

Stage-related differences in rat seminiferous tubule contractility *in vitro* and their response to oxytocin

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Abstract

Oxytocin (OT) is present in the mammalian testis and has been shown to play a role in the modulation of seminiferous tubule contractility and steroidogenesis. However, stage-specific effects of the peptide have not been previously investigated. In this study, computer-assisted analysis and time-lapse videomicrography were used to investigate basal contractility and the response to OT of seminiferous tubules at specific stages of the spermatogenic cycle. Adult rat testes were placed in fresh oxygenated DMEM F12 medium, decapsulated, and the tubules gently teased apart. Stages were identified by transillumination and a 10 mm section of tubule at each of stages IV-V, VII-VIII and XIII-I was placed in a microslide chamber and perfused with medium. After a control period of 3 h, OT (2 nM) was given for 1 h, followed by another control period of 1 h. The experiment was repeated using tubules

from different rats and data were analysed to give arbitrary units of tubule contractility. Contractility was observed in all the tubules studied and the contractile activity was shown to vary depending on the stage of the spermatogenic cycle. Mean basal contractility at stages VII-VIII, the time when sperm are shed from the epithelium, was significantly lower than that at stages IV-V and XIII-I. The response of the tubules to OT was also stage-dependent, with the peptide producing the largest increases in contractile activity at stages VII-VIII and having no effect at stages IV-V. We postulate that these stage-specific differences in basal and OT-stimulated contractility may be important in co-ordinating the movement of developing germ cells towards the lumen of the seminiferous epithelium and in the process of spermiation.

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Introduction

Spermatogenesis is an organised process, which in the rat occurs in an ordered fashion, producing 14 distinct histological stages arranged linearly along the seminiferous tubule (Leblond & Clermont 1952). During their development, the germ cells not only complete a series of mitotic and meiotic divisions, but also have to move from the base of the tubule to the luminal surface and undergo major morphological changes. Non-motile spermatozoa are shed from the seminiferous epithelium at the end of stage VIII and are transported through the tubular lumen to the rete testis. A number of mechanisms have been hypothesised to be involved in the shedding of sperm from the seminiferous epithelium (spermiation). These include the formation of tubulobulbar complexes (Gravis 1980), the breakdown of junctional specialisations between the germ cells and the supporting Sertoli cells (Romrell & Ross 1979) and the involvement of proteases at stages VII-VIII of the cycle (Lacroix *et al.* 1981, Vihko *et al.* 1984, Erickson-Lawrence *et al.* 1991).

The movement of spermatozoa from the testis to the epididymis, where they become motile, has been

postulated to occur by a combination of secretion and flow of fluid from the seminiferous tubules and contractile activity of the tubules themselves. Each tubule is surrounded by a layer of smooth muscle-like cells, myoid cells, which produce contractile movements of the tubule (Roosen-Runge 1951). More recently these movements have been characterised and two types of contractile activity have been demonstrated (Worley *et al.* 1984), type A comprising continual, 'vibratory' movements involving small segments of the tubule wall, and type B which are intermittent contractions of large segments of the tubule. These latter contractions are accompanied by movement of the luminal contents and are thought to be involved in transporting spermatozoa from the testis. The function of the type A movements is unknown, although these movements may assist in the translocation and subsequent shedding of germ cells from the seminiferous epithelium. Such studies were performed *in vitro* and it is possible that contractility of tightly packed seminiferous tubules *in vivo* may not be as great. The testis of the rat is not very fibrous when compared with that of the ram, where the testis consists of a very fibrous interstitium, yet, in the ram, movements of the

seminiferous tubules contained within whole pieces of excised testis can be observed *in vitro* (G C Harris, unpublished observations). Furthermore, in the rat, contractions have been observed in gently teased out seminiferous tubules *in vivo* (H D Nicholson, unpublished observations), indicating that the movement observed in such studies *in vitro* is not merely an artefact caused by excision and isolation of the seminiferous tubule in a perfusion chamber.

