Endogenous energy production by mature boar spermatozoa

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When incubated in the absence of exogenous substrates, washed boar spermatozoa maintained a high energy charge potential (ECP) for at least 5 h. Addition of 3-chloro-1-hydroxypropanone, an inhibitor of triosephosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase, at any time caused the ECP to decline and fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glycerol to accumulate. There appear to be two endogenous substrates that are degraded ultimately to produce the triosephosphates which allow the cells to produce lactate for the mitochondrial synthesis of ATP. One substrate generates minor amounts of glycerol 3-phosphate whereas the other substrate degrades to glycerol and may be di-glycerides, or tri-glycerides, or both.

Introduction

ATP is necessary for many purposes within spermatozoa, such as the maintenance of the intracellular milieu by the active extrusion of ions and modifications to the structure and function of the cell during maturation, capacitation and fertilization, but the process of motility accounts for most of the ATP that is consumed by mature spermatozoa (Bohnsack and Halangk, 1986; Ford and Rees, 1990; Mita *et al.*, 1991; Hofmann *et al.*, 1992). Although there are marked species differences in the manner by which spermatozoa generate ATP, boar spermatozoa are restricted to fructose, glucose, glycerol, glycerol 3-phosphate and lactate as metabolic substrates and, in this species at least, spermatozoa are totally reliant on the glycolytic pathway for the ultimate production of acetyl CoA for the mitochondrial generation of ATP (Jones and Chantrill, 1989).

Once in the female reproductive tract, spermatozoa must maintain their motility and for this it is generally assumed that substrates for ATP generation are obtained from tract secretions. While this may be so, Tash and Mann (1973) observed that thrice-washed ram spermatozoa had poor motility and produced low concentrations of cyclic AMP when incubated under anaerobic conditions for 1 h, whereas a further hour under aerobic conditions with no added substrate resulted in good motility and high concentrations of cyclic AMP. Similarly, when washed ram spermatozoa were incubated for 5 h at 16°C with no exogenous substrate, they maintained high concentrations of cyclic AMP and had good motility. Mann and Lutwak-Mann (1981) later commented that "In the total absence of either seminal plasma or extraneous metabolizable substrate, spermatozoa retain their motility provided oxygen is available; owing to endogenous respiration they continue to take up oxygen, albeit for a shorter period than in whole semen." Washed mature spermatozoa from rhesus monkey ejaculates, when incubated in a substrate-free medium, were completely immotile at the end of a 1.5 h anaerobic period but

motility was restored to almost normal values by aeration (Hoskins and Patterson, 1968).

While examining the effects of chemical inhibitors on the metabolic activity of washed boar spermatozoa (Jones and Porter, 1995), we observed that these cells could maintain high concentrations of ATP for prolonged periods *in vitro* when incubated under aerobic conditions in the absence of exogenous substrates. The results of our investigations into this phenomenon, by the judicious use of specific metabolic inhibitors, are presented here.

Materials and Methods

Chemicals and substrates

L- α -Glycerophosphorylcholine cadmium chloride complex grade 1, bovine serum albumin (BSA) and D,L-glyceraldehyde were obtained from Sigma (St Louis, MO) and D,L-carnitine chloride from Merck (Darmstadt). 3-Chloro-1-hydroxypropanone (CHOP) was prepared from 3-chloro-1-hydroxypropanone dimethyl acetal by the procedure of Jones *et al.* (1986). Neutralized solutions of CHOP (10 mmol l^{-1}) were prepared immediately before use and added to incubations at a final concentration of 0.5 mmol l^{-1} . 3-Bromopyruvate hydrate, supplied by Aldrich Chemical Co. (Milwaukee, WI), was recrystallized several times from dry chloroform, m.p. 54.7°C. Aqueous solutions were neutralized with solid NaHCO_3 and added to incubations as sodium bromopyruvate (BPA) at a final concentration of 0.5 mmol l^{-1} . (R,S)- α -bromohydrin phosphate biscyclohexylamine salt was prepared to established purity by the method of Jones and Gillan (1996a), 1,6-dichloro-1,6-dideoxy-D-fructose according to Jones and Morin (1995) and (R,S)-3-chlorolactaldehyde by the method of Williams *et al.* (1960). Etomoxir (sodium 2-[6-(4-chlorophenoxy)-hexyl]-oxirane-2-carboxylate) was a gift from G. J. Cooney (Department of Endocrinology, University of Sydney) and penicillin G (Calbiochem, La Jolla, CA), ampicillin (Alphapharm, Glebe NSW) and tetracycline (Boehringer

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Mannheim Australia, Castle Hill, NSW) were purchased from the manufacturers. All enzymes and substrates were supplied by Boehringer Mannheim Australia and compounds were prepared as aqueous solutions and used at concentrations indicated in the text. All other chemicals and reagents were of analytical grade and all solutions were prepared in water purified by reverse osmosis.

