Nongenomic effect of testosterone on chloride secretion in cultured rat efferent duct epithelia

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Leung, G. P. H., S. B. Cheng-Chew, and P. Y. D. Wong. Nongenomic effect of testosterone on chloride secretion in cultured rat efferent duct epithelia. Am J Physiol Cell Physiol 280: C1160–C1167, 2001.—Short-circuit current (Isc) technique was used to investigate the role of testosterone in the regulation of chloride secretion in cultured rat efferent duct epithelia. Among the steroids tested, only testosterone, and to a lesser extent, 5α-dihydrotestosterone (5α-DHT), reduced the basal and forskolin-induced Isc in cultured rat efferent duct epithelia when added to the apical bathing solution. Indomethacin, a 3α-hydroxysteroid dehydrogenase, did not affect the inhibitory effect of 5α-DHT. The effect of testosterone occurred within 10–20 s upon application and was dose dependent with apparent IC50 value of 1 μM. The effect was abolished by removal of Cl− but not HCO3− from the normal Krebs-Henseleit solution, suggesting that testosterone mainly inhibited Cl− secretion. The efferent duct was found to be most sensitive to testosterone, while the caput and the cauda epididymis were only mildly sensitive. Cyproterone acetate, a steroidal antiandrogen, or flutamide, a nonsteroidal antiandrogen, did not block the effect of testosterone on the forskolin-induced Isc, nor did protein synthesis inhibitors, cycloheximide, or actinomycin D. However, pertussis toxin, a Gi protein inhibitor, attenuated the inhibition of forskolin-induced Isc by testosterone. Testosterone caused a dose-dependent inhibition of forskolin-induced rise in cAMP in efferent duct cells. It is suggested that the rapid effect of testosterone was mediated through a membrane receptor that is negatively coupled to adenylate cyclase via Gi protein. The role of nongenomic action of testosterone in the regulation of electrolyte and fluid transport in the efferent duct is discussed.

testosterone; chloride secretion; efferent duct

It can hardly be overemphasized that the male posttesticular duct system plays an indispensable role in male reproduction. The efferent duct, or ductuli efferentes, forms the initial portion of the duct where the bulk of the testicular fluid is reabsorbed (7). Water reabsorption results in an increase in the concentration of spermatozoa and also of the constituents that are essential for the maintenance of epididymal integrity and functions. Although the efferent duct is engaged in net water reabsorption (7, 16), electrolyte secretion is also known to take place (6, 23). Secretion of anions may act as a counterbalance to absorption, thereby exerting a fine control over net water movement across the ductules. It is generally held that in the epididymis, unlike absorption which is held at a tonic rate, secretion is subject to short-term neurohumoral regulation (39, 40). Rapid changes in secretion will have impact on the fluidity of the epididymal microenvironment in which maturing spermatozoa are bathed (40).

Androgens are essential for the normal growth and differentiation of the male reproductive tract during sexual development (12, 18, 36). The acquisition of fertilizing capacity of spermatozoa in epididymis also depends on androgens (4, 25, 28). It is generally accepted that androgens bind to intracellular androgen receptors resulting in mRNA and protein synthesis (32). Nevertheless, rapid responses to androgens have been observed in many nonepithelial tissues that cannot be explained by involvement of mRNA and protein synthesis (2, 5, 8, 13, 30). These rapid, nongenomic effects are also seen for other steroid hormones (for review, see Ref. 31). Recently, unconventional membrane receptors for testosterone have been visualized under confocal microscopy (1).

It has been shown that fluid reabsorption in efferent duct and epididymis is androgen dependent (15, 41). Methods for the isolation and culture of epithelial cells from efferent duct have already been described (34). Epithelia so-derived display apical/basolateral polarity and possess morphological features and secretory functions of the intact efferent duct in vivo. In the present study, we have elucidated the nongenomic effect of testosterone on chloride secretion in cultured rat efferent duct epithelia. The implications of this effect in relation to male reproduction are discussed.

