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Effects of sex hormones on fluid and solute transport in Madin-Darby canine kidney cells

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Effects of sex hormones on fluid and solute transport in Madin-Darby canine kidney cells. Polycystic kidney disease progresses more rapidly in men than in women. To investigate the basis for this sexual dimorphism, we exposed Madin-Darby canine kidney (MDCK) cells grown on collagen-coated cell culture inserts to control media, or to estradiol or testosterone (1 nM–1 μM). Compared to control and estradiol-treated cells, testosterone stimulated fluid secretion in a dose-dependent manner, enhancing fluid secretion 4.8-fold at 1 nM and 19.7-fold at 1 μM (0.59 ± 0.18 vs. 0.03 ± 0.01 μl/cm^2/hr, $P < 0.001$). Chloride transport paralleled fluid secretion. Testosterone increased cellular cyclic AMP levels 3.2-fold at 1 nM and 12.3-fold at 1 μM (81.3 ± 30.7 vs. 6.6 ± 3.3 pmol/mg protein, $P < 0.001$). GDPβS (500 μM), an inhibitor of Gs, and 2′,3′-dideoxyadenosine (10 μM), an inhibitor of the catalytic subunit of adenylate cyclase, suppressed testosterone-induced fluid and solute secretion. Neither testosterone nor estradiol had any effect on microsomal Na,K-ATPase activity, cellular proliferation or cellular total protein content. Our studies show that testosterone stimulates fluid secretion and solute transport by MDCK cells by increasing cAMP generation. *In vivo*, testosterone may contribute to cyst expansion by enhancing fluid secretion. This observation may help explain the worse prognosis of polycystic kidney disease observed in men.

Gender influences both the renal and extrarenal manifestations of autosomal dominant polycystic kidney disease (ADPKD) [1–3]. Observations in experimental animals and in humans suggest that the progression of renal cystic diseases to end-stage renal failure is markedly influenced by gender [1–5]. Nearly all studies have found that the rate at which renal failure progresses in ADPKD is more rapid in men than in women [1–3]. Sexual dimorphism is also a feature of renal cystic disease in the HAN:SPRD *cy* rat, a model of autosomal dominant renal cystic disease [4]. In this model, castration ameliorates and administration of exogenous testosterone accelerates the course of renal disease, whereas ovariectomy has no effect [5]. Sex hormones may directly influence many of the cellular processes implicated in the development of renal cystic disease including cellular proliferation, matrix accumulation and fluid secretion [6–8]. The present study focused on the effects of sex hormones on fluid secretion by Madin-Darby canine kidney (MDCK) cells grown on collagen-coated cell culture inserts. This system represents a model of fluid secretion by cystic renal epithelia [9].

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Renal volume in ADPKD, which reflects the number and size of renal parenchymal cysts, is correlated with the rapidity of renal functional deterioration [2]. In this context, renal volume is greater in normotensive men than in normotensive women with ADPKD, which parallels the faster rate of progression to end-stage renal failure in men [1–3, 10]. It has been proposed that fluid secretion by renal cysts may be driven by cAMP-stimulated chloride transport, which in turn is dependent on the sodium gradient established by Na,K-ATPase [9, 11–15]. Since testosterone has been shown to stimulate cAMP generation and to enhance Na,K-ATPase activity in numerous cell types [16–22], we tested the hypothesis that sexual dimorphism in ADPKD is related to the ability of testosterone to enhance cyst expansion by stimulating fluid secretion via increased cAMP generation and/or Na,K-ATPase activity. Our studies show that testosterone stimulates fluid secretion and solute transport by MDCK cells by increasing cAMP generation. Thus, testosterone may contribute to cyst expansion in ADPKD by enhancing fluid secretion. This observation may help explain the worse prognosis of polycystic kidney disease observed in men.

Methods

Measurement of fluid secretion by MDCK monolayers

Fluid secretory rate was assessed by measuring the quantity of fluid that accumulated beneath a layer of mineral oil overlying the apical surface of a MDCK cell monolayer [9, 23]. Wild-type MDCK cells (CCL 34; American Type Culture Collection, Rockville, MD, USA) were grown in plastic flasks in DMEM/Ham's FI2 (1:1, vol:vol) media supplemented with 10 mM HEPES, 40 mM $NaHCO_3$, 100 units/ml penicillin G and 5% FBS. Cells were incubated at 37°C in 5% CO_2/air. Cell culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA) coated with type I collagen (rat tail; Sigma Chemical Co., St. Louis, MO, USA) were seeded with 1×10^6 MDCK cells on their upper surface. After six days, media on the upper surface of the inserts was replaced with 1.5 ml hydrated mineral oil; 1.5 cc of media remained in the outer chamber. Three groups of cells were studied in defined media: (a) control media containing ethanol vehicle, (b) media supplemented with estradiol (1 nM–1 μM) and (c) media supplemented with testosterone (T) (1 nM–1 μM). Fluid and oil on the upper surface were aspirated after 48, 72 and 96 hours, and centrifuged at 2,500 rpm. The quantity of fluid secreted was quantified with a calibrated glass microcapillary tube (Drummond Scientific Co., Broomall, PA, USA). Fluid secretory rate was expressed as volume per cm^2 per hour.