A variety of substances have been shown to affect myoid cell activity, including oxytocin (OT) (Worley *et al.* 1984), vasopressin (Pickering *et al.* 1989), prostaglandins (Buhrlay & Ellis 1975) and endothelin (Filippini *et al.* 1993, Tripiciano *et al.* 1996). OT is produced by Leydig cells (Nicholson & Hardy 1992) and increases the contractility of seminiferous tubules *in vitro* (Niemi & Korman 1965) in a dose-dependent manner (Worley *et al.* 1988) at concentrations similar to those found in the testis (Pickering *et al.* 1989). Selective Leydig cell depletion with the drug ethane dimethane sulphate reduces testicular OT and decreases seminiferous tubule contractility *in vitro*, but contractility can be restored by treatment with OT (Nicholson *et al.* 1987). In addition, treatment of hypogonadal mice with luteinising hormone or testosterone results in the appearance of both testicular OT and tubular contractility (Nicholson *et al.* 1986). This, and the evidence that testicular OT is regulated by the seminiferous epithelium (Nicholson *et al.* 1994), suggests a physiological role for OT in the regulation of tubule contractility. Additionally, although vasopressin has been shown to be at least ten times more potent in stimulating rat seminiferous tubule contractility *in vitro* than OT (Harris & Nicholson 1998), it was shown in the same study, by the use of specific OT and vasopressin antagonists, that vasopressin acts via V_{1a} receptors to stimulate contractile activity, whilst OT may act via a receptor which differs from the classical V_{1a} and uterine-type OT receptor. This may also explain the current inability to localise OT receptors on the seminiferous epithelium.

In the epididymis and ductus deferens it has been postulated that OT-induced contractile activity is important in sperm transport (Voglmayr 1975), but more recent evidence suggests that OT may play another role in the testis. Preliminary studies *in vivo* have shown that OT may also affect spermiation, since administration of the peptide advanced, and treatment with an OT antagonist delayed, the onset of spermiation in the pubertal rat (Frayne *et al.* 1996). If tubule contractility and testicular peptides, such as OT, are involved in the movement and shedding of germ cells, then one would expect these parameters to change during the spermatogenic cycle. We have used computer-assisted analysis and time-lapse videomicrography to investigate basal contractility and the response to OT of seminiferous tubules at specific stages of the spermatogenic cycle.

Materials and Methods

Preparation of seminiferous tubules

Adult male Sprague–Dawley rats were killed by cervical dislocation. Testes were removed and decapsulated. The seminiferous tubules were submerged in pre-oxygenated Dulbecco's modified Eagle's medium (DMEM F12; Gibco Ltd, Paisley, Strathclyde, UK) supplemented with penicillin and streptomycin (50 U ml^{-1} and 50 mg ml^{-1} respectively, Sigma Chemical Co., Poole, Dorset, UK) at 34°C , and gently teased apart. Stages of the spermatogenic cycle were identified by transillumination as described by Parvinen & Ruokonen (1982), and approximately 10 mm of tubule at each of stages IV–V, VII–VIII and XIII–I was excised and drawn into a microslide chamber and then perfused with fresh DMEM F12 medium (with antibiotics) at 1.25 ml h^{-1} using a peristaltic pump.

The perfusion chamber

A microslide (Camlab Ltd, Cambridge, Cambs, UK) with an inner path length of $400 \mu\text{m}$ was used for containment of the seminiferous tubule and was connected to silicone tubing (Anachem, Luton, Beds, UK). The entire apparatus was enclosed in a cabinet held at the temperature of 34°C and suspended on an anti-vibration table (U-frame Isolator, Ealing Electro-optics, Ealing, UK).

Peptides

All peptides used in this study were first dissolved in distilled water and then diluted in culture medium to the appropriate concentration. OT (Cambridge Research Biochemicals, Cambridge, Cambs, UK) was given at a concentration of 2 nM in the perfusion chamber. The OT antagonist desGly-NH₂,d(CH₂)₅[p-Tyr²,Thr⁴]OVT and the vasopressin antagonist Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH₂ used in this study were kindly donated to us by Dr M Manning (Medical College of Ohio, Toledo, OH, USA) and were used at doses similar to their *in vitro* pA₂ values in rat uterus and liver respectively (Schmidt *et al.* 1991, Manning *et al.* 1995).