MATERIALS AND METHODS

Tissue culture technique. All experiments were carried out according to the guidelines laid down by the Laboratory Animal Services Centre of the Chinese University of Hong Kong. The procedures of primary cultures of rat efferent duct epithelial cells were modified from Rozewicka et al. (34). Immature male Sprague-Dawley rats weighing 150 g were used as a source of efferent duct. Adult rats were not used, as...
it has been shown previously that the presence of spermatozoa prevents the plating of epithelial cells, rendering formation of monolayers difficult. Rats were killed by asphyxiation with a rising concentration of CO\(_2\). The lower abdomens were opened, and the efferent ducts were isolated and microdissected under sterile conditions to remove fat and connective tissue. The ductules were cut into several small segments, transferred to Hanks' balanced salt solution (HBSS) containing 0.1% (wt/vol) trypsin and 0.2% (wt/vol) collagenase I, and incubated in a water bath at 32°C for 1 h with vigorous shaking (150 strokes/min). Then, the tissue was separated by low-speed centrifugation (800 \(g\), 5 min). The supernatant was discarded, and the pellet resuspended in HBSS containing 0.2% (wt/vol) collagenase I for 30 min at 32°C with vigorous shaking. After centrifugation at 800 \(g\) for 5 min, cell plaques were resuspended in HBSS containing 0.2% (wt/vol) collagenase I and subjected to repeated pipetting for 15 min. The cells were centrifuged at 800 \(g\) for 5 min and resuspended in Eagle's minimum essential medium (MEM) containing non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), glutamine (4 mM), 5α-dihydrotestosterone (5α-DHT, 1 nM), 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 \(\mu\)g/ml). The cell suspension was decanted and seeded into the wells of Matrigel-coated Millipore filter assemblies with a diameter of 0.2 cm\(^2\) (cell concentration 5 \(\times\) 10\(^4\) cell plaques/ml, plating density 2.5 \(\times\) 10\(^4\) cell plaques/cm\(^2\) filter) floating on 15 ml of culture medium. Cultures were incubated for 5 days at 32°C in 5% CO\(_2\). Thereafter, the monolayers reached confluence and were ready for the measurement of short-circuit current (\(I_{sc}\)). Sample cultures were stained with toluidine blue and the morphological examination was made under light microscopy.

The procedures of primary cultures of rat caput and cauda epididymis have been described previously (9, 39). Briefly, the tissues were dissected out, finely chopped with scissors, and then digested with 0.25% (wt/vol) trypsin followed by 0.1% (wt/vol) collagenase I. Epithelial cells were seeded into the wells of Millipore filter assemblies floating on 15 ml of culture medium. Cultures were incubated for 3 days at 32°C in 5% CO\(_2\).

\(I_{sc}\) measurement. Confluent monolayers were washed three times with Normal Krebs-Henseleit solution to get rid of any sex hormone-binding globulin that may be present in culture medium. They were then clamped between two halves of Ussing chambers with a 0.6-cm\(^2\) window. The tissue was short-circuited by the use of a voltage-clamp amplifier (model DVC 1000; World Precision Instruments, New Haven, CT).

![Fig. 1. Light microscopic photograph of a confluent rat efferent duct epithelial monolayer grown on Millipore filter 5 days in culture. Arrows, cells with large nuclei and stereocilia at the apical border; f, Millipore filter.](http://example.com/fig1.png)

The \(I_{sc}\) was displayed on a pen recorder. Transepithelial resistance was obtained from Ohm's law by clamping the tissue intermittently at a voltage at 0.1 mV displaced from zero. The two channels of the amplifier were mostly used simultaneously on parallel monolayers so that studies could be made under control and experimental conditions. In most situations, monolayers were bathed on both sides with Krebs-Henseleit solution, gassed with 95% O\(_2\)-5% CO\(_2\), and warmed to 32°C.

