The concerted effect of α -chlorohydrin and glucose on the ATP concentration in spermatozoa is associated with the accumulation of glycolytic intermediates

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Summary. In the absence of a glycolysable sugar the effect of 1 mM-*RS*- α -chlorohydrin on the ATP concentration in ram or boar spermatozoa was relatively small but the addition of 0.10 or 0.03 mM-glucose initiated a rapid loss of ATP. When the spermatozoa were incubated with 0.05 mM-*RS*- α -chlorohydrin, the addition of 1.0 mM (ram) or 0.06 mM (boar)-glucose was required to produce ATP dissipation. In ram spermatozoa treated with 0.05 or 1.00 mM-*RS*- α -chlorohydrin, ATP loss was caused by 10 mM-fructose or 10 mM-mannose but not by 10 mM-glycerol or 10 mM-inositol. In boar spermatozoa incubated with 1 mM-*RS*- α -chlorohydrin the addition of 10 mM-L-lactate plus 1.0 mM-pyruvate protected the spermatozoa against the ability of 1.0 mM-glucose to produce a decline in ATP concentration.

Every combination of treatments capable of inducing a marked decline in ATP concentration also caused a dramatic (20–100-fold) increase in the concentration of fructose 1,6-bisphosphate. An increase in fructose 1,6-bisphosphate concentration was never observed when the ATP concentration was unaffected. We conclude that it is very probable that the concerted effect of α -chlorohydrin and glycolysable sugar is responsible for the contraceptive action of α -chlorohydrin *in vivo* and that fructose 1,6-bisphosphate is implicated in its mechanism of action.

Introduction

α -Chlorohydrin and the 6-chloro-6-deoxysugars are effective male contraceptives in many species (see Ford, 1982; Jones, 1983). Their mode of action probably relies on the inhibition of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) in spermatozoa which blocks the glycolytic pathway in these cells (Brown-Woodman, Mohri, Suter & White, 1978; see Ford, 1982). Ford & Harrison (1985) have demonstrated that α -chlorohydrin has relatively little effect on the energy balance of spermatozoa unless glucose is added, when the concentration of ATP in the cells decreases dramatically. In the present study the conditions required for glucose-dependent ATP dissipation to occur have been investigated further and the phenomenon has been correlated with changes in the concentrations of glycolytic intermediates. A preliminary account of some of the results has appeared (Ford & Harrison, 1984).

Materials and Methods

Materials

Ram testes and epididymides were obtained from local abattoirs (Alf Meade Ltd, The Abattoirs, Reading; Brooks (Newbury) Ltd, Newbury). The sperm-rich fraction of boar semen

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was generously donated by the Ministry of Agriculture, Fisheries and Food, Pig Breeding Centre, Shinfield, Reading. The spermatozoa were prepared and suspended in PBS buffer as described previously (Ford & Harrison, 1985).

Enzymes and biochemicals were supplied by Sigma (London) Chemical Co. Ltd, Poole, Dorset BH17 7NH, U.K. or by Boehringer Corporation (London) Ltd, Lewes BN17 1LG, U.K. *RS- α* -Chlorohydrin was supplied by Koch-Light Ltd, Colnbrook SL3 0BZ, U.K. and was redistilled under reduced pressure before use. Other reagents were purchased from Fisons Scientific Apparatus, Loughborough LE11 0RG, U.K.

The interactions of α -chlorohydrin concentration and glucose concentration

Ram spermatozoa. The experiment was done on three occasions; on each a 21-ml portion of the sperm suspension ($1.4 \pm 0.15 \times 10^8$ spermatozoa/ml) was incubated with (A) 0, (B) 0.05, (C) 1.0 mM-*RS- α* -chlorohydrin for 10 min at 34°C in a shaking water bath. Then five 3.9-ml portions were taken from A, B and C and glucose was added to give final concentrations of 0, 0.01, 0.10, 1.00 and 10.00 mM in a final volume of 4.0 ml in a 10 ml conical flask. At the same time 1.0-ml samples were taken from A, B and C and mixed with 0.5 ml 1.0 M-perchloric acid. The 15 portions continued to incubate at 34°C and 1.0-ml samples were taken and mixed with perchloric acid 10, 30 and 60 min after the addition of glucose.

