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Use of NAD(P)H and flavoprotein fluorescence signals to characterize the redox state of pyridine nucleotides in epididymal bull spermatozoa

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The redox behaviour of the NAD(P) system and flavoproteins was registered by simultaneous fluorescence measurements in epididymal bull spermatozoa. The flavoprotein fluorescence signal can nearly exclusively be attributed to an NAD-linked enzyme, α-lipoamide dehydrogenase ($E_{m7.4} = -286$ mV). A comparison of intact with digitonin-permeabilized spermatozoa revealed that about 50% of the total NAD(P)H fluorescence signal was of mitochondrial origin. Under equilibrium conditions, the midpoint potentials of the NAD(P)H fluorescence signal of both compartments were almost identical (−300 mV). When lactate was present as substrate, 1 mM caffeine increased respiration oxidizing the NAD(P)H system in both mitochondria and cytosol. This indicates a close relationship of the two NAD pools in spermatozoa.

Introduction

The energy metabolism of bull spermatozoa is supplied mainly by substrates of carbohydrate metabolism. Depending on the surrounding milieu (epididymal plasma, seminal plasma, fluids of the female genital tract), major substrates of spermatozoa are fructose, glucose, lactate or pyruvate [1]. Under aerobic conditions, these substrates yield reducing equivalents to maintain ATP production of mitochondria [1]. For this reason, the redox state of the NAD(P) system was determined as an indicator of the energy state of spermatozoa [2–4] and can be registered continuously by a relatively simple fluorescence measurement of reduced pyridine nucleotides [4]. The overall fluorescence signal, however, does not distinguish mitochondrial from cytosolic pyridine nucleotides.

In liver mitochondria, the main part of flavoprotein fluorescence is due to α-lipoamide dehydrogenase [5]. The fluorescence signal of this flavin was found to be closely related to the mitochondrial NAD(P) system [6,7]. This signal may hence be used as indicator for the redox state of the mitochondrial pyridine nucleotides [5].

The present work is designed to investigate the suitability of fluorescence signals of flavoproteins and NAD(P)H for calculating redox states of mitochondrial and cytosolic pyridine nucleotides. To this end, fluorescence signals of the two fluorochromes were registered simultaneously in both intact and digitonin-treated spermatozoa to gain information about the shares of mitochondrial and cytosolic fluorochromes in the measured fluorescence signals. Midpoint potentials of fluorochromes were determined by titration with the substrate couples lactate/pyruvate and β-hydroxybutyrate/acetoacetate. In intact epididymal spermatozoa, different states of activity were adjusted by caffeine, which increases motility and respiration [8,9]. This led, in the presence of lactate as substrate, to an oxidation of the mitochondrial and the cytosolic NAD system, indicating that the two pools are closely related to each other.

Materials and Methods

Spermatozoa were prepared from caudae epididymidis of slaughtered bulls as previously described [10]. For incubating intact spermatozoa, the medium contained 140 mM NaCl, 6 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris and 10 mM Hepes (pH 7.4). Preparation and

Abbreviation: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

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incubation of permeabilized cells were performed in a medium of 110 mM sucrose, 60 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM EDTA and 60 mM Tris (pH 7.4). After permeabilization of spermatozoa with 5 μg digitonin per μl cells [10], the cells were washed twice and kept at 0–4°C until use.

Concentrations of spermatozoa were assayed by using a spermatocrit procedure and were calculated as μl packed cells [11]. Fluorescence recording and measurement of respiration were carried out simultaneously in a light-screened thermostated and stirred oxygraph vessel. Samples were excited at 366 nm and 436 nm for measuring NAD(P)H and flavoprotein fluorescence, respectively. The fluorescent light was measured at 480 nm and at 546 nm. Two-channel fluorescence measurements were conducted with a multichannel surface fluorimeter which was equipped with light-guides [12]. A stabilized xenon lamp XBO 101 (Narva, Berlin) was used as light source. Interference and cut-off filters were purchased from Carl Zeiss Jena. When fluorescence data had to be quantified (Tables I and II and Fig. 3), the fluorescence scale was defined as the range of values between 0% (uncoupled conditions) and 100% (in the presence of 10 mM lactate and 5 mM KCN).

To determine adenine nucleotides, samples were taken from the oxygraphic vessels and quenched in a phenol/chloroform/isoamyl alcohol mixture [13]. Adenine nucleotides were assayed by standard enzymic procedures [14].

