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Control of anion and fluid secretion by apical P2-purinoceptors in the rat epididymis

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1 Exogenous adenosine triphosphate (ATP) stimulated the short circuit current (SCC) in primary monolayer cultures of rat epididymal cells when added to the apical but not to the basolateral side of the monolayers. Half-maximal stimulation was achieved at 5×10^{-8} M ATP.

2 The increase in SCC induced by ATP was dependent on the presence of extracellular Cl in the bathing solutions.

3 The effects of other adenosine derivatives, and purine and pyrimidine nucleotides were studied. Their orders of potency in stimulating SCC were: ATP > adenosine diphosphate ≫ adenosine monophosphate, adenosine, and ATP > inosine triphosphate > guanosine triphosphate > cytidine triphosphate. These results indicate that ATP interacts with a P_2-purinoceptor at the apical membrane of the epididymal cells.

4 The SCC response to ATP was not blocked by 8-phenyltheophylline, a P_1-purinoceptor antagonist or by propranolol. Although pretreatment of the cultures with piroxicam abolished the SCC response to bradykinin, it did not affect the response to ATP. This indicates that the SCC response to ATP was not mediated by an increase in the synthesis of prostaglandins.

5 Serosal to mucosal Cl flux (J_{s-m} Cl) and net water flux were measured in the luminally perfused rat epididymis *in vivo*. ATP (1 μM) added to the luminal perfusion solution caused an increase in J_{s-m} Cl and net water secretion by the epididymal duct.

6 Since spermatozoa contain a high concentration of ATP, it is proposed that ATP released from spermatozoa may affect anion and fluid secretion by the epididymis. The control of secretion via the apical purinoceptors offers a means by which spermatozoa regulate the fluidity of their own environment.

Introduction

The epididymis is an absorptive as well as a secretory epithelium. Under normal conditions, there is a net absorption of electrolytes and water across the duct and this accounts for the reabsorption of a major part of the testicular fluid (Wong & Yeung, 1978). However, under other conditions the epididymis may be stimulated to secrete fluid and, as in other secretory epithelia, active transport of anions seems to be the driving force for fluid secretion (Wong, 1986).

Monolayer cultures of rat epididymal cells have been shown to secrete chloride and bicarbonate concurrently when stimulated with secretory agonists (Cuthbert & Wong, 1986; Wong & Chan, 1988; Wong, 1988a,b). Secretion involves the simultaneous activities of a Na/K/Cl symport, a Na/H exchanger and a Cl/HCO_3 exchanger at the basolateral membrane. These carriers actively maintain high Cl and HCO_3 activities above electrochemical equilibrium. Exit of anions across the apical membrane is through diffusible channels which are blocked by anion channel blockers (Wong, 1988b; Huang & Wong, 1988).

Adrenaline added to the basolateral side of the epithelium stimulates anion secretion (Wong, 1988a). It has been proposed that adrenergic stimulation of anion (and fluid) secretion by the epididymis may play a role in seminal emission (Wong & Chan, 1988). During ejaculation there is contraction of the smooth muscle surrounding the epididymal duct, although ductal contraction alone may be insufficient to eject the viscous epididymal content. However, if fluid secretion also takes place during the reflex, the addition of watery secretion into the ductal lumen would help to wash out the spermatozoa.

Recently, we have been interested in agents that affect secretion by acting at the apical side of the epididymal cells. The epididymal lumen is normally filled with spermatozoa and it is possible that spermatozoa themselves can affect secretion. Since spermatozoa are known to contain a high concentration of ATP (Calamera *et al.*, 1979; Gottlieb *et al.*, 1987), which may be released into the lumen during their transit through the epididymis, we have studied the effects of exogenous ATP on anion secretion by primary monolayer cultures of rat epididymal cells.

Methods

Cell culture and short circuit current measurement

Primary monolayer cultures of rat epididymal cells were grown on millipore filters coated with collagen by methods previously described (Cuthbert & Wong, 1986; Wong, 1988a,b). After 4 days of culture, the monolayers became confluent and were ready for the measurement of electrogenic ion transport using the short circuit current (SCC) technique.