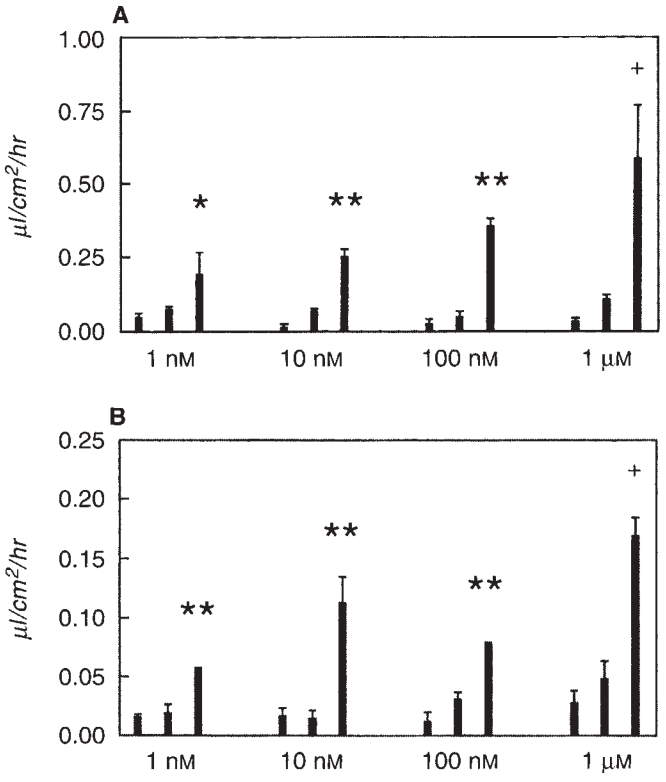

Fig. 1. *Fluid secretion by Madin-Darby canine kidney cells incubated with control media, estradiol or testosterone.* **A.** Values obtained at 48 hours. **B.** Values obtained at 96 hours. Abbreviations are: C, control; E, estradiol; T, testosterone. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{+}P < 0.001$.

Table 1. Composition of secreted fluid

		[Sodium]	[Chloride]
Basolateral solution		172 ± 1	152 ± 1
Control[a]		175 ± 6	158 ± 5
Estradiol[b]		174 ± 5	159 ± 8
Testosterone	1 nM	178 ± 7	152 ± 7
	10 nM	176 ± 6	157 ± 6
	100 nM	177 ± 7	157 ± 10
	1 μM	174 ± 4	147 ± 10

P = NS. Subgroups did differ from one another.
[a] Mean of subgroups 1 nM to 1 μM

Sodium and chloride concentrations were measured in the collected fluid and in aliquots of media in the outer chamber of the wells. Chloride transport was calculated by multiplying the fluid secretory rate by the chloride concentration of the secreted fluid and was expressed as nEq per cm^2 per hour.

Measurements of fluid secretory rate were repeated in the presence and absence of ouabain (10^{-6} M; Sigma), 2,3 dideoxyadenosine (DDA), an inhibitor of the catalytic subunit of adenylate cyclase (10^{-5} M; Sigma), or guanosine 5′-O-(2-thiodiphosphate) (GDPβS), an inhibitor of Gs (500 μM; Boehringer Mannheim, Mannheim, Germany). All agents were added to the outer chamber of the wells.

We also assessed the effects of estradiol and testosterone on cellular proliferation and total protein content in cultured MDCK cells. Cellular proliferation was measured by ^{3}H thymidine incorporation by techniques previously described in detail by our laboratory [6]. Cellular protein was measured by colorimetric assay (Bio-Rad Protein Assay, Richmond, CA, USA).

Measurement of cellular cAMP

To measure cellular cAMP levels, MDCK cells were incubated for 72 hours at 37°C in 5% CO_2/air with control media, estradiol-supplemented media (1 nM, 1 μM), or T-supplemented media (1 nM, 1 μM). The cells were pelleted by centrifugation and cAMP extracted with 80% methanol [24]. After evaporation to dryness, 0.4 ml of 0.05 M sodium acetate, pH 6.2 was added. cAMP was measured with a radioimmunoassay kit (New England Nuclear, Boston, MA, USA) and cellular protein was measured by colorimetric assay (Bio-Rad).