Stage-dependent changes in basal and OT-stimulated contractility

Segments of tubules were prepared as above and perfused for 3 h in control medium before perfusion with medium containing OT (2 nM) for 1 h. This was followed by perfusion for a further hour with control medium. The experiment was repeated using tubules from different animals. Stage VII–VIII tubules were also treated with 8 nM and 20 nM OT.

The effect of OT and vasopressin antagonists on basal tubule contractility

To investigate whether basal contractility might be due to the effects of endogenous neurohypophysial peptides, tubules were prepared as above and after a 3 h control period perfused with the OT antagonist, desGly-NH₂,d(CH₂)₅[D-Tyr²,Thr⁴]OVT (2 µM) and the vasopressin antagonist Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH₂ (20 nM) for 1 h.

Basal contractility and spermiation

In order to investigate whether shedding of sperm was related to changes in contractility, basal contractility was examined at stage IX, i.e. after shedding of sperm, and then from the adjacent stage VIII of the same tubule. Measurement of basal contractility at stage IX was performed for approximately 40 min, followed by a further 40 min measurement at the adjacent stage VIII of the same tubule. Recordings were taken 50 µm either side of the stage VIII/stage IX boundary, which was easily distinguished by the abrupt change from dark to light lumen associated with spermiation.

Contractility measurement

Seminiferous tubule contractility was monitored as previously described by Worley & Leendertz (1988). Briefly, ×12 time-lapse videomicrographic recordings were performed on tubules *in vitro*. Movement was quantified using an electronic bar, superimposed on a vertically positioned tubule, which measured the changes in contrast which occurred on a video screen when the tubule moved laterally. These changes in contrast were converted to voltages and recorded by a computer. The amplitude and duration of contractions were analysed to give arbitrary units (a.u.) of contractile activity, such that 10 a.u. represented a 10-fold increase in activity compared with 1 a.u. This method was very sensitive, with movements of <0.5 µm being detectable by the equipment. Mean contractility was calculated over 5 min intervals unless otherwise stated. Linear interpolation of basal contractility, as described in Harris & Nicholson (1998), was used to measure the response to OT to take into account any shifting baseline of tubule contractility. The diameter of each seminiferous tubule was also measured to investigate whether the size of the tubule was related to its contractile activity.

Data analysis

Data were expressed as means ± s.e.m. In all experiments, data were normally distributed and possessed similar variance. Responses to peptides were expressed as both the ratio and difference between the 'actual contractility

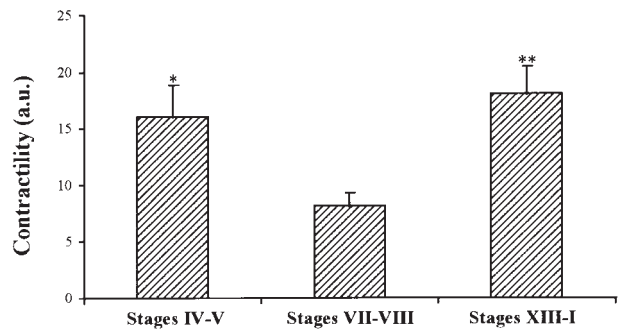


Figure 1 Mean basal contractility of seminiferous tubules at different stages of the spermatogenic cycle. Stages IV-V and XIII-I, $n=6$; stages VII-VIII, $n=15$. * $P<0.05$, ** $P<0.01$ vs stages VII-VIII.

during treatment' (a.u.) compared with the 'linearly interpolated contractility during treatment' (a.u.). Data were expressed in both ways to allow for variations in basal contractility that occurred between tubules at different stages of the spermatogenic cycle (ANOVA, $P<0.05$). Student's paired *t*-tests were performed to test for significant OT-induced increases in contractility. Student's *t*-tests and ANOVA were used to compare basal contractility with the response to OT at different stages of the spermatogenic cycle. Values were considered to be significantly different if $P<0.05$.

Results

Basal contractility of seminiferous tubules

Basal contractile activity was observed in all of the tubules examined. Contractility varied significantly (ANOVA, $P=0.001$) between tubules at different stages of the spermatogenic cycle (Fig. 1), with basal contractile activity being significantly lower at stages VII-VIII compared with stages IV-V ($P<0.05$) and XIII-I ($P<0.01$), and highest at stages XIII-I.