Chemicals. DL-β-Hydroxybutyrate, sodium pyruvate and enzymes were purchased from Boehringer (Mannheim), lithium acetoacetate and rotenone from Serva (Heidelberg), L(+)-lactic acid from Sigma (St. Louis), sodium dithionite from Ferak (Berlin) and caffeine from Arzneimittelwerk (Dresden). The uncoupler TTFB was a kind gift by Dr. Beechey (Aberystwyth). All other chemicals were of analytical grade.

Results and Discussion

Characterization of NAD(P)H and flavoprotein fluorescence signals

In Fig. 1 traces are shown which were obtained by simultaneous registration of NAD(P)H and flavoprotein fluorescence in intact spermatozoa. The uncoupler TTFB causing a rapid decrease of the NAD(P)H fluorescence signal also produced an increase of the flavoprotein fluorescence signal. This indicates that there was an oxidation of both fluorochromes. The higher degree of reduction observed before the addition of TTFB was obviously caused by endogenous substrates that were present in the washed epididymal spermatozoa. Addition of lactate and rotenone provoked an instantaneous reduction of both pyridine nucleotides and flavoproteins measured as increase and decrease of the NAD(P)H and the flavin fluorescence signal, respectively. This state of reduction of both fluorochromes was increased only slightly by cyanide. Octanoate as additional substrate did not have any effect on the fluorescence signals. In order to obtain fluorescence signals of fluorochromes present only in mitochondria, experiments shown in Fig. 2 were carried out with washed digitonin-permeabilized spermatozoa of the same sperm suspension as seen in Fig. 1. The effectors used in permeabilized spermatozoa produced reactions which were similar to those used in intact spermatozoa. As there was no effect of TTFB on the fluorescence signals, pyridine nucleotides and flavoproteins may have

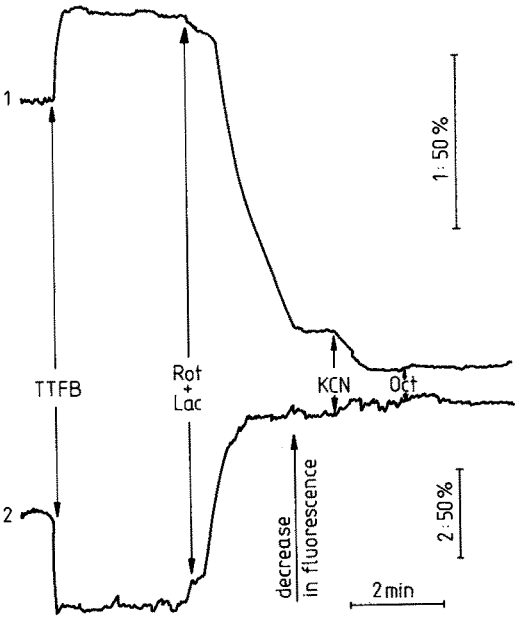

Fig. 1. NAD(P)H (1) and flavoprotein (2) fluorescence of intact spermatozoa. Changes of fluorescence signals of intact spermatozoa (35 μl cells per ml) resulting from additions of: 10 μM TTFB, 10 μM rotenone (Rot), 10 mM lactate (Lac), 5 mM KCN and 1 mM octanoate (Oct).

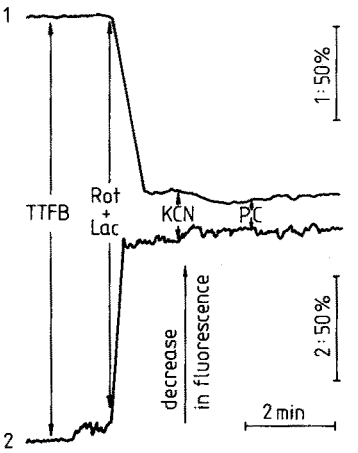

Fig. 2. NAD(P)H (1) and flavoprotein (2) fluorescence of digitonin-permeabilized spermatozoa. Washed digitonin-permeabilized spermatozoa (30 μl cells per ml) were investigated as shown in Fig. 1. The concentration of palmitoylcarnitine (PC) was 50 μM.

been in a completely oxidized state before the uncoupler was added, suggesting that the cause of this was the absence of endogenous substrates in this preparation. The reducing effect of lactate can be explained by an action of intramitochondrial lactate dehydrogenase [3,4]. Palmitoylcarnitine, which does not need any further activation to be oxidized, had, like octanoate, no effect on the fluorescence signals. In contrast to bovine spermatozoa, the flavoprotein fluorescence of rat liver mitochondria showed a remarkable response to the addition of fatty acids. This was attributed to the reduction of the electron transfer flavoprotein of β-oxidation [5]. On the other hand, bovine sperm mitochondria showed low respiration rates with fatty acids as substrates [15]. From this may be drawn the conclusion that the electron transfer flavoprotein of β-oxidation does not contribute to the flavoprotein fluorescence signal in bovine spermatozoa. Having tested the substrates of other flavoproteins (succinate, α-glycerophosphate), we found that they also did not exert any effect on the flavoprotein fluorescence signal in the rotenone- and cyanide-inhibited states (results not shown). Due to the close relation to pyridine nucleotide reduction, the detected flavoprotein species seems to be identical with α-lipoamide dehydrogenase, which is a constituent of the oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes [6,7].

