Stimulation of protein secretion in the initial segment of the rat epididymis by fluid from the ram rete testis

S. Sujarit, R. C. Jones, B. P. Setchell*, G. Chaturapanich, M. Lin and J. Clulow

*Department of Biological Sciences, The University of Newcastle, New South Wales 2308, Australia; and *Department of Animal Sciences, Waite Agricultural Research Institute, University of Adelaide, South Australia 5064, Australia*

Summary. Zone 1A of the ductus epididymidis was perfused with ovine rete testis fluid (nRTF) and modifications of it, and a synthetic medium (sRTF) based on the inorganic composition of nRTF. There was little fluid transport by the duct mucosa and nRTF stimulated protein secretion. The secretagogue activity was not extracted by charcoal, was sensitive to protease digestion and was present in a portion of nRTF with a molecular weight of $> 10\,000$. The addition of bovine serum albumin to the sRTF stimulated protein secretion, but not to the same extent as equal amounts of protein in nRTF.

Polyacrylamide gel electrophoresis of the perfusates showed that proteins with molecular weights of 19 000 (all rats studied), and 22 000, 30 000 and 60 000 (at least half the rats studied) were secreted into the perfusion fluids as well as some blood proteins, but the pattern of secretion was not affected by the composition of the perfusion fluid.

Keywords: initial segment; rete testis fluid; secretagogue; rat

Introduction

Benoit (1926) recognized that there is an initial segment in the epididymis of mammals which is characterized by a tall secretory epithelium with long stereocilia, a wide diameter and a low concentration of spermatozoa in the lumen. The segment is dependent on a luminal connection with the testis even when systemic androgen concentrations are high (Gustafsson, 1966; Moniem *et al.*, 1978; Fawcett & Hoffer, 1979; Nicander *et al.*, 1983), and it is considered that the segment plays an important role in extratesticular sperm maturation (Jones *et al.*, 1987; Jones & Clulow, 1987b).

Protein secretion is one of the major functions of the initial segment epithelium; the secretion is dependent on a luminal connection with the testis and some of it becomes associated with the plasmalemma of spermatozoa (Fawcett & Hoffer, 1979; Jones *et al.*, 1980b; Jones & Brown, 1982).

This report describes the use of a microperfusion technique to study protein secretion by the initial segment of the rat and the role of fluid collected from the rete testis in regulating the secretion.

Materials and Methods

Microperfusion. Mature, male Wistar rats (320–460 g) were anaesthetized with sodium 5-ethyl-5(1-methyl propyl)-2-thiobarbiturate (Inactin, Byk Gulden Pharmaceuticals, Konstanz, West Germany) initially administered intraperitoneally at a dose of 100 mg/kg body mass and subsequently via a cannula in the jugular vein. The rats were supported on a heated table and maintained at 35–36°C.

The ductus epididymidis was cannulated using 9-0 silk thread (Ethicon, Inc., Johnson & Johnson, Sydney, Australia), and polyethylene tubing (o.d. 0.61 mm, i.d. 0.28 mm; Dural Plastics & Engineering, Auburn, NSW,

Australia) drawn to a tip diameter of 100–120 μm and supported by a micromanipulator. The proximal cannula was inserted near the proximal end of zone 1A and the distal cannula was inserted into zone 1B (near the proximal end) of the epididymis (Reid & Cleland, 1957) so that 15 ± 1.6 cm (mean $\pm$ s.e. from 29 rats) of the duct were perfused. The temperature of the perfused duct was maintained at 32–33°C by dropping warm paraffin oil (Fisher Scientific Co., Springfield, NJ, USA) onto its surface from an oil heater.

The duct was perfused at a rate of 0.5 $\mu\text{l}/\text{min}$ (Model 341A Syringe pump, Sage Instruments, Cambridge, MA, USA) and the perfusate emerging from the distal cannula was collected into a microhaematocrit tube under water saturated paraffin oil stained with Sudan black. The first sample was collected 1.5 h after perfusion began when all spermatozoa had been flushed from the duct, and samples were accumulated over 2-h intervals during a 7.5-h period of perfusion, then stored at -20°C until analysis.