Perfusion with the OT antagonist desGly-NH₂,d(CH₂)₅[D-Tyr²,Thr⁴]OVT (2 µM) and the vasopressin antagonist Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH₂ (20 nM) did not affect basal contractility (Fig. 2) at stages IV-V and XIII-I. Separate incubation with each of these antagonists has also been shown to specifically block OT- and vasopressin-induced increases in contractility at stages VII-VIII, whilst again having no effect on basal contractility (Harris & Nicholson 1998).

Basal contractile activity was found to alter around the time of spermiation. Tubules exhibited greater basal contractile activity at stage IX, immediately after sperm had been shed, than at stage VIII for the same tubule (Fig. 3). The site of spermiation did not change during any of the experiments.

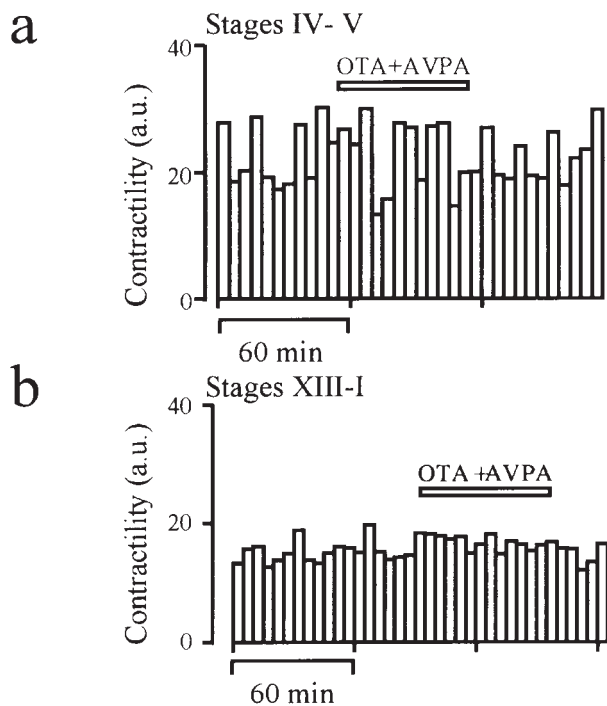


Figure 2 Representative contractility recordings showing the lack of effect of OT (OTA, 2 μ M) and vasopressin (AVPA, 20 nM) antagonists on seminiferous tubule contractility at (a) stages IV-V and (b) stages XIII-I of the spermatogenic cycle. Each bar represents the mean contractility of the tubule over a 5 min period.

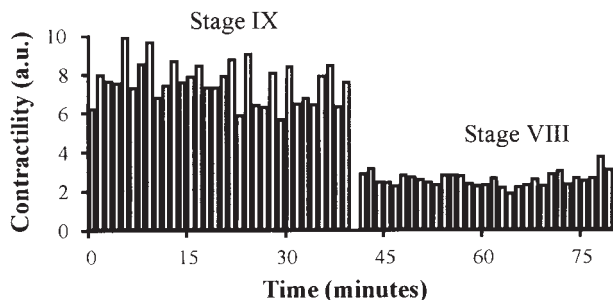


Figure 3 Representative trace of seminiferous tubule contractility at stage IX and stage VIII of the spermatogenic cycle within an individual tubule. Each bar represents the mean contractility of the tubule over a 1.25 min period.

The diameter of the seminiferous tubules also varied with the stage of the spermatogenic cycle (Fig. 4). The tubular diameters were significantly larger at stages VII-VIII compared with stages IV-V and XIII-I (ANOVA, $P < 0.001$). In Fig. 5, the basal contractility is plotted against tubule diameter. No significant correlation was seen between contractile activity and tubule diameter when all the tubules were considered. However, tubules at stages XIII-I showed a significant positive correlation ($P < 0.05$) of tubule diameter with basal contractility.