Collection and preparation of spermatozoa

Cauda epididymides, removed from the testis-epididymis complex of mature boars (*Sus scrofa*: various crosses of Duroc, Hampshire, Landrace and Large White) 15–20 min after slaughter at the abattoir, were immediately placed in Dewar flasks containing phosphate-buffered saline (PBS) at 34°C. (PBS consisted of NaCl (139 mmol l⁻¹), KCl (4.7 mmol l⁻¹), MgSO₄ (1.2 mmol l⁻¹), Na₂HPO₄ (3.1 mmol l⁻¹), NaH₂PO₄ (0.8 mmol l⁻¹) and CaCl₂ (2.5 mmol l⁻¹) adjusted to pH 7.4.) The sealed flasks were transported to the laboratory where the spermatozoa were flushed with PBS at 34°C from incisions made essentially in the third and fourth segments of the cauda according to the classification of Holtz and Smidt (1976). The sperm suspension, usually from 10–20 boars, was centrifuged (2000 g for 10 min), the supernatant solution discarded and the cells aspirated from any denser erythrocytes and resuspended in PBS at 34°C. This procedure was repeated twice and the spermatozoa were finally prepared as a 10% (v/v) suspension in PBS at 34°C. The concentration of spermatozoa in this suspension was of the order of 10⁷ cells ml⁻¹ containing 15–25 mg protein ml⁻¹.

Incubations with spermatozoa

Incubations were performed at 34°C (boar scrotal temperature) in either 25 ml (short-term) or 50 ml (long-term) open conical flasks shaken at 120 cycles min⁻¹ with air as the gas phase. Long-term incubations contained 35 ml (approximately 3–4 × 10⁸ cells) of the 10% sperm suspensions with no exogenous substrates and, where applicable, 1 ml of the appropriate inhibitor. Samples (1 ml) were removed at zero time and at hourly intervals and added to 3 mol HClO₄ l⁻¹ (100 µl). Short-term incubations began at zero time and at hourly intervals by taking 5 ml (approximately 5 × 10⁷ cells) of the long-term incubations, adding this to the appropriate inhibitor (250 µl) to a final concentration of 0.5 mmol l⁻¹ and incubating them for 1 h. Samples (1 ml) were removed every 15 min for the following hour and added to 3 mol HClO₄ l⁻¹ (100 µl). Neutralized, deproteinized and desalted incubation solutions (Stevenson and Jones, 1982) were used for assay of glycerol 3-phosphate and lactate (Lang, 1985), ATP (Jaworek and Welsch, 1985a), ADP and AMP (Jaworek and Welsch, 1985b), glycerol (Wieland, 1984), glyceraldehyde (Goedde and Langenbeck, 1984), fructose-1,6-bisphosphate, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Michal, 1985) using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto). Spermatozoa were prepared (Chan and Exton, 1976) and assayed for glycogen content by the procedure of Hugget and Nixon (1957) and protein was estimated (Lowry *et al.*, 1951) on freeze-thawed samples using BSA as the standard.

The adenine nucleotide pool is defined as [ATP] + [ADP] + [AMP] and energy charge potentials (ECPs) were calculated according to the method of Atkinson and Walton (1967), where