**Measurement of cAMP.** Efferent duct monolayers from rats were grown on 24-well plates (Costar, Cambridge, MA). After reaching confluence, they were washed twice with Krebs-Henseleit solution and then incubated in 0.5 ml of the same buffer containing isobutyylmethylxanthine (IBMX) (1 mM) for 10 min at 32°C. Forskolin and testosterone were added to the wells and incubated for a further 10 min. The reaction was terminated by adding 10 \(\mu\)l of 60% (wt/vol) perchloric acid to each well. The content of each well was mixed thoroughly and transferred to a 1.5-ml microcentrifuge tube and was then centrifuged at 10,000 \(g\) for 5 s. Supernatant (300 \(\mu\)l) was neutralized by KOH (1 M). The mixture (100 \(\mu\)l) was taken and assayed for cAMP by an immunoassay kit. The principle of the assay is based on the competition between cAMP in the sample and a fixed amount of alkaline phosphatase-labeled cAMP for binding to a specific antibody. The concentration of cAMP in the sample is inversely proportional to the alkaline phosphatase activity.
Solutions. Krebs-Henseleit solution had the following composition (in mM): 117 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄·7H₂O, 2.56 CaCl₂·2H₂O, 24.8 NaHCO₃, and 11.1 glucose. This solution had a pH of 7.4 when bubbled with 95% O₂-5% CO₂. In Cl⁻-free solution, NaCl, KCl, and CaCl₂ were replaced by sodium gluconate, potassium gluconate, and calcium gluconate, respectively. When HCO₃⁻-free solution was used, NaHCO₃ was replaced with NaCl, and the solution was buffered with 10 mM HEPES at pH 7.4, gassed with 100% O₂.

Materials. MEM, fetal bovine serum, and nonessential amino acids were purchased from GIBCO. Penicillin/streptomycin, HBSS, sodium pyruvate, trypsin, collagenase I, forskolin, chlorophenylthio-cAMP, aldosterone, estrogen, cyproterone acetate, flutamide, cycloheximide, actinomycin D, pertussis toxin, IBMX, and indomethacin were from Sigma. Dexamethasone was from Research Biochemicals International. Testosterone and 5α-DHT were from Fluka. Matrigel was purchased from Collaborative Biochemical (Bedford, MA). The immunoassay kit for cAMP measurement was bought from R & B Systems (Minneapolis, MN). Steroids were dissolved in 95% ethanol. It was found that solvent alone did not affect the Iₛₑ.

Statistical analysis. Results are expressed as means ± SE. Comparisons between groups of data were made by the Student's unpaired t-test. P < 0.05 was considered statistically significant.

RESULTS

Formation of polarized epithelia on permeable supports. Light microscopic study showed that efferent duct epithelial cells grown on Millipore filters formed confluent monolayers 5 days in culture (Fig. 1). Epithelial polarity was established with stereocilia appearing on the apical side of the cells.

Basal and forskolin-stimulated Iₛₑ in rat efferent duct epithelia. When bathed in normal Krebs-Henseleit solution, cultured efferent duct epithelia exhibited a transepithelial potential difference of 0.7 ± 0.03 mV (n = 189 epithelia), a basal Iₛₑ of 7.10 ± 0.4 μA/cm² (n = 189), and a transepithelial resistance of 157.4 ± 6.3 Ω·cm² (n = 189), when calculated from transient current changes elicited by intermittent voltage pulses.