Epididymal monolayers (area 0.4 cm^2) were clamped between the two halves of the Ussing chambers with a 0.7 cm^2 window. The tissue was short circuited (voltage clamped at zero potential) using W-P Instruments Dual Voltage-Current Clamp Amplifier (DVC 1000) and the short circuit current displayed on a pen recorder (Kipp and Zonen). The clamp can be used to set the potential, intermittently, at a voltage displaced from zero (usually 0.3 mV), the resulting current change allowing calculation of the resistance from the ohmic relationship. Very often, the two channels of the amplifier were used simultaneously on parallel monolayers so that studies could be made under control and experimental conditions.

Luminally perfused rat epididymis

In another set of experiments, serosal to mucosal Cl flux (J_{s-m}) was measured in the luminally perfused cauda epididymis of anaesthetized rats according to the method described previously (Wong, 1988b). After cannulation of the epididymal duct and vas, the epididymal content was flushed out with Krebs Henseleit solution and the sperm-free epididymal duct (about 20 cm long) was perfused intermittently with Krebs Henseleit solution using the 'stop flow' method (Wong, 1988b). ^{36}Cl ($Na^{36}Cl$, 7.5 μCi, specific activity: 150 $\mu Ci\,ml^{-1}$) was injected intravenously. The ^{36}Cl activity in the 5 min stop-flow sample was taken as a measure of blood to lumen flux (serosal to mucosal flux, J_{s-m}) of chloride which was expressed in nmol $cm^{-1}\,min^{-1}$ (Wong, 1988b).

Net water transport across the luminally perfused rat epididymis was also measured using inulin as an intraluminal volume marker (Wong & Yeung, 1978). [^{3}H]-inulin (125 nCi ml^{-1}, Amersham, specific activity: 122 $\mu Ci\,mg^{-1}$) was added to the Krebs Henseleit solution which was used to perfuse the lumen of the cauda epididymis. Perfusion was carried out at a rate of 1 $\mu l\,min^{-1}$. The activities of radioactive inulin in both the perfusate and the original perfusion solution were measured and their ratio was used to calculate net water flux which was expressed in nl $cm^{-1}\,min^{-1}$ (Wong & Yeung, 1978).

Solutions and chemicals

The Krebs Henseleit solution used had the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.56, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8, glucose 11. The solution had a pH of 7.4 when bubbled with 95% O_2 and 5% CO_2. In some experiments, a Cl-free solution was used in which NaCl was replaced by Na gluconate and KCl was substituted by K gluconate and $CaCl_2$ by $CaSO_4$.

Adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), adenosine, guanosine 5'-triphosphate (GTP), inosine 5'-triphosphate (ITP), cytidine 5'-triphosphate (CTP), piroxicam, 8-phenyltheophylline and lysylbradykinin were purchased from Sigma Chemical Co. (St Louis, MO). Propranolol was a gift from ICI. Adrenaline was obtained from David Bull Laboratories.

Statistical analysis

Comparisons between groups of data were made by Student's *t*-test (one-tailed, paired or unpaired).

Results

Effect of ATP on the short circuit current

When first clamped in the Ussing chamber, epithelial monolayers formed from the rat cauda epididymis have a transepithelial potential of 2–4 mV, a short circuit current of 1–2 $\mu A\,cm^{-2}$ and a transepithelial resistance of about 500 $\Omega\,cm^2$ (Wong, 1988a,b). In order to avoid excessive washing of the cell monolayers (a process found to compromise the integrity of the monolayers), the effect of different doses of ATP on the SCC was studied using a cumulative method. ATP added to the basolateral side of the monolayers had no effect on the SCC. However, when added apically, it caused an increase in the SCC. The time course of the response was complex: a spike preceded the maximal response, thereafter there was a gradual decline in the SCC. The response to ATP was dose-dependent. At low doses, the response to ATP was small and transient. At

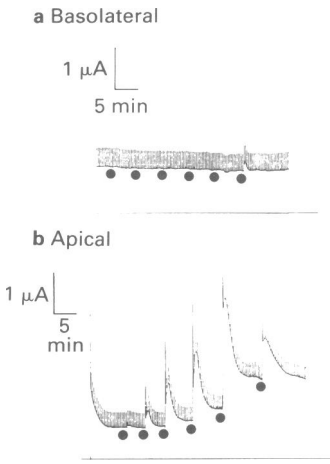

Figure 1 Short circuit current (SCC) of 2 separate epididymal monolayers, area 0.4 cm^2, recorded simultaneously. ATP was added to the basolateral (a) or apical (b) side at the time shown by the dots to produce the following final concentrations: 10^{-8} M, 2.5×10^{-8} M, 5×10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M. Transient current pulses were the result of intermittently clamping the potential at 0.3 mV. The horizontal lines indicate zero SCC. These records are representative of 4 different sets of experiments.