$$\text{ECP} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

Acid hydrolyses

Samples of L-α-glycerophosphorylcholine (GPC) (5 mmol l⁻¹, 20 ml), liberated from its cadmium chloride complex by the supplier's procedure, were incubated with HCl (2.5 mol l⁻¹, 10 ml) at various temperatures (60°–75°C) for 3 h. Aliquots (750 µl) were removed every 30 min, NaOH (2 mol l⁻¹, 250 µl) added, the cooled solutions neutralized and desalted and then assayed for glycerol 3-phosphate. The conditions for the maximum production of glycerol 3-phosphate, and its least hydrolysis, were determined to be 65°C for 1.5 h. These conditions were used for the production of glycerol 3-phosphate in hydrolysates as a modification to the procedure of Cooper *et al.* (1988). Samples (1 ml) of incubation suspensions were centrifuged (2000 g for 5 min) and the supernatant solution removed. The pellets were disrupted by sonic oscillation using a Branson B-12 Sonifier (Branson Sonic Power Company, Danbury CT), hydrolysed by the above procedure, the cooled suspensions centrifuged (2000 g for 5 min) and the supernatant solutions neutralized, desalted and assayed for glycerol 3-phosphate.

Saponification of sperm pellets

Sperm suspensions were incubated for 0 and 5 h in the absence of exogenous substrates and samples (1 ml) added to pre-weighed centrifuge tubes. After centrifugation (2000 g for 10 min) and removal of the supernatant solution, the wet pellets were weighed and saponified at 70°C for 15 min with KOH (1 mol l⁻¹, 3 ml). Ethanol (95%, 2 ml) was added and heating continued for a further 2 h. To the cooled mixture was added H₂SO₄ (9 mol l⁻¹, 0.5 ml) and, after centrifugation (2000 g for 5 min), the decanted supernatant solutions were neutralized, desalted and assayed for glycerol.

Results

When incubated at 34°C in the absence of exogenous substrates, washed boar spermatozoa maintained a reasonably high energy charge potential (ECP) (0.5–0.8) over 5 h (Fig. 1). When incubations were performed in the presence of 5 mg ml⁻¹ of penicillin G, ampicillin or tetracycline, the ECP was maintained indicating that it was not due to bacterial contamination. The concentration of lactate decreased from an initial value of 11 nmol mg⁻¹ protein over the first hour but remained constant at 2–4 nmol mg⁻¹ protein from 1 to 5 h (Fig. 2); there was no effect when glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase were inhibited by the addition of CHOP. Incubations performed in the presence and absence of 1,6-dichloro-1,6-dideoxy-D-glucose (10 mmol l⁻¹),

Fig. 1. The energy charge potential (ECP) of boar spermatozoa incubated (●) without exogenous substrate at 34°C for 5 h ($n = 10$) and the effect of (○) 3-chloro-1-hydroxypropanone (CHOP) added each hour ($n = 6$).

Fig. 2. Concentration of lactate in boar spermatozoa incubated without exogenous substrate at 34°C for 5 h (●) ($n = 6$).

which also inhibits these two enzymes and transiently inhibits aldolase, maintained high ECPs over 1 h in its absence (0.87 ± 0.02 , $n = 4$) and slightly lower in its presence (0.53 ± 0.04 , $n = 4$) and when freshly washed spermatozoa were assayed, glycogen was shown to be absent. The presence of etomoxir ($10 \mu\text{mol l}^{-1}$), an inhibitor of fatty acid transport into mitochondria, had no effect on the ECP over 5 h.

Samples removed from 5 h incubations at each hour and incubated for a further hour in the presence of CHOP (0.5 mmol l^{-1}) showed that the ECP was unable to be sustained, declining to 0.1–0.25 (Fig. 1). Detectable concentrations of the glycolytic intermediates fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate were present over the 5 h but when CHOP was added, the concentrations of fructose-1,6-bisphosphate and dihydroxyacetone phosphate were increased (Fig. 3).

Free glycerol 3-phosphate was not detected at any time over the 5 h incubation and did not accumulate when hourly samples were incubated with CHOP. Samples that were removed at hourly intervals, centrifuged, and the pellet hydrolysed and assayed, did not show a decline in the concentration of glycerol 3-phosphate which remained at 11–12 nmoles

mg^{-1} protein. This was also the case when hourly samples were incubated with CHOP.

Incubations performed in the presence of (*R,S*)- α -bromohydrin phosphate (5 mmol l^{-1}), an inhibitor of glycerol 3-phosphate dehydrogenase, showed a gradual decline in the ECP over 5 h, from 0.9 to 0.5 but the addition of CHOP each hour decreased this value to 0.1–0.2 ($n = 6$). Incubations performed in the presence of D,L-carnitine chloride (4 mmol l^{-1}) maintained a high ECP but the addition of CHOP each hour decreased this to 0.1–0.2 ($n = 5$).