Fig. 3. Effect of testosterone on forskolin-induced Iₛₑ. Forskolin (10 μM) was added to the basolateral side (bl) followed by testosterone (0.1, 1, and 10 μM) added sequentially to the apical side (ap). The record is representative of 5 different experiments. Horizontal line indicates zero Iₛₑ. Inset: concentration-inhibition curves for testosterone on forskolin-induced Iₛₑ in rat efferent duct epithelia (○), caput epididymal epithelia (●), and cauda epididymal epithelia (□). Each point shows the mean ± SE of 4 experiments.
Forskolin (10 μM), an adenylate cyclase activator, added to the basolateral side caused a rise in $I_{sc}$ that reached a peak level after 3 min, $I_{sc} = 6.0 ± 0.3 \mu A/cm^2$ ($n = 147$ epithelia). The current then stabilized at a lower level, $I_{sc} = 3.5 ± 0.2 \mu A/cm^2$ ($n = 147$) after about 15 min (Fig. 2). Experiments with ion transport inhibitors were carried out to investigate the ionic basis of the forskolin-induced $I_{sc}$. At the plateau phase of the response, addition to the apical side of amiloride and benzamil, both of which are inhibitors of apical epithelial Na⁺ channels, and phloridzin, an inhibitor of the Na⁺-glucose cotransporter, had no effect on $I_{sc}$. In contrast, diphenylamine-2-carboxylate (DPC), an anion channel blocker, resulted in dose-dependent inhibition of $I_{sc}$ (Fig. 2A). The results suggested that the major part of forskolin-induced $I_{sc}$ was due to anion secretion. Figure 2B shows basolateral addition of bumetanide, an inhibitor of Na⁺-K⁺-2Cl⁻ symport, produced a dose-dependent inhibition of the $I_{sc}$. Addition of acetazolamide, a carbonic anhydrase inhibitor, SITS, an inhibitor of the Cl⁻/HCO₃⁻ exchanger, or amiloride, an inhibitor of the Na⁺/H⁺ exchanger, produced only a negligible inhibition of the $I_{sc}$ when compared with bumetanide.

Effect of steroid hormones on basal and forskolin-induced $I_{sc}$. Different steroid hormones were screened for their effects on anion secretion by the efferent duct epithelial cells. In each case, $I_{sc}$ was stimulated by forskolin (10 μM), which increases intracellular cAMP. At the plateau phase of the response, addition of testosterone to the apical side inhibited $I_{sc}$ within 10–20 s. The inhibition was dose dependent, with the IC₅₀ value at $1 \mu M$ and maximal inhibition at 10 μM testosterone (Fig. 3, inset). In contrast, only 20% of forskolin-induced $I_{sc}$ could be reduced by 10 μM of 5α-DHT (Fig. 4D). Testosterone and 5α-DHT also inhibited the basal $I_{sc}$ by 37% and 11%, respectively (results not shown). Aldosterone, dexamethasone, estrogen, and dehydroisoandrosterone (a weak androgenic steroid) did not affect the basal $I_{sc}$ (results not shown) nor the forskolin-induced $I_{sc}$ (Fig. 4, A–C and E). Basolateral application of testosterone or 5α-DHT had no effect on forskolin-induced $I_{sc}$ (results not shown).

Figure 3, inset, shows the degree of inhibition by testosterone of the forskolin-induced anion secretion was dependent on the regions from which the epithelia were derived. In the efferent duct, the forskolin-induced $I_{sc}$ could be completely abolished by apical application of 10 μM testosterone. However, in epithelia derived from the rat caput or cauda epididymis, the same concentration of testosterone inhibited the forskolin-induced $I_{sc}$ by only 20%.

To elucidate whether the low efficacy of 5α-DHT was due to the rapid metabolism by 3α-hydroxysteroid de-
hydrogenase (3α-HSD), the effect of indomethacin, a potent 3α-HSD inhibitor (17, 29), was studied. As shown in Fig. 5, indomethacin (100 μM) did not affect the inhibitory effect of 5α-DHT.

**Effect of testosterone on forskolin-induced I_{sc} in Cl⁻-free or HCO₃⁻-free solution.** Tissues were bathed in Cl⁻-free solution and stimulated with forskolin. At the steady state of the response, testosterone added apically had no effect on the current (Fig. 6B). However, in the absence of HCO₃⁻, apical addition of testosterone (10 μM) completely inhibited the forskolin-induced I_{sc} (Fig. 6C).