A comparison of the fluorescence signals of intact and permeabilized spermatozoa from the same preparation permits quantification of the contributions of mitochondrial and cytosolic fluorochromes to the total fluorescence of the cells. These fluorescence signals were obtained from the span between the totally oxidized and totally reduced state which can be adjusted by uncoupling substrate-free incubated spermatozoa and by adding lactate in the presence of cyanide, respectively. Comparing the measured fluorescence signals of intact and permeabilized spermatozoa of four specimens quantitatively, the flavoprotein fluorescence signals of both preparations were found to differ by about $\pm 10\%$ only. Taking into account that permeabilized spermatozoa contain the same quantity of mitochondria as intact cells [10], the flavoprotein fluorescence signal is considered to be solely of mitochondrial origin. Moreover, a comparison of NAD(P)H fluorescence signals seen in Figs. 1 and 2 showed that washed permeabilized cells contained smaller amounts of pyridine nucleotides. An exact estimation of the share of mitochondrial NAD(P)H fluorescence in the overall signal of intact cells should include that (i) not all cells of a freshly prepared sperm suspension have an intact plasma membrane and (ii) the digitonin procedure does in some cases not remove the plasma membrane completely. The intactness of the plasma membrane and, accordingly, the loss of low-molecular compounds can be quantified via succinate exclusion [10,11]. When applying this test, we found that 82 to 89% of fresh and untreated spermatozoa and 9 to 19% of digitonin-permeabilized cells (four preparations) possessed a plasma membrane which was impermeable to succinate. These values enabled us to extrapolate to 0 and to 100% intactness of plasma membrane, 0% representing the mitochondrial compartment and 100% the whole cell. We found that $50.6 \pm 4.3\%$ ($n = 4$) of the NAD(P)H fluorescence signal can be attributed to the mitochondrial pool. This value is somewhat higher than that reported by Milkowski and Lardy [4] for the NAD(P)H fluorescence, but is comparable to the NAD(P) content determined enzymatically in this work. A significant contribution of NADPH to the pyridine nucleotide fluorescence may be ruled out because the NADP(H) content of spermatozoa is extremely low [3,4,16].

Determination of electrochemical properties of the NAD(P)H and the flavoprotein fluorescence signal

When using fluorescence signals of NAD(P)H and flavoproteins as indicator systems for the redox states of cytosolic and mitochondrial NAD systems in intact spermatozoa, the midpoint potentials of the fluorochromes detected should be known. For this purpose, the ratios of oxidized to reduced portions of fluorescence signals were determined by titrations with the substrate couples lactate/pyruvate and β-hydroxybutyrate/acetoacetate in the cyanide-inhibited and uncoupled state [5]. Oxidative titration experiments were done in the presence of 10 mM lactate or β-hydroxybutyrate adding increasing amounts of pyruvate or acetoacetate, respectively. For reductive titration experiments the procedure was reversed, and both titration experiments achieved values which can be presented by the same Nernst curve. In Fig. 3, the redox potentials depicted on the abscissa were calculated from the Nernst equation as well as the $E_{m7.4}$ values of -235 mV and -286 mV for the redox couples lactate/pyruvate and β-hydroxybutyrate/acetoacetate, respectively. With the β-hydroxybutyrate/acetoacetate couple, the titrable span of fluorescence changes was 50 to 60% of that obtained with the lactate/pyruvate couple. Due to the occurrence of a mitochondrial isoenzyme of lactate dehydrogenase in spermatozoa [3,4], titrations with lactate/pyruvate included cytosolic as well as mitochondrial pyridine nucleotides. This indicates that 50 to 60% of the NAD(P)H fluorescence signal is of mitochondrial origin, which agrees with the estimation of the contribution of the mitochondrial compartment (cf. Figs. 1 and 2). In Table I, the $E_{m7.4}$ values determined in various preparations of intact and permeabilized spermatozoa are listed. In permeabilized cells, where the NAD(P)H fluorescence signal is assumed to be solely of mitochondrial origin, both NAD(P)H and flavoprotein fluorescence were completely titrable with lactate/pyruvate as well as with β-hydroxybutyrate/acetoacetate. More-

TABLE I

Midpoint potentials of NAD(P)H and flavoprotein fluorescence signals in intact and in permeabilized spermatozoa

Suspensions of intact (17–62 μml cells per ml) and permeabilized (23–37 μl cells per ml) spermatozoa were investigated as described in Fig. 3. Given are means ± S.D. of (n) titrations.