When hourly samples of incubations were assayed for glyceraldehyde, none could be detected. However, in the presence of CHOP, 'glyceraldehyde' appeared to accumulate. This was subsequently shown to be an artefact since the assay was not specific for glyceraldehyde; the alcohol dehydrogenase had a much greater affinity for the (*S*)-3-chlorolactaldehyde produced from CHOP rather than D-glyceraldehyde. No 'glyceraldehyde' could be detected when incubations were performed in the presence of bromopyruvate, an inhibitor of glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase.

Glycerol was detected during the 5 h incubation in variable amounts and when hourly samples were incubated in the presence of CHOP, the amount of glycerol increased (Fig. 4). Saponification of sperm pellets showed that the amount of glycerol produced from those incubated for 0 h (12.4 ± 0.6) in the absence of CHOP had significantly decreased ($P < 0.01$) in those incubated for 5 h ($9.3 \pm 0.4 \text{ nmol mg}^{-1}$ protein, $n = 5$).

Discussion

After studying an egg yolk buffer medium in an attempt to determine which constituents were responsible for promoting and maintaining the motility of bull spermatozoa for long periods, Lardy and Phillips (1941) reported that "... fractional studies showed that the protein and neutral fat portion of the egg yolk had no beneficial effect in preserving motility of spermatozoa in buffered medium. In fact, the neutral fat rendered the spermatozoa immotile. However, the phospholipid fraction of the egg yolk greatly prolonged the motility of spermatozoa in a buffered medium." Early work on energy production by spermatozoa suggested the presence of an endogenous metabolic substrate thought to be a lipid but later reported to be a plasmalogen (Harrison, 1977), whereas ram spermatozoa incubated in the absence of exogenous substrates showed little or no detectable changes in any phospholipid fraction (Darin-Bennett *et al.*, 1973). Harrison (1977) discounted the use of phospholipids as endogenous substrates and favoured the role of acetyl carnitine as a storage form of acetyl units. Subsequently, Mita and Ueta (1990) showed that sea urchin spermatozoa can maintain their motility in sea water by hydrolysing endogenous phospholipids and producing ATP from the resulting fatty acids and glycerol 3-phosphate.

The prolonged fertilizing life of spermatozoa in bats (Racey, 1979), hares (Martinet and Raynaud, 1975) and mice (Ullman, 1976) indicates that spermatozoa can remain viable for long periods. While this probably occurs by the use of metabolic substrates obtained from the female reproductive tract, the spermatozoa of many species may have the ability to

Fig. 3. Concentrations of (a) fructose-1,6-bisphosphate, (b) dihydroxyacetone phosphate and (c) glyceraldehyde 3-phosphate in boar spermatozoa incubated without exogenous substrate at 34°C for 5 h (●) and the effect of (○) 3-chloro-1-hydroxypropanone (CHOP) added each hour ($n = 6$).

Fig. 4. Concentration of glycerol in boar spermatozoa incubated without exogenous substrate at 34°C for 5 h (●) and the effect of (○) 3-chloro-1-hydroxypropanone (CHOP) added each hour ($n = 10$). Values are compared with the zero time values which were 270–3100 pmol mg⁻¹ protein.

metabolize an endogenous substrate when no exogenous substrates are available but this substrate (or substrates) may differ from species to species. The present study has shown that washed boar spermatozoa can maintain a high energy charge potential (ECP) for at least 5 h at 34°C when incubated under aerobic conditions in the absence of any exogenous substrate. Boar spermatozoa do not oxidize short- or medium-chain fatty acids (Jones and Chantrill, 1989) and the present study has shown that incubations performed in the presence of the long-chain fatty acid transport inhibitor etomoxir (Wolf *et al.*, 1982) were still able to maintain high ECPs. In the event that the inability of the cells to oxidize fatty acids was due to the washing procedure depleting their carnitine content, incubations were performed in the presence of exogenous carnitine. Addition of CHOP at any period immediately lowered the ECP suggesting that lack of carnitine was not a factor contributing to this inability. Thus the endogenous substrate, which cannot be a ketogenic amine (Jones and Gillan, 1996b), must channel carbon atoms to some point in the glycolytic pathway to produce the lactate which is essential for the mitochondrial production of ATP (Jones, 1997).