Boar spermatozoa. The experiment was done with 2 semen samples, and each time 11-ml portions of sperm suspension (0.58 or 0.66×10^8 spermatozoa/ml) were incubated with (A) 0, (B) 0.05 and (C) 1.0 mM-*RS- α* -chlorohydrin at 34°C in a shaking water bath for 10 min. Then five 1.8-ml portions were removed from A, B and C to 10-ml conical flasks and glucose was added to give final concentrations of 0, 0.03, 0.06, 0.12 and 1.00 mM. At the same time 0.5-ml samples of A, B and C were mixed with 1.0 ml 0.5 mM-perchloric acid. The 15 portions continued to incubate at 34°C and 0.50 ml samples were taken and mixed with acid 10, 30 and 60 min after the addition of glucose.

The effect of different sugars on ram spermatozoa

This experiment was done on 4 occasions: 22-ml portions of the sperm suspension ($1.6 \pm 0.17 \times 10^8$ spermatozoa/ml) were incubated with (A) 0, (B) 0.05 and (C) 1.0 mM-*RS- α* -chlorohydrin for 10 min at 34°C in a shaking water bath. Then five 3.9-ml portions were taken from A, B and C into 10-ml conical flasks and 0.10 ml PBS buffer, 0.10 ml 0.4 M-D-fructose, 0.10 ml 0.4 M-glycerol, 0.10 ml 0.4 M-D-mannose or 0.10 ml 0.4 M-*myo*-inositol was added. At the same time 1.0-ml samples were taken from A, B and C and mixed with 0.50 ml 1.0 M-perchloric acid. The 15 portions continued to incubate at 34°C and 1.0-ml samples were removed 10, 30 and 60 min after the addition of substrates.

Protection by lactate plus pyruvate against the concerted effect of α -chlorohydrin and glucose in boar spermatozoa

Boar spermatozoa (1.4 or 1.2×10^8 /ml) were incubated with 1.0 mM-*RS- α* -chlorohydrin for 10 min at 34°C in a shaking water bath. Then the following additions were made to 4.5-ml portions in 10-ml conical flasks: (1) none, (2) 10 mM-L-lactate plus 1.0 mM-pyruvate, (3) 1.0 mM-D-glucose, (4) 1.0 mM-D-glucose followed by 10 mM-L-lactate plus 1.0 mM-pyruvate after 10 min, (5) 1.0 mM-D-glucose plus 10 mM-L-lactate plus 1.0 mM-pyruvate. Then 1.0-ml samples were taken and mixed with 0.5 ml 1.0 M-perchloric acid from the original suspensions when it was divided and from each flask at 10, 30 and 60 min.

Assay procedures and evaluation of results

The perchloric acid extracts were neutralized and assays of adenine nucleotides and glycolytic intermediates were done as described previously (Ford, Harrison & Waites, 1981; Ford & Harrison, 1981). The absolute concentration of ATP was seriously underestimated in the incubations which contained mannose because this sugar has a very high affinity for the hexokinase used in the assay. Therefore in this experiment the concentration of ATP is expressed as a percentage of the control. The effects of α -chlorohydrin and the other treatments were analysed by a multi-factorial analysis of variance and individual differences were evaluated by a *t* test using the standard error of difference of the means. The calculations were done by a computer program 'GENSTAT' (Rothamsted Experimental Station) provided by the Department of Applied Statistics, University of Reading.

Results*The interaction of α -chlorohydrin concentration and glucose concentration*

Ram spermatozoa. The spermatozoa produced a significant amount of lactate when 1.0 or 10.0 mM-D-glucose was present. This was partly inhibited by 0.05 mM- α -chlorohydrin and completely inhibited by 1.0 mM- α -chlorohydrin (Figs 1b, c).

The ATP concentration declined steadily from about 30 nmol/10⁸ spermatozoa after 10 min incubation to about 12 nmol/10⁸ spermatozoa after 60 min in spermatozoa incubated with 0 or 0.01 mM-glucose. There was no effect of 0.05 mM- α -chlorohydrin on the rate of decline but 1.0 mM- α -chlorohydrin produced a significantly lower concentration after 30 min (*P* < 0.05).

Fig. 1. The (a) production of lactate by and (b) concentration of ATP in ram spermatozoa incubated with α -chlorohydrin for 10 min at 34°C before the addition of 0 (○), 0.01 (△), 0.10 (□), 1.0 (●) or 10.0 (▲) mM-glucose at time = 0 and incubation for a further 60 min. Mean of 4 experiments.