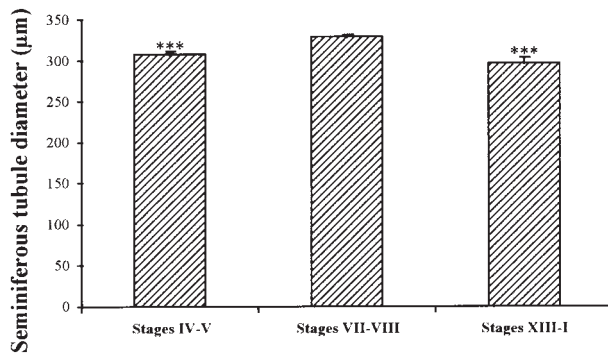


Figure 4 Seminiferous tubule diameter at stages IV-V ($n=8$), VII-VIII ($n=91$) and XIII-I ($n=9$) of the spermatogenic cycle. Data are expressed as means \pm S.E.M. *** $P < 0.001$ vs stages VII-VIII.

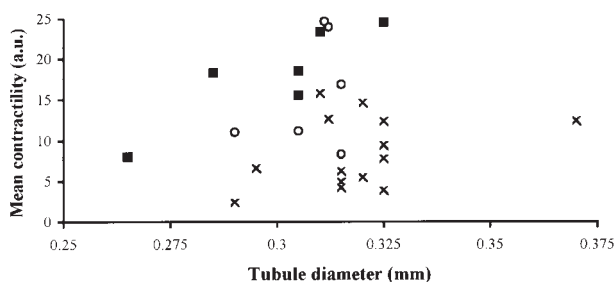


Figure 5 Scatter diagram of tubule diameter vs basal contractility. ○, stages IV-V ($n=6$); ×, stages VII-VIII ($n=15$); ■, stages XIII-I ($n=6$).

Effects of OT on seminiferous tubule contractility

OT (2 nM) significantly increased tubule contractility at stages VII-VIII ($P < 0.001$) and XIII-I ($P < 0.05$) compared with the control period (Fig. 6). The increase in contractility was larger at stages VII-VIII (33%) than at stages XIII-I (7%). These effects were seen when the results were expressed as the increase in contractility (Fig. 6a), and as the relative contractile response (Fig. 6b) compared with the activity during the control period. Furthermore, OT increased seminiferous tubule contractility in a dose-dependent manner at stages VII-VIII (Fig. 7). Stages IV-V did not show a significant response to OT (2 nM). The relative contractile response to OT (2 nM) at stages VII-VIII was significantly higher ($P < 0.05$) than that at stages XIII-I (Student's unpaired t -test).

To investigate whether the response to OT was determined by the initial contractile activity of the tubule, the basal contractility of seminiferous tubules was plotted against the relative contractile response (Fig. 8a) and OT-induced increase in contractility (Fig. 8b). There was no significant correlation between basal contractility and OT-induced contractile responses at any of the stages of the spermatogenic cycle examined, regardless of whether the data were expressed as the increase in contractility or the relative contractile response.

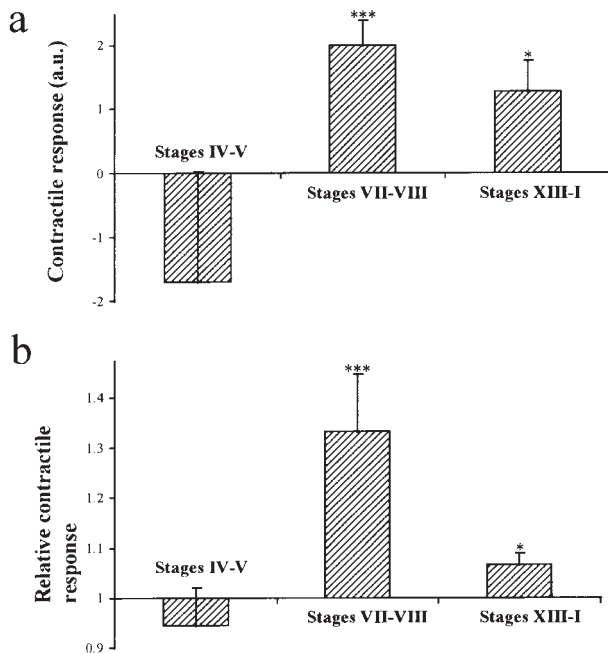


Figure 6 The effects of OT (2 nM) on seminiferous tubule contractility at stages IV-V ($n=6$), VII-VIII ($n=15$) and XIII-I ($n=6$) of the spermatogenic cycle. Data were analysed by linear interpolation. Responses are represented as (a) differences in contractility and (b) relative contractility. Data are expressed as means \pm S.E.M. * $P<0.05$, *** $P<0.001$ vs linearly interpolated control.

