the adenine nucleotide translocator in intact spermatozoa.

It follows from the estimated distribution of adenine nucleotides that the cytosolic [ATP]/[ADP] ratio must be higher than the total cellular ratio. However, cytosolic [ATP]/[ADP] ratios from 10 to 20 which were calculated as shown in Table II, did not correspond to the high extramitochondrial ratios that must be expected from the dependence of respiration of digitonin-treated spermatozoa (cf. Fig. 4). Similar situations were experienced for other intact cells [20–23]. This discrepancy has been discussed in terms of a binding of adenine nucleotides, especially of ADP [24–26]. Due to the particular structure of spermatozoa, there may also exist diffusion-dependent concentration gradients of adenine nucleotides within the cell. Production of ATP by mitochondria in the midpiece and ATP consumption by dynein ATPase along the whole sperm tail might lead to higher [ATP]/[ADP] ratios in the surroundings of mitochondria than that indicated by the average value calculated for the cytosolic compartment.

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