Fig. 2. The concentration of fructose 1,6-bisphosphate in ram spermatozoa incubated with α -chlorohydrin for 10 min at 34°C before the addition of 0 ($\circ$), 0.01 ($\triangle$), 0.10 ($\square$), 1.0 ($\bullet$) or 10.0 ($\blacktriangle$) mM-glucose at time = 0 and incubation for a further 60 min. Mean of 4 experiments.

although it had no significant effect at 10 or 60 min (Fig. 1c). With 0.05 mM- α -chlorohydrin, the presence of 1.0 or 10.0 mM-glucose produced a marked decrease in ATP concentration compared to spermatozoa incubated with α -chlorohydrin alone or with glucose alone ($P < 0.05$, $P < 0.001$ respectively). The difference was most marked after 30 min (Fig. 1b). With 1.0 mM- α -chlorohydrin, 0.1 mM-glucose also produced a significant decrease in ATP concentration ($P < 0.01$, $P < 0.001$ relative to α -chlorohydrin alone, glucose alone) and the difference was most pronounced after 10 min (Fig. 1c). The interaction between glucose concentration and α -chlorohydrin concentration was significant ($P < 0.001$) in the analysis of variance. In the absence of α -chlorohydrin, 0.1–10 mM-glucose appeared to enhance the ATP concentration in the spermatozoa (Fig. 1a) but this narrowly failed to be significant at the $P < 0.05$ level.

The concentration of fructose 1,6-bisphosphate always remained < 1.0 nmol/ 10^8 spermatozoa when no α -chlorohydrin was present although there was a measurable increase when glucose was added. When 0.05 mM- α -chlorohydrin was present together with 1.0 or 10 mM-D-glucose, fructose 1,6-bisphosphate accumulated during the incubation, reaching a concentration of about 25 nmol/ 10^8 spermatozoa after 30 min; no such accumulation occurred with 0.01 or 0.10 mM-glucose (Fig. 2). With 1.0 mM- α -chlorohydrin, fructose 1,6-bisphosphate accumulated very rapidly in the presence of 0.1 as well as 1.0 and 10.0 mM-glucose, reaching a concentration of about 20 nmol/ 10^8 spermatozoa after 10 min. There was a small increase ($P < 0.01$) even with 0.01 mM-glucose (Fig. 2). The interaction between glucose concentration and α -chlorohydrin concentration was significant ($P < 0.001$).

The changes in the concentration of triose phosphates (glyceraldehyde 3-phosphate plus dihydroxyacetone phosphate) were broadly similar to those of fructose 1,6-bisphosphate except that the maximum concentration reached was about 10 nmol/ 10^8 spermatozoa. The presence of α -chlorohydrin and glucose together had no significant effect on the concentration of glucose 6-phosphate.

Boar spermatozoa. The effect of α -chlorohydrin and glucose was qualitatively similar to that observed with ram spermatozoa. With 0.05 mM- α -chlorohydrin the presence of > 0.06 mM-glucose produced a marked decline in ATP concentration ($P < 0.05$, $P < 0.01$ versus no glucose or no

Fig. 3. The concentration of (a) ATP and (b) fructose 1,6-bisphosphate in boar spermatozoa incubated with α -chlorohydrin for 10 min at 34°C before the addition of 0 (○), 0.03 (△), 0.06 (□), 0.12 (●) or 1.00 (▲) mM-glucose at time = 0 and incubation for a further 60 min. Mean of 2 experiments. The concentration of fructose 1,6-bisphosphate was always < 1 nmol/10⁸ spermatozoa in the absence of α -chlorohydrin.

α -chlorohydrin respectively) (Fig. 3a). With 1.0 mM- α -chlorohydrin, 0.03 mM-glucose also produced the loss of ATP ($P < 0.01$, $P < 0.001$ versus no glucose or no α -chlorohydrin respectively) (Fig. 3a). Once again fructose 1,6-bisphosphate accumulated in the spermatozoa under the conditions that gave rise to ATP dissipation but did not when no concerted effect of α -chlorohydrin and glucose was observed (Fig. 3b). The extent of fructose 1,6-bisphosphate accumulation was inversely related to the amount of ATP which was lost. There was significant ($P < 0.001$) interaction between glucose and α -chlorohydrin concentration in their effects on both ATP and fructose 1,6-bisphosphate concentration.