higher doses, the SCC stabilized at a value above the basal level (Figure 1). In other experiments in which a single dose of ATP was added, the plateau phase was continuous for at least 10 min before it finally declined (results not shown). If maximum peak increments of SCC (measured above the immediately preceding basal level) were plotted against ATP concentration, a sigmoidal curve was obtained with a 50% maximum stimulation at $5.01 \pm 0.06 \times 10^{-8}$ M (mean ± s.e.; Figure 3).

The SCC response to ATP was dependent on the presence of extracellular Cl (Figure 2). In normal Krebs Henseleit solution, the increase in SCC (ΔSCC, measured at the peak of response) by ATP (1 μM) was 7.34 ± 0.51 μA cm^{-2} (mean ± s.e.mean, $n = 10$), and in Cl-free solution, the increase in current was reduced to 1.12 ± 0.13 μA cm^{-2} ($n = 4$). The difference was statistically significant ($P < 0.001$).

Specificity of the response

The effects of other adenosine nucleosides/nucleotides were studied. When added apically, ADP stimulated the SCC with an apparent EC_{50} value of $1.7 \pm 0.04 \times 10^{-6}$ M (mean ± s.e.), AMP and adenosine were poorly active (Figure 3). The order of potency was therefore ATP > ADP ⩾ AMP, adenosine, similar to that found for P_2-purinoceptors

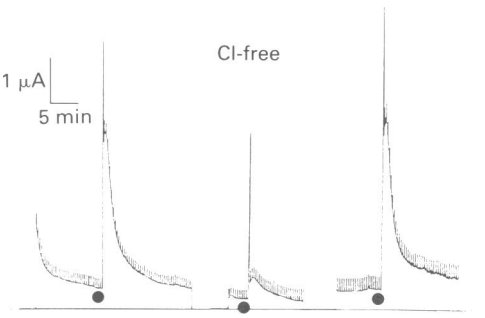

Figure 2 Short circuit current (SCC) responses to ATP (1 μM) (dot) applied to the apical side in a single epididymal monolayer, area 0.4 cm^2. During the gaps in the record, the tissue was washed free of ATP. In the first and third sections the tissue was bathed in Krebs Henseleit solution while in the middle section the tissue was bathed in chloride-free solution. Transient current pulses were the result of intermittently clamping the potential at 0.3 mV. The horizontal line indicates zero SCC. These records are representative of 4 different sets of experiments.

(Burnstock, 1978). The effects of other purine and pyrimidine triphosphates were also studied. ITP, GTP and CTP all stimulated SCC with 50% maximum stimulation at $3.01 \pm 0.04 \times 10^{-7}$ M, $2.00 \pm 0.03 \times 10^{-6}$ M, and $3.90 \pm 0.03 \times 10^{-5}$ M, respectively. The responses to all these compounds displayed apical 'sidedness', had a similar time course to the ATP stimulated SCC and a similar dependence on extracellular Cl.

Effects of inhibitors on the SCC response to ATP

Since stimulation of SCC by ATP in monolayer cultures of rat epididymal cells appeared to be mediated by a P_2-purinoceptor, we examined the effect of 8-

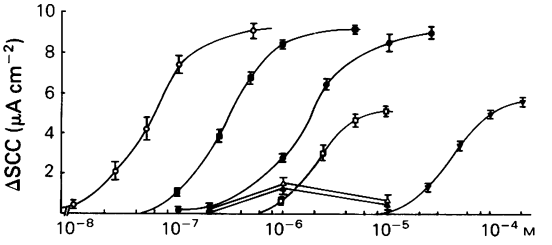

Figure 3 Effects of ATP (○), inosine 5'-triphosphate (■), ADP (●), GTP (□), cytidine 5'-triphosphate (▼), AMP (△) and adenosine (▲) on the short circuit current (SCC) of cultured epididymal monolayers, area 0.4 cm^2. Drugs were added to the apical side. ΔSCC is the change in current measured at the peak of the response. Each point shows the mean of 3 to 5 experiments; vertical lines indicate s.e.mean.