Preparation	Redox couple	$E_{m7.4}$ (mV)	
		pyridine nucleotides	flavoproteins
Permeabilized spermatozoa	lactate/pyruvate	−290 ± 4 (4)	−267 ± 3 (4)
	β-hydroxybutyrate/acetoacetate	−298 ± 4 (3)	−281 ± 8 (3)
Intact spermatozoa	lactate/pyruvate	−281 ± 4 (3)	−257 ± 2 (3)
	β-hydroxybutyrate/acetoacetate	−306 ± 3 (2)	−281 ± 8 (2)

over, the $E_{m7.4}$ value of the NAD-linked flavoprotein fluorescence signal appears to be about 20 mV more positive than that of the NAD(P)H system. This was found in rat liver mitochondria, too [5], which provides further evidence for its identity with α-lipoamide dehydrogenase [6,7]. The difference in the $E_{m7.4}$ values found with lactate/pyruvate and β-hydroxybutyrate/acetoacetate in intact cells (cf. Fig. 3) was also observed in permeabilized cells. As these preparations contain the mitochondrial NAD(P) only, the deviation could be caused by pyruvate which is not only the oxidant in the lactate dehydrogenase reaction but may also act as reductant in the pyruvate dehydrogenase reaction leading to a positive shift of the calculated midpoint potentials. Similar positive shifts of $E_{m7.4}$ values were observed in the flavoprotein fluorescence signal. The larger positive shift of $E_{m7.4}$ values in intact spermatozoa after lactate/pyruvate titration can be explained by the higher amount of endogenous substrates (cf. Fig. 1). Thus, the conclusion may be drawn that both the mitochondrial and the cytosolic fraction of the NAD(P) system show nearly the same midpoint potentials. $E_{m7.4}$ values of −300 mV for both cytosolic and mitochondrial NAD(P)H signals and those of −281 mV for the flavoprotein signal were therefore used in further experiments to quantitatively calculate actual redox states.

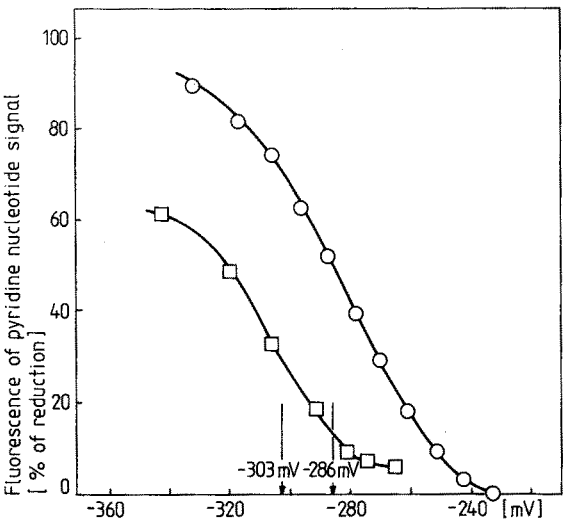

Fig. 3. Redox titration of NAD(P)H fluorescence signal of intact spermatozoa. Intact spermatozoa (17.3 μl cells per ml) were incubated with 10 μM TTFB and 5 mM KCN. Oxidative titration experiments shown for the substrate couples lactate/pyruvate (○) and β-hydroxybutyrate/acetoacetate (□) were carried out adding 10 mM lactate or 20 mM DL-β-hydroxybutyrate and titrating increasing amounts of pyruvate or acetoacetate, respectively. Midpoint potentials were obtained by the least-square regression of data points with the Nernst equation and $n = 2$:

$$E_h = E_m + \frac{RT}{nF} \cdot \ln \frac{[ox]}{[red]}.$$

Effect of caffeine on NAD(P)H and flavoprotein fluorescence signals

The stimulating effects of caffeine on motility and respiration were used to observe changes in the redox state of cytosolic and mitochondrial pyridine nucleotides under physiological conditions. Caffeine is thought to be an appropriate effector for studying spermatozoa in different states of activity because it affects primarily the dynein-tubulin system and not the mitochondrial energy metabolism [8,9].