The effect of different sugars

The presence of 10 mM-fructose or 10 mM-mannose caused the ATP concentration in ram spermatozoa incubated with 0.05 or with 1.0 mM- α -chlorohydrin to decline more rapidly than when no substrate was present (fructose, $P < 0.001$, with 0.05 and 1.0 mM- α -chlorohydrin; mannose, $P < 0.05$ and $P < 0.01$, with 0.05- and 1.0 mM- α -chlorohydrin respectively); 10 mM-glycerol or 10 mM-myoinositol had no effect. There was a deleterious effect of 1.0 mM- α -chlorohydrin but not 0.05 mM- α -chlorohydrin on the ATP concentration in the spermatozoa even when no substrate was added ($P < 0.001$) (Fig. 4).

Fig. 4. The concentration of ATP in ram spermatozoa incubated with α -chlorohydrin for 10 min at 34°C before the addition of 10 mM-fructose (Δ), 10 mM-glycerol (∇), 10 mM-mannose ($\square$), 10 mM-inositol ($\bullet$) or an equivalent volume of buffer ($\circ$) at time = 0 and incubation for a further 60 min. Mean of 4 experiments. The results are expressed as a percentage of the value in the incubation with the same sugar and no α -chlorohydrin.

Fig. 5. The concentration of triose phosphates and of fructose 1,6-bisphosphate in ram spermatozoa incubated with α -chlorohydrin for 10 min at 34°C before the addition of 10 mM-fructose (Δ), 10 mM-glycerol (∇), 10 mM-mannose ($\square$), 10 mM-inositol ($\bullet$) or an equivalent volume of buffer ($\circ$) at time = 0 and incubation for a further 60 min. Mean of 2 experiments.

The concentration of fructose 1,6-bisphosphate and of triose phosphates was dramatically increased in spermatozoa incubated with α -chlorohydrin and fructose or mannose. Neither glycerol nor inositol had any effect on the fructose 1,6-bisphosphate concentration but glycerol produced a marked accumulation of triose phosphates in the presence of 1.0 mM- α -chlorohydrin (Fig. 5).

Protection by lactate and pyruvate against the concerted effect of α -chlorohydrin and glucose

When no substrate was present the ATP concentration in boar spermatozoa incubated with 1.0 mM- α -chlorohydrin declined from about 15 to about 10 nmol/ 10^8 spermatozoa during a 1-h incubation. This decline was markedly accelerated by 1 mM-glucose but was prevented by 10 mM-lactate plus 1 mM-pyruvate. When 1 mM-glucose, 10 mM-lactate and 1 mM-pyruvate were all present the ATP concentration declined by about the same amount as when no substrate was present. If 10 mM-lactate plus 1 mM-pyruvate was added after 10 min incubation with glucose the initial decline in ATP concentration was halted ($P < 0.05$ for all treatments versus no substrate) (Fig. 6).

Fructose 1,6-bisphosphate accumulated to a high concentration when 1 mM-glucose was the only substrate ($P < 0.001$) but to a much smaller extent when lactate and pyruvate were also present ($P < 0.05$). When lactate and pyruvate were added after 10 min some of the fructose 1,6-bisphosphate which had accumulated was lost ($P < 0.05$).

Fig. 6. The concentration of ATP and of fructose 1,6-bisphosphate in boar spermatozoa incubated with 1 mM- α -chlorohydrin before the addition of the substrate at time = 0 and further incubation for 60 min. In the final combination lactate and pyruvate were added after 10 min incubation with glucose. Mean of 2 experiments.

Discussion

These results confirm that glucose and other glycolysable sugars precipitate a rapid decline in the ATP concentration in spermatozoa exposed to low (≥ 0.05 mM) concentrations of α -chlorohydrin. Only low concentrations of glucose are required and it is very probable that the conditions required for ATP dissipation occur when spermatozoa from animals treated with α -chlorohydrin or with 6-chloro-6-deoxysugars are mixed with seminal plasma at ejaculation and that this provides the basis for the contraceptive action of these compounds. One objection to this theory is that 10 mM-lactate plus 1 mM-pyruvate can protect boar spermatozoa against the worst effects of α -chlorohydrin and glucose (Fig. 6). Boar semen contains 2–3 mM-lactate (Mann & Lutwak-Mann, 1981) and this could be sufficient to have a similar protective effect. Nevertheless, boars given α -chlorohydrin at 5 mg/kg/day for ≥ 5 days were infertile (Johnson & Pursel, 1973).