Table 1 Effect of 8-phenyltheophylline (10 μM), propranolol (1.7 μM) or piroxicam (10 μM) on the ATP (1 μM), adrenaline (0.23 μM) or bradykinin (0.1 μM) induced rise in the short circuit current (SCC)

Agent	*ΔSCC*	*Number of experiments*
ATP (apical)	7.52 ± 0.50	4
ATP (apical) + 8-phenyltheophylline (apical)	7.43 ± 0.53	4
ATP (apical)	7.39 ± 0.46	6
ATP (apical) + piroxicam (both sides)	6.94 ± 0.64	6
Bradykinin (basolateral)	6.50 ± 0.42	4
Bradykinin + piroxicam (both sides)	0*	4
ATP (apical)	7.45 ± 0.35	4
ATP (apical) + propranolol (apical)	7.53 ± 0.42	4
Adrenaline (basolateral)	10.35 ± 1.61	6
Adrenaline (basolateral) + propranolol (basolateral)	0.20 ± 0.02*	6

Results are expressed as the change in current, ΔSCC ($\mu A\,cm^{-2}$) measured at the peak of the response. Inhibitors were added to the bath 2 min (8-phenyltheothylline and propranolol) or 15 min (piroxicam) before addition of the agonists. Each value shows the mean ± s.e.mean of the number of experiments shown.
* $P < 0.001$ compared to the control of the respective group (Student's unpaired t test).

phenyltheophylline, a P_1-purinoceptor antagonist, on the SCC. 8-Phenyltheophylline was found to have no significant effect on the SCC induced by ATP (1 μM) (Table 1). Similarly, the β-adrenoceptor antagonist, propranol, was without effect on the ATP-induced SCC (Table 1), but did inhibit the increase in SCC caused by adrenaline (0.23 μM). The prostaglandin synthesis inhibitor piroxicam (10 μM) was also without effect on the response to ATP, but did inhibit the increase in SCC induced by bradykinin (0.1 μM) (Table 1).

Effect of ATP on serosal to mucosal Cl and net water flux across the intact epididymis

The ATP stimulated rise in the SCC was predominately due to an increase in net Cl secretion from the basolateral to the apical side. This contention was supported by measuring Cl flux across the intact epididymis luminally perfused with Krebs Henseleit solution *in vivo*. Figure 4 shows the J_{s-m} (serosal to mucosal) Cl flux. Addition of ATP (1 μM) to the luminal perfusion solution caused a significant increase in the J_{s-m}.

Net water flux across the luminally perfused epididymis was also measured using inulin as an intraluminal volume marker (Wong & Yeung, 1978).

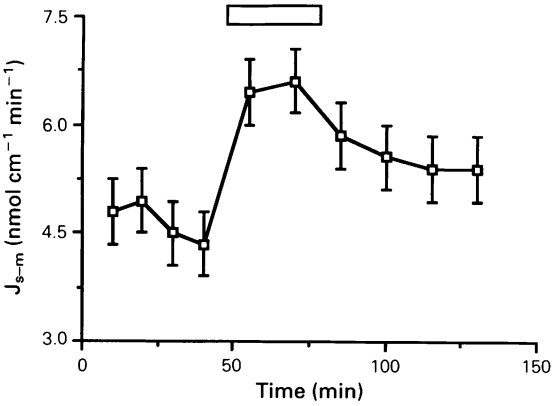

Figure 4 Serosal to mucosal chloride flux (J_{s-m}) across the rat cauda epididymis *in vivo*. During the period shown by the bar, ATP (1 μM) was added to the luminal perfusion solution. Each point shows the mean of 4 determinations; vertical lines indicate s.e.mean. The fluxes measured during application of ATP (period shown by the bar) were significantly different from the flux measured just before application, $P < 0.005$ (Student's paired t test).

Under basal conditions, the epididymis reabsorbs water at a rate of about 15 $nl\,cm^{-1}\,min^{-1}$. ATP (1 μM) added to the luminal perfusion fluid reversed the absorptive flux to a net secretory flux. When ATP was removed from the perfusion solution, net water reabsorption returned to normal (Figure 5).