Discussion

We have used a sensitive method to measure seminiferous tubule contractility *in vitro*, and have demonstrated for the first time that basal contractility varies during the spermatogenic cycle of the rat. Interestingly, basal contractility was lower *in vitro* at stages VII-VIII, i.e. at the time sperm are shed from the seminiferous epithelium. These findings support earlier work, where the presence or absence of any contractile activity of individual tubules was studied under real time (Suvanto & Kormanio 1970). These workers suggested that a higher proportion (although not significant) of tubules at stage V of the spermatogenic cycle

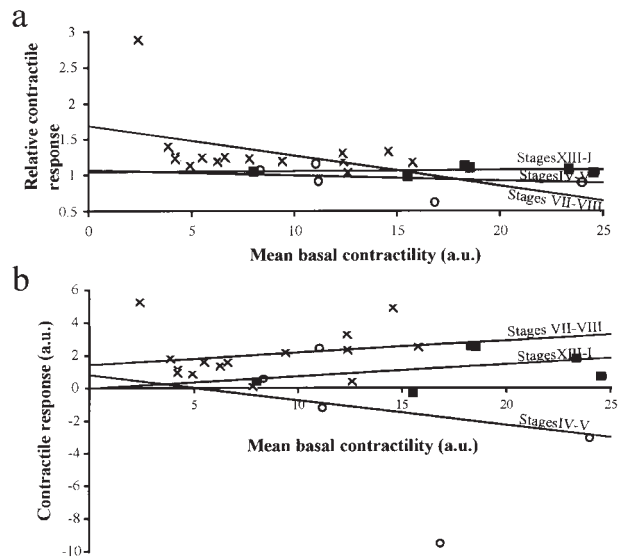


Figure 8 Scatter diagrams of mean basal contractility vs (a) relative contractile response and (b) contractile response (a.u.). \circ , stages IV-V ($n=9$); \times , stages VII-VIII ($n=15$); \blacksquare , stages XIII-I ($n=9$).

exhibited some contractile activity compared with stages VII-VIII. In our study, the use of time-lapse videomicrography enabled more accurate analysis and resulted in the detection of significant differences in tubule contractility.

The mechanisms for the differences in basal contractility are unclear. Intraluminal pressure does not seem to play a major role in basal contractility, as seminiferous tubules that lost luminal contents when they were excised did not display a difference in basal contractility compared with tubules which had been excised so that the luminal fluid remained sealed in the tubule segment. Instead, contractility appeared to be a genuine, inherent characteristic of the stage of tubule removed. However, the effects of external pressure created by muscle tone in the testicular capsule of the testis *in vivo* on basal contractility of seminiferous tubules *in vitro* are not known and this may play a significant role.

We have shown that the decrease in basal contractility at stages VII-VIII of the spermatogenic cycle is not simply

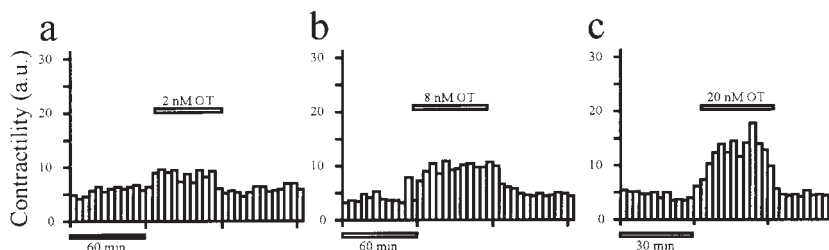


Figure 7 Representative contractility recordings of seminiferous tubules at stages VII-VIII of the spermatogenic cycle showing the effect of (a) 2 nM ($n=15$), (b) 8 nM ($n=6$) and (c) 20 nM ($n=8$) OT. Each bar represents the mean contractility of the tubule over a 5 min period.