All the conditions which produced a decline in the ATP concentration relative to the appropriate controls also produced an accumulation of fructose 1,6-bisphosphate and of triose phosphates. It is possible that these intermediates are responsible in some way for the dissipation of ATP. Large amounts of fructose 1,6-bisphosphate did not accumulate when no sugar-dependent decline in ATP concentration occurred. By contrast, triose phosphates accumulated in spermatozoa incubated with 1.0 mM- α -chlorohydrin and 10.0 mM-glycerol even though the glycerol had no effect on the ATP concentration. Therefore, fructose 1,6-bisphosphate is more likely than glyceraldehyde 3-phosphate or dihydroxyacetone phosphate to be involved in ATP dissipation.

The accumulation of the intermediates is a consequence of the inhibition of glyceraldehyde 3-phosphate dehydrogenase in the first instance but because such large amounts build up it is probable that phosphofructokinase (EC 2.7.1.11) is activated by the increases in the concentrations of ADP, AMP and possibly fructose 1,6-bisphosphate and by the decrease in ATP concentration. This would be consistent with the properties of phosphofructokinase from rhesus monkey spermatozoa (Hoskins & Stephens, 1969; Hoskins, Stephens & Casillas, 1971) and would explain the lack of accumulation of glucose 6-phosphate. The addition of pyruvate and lactate would provide the metabolic energy to convert AMP and ADP to ATP and to decrease the activation of phosphofructokinase. The accumulation of fructose 1,6-bisphosphate would then be decreased to prevent it from exerting its deleterious effect on energy balance in the spermatozoon. The addition of 10 mM-lactate plus 1 mM-pyruvate did not affect the concentration of citrate, a powerful potential inhibitor of phosphofructokinase (+ glucose, 41 ± 5.0 nmol citrate/ 10^8 spermatozoa; + glucose + lactate + pyruvate, 36.8 ± 3.4 nmol citrate/ 10^8 spermatozoa: mean $\pm$ s.e.m., $n = 4$).

It is unclear whether the accumulation of glycolytic intermediates is a vital component of the effect of α -chlorohydrin and glucose on the energy balance of spermatozoa or merely a side-effect. However, two possibilities are worthy of further investigation.

(1) The high concentrations of fructose 1,6-bisphosphate might induce a high rate of futile substrate cycling (see Katz & Rognstad, 1976) in the glycolytic pathway. This process has been demonstrated in bull spermatozoa (Hammerstedt & Lardy, 1983) but here high concentrations of fructose 1,6-bisphosphate produced by stimulation of the glycolytic flux did not increase 'futile' cycling. However, the situation may be very different in the present experiments in which glycolysis is strongly inhibited.

(2) The entry of inorganic phosphate into spermatozoa is limited (Babcock, First & Lardy, 1975). In boar spermatozoa exposed to glucose and to α -chlorohydrin the phosphate released by the conversion of ATP to ADP and AMP is consumed in the synthesis of glycolytic intermediates. Thus insufficient inorganic phosphate may be available to permit the resynthesis of ATP by oxidative phosphorylation. In support of this the rate of oxygen uptake in boar spermatozoa incubated with 1 mM-glucose and 1 mM α -chlorohydrin for 30 min is less than in spermatozoa incubated with 1 mM-glucose alone (0.58 ± 0.09 versus 0.87 ± 0.16 nmol O_2 /min/ 10^8 spermatozoa respectively; mean $\pm$ s.e.m., $n = 7$, $P < 0.05$, two-way analysis of variance). However, it is not clear how such an effect could be reversed by the addition of lactate and pyruvate.

Further investigation of this phenomenon may yield information about the regulation of energy metabolism in spermatozoa as well as illustrating how these cells are vulnerable to contraceptive action. It may also be relevant to understanding of similar events in isolated spermatids when the addition of glucose initiates a rapid conversion of ATP to AMP with a concomitant accumulation of fructose 1,6-bisphosphate (Nakamura, Fujiwara, Yasumasu, Okinaga & Arai, 1982; Nakamura, Okinaga & Arai, 1984; J. A. Grootegoed, personal communication).

This work was supported by Grant no. G822472SB from the Medical Research Council.

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Received 22 October 1985