Discussion

Exogenous ATP has been found to be a potent stimulus for secretion in a number of secretory tissues. For example, surfactant secretion from primary cultures of alveolar type II cells (Rice & Singleton, 1986), insulin secretion from the isolated perfused pancreas (Loubatiéres-Mariani *et al.*, 1979), and amylase secretion from parotid acinar cells (Gallacher, 1982) are found to be stimulated by ATP. Similarly, in secretory epithelia like the intestinal mucosa (Kohn *et al.*, 1970; Field, 1971), corneal epithelium (Spinowitz & Zadunaisky, 1979), and cultured MDCK cells (Simmons, 1981), ATP has been found to stimulate active chloride transport. In all cases, the ATP-induced secretion appears to be mediated by P_2-purinoceptors on the basis of agonist potency and blockade by specific antagonists.

In accord with these actions of ATP, our present study showed that ATP stimulated the short circuit current (SCC) in monolayer cultures of rat epididymal cells grown on a previous support. SCC has been used to measure electrogenic anion secretion in monolayer cultures of rat epididymal cells

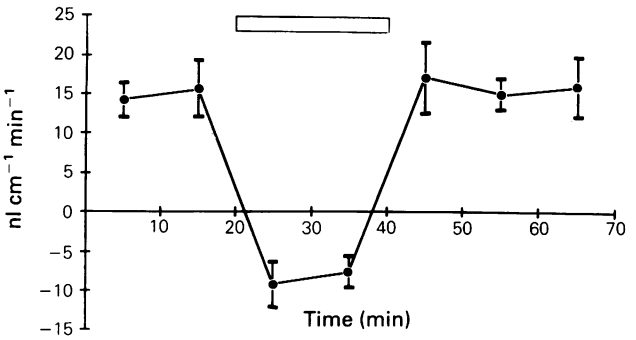

Figure 5 Net water flux across the luminally perfused cauda epididymis of the rat, *in vivo*. Values above the zero line indicate net absorption and values below it indicate net secretion. ATP (1 μM) was added to the luminal perfusion solution during the period shown by the bar. Each value shows the mean of 5 determinations; vertical lines indicate s.e.mean. The fluxes measured during application of ATP (period shown by the bar) were significantly different from the flux measured just before application, $P < 0.005$ (Student's paired t test).

(Cuthbert & Wong, 1986; Wong, 1988a,b). Although direct measurement of Cl fluxes in the epididymal cell monolayers is difficult, there is evidence that the increase in the SCC by ATP was caused by an increase in net chloride secretion. First, the ATP-induced increase in SCC was dependent on extracellular Cl. When Cl was substituted by impermeant gluconate, the increase in SCC by ATP was greatly diminished; the remaining current was probably due to bicarbonate secretion (Figure 2) (see Cuthbert & Wong, 1986; Wong 1988a). Secondly, in the intact epididymis luminally perfused *in vivo*, ATP added to the lumen stimulated the blood to lumen Cl flux (Figure 4). The same concentration of ATP in the luminal fluid also induced a net water secretion (Figure 5). Fluid transport in the epididymis is similar to the gut in that there are large fluxes of electrolytes moving across the epithelium in both directions. Under basal conditions, the absorptive flux predominates over that of secretion and the epididymis undergoes a net water reabsorption. This accounts for the loss of a major part of the testicular fluid as it flows down the epididymis (see Cooper, 1986). However, if the secretory flux is increased to a level above that of absorption, net water secretion ensues. The stimulation of water secretion by ATP may well be the result of an increased NaCl secretion, driven primarily by active anion secretion.