due to the increase in tubule diameter that occurs at these stages. In contrast, larger tubules if anything possessed greater basal contractility (Fig. 5). It is more likely that basal contractility depends on the increased resistance to bending forces of seminiferous tubules at stages VII–VIII, which may be associated with the maturation and enlargement of developing spermatids. Increased resistance to bending is not simply caused by an increased width of the seminiferous epithelium, as at stages VII–VIII, accompanying the increase in both lumen and tubule diameter, there is a significant decrease in seminiferous epithelium area (Wing & Christensen 1982, Sharpe 1989). The hypothesis that basal contractility is reduced by the presence of elongate spermatids is supported by the fact that there is higher basal contractility at stage IX of the spermatogenic cycle compared with stage VIII (Fig. 3), as little as 50 µm either side from the site of spermiation. Hence, the shedding of spermatozoa may cause a decrease in ‘tubule stiffness’ and the associated increase in basal contractility. The differences in contractility between stages VIII and IX can be seen further from the site of spermiation, suggesting that the increase in contractility at stage IX is not a remnant of contractility that began in late stage VIII, which may have led to spermiation.

OT and vasopressin are unlikely to be responsible for producing basal contractility, since the peptides are produced by Leydig cells, which were not present in the perfusion chamber. Furthermore, treatment with an OT or vasopressin antagonist had no effect on basal contractility at any stage of the spermatogenic cycle. Since only the seminiferous tubules are included in our preparation, changes in contractile activity are likely to be mediated by factors produced by the seminiferous epithelium, or may be due to the physical properties of the tubules themselves at different stages of spermatogenesis. Many local factors are produced by the testis and it is possible that paracrine factors produced by Sertoli or germ cells may affect myoid cell contractility in a stage-dependent fashion (Skinner 1991).

When considering the relevance of these findings it is interesting to observe that basal contractility is maximal at stages IV–V, a time when spermatids begin their translocation from deep within the seminiferous epithelium towards the lumen surface. The accompanying increase in contractility of the tubule wall may, therefore, help in the movement of spermatids. Conversely, at stages VII–VIII, when spermatids are nearing the lumen in preparation for spermiation, factors which would aid shedding of sperm, such as basal contractility and possibly speed of fluid flow, are lowest (the increased luminal size at stages VII–VIII would theoretically give rise to a slower ‘speed’ of fluid flow through this region, although this has not as yet been measured and there may be other factors involved). It is possible that reduced contractility is important in allowing final maturation of the sperm, so that elimination of excess cytoplasm occurs and premature shedding is avoided.

The response of tubules to OT also varied during the spermatogenic cycles. Whilst OT produced a significant increase in contractility at both stages VII–VIII and XIII–I, the response was greater at VII–VIII. The fact that OT produced the highest response in tubules with the lowest basal contractility raises the possibility that OT may induce an equal physiological response in myoid cells at differing stages, but that the response is less clearly seen in tubules which already have a higher basal contractility. However, Fig. 8, which compares the contractile response with basal contractility, suggests that the stage-related effects of OT are due to more than this factor alone. The differential response to OT may reflect alterations in receptor density and this may be further modulated *in vivo* by the changes in the local production of the peptide by Leydig cells. Although there is no firm evidence that OT is secreted in a stage-dependent manner, the fact that germ cells, and in particular pachytene spermatocytes, can influence OT secretion suggests that this might be the case (Nicholson *et al.* 1994). The observation that OT has its greatest effect at stages VII–VIII raises the possibility that the peptide may be secreted at a specific point during stage VIII, to provide an increase in contractility to facilitate shedding of the spermatozoa and their subsequent transport along the tubule. Indeed, this hypothesis would agree with evidence indicating that OT appears in the rat testis just before the first sperm are shed, and that the timing of spermiation can be altered by the administration of OT or a specific antagonist *in vivo* in the pubertal rat (Frayne *et al.* 1996). However, no noticeable change in the site of spermiation occurred in the tubules whilst studied *in vitro*, even after treatment with OT.

In conclusion, we have shown that basal contractility varies during the spermatogenic cycle of the rat and we hypothesise that this contractile activity may play a role in the movement of germ cells within the seminiferous epithelium. Furthermore, the demonstration that the response to the testicular peptide OT depends on the stage of spermatogenesis, being greatest at stages VII–VIII, suggests a possible stage-specific role for the peptide in tubular contractility.

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