As with human spermatozoa (Mann and Rottenberg, 1966), boar spermatozoa contain no detectable quantity of glycogen

and incubations performed in the presence of 1,6-dichloro-1,6-dideoxy-D-fructose, which inhibits glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase and aldolase (Jones and Morin, 1995), showed only a moderate effect on the ECP over 1 h. Addition of CHOP, an inhibitor of both glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase (Jones and Cooney, 1987), decreased the ECP at any time over a 5 h incubation. The inhibitory action of CHOP localized the point at which the exogenous carbon atoms were entering the glycolytic pathway to be at or before the formation of the triosephosphates, since there was an accumulation of dihydroxyacetone phosphate and fructose-1,6-bisphosphate after its addition. Incubations to which (*R,S*)- α -bromohydrin phosphate was added maintained a relatively high ECP but not as high as that maintained in its absence. As this compound inhibits the mitochondrial membrane-bound glycerol 3-phosphate dehydrogenase which converts glycerol 3-phosphate to dihydroxyacetone phosphate (Jones and Gillan, 1996b), it was deduced that a contributing though minor source of a metabolic substrate was producing glycerol 3-phosphate. Glycerol 3-phosphate is rapidly oxidized by boar spermatozoa and its presence in washed cells or semen cannot be detected (Jones and Gillan, 1996a). Possible precursors of glycerol 3-phosphate are membrane phospholipids or glycerylphosphorylcholine, which is present in boar semen to the extent of 1.3% (Jones, 1978). However, no significant changes could be detected in the concentration of glycerol 3-phosphate when cells were hydrolysed after being incubated for 5 h.

Another possibility was that a metabolic substrate was entering the glycolytic pathway as glyceraldehyde 3-phosphate. The metabolism of glycerol by boar spermatozoa has been shown to be as glycerol $\rightarrow$ glyceraldehyde $\rightarrow$ glyceraldehyde 3-phosphate (Jones *et al.*, 1992) so that inhibition of glyceraldehyde 3-phosphate dehydrogenase and triosephosphate isomerase by CHOP may have led to the accumulation of glyceraldehyde 3-phosphate. This was not the case but when glyceraldehyde was assayed, there appeared to be an increase in its concentration. However, further investigation revealed that this was incorrect since the non-specific assay for glyceraldehyde was estimating (*S*)-3-chlorolactaldehyde, the metabolite of CHOP which is the actual metabolic inhibitor (Jones *et al.*, 1986). When stage 2 of

the glycolytic pathway was inhibited by 3-bromopyruvate (Jones *et al.*, 1995), which does not produce an aldehyde, there was no detectable aldehydic material. With either inhibitor there was an increase in the concentration of glycerol and the diglyceride/triglyceride concentration of the cells, as measured by saponification and assay of the glycerol produced, had declined after the 5 h incubation. Triglycerides have been identified as metabolic energy sources only in one species of sea urchin spermatozoa (Mita and Nakamura, 1993). *G. crenularis* spermatozoa contain lipid globules within their mid-pieces and, when incubated in sea water with no substrate for 1 h, these globules were reduced in size, some fatty acids were oxidized but there was also an increase in the concentration of free fatty acids. Boar spermatozoa are known to possess a pool of diglycerides that are actively involved in phospholipid synthesis (Vazquez and Roldan, 1997) and it may be that this is the source of the glycerol.

We conclude that there are at least two endogenous materials that washed boar spermatozoa can mobilize when they are incubated in the absence of any exogenous substrate. One material is degraded to glycerol 3-phosphate, although this appears to be a minor contributor to the production of ATP. Inhibition of the oxidation of glycerol 3-phosphate by the presence of (*R,S*)- α -bromohydrin phosphate lowered the ECP to a slight degree, whereas the further addition of CHOP immediately caused the ECP to decline rapidly. The major contributor is mobilized to produce glycerol which provides the carbon atoms for the production of lactate. If this glycerol-producing material is di- or tri-glycerides, as data from saponification studies suggest, then this raises an interesting question. Why do boar spermatozoa break down these substrates and only oxidize the glycerol? Further studies are in progress to identify these endogenous materials and when this is done, the question may be answered.

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