Our study of agonist potency showed that among the adenosine derivatives, ATP was the most potent with adenosine the least potent in stimulating SCC. This order of potency: ATP > ADP $\gg$ AMP, adenosine, together with the order of potency found for the other purine and pyrimidine nucleotides: ATP > ITP > GTP > CTP, suggest that P_2-purinoceptors mediate the secretory response to ATP (Burnstock, 1978). This contention was supported by the finding that 8-phenyltheophylline, a potent antagonist of the P_1-purinoceptor (Smellie *et al.*, 1979; Griffith *et al.*, 1981) did not prevent the rise in SCC caused by ATP (Table 1). Propranolol which inhibited the secretory response to adrenaline (Table 1, Wong & Chan, 1988) was without effect on the ATP-induced rise in the SCC. We have previously found that anion secretion stimulated by kinins (Cuthbert & Wong, 1986) and angiotensin (Wong, 1988a) was mediated by an increase in prostaglandin synthesis. However, the response to ATP did not appear to be mediated by prostaglandins, since pretreating the tissues with piroxicam, a potent cyclo-oxygenase inhibitor which prevents eicosanoid formation, did not affect the SCC response to ATP (Table 1). Our results differ from those of Simmons (1981) who showed that the ATP-induced anion secretion in cultured MDCK cells was reduced after treating the cells with indomethacin, another inhibitor of prostaglandin synthesis.

The sensitivity of the epididymal cells to ATP displayed 'sidedness' in that apical application stimulated secretion whereas basolateral application was without effect (Figure 1). This finding is different from that in the intenstinal tract (Kohn *et al.*, 1970; Korman *et al.*, 1982) and cultured MDCK cells (Simmons, 1981) in that exogenous ATP stimulated anion secretion in these tissues when added apically or basolaterally. Our results, however, raise an interesting possibility that in the epididymis the purinoceptors are located at the apical but not at the basolateral membrane. Epithelia are polar structures and it may be possible that the distribution of receptors controlling secretion also exhibits apical/serosal 'sidedness'. This apical distribution of purinoceptors is in contrast to the receptors for prostaglandin, bradykinin, adrenaline, 5-hydroxytryptamine, angiotensin and vasopressin which have been demonstrated to be present predominantly at the

basolateral membrane of the epididymal cells (Cuthbert & Wong, 1986; Wong & Chan, 1988; Wong, 1988a). Many of these basolateral receptors utilize cyclic AMP as an intracellular second messenger (see Cuthbert & Wong, 1986; Wong, 1988a). At present, the signal transduction mechanism which mediates the response to apical ATP remains unknown, although ATP in concentrations which stimulate SCC was found to increase the intracellular concentration of cyclic AMP in cultured epididymal cells (Wong & Huang, unpublished).

What is the role of the apical purinoceptors in the control of anion and fluid secretion in the epididymis? The epididymal lumen is normally filled with spermatozoa and it is possible that spermatozoa can regulate the fluidity of their environment by feedback onto the epididymal cells. Such a local control would require a factor(s) that is (are) released from spermatozoa. Secondly, there must be a receptor(s) for such a factor(s) at the apical (not basolateral) membrane, and lastly, interaction of the receptor(s) with such a factor(s) should affect anion and fluid secretion. These criteria for a local control factor may be met by ATP which is present in spermatozoa at a high concentration (Calamera *et al.*, 1979; Gottlieb *et al.*, 1987). In the rat, spermatozoa flushed out from the cauda epididymis were found to contain 7.2 mmol ATP l^{-1} sperm cells (Wong, unpublished observation). It is possible that degenerating spermatozoa release ATP which stimulates fluid secretion locally. An increase in fluidity in the vicinity of the sperm would help reabsorption of the sperm fragments by the epididymal cells. This mechanism may be involved in the disposition of unejaculated aged sperm by the cauda epididymis.

It appears that in the epididymis, various secretory agonists affect anion secretion by acting either from the basolateral or the apical side of the epithelium. Those that act basolaterally include the neurotransmitters (noradrenaline), blood-borne hormones and humoral agents which are produced locally in the epididymis (prostaglandins, angiotensin, bradykinins, etc.). The high concentration of ATP present in spermatozoa may act on the purinoceptors located at the apical membrane. This local control via the apical purinoceptors offers a means by which spermatozoa control the fluidity of their own environment. Under normal conditions, fluid transport in the epididymis is precisely controlled so that there is a balance between absorption and secretion. This balance is important to maintain a normal fluidity within the sperm compartment. Any disturbance in the control would lead to an abnormal fluid environment which may have adverse effects on sperm functions and hence fertility. The obstructive azoospermia seen in cystic fibrosis, Young's syndrome and other cases of unexplained male infertility may well be the result of a defective secretory mechanism in the epididymis.

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