Perfusion fluids. [^3H]Inulin (sp. act. 1 $\mu\text{Ci}/\mu\text{l}$; Amersham, Bucks, UK) was added to determine fluid transport across the duct mucosa and so correct for its effect on differences in protein concentration between the perfusion fluid and the perfusate. Fluid transport was expressed as the inulin ratio, calculated as the number of scintillation counts per minute in the perfusate divided by the number of counts in the perfusing fluid.

Rete testis fluid (nRTF) was collected from the ram by cannulation (Suominen & Setchell, 1972), and stored at -20°C until use. Fluid from the ram rather than the rat was used in order to obtain a sufficient volume for the work. Steroid-free nRTF was prepared using charcoal and dextran T70 (Pharmacia, Uppsala, Sweden) to extract the steroid (Tsonis *et al.*, 1983). High and low molecular weight (M_r) fractions of nRTF were prepared using an ultrafiltration membrane with a cut-off level of M_r 10 000 (Centricon 10 Microconcentrator, Amicon Scientific Australia; Division of W. R. Grace & Co., Fawkner, Victoria). The protein content of the nRTF was concentrated 5-fold in the high M_r fraction of nRTF. Protease-digested samples of nRTF were prepared using trypsin (type X11-S, bovine pancreas; Sigma Chemical Co., St Louis, MO, USA) and α -chymotrypsin (type VI, Sigma Chemical Co.), and trypsin inhibitor (Type I-S, Sigma Chemical Co.) as described by Feig *et al.* (1980). All solutions were adjusted to pH 7.1 by bubbling 5% carbon dioxide in air through them.

A synthetic solution (sRTF) was prepared containing the same concentrations of inorganic electrolytes as nRTF (Setchell *et al.*, 1969). It contained 118 mm-sodium chloride, 13 mm-potassium chloride, 1.05 mm-calcium chloride, 0.34 mm-magnesium sulphate, 0.075 mm-sodium dihydrogen phosphate, 8 mm-sodium bicarbonate, 20 mm-Hepes (Sigma), and 0, 1.3 or 3.4 μg bovine serum albumin/ μl (BSA, Fraction V: Commonwealth Serum Laboratory, Melbourne, Australia). The pH was adjusted to pH 7.1 with sodium hydroxide and the final osmolality was 290 mosmol/kg.

Analyses of perfusates. [^3H]Inulin was determined by scintillation spectroscopy and protein was determined quantitatively using Coomassie blue (Bradford, 1976). The rate of secretion of protein was calculated from the difference in protein concentration in the perfusion fluid and the collectate, and the estimate was corrected, using the inulin ratio, for fluid transport across the mucosa of the epididymal duct. Some samples of nRTF and blood plasma, and perfusates of nRTF (4 rats) and sRTF (4 rats) were subjected to denatured, continuous gradient polyacrylamide gel electrophoresis (Jones, 1987).

Structure of epithelium. Some of the rats in which the ductus epididymidis was perfused with nRTF and sRTF were fixed by perfusing fixatives through the vascular system (Jones *et al.*, 1984) and samples of the ductus epididymidis in zone 1A were taken from the perfused duct and contralateral epididymis. The samples were post-fixed (Jones *et al.*, 1984), embedded in Spurr's medium and sectioned for light and electron microscopy.

Statistical analysis. Analysis of variance was used to test the statistical significance of the effects of treatment using the variance between animals within treatments as the denominator in the F-tests. Results are presented as means $\pm$ s.e. and the standard errors were calculated from the variance between animals.

Elemental analysis. Samples of perfusion fluid and perfusate from ducts perfused with nRTF (5 rats) and sRTF (5 rats) were analysed for elemental concentrations using electron probe microanalysis (Jones & Clulow, 1987a).

Results

Perfusing the duct for 7.5 h with sRTF or nRTF had no effect on the structure of the duct mucosa (Fig. 1).

The mean ($\pm$ s.e.) inulin ratios for the 8 perfusion fluids shown in Table 1 were respectively 0.92 ± 0.01 , 0.96 ± 0.05 , 0.92 ± 0.03 , 0.96 ± 0.06 , 0.96 ± 0.01 , 1.13 ± 0.10 , 1.00 ± 0.03 and 0.95 ± 0.01 . However, the differences between perfusion fluids were not statistically significant. Further, except for potassium in sRTF there was no significant change during perfusion in the concentration of any of the 7 elements examined (sodium, potassium, chlorine, calcium, magnesium, phosphorus and sulphur). The potassium concentration was 13.46 ± 0.87 in sRTF and 9.57 ± 0.78 in the perfusate ($P < 0.05$).