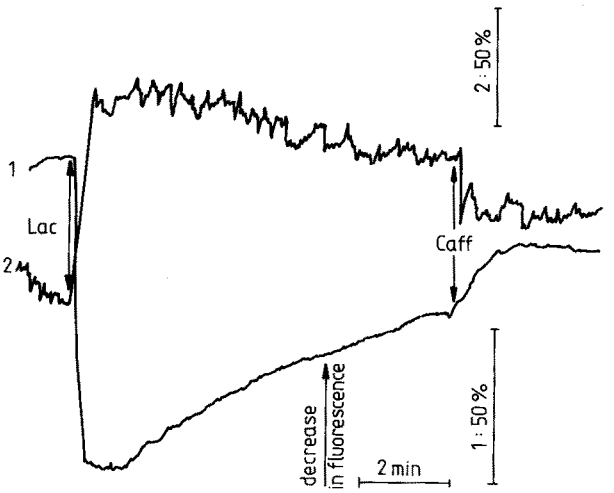

Fig. 4. Effect of caffeine on the NAD(P)H (1) and flavoprotein (2) fluorescence in intact spermatozoa. 10 mM lactate and 1 mM caffeine were added to the suspension containing 70 μl cells per ml.

TABLE II

Effect of caffeine on respiration, [ATP] / [ADP] and the fluorescence signals of NAD(P)H and flavoproteins

Incubations were performed with four sperm preparations (37–70 μl cells per ml) as shown in Fig. 4. Respiration and fluorescence signals were registered simultaneously. Immediately before and 4 min after addition of 1 mM caffeine, samples were taken and quenched for determination of adenine nucleotides (cf. Materials and Methods). Percentages of reduction of pyridine nucleotides and flavoproteins were calculated in relation to the total span between oxidized (+10 μM TTFB) and reduced state (+10 mM Lac, +5 mM KCN; cf. Fig. 1). Given are means ± S.D.

Incubation	Respiration (nmol O_2 per min per μl cells)	[ATP]/[ADP]	Fluorescence (% of totally reduced state) of	
			NADH	flavoproteins
− Caffeine	0.52 ± 0.10	7.52 ± 1.37	49.5 ± 6.8	72.8 ± 5.4
+ Caffeine	1.16 ± 0.21	4.53 ± 1.37	25.5 ± 2.5	49.6 ± 11.6

When lactate was added to normoxic epididymal spermatozoa, a large increase of the NAD(P)H fluorescence together with a decrease of the flavoprotein fluorescence was found (see Fig. 4). Actually, this initial state of reduction did not remain constant because of the accumulation of pyruvate that is produced intramitochondrially by lactate dehydrogenase. After a certain time (8 to 10 min), however, a new steady state of fluorescence was observed. In this state, caffeine was added and produced a measurable decrease of NAD(P)H as well as an increase of flavoprotein fluorescence signals. In Table II, the quantitative data on respiration, [ATP]/[ADP] and fluorescence signals are summarized. As shown, caffeine stimulated respiration 2-fold, paralleled by a drop in the ATP/ADP ratio. The measured fluorescence signals can be used to calculate the reduction state of mitochondrial pyridine nucleotides by applying the flavoprotein fluorescence signal and the midpoint potentials of both fluorochromes (cf. Table I). From these calculations it can be evaluated that the mitochondrial NAD(P)H signal was reduced by 39% before and by 19% after caffeine addition (average of four experiments). The reduction state of cytosolic pyridine nucleotides is not available directly. For the total NAD(P)H fluorescence measured, the following equation is applied:

$$r_t = r_c \cdot p_c + r_m \cdot p_m$$

r_t, r_c and r_m corresponding to the percentage of reduction of the total, the cytosolic and the mitochondrial NAD(P)H signals, respectively, p_c and p_m to the cytosolic and the mitochondrial portions of this signal. It follows that:

$$r_c = \frac{r_t - r_m \cdot p_m}{1 - p_m}$$

A calculation of the redox state of the cytosolic NAD(P)H signal however is more difficult as there are uncertainties (i) in the determination of the true cytosolic $E_{m7.4}$ value and (ii) in the estimation of the real share of cytosolic NAD(P)H in the amount of fluorescence. Taking into consideration that 50% of the NAD(P)H fluorescence signal are of cytosolic origin and that the cytosolic $E_{m7.4}$ value is about −300 mV, the reduction state of the cytosolic NAD(P)H signal could be calculated to be 60% before and 32% after caffeine addition. These results may be interpreted as a linkage of cytosolic and mitochondrial pools of reducing equivalents. In correspondence to Milkowski and Lardy [4], this might be caused by extra- and intramitochondrial localization of lactate dehydrogenase in spermatozoa as well as by a pyruvate/lactate transport functioning across the mitochondrial inner membrane.

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