**Effects of testosterone receptor antagonists, protein synthesis inhibitors, and pertussis toxin.** To verify whether the inhibitory effect of testosterone was mediated through the classic testosterone receptors, the effect of anti-androgens were studied. Cyproterone acetate (100 μM), a steroidal anti-androgen, or flutamide, a nonsteroidal anti-androgen (100 μM), did not affect the inhibition of I_{sc} by testosterone (Fig. 7, A and B). Figure 8, A and B, shows that pretreatment for 2 h with actinomycin D (10 μg/ml) and cycloheximide (100 μg/ml), inhibitors of DNA transcription and mRNA translation, respectively, were unable to prevent the inhibitory effect of testosterone. To determine the involvement of G protein in testosterone action, epithelia were pretreated with pertussis toxin (100 ng/ml) for 6 h before addition of testosterone. Figure 9 shows that the concentration-inhibition curve for testosterone was shifted to the right, with the apparent IC₅₀ value shifted from 1 to 40 μM after pretreatment with pertussis toxin.

**Effect of testosterone on intracellular cAMP.** Immunnoassays were performed to study the effect of testosterone on intracellular cAMP level in the efferent duct epithelium. Under basal condition, the intracellular cAMP content was 45.8 ± 17.2 pmol/well (mean ± SE, n = 7). Stimulating the tissues with forskolin (10 μM) led to a rise in intracellular cAMP content to 140.7 ± 11.7 pmol/well (mean ± SE, n = 4) (P < 0.01). Testosterone (10 μM) did not affect the basal intracellular cAMP but significantly inhibited the forskolin-induced rise of cAMP in a concentration-dependent manner (Fig. 10).

**DISCUSSION**

The primary function of the efferent duct in vivo is to reabsorb a major portion of fluid flowing down from the
testis (7). However, previous work in our laboratory has shown that efferent duct epithelial cells are also capable of secreting chloride electrogenically in the presence of humoral agents that increase intracellular cAMP (6, 23). The present studies with forskolin, ion removal (Fig. 6), and transport inhibitors (Fig. 2) confirm these results. Several steroid hormones were investigated for their effects on anion secretion by the efferent duct. Among the steroids tested, only testosterone and 5α-DHT inhibited the basal (results not shown) and forskolin-stimulated $I_{sc}$ (Figs. 3 and 4D).

This inhibitory effect was dose dependent (Fig. 3, inset) and highly specific for testosterone, as dehydroisoandrosterone, a weak androgenic steroid, and other non-androgenic but structurally related steroids could not mimic the effect of testosterone (Fig. 4). Although the effect of testosterone was mostly seen when the agent was added to the apical side of the epithelium, a basolateral action of the hormone could not be excluded.

Testosterone is highly lipophilic, and it is possible that
the Matrigel-coated Millipore filters may have prevented testosterone from reaching the basolateral membranes of the cells. Ion replacement experiments indicated that the fall in $I_{sc}$ after testosterone was due to a decrease in chloride secretion, since the inhibition was prevented by removal of chloride from the bathing solution (Fig. 6).

In the epididymis, testosterone is converted by 5α-reductase to 5α-DHT, which has a greater affinity for the nuclear androgen receptors (14). However, in the present work, 5α-DHT was found to be less effective than testosterone in inhibiting the forskolin-induced anion secretion (Fig. 4D). The inability of indomethacin, a 3α-HSD inhibitor, to augment the inhibition by 5α-DHT (Fig. 5) indicates that the low efficacy of 5α-DHT was not due to rapid metabolism of 5α-DHT by 3α-HSD. It seems that a testosterone-binding site other than the classic nuclear androgen receptor is involved in the inhibition of secretion by testosterone. It is of interest that testosterone has a greater effect on the efferent duct than on the caput and cauda epididymidis (Fig. 3, inset). This regional difference can be ascribed to a higher 5α-reductase activity in the epididymis to metabolize testosterone (32, 33), or alternatively, it could be due to a regional difference in the distribution of the nonclassic testosterone-binding site.