Fig. 1. Light micrographs of cross-sections of zone 1A of the ductus epididymidis of (a) an untreated duct and (b) a duct which had been perfused for 7.5 h with sRTF. Spurr's resin, toluidine blue staining, $\times 250$.

Table 1 summarizes the effects on net protein secretion by the ductus epididymidis of perfusing the ductus with nRTF, sRTF and various modifications of the fluids. The mean secretion rate into each of the perfusing fluids was much the same during the whole period of perfusion. The mean rate of protein secretion was approximately 3–4 times greater into the ducts perfused with nRTF, the steroid-extracted nRTF and the high M_r fraction of nRTF than with the low M_r fraction of nRTF, the protease digested nRTF or the sRTF ($P < 0.001$). Protein secretion into the unmodified nRTF was much the same as into the steroid-free nRTF, but was about 1.5 times greater into the duct perfused with the high M_r fraction of nRTF (in which the protein was concentrated; see 'Materials and Methods') than into unmodified nRTF ($P < 0.05$).

Table 1. Rate of protein secretion (ng/min) into zone 1A of the rat epididymis during perfusion for 7.5 h with nRTF, sRTF or various modifications of the fluids

Treatment	No. of animals	Time (h) after perfusion started			Mean for 7.5 h
		1.5-2.5	2.5-5.5	5.5-7.5	
nRTF					
(1) Unmodified	7	76 ± 17	105 ± 29	97 ± 19	93 ± 22
(2) Steroid-extraction	4	78 ± 32	123 ± 67	96 ± 37	99 ± 45
(3) High M_r -fraction	5	143 ± 44	156 ± 34	137 ± 39	145 ± 39
(4) Low M_r -fraction	3	18 ± 2	19 ± 4	20 ± 4	19 ± 3
(5) Protease digested	3	36 ± 18	38 ± 25	50 ± 10	41 ± 18
sRTF					
(6) 0.0 µg BSA/µl	8	27 ± 5	27 ± 3	22 ± 3	25 ± 4
(7) 1.3 µg BSA/µl	6	21 ± 10	77 ± 30	37 ± 22	45 ± 21
(8) 3.4 µg BSA/µl	5	72 ± 18	123 ± 14	112 ± 5	102 ± 12

Values are means ± s.e.

The inclusion of BSA in the sRTF produced a higher rate of protein secretion into the lumen than was obtained by perfusing sRTF containing no BSA ($P < 0.01$). However, Fig. 2 shows that BSA did not stimulate protein secretion as much as nRTF (unmodified nRTF and the high M_r fraction of nRTF) which contained an equivalent amount of protein ($P < 0.01$).

Fig. 2. Protein secretion into zone 1A of the ductus epididymidis during perfusion with unmodified nRTF and the high M_r fraction of nRTF (—■—) or sRTF containing 1.3 or 3.4 µg BSA/µl (—□—). The number of animals is shown in parentheses.

Polyacrylamide gel electrophoresis of perfusates (Fig. 3) from 4 animals perfused with nRTF and 4 perfused with sRTF showed that the ductus secreted 3 proteins which were not present in blood. One of these (of M_r 19 000) formed a dense band in all gels; a protein of M_r 22 000 also

formed a dense band in gels, but only for perfusates of 2 rats perfused with nRTF and 2 rats perfused with sRTF; and a protein of M_r 60 000 formed a moderately dense band in gels for perfusates from 1 rat perfused with nRTF and 3 rats perfused with sRTF. In addition a moderately dense band corresponding to a protein of M_r 30 000, which was not present in samples of blood, was present in all gels of nRTF, and in gels of perfusates from 2 rats (of the 4 rats examined) which were perfused with sRTF. Several proteins with the same molecular weight as blood proteins were present in gels of perfusates (Fig. 3), including one with an M_r of 83 000 which was present in perfusates of 3 animals perfused with sRTF and 3 perfused with nRTF. It was interpreted that there was no difference in the pattern of protein secretion into the two perfusion fluids.

Fig. 3. Photograph of denatured linear-gradient polyacrylamide gel of blood plasma (B), nRTF (1) and perfusates after perfusing zone 1A of the epididymis with nRTF (2) or sRTF (3). Gels were loaded with 0.25 μ g protein for B, lanes 1 and 3 or 0.125 μ g protein for lane 2. Silver stain.

Discussion

As Nicander *et al.* (1983) indicated that apoptosis of the duct epithelium starts in zone 1A of the rat epididymis 6 h after efferent duct ligation, yet in the studies described in this report there was no effect of perfusing the duct with sRTF, it is suggested that the perfusion somehow delays the regression associated with preventing testicular fluid from entering the initial segment.

It is concluded from the studies described in this report that sheep rete testis fluid contains a factor or factors which stimulates protein secretion by the epithelium lining zone 1A of the ductus epididymidis. The activity is probably due to a protein or proteins as it is in a fraction with a molecular weight of > 10 000 and is sensitive to proteases. Some of the activity may be due to

albumin which is present in rete testis fluid (Koskimies & Kormano, 1975; Skinner *et al.*, 1987), but it would not account for much of the activity observed in the nRTF used in these studies as albumin represents only 11–17% of the protein present in the fluid (Koskimies & Kormano, 1973; Skinner *et al.*, 1987) and the potency of albumin is less than one-third of the protein in nRTF (Fig. 2). Albumin is also transported across the duct mucosa from blood into the lumen of the ductus epididymidis (see Fig. 3 of this study; Brooks & Higgins, 1980). Several other biologically active macromolecules have been identified in rete testes fluid, including androgen-binding protein (Griswold, 1986), seminiferous growth factor (Feig *et al.*, 1980), α_2 -macroglobulin (Johnson & Setchell, 1968) and cellular retinol binding protein (Kato *et al.*, 1985), but it is premature to speculate on their involvement in regulating protein secretion in the epididymis.

The finding that nRTF stimulates protein secretion by zone 1A of the epididymis of the rat supports studies on the effects of efferent duct ligation (Brooks & Higgins, 1980; Jones *et al.*, 1980a, b) which showed that protein synthesis and secretion in the initial segments of the rat epididymis are altered by preventing the testicular effluent from flowing into the ductus. However, it should not be assumed that the perfusion studies described in this report are examining the same response that was studied following efferent duct ligation as the latter studied protein secretion at a time when there would be considerable intracellular structural reorganization of the principal cells, whereas there was no such change during the period of perfusion described in this report (see Fig. 1). Also, Jones *et al.* (1980b) found that the main effect of efferent duct ligation was on the synthesis of specific proteins (M_r 23 000 and 80 000) whereas in this study the secretagogue effect on nRTF seemed to be mainly on the rate of protein secretion.

It is also concluded from the present studies that zone 1A of the epididymis of the rat secretes protein with an M_r of 19 000 which is not present in blood. This protein probably corresponds to the 2 proteins, with M_r values of 18 500 and 19 000, described by Jones *et al.* (1980a). The duct sometimes secretes other proteins which are not present in blood (M_r 22 000, M_r 30 000). It is suggested that the protein with a M_r of 22 000 corresponds to the protein with an M_r of 23 000 which Jones *et al.* (1980b) found to be synthesized by the 'initial segment' of the rat, and probably the protein with an M_r of 30 000 corresponds to the protein with an M_r of 32 000 which Jones *et al.* (1980a) found to be synthesized by the 'caput epididymidis' of the rat. Jones *et al.* (1980a, b) also found that the 'initial segment' secretes a protein of M_r 80 000 and it is suggested that this corresponds to the protein of M_r 83 000 which was secreted into most perfusion fluids.

The finding in this study that there is little net fluid transport across the duct mucosa of zone 1A is in agreement with our earlier work (Jones & Jurd, 1987) which showed that the fluid which was reabsorbed between the rete testis and zone 2 of the ductus epididymidis (84% of the fluid: Hinton *et al.*, 1980) could be accounted for by reabsorption in the ductuli efferentes as they were estimated to reabsorb 83–92% of the fluid leaving the rete testis. Although Djakiew *et al.* (1986) recorded an increase in spermatocrit value in micropuncture samples taken from zones 1A and 2, this should be interpreted with some caution as micropuncture samples of zone 1 are contaminated with stereocilia (Jones *et al.*, 1987) and result in a diffuse boundary between the sperm and supernatant layers in a spermatocrit.

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