The effect of testosterone occurred within 10–20 s, too rapid to have been attributed to the genomic action of steroid hormones. This notion was supported by the lack of effect of actinomycin D, a DNA transcription inhibitor, or cycloheximide, a mRNA translation inhibitor (Fig. 8). Moreover, the inability of the classic nuclear testosterone receptor antagonists, flutamide and cyproterone acetate, to block the effect of testosterone (Fig. 7) further indicated that testosterone may act on receptors that are distinct from the classic nuclear androgen receptors. Preincubation of the efferent duct epithelium with pertussis toxin, a G$i$ protein inhibitor, attenuated the effect of testosterone (Fig. 9). Lieberherr and Grosse (24) found in osteoblasts the rapid increase of intracellular calcium by androgens was inhibited by pertussis toxin, suggesting that the effects of androgens were mediated through a G$i$ protein-coupled membrane receptor. In the efferent duct, testosterone was found to inhibit the forskolin-induced rise of intracellular cAMP (Fig. 10), prompting speculation that testosterone acted on membrane receptors negatively linked to adenylate cyclase via G$i$ protein, as first proposed by Ravindra and Aronstam (30).

There is strong evidence that sex steroids are of central importance in regulating fluid reabsorption in the efferent duct (15, 19). Using in vivo microperfusion technique, Hansen et al. (15) demonstrated that in the rat, estrogen reduced fluid reabsorption substantially, causing a 2.5-fold increase in fluid flow through the efferent duct. However, when fluid reabsorption was measured in closed segments of the efferent duct isolated from the estrogen receptor knockout mice (ERKO) in vitro, Hess et al. (19) found no fluid reabsorption by the ducts. In addition, in normal mice, the anti-estrogen, ICI-182,780, was found to greatly reduce fluid reabsorption. Further investigations are required to resolve the controversy over the control of fluid reabsorption by estrogen. In our experiments, we did not find any effect of estrogen on chloride secretion by cultured rat efferent duct epithelia (Fig. 4).

Systemic administration of testosterone in the rat has been reported to cause a small increase in fluid reabsorption in the efferent duct (15). This effect could be due to an increase in sodium reabsorption, as the Na$^+$/K$^+$-ATPase activity of the efferent duct is known to be regulated by androgens (21). In the renal proximal tubule, a tissue that resembles the efferent duct, expression of the brush-border Na$^+$/H$^+$ exchanger was found to be increased by testosterone (26). In addition to its effect on sodium reabsorption, we propose that testosterone decreases electrogenic chloride secretion via a rapid nongenomic effect. Little is known about the physiological role of the local effect of testosterone in efferent duct. Vreeburg (38) and Turner et al. (37) reported that the rat seminiferous tubular fluid has a total testosterone concentration of 82 and 62 nmol, respectively, falling short of 100 nM free testosterone required to inhibit chloride secretion in the efferent duct (Figs. 3 and 6A). It is therefore not known whether the effect observed is of physiological relevance. In certain diseases such as Leydig cell tumor, production of testosterone may be greatly increased (10, 35). Most of the men with such tumors have difficulty to father children because of poor semen quality (low sperm count and poor sperm quality) (3). In addition to the impairment of spermatogenesis (20), high testicular testosterone might also inhibit electrolyte transport in the efferent duct, and this could have contributed to infertility in this case. The effect of testosterone on efferent duct electrolyte transport may therefore have a pathological role to play. It would be of interest to speculate a synergism between the genomic and nongenomic actions of testosterone in regulating fluid transport. In the efferent duct, we found testosterone decreases intracellular cAMP, which, in the renal tubule, downregulates the Na$^+$/H$^+$ exchanger (22) known to be regulated by androgens (26). There is increasing evidence that cAMP is able to reduce (11) or enhance (27) the expression of some androgen-dependent genes.

These findings therefore support a possible interaction between the genomic and nongenomic actions of testosterone and open up new areas of investigation into the hormonal regulation of the male reproductive system.

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