Measurement of Na,K-ATPase activity

MDCK cells were incubated for 72 hours at 37°C in 5% CO_2/air with control media, estradiol-supplemented media (1 nM–1 μM), or T-supplemented media (1 nM–1 μM). Na,K-ATPase activity was then measured in microsomal fractions [25, 26]. Microsomal fractions were prepared by scraping cells into 1 ml PBS at 4°C. The cells were centrifuged at 800 rpm at 4°C and the pellet resuspended in buffer A (5 mM Tris-HCl, pH 7.4, 0.25 M sucrose) with 0.2 mM $MgSO_4$ at 4°C. The cells were mechanically homogenized and centrifuged at 3,000 rpm for five minutes at 4°C. The homogenization procedure was repeated and the microsomal pellet resuspended in buffer A. Reactions were performed in duplicate in the presence and absence of ouabain (2.5 mM). Microsomal fractions were incubated with 100 mM NaCl, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM Tris-ATP, 1 mM Tris (cyclohexylammonium) phosphoenolpyruvate, 30 mM imidazole-HCl (pH 7.3), 0.15 mM NADH, 50 μg/ml LDH and 30 μg/ml pyruvate kinase. ATPase activity was calculated from the rate of change in OD measured at 340 nm at 37°C and is expressed as fmol NADH oxidized/gram protein/hour. The difference between activity in the absence and presence of ouabain represents specific Na,K-ATPase activity.

Results

Testosterone stimulated fluid secretion by MDCK cell monolayers at every concentration tested during the first 48 hours of exposure (Fig. 1A). At a concentration of 1 nM, testosterone increased fluid secretion 4.8-fold compared to untreated cells (0.19 ± 0.08 vs. 0.04 ± 0.02 μl/cm^2/hr, $P < 0.05$, ANOVA with Scheffe's correction). At a concentration of 1 μM, testosterone increased fluid secretion 19.7-fold compared to untreated cells (0.59 ± 0.18 vs. 0.03 ± 0.01 μl/cm^2/hr, $P < 0.001$). At a concentration of 1 nM, the increase in fluid secretory rate elicited by testosterone reached 24.1% of the maximum increase elicited by forskolin (10 μM). At a concentration of 1 μM, the increase in fluid secretory rate elicited by testosterone achieved 68.7% of the maximum increase elicited by forskolin (10 μM).

Chloride and sodium concentrations were not significantly higher in secreted fluid from control, estradiol-, and testosterone-treated monolayers than in the basolateral bathing solution (Table 1). However, transepithelial chloride transport and fluid secretion rose in parallel (Fig. 2A). At a concentration of 1 nM, testosterone increased chloride transport 4.3-fold compared to untreated cells (6.58 ± 0.51 vs. 28.67 ± 5.62 nEq/cm^2/hr, $P <$

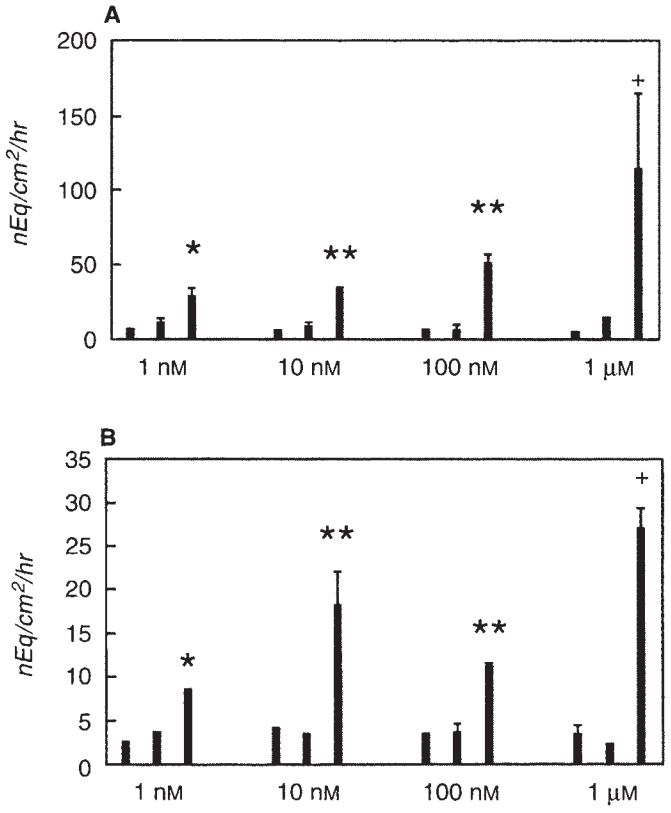

Fig. 2. *Chloride transport by Madin-Darby canine kidney cells incubated with control media, estradiol or testosterone.* **A.** Values obtained at 48 hours. **B.** Values obtained at 96 hours. Abbreviations are: C, control; E, estradiol; T, testosterone. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{+}P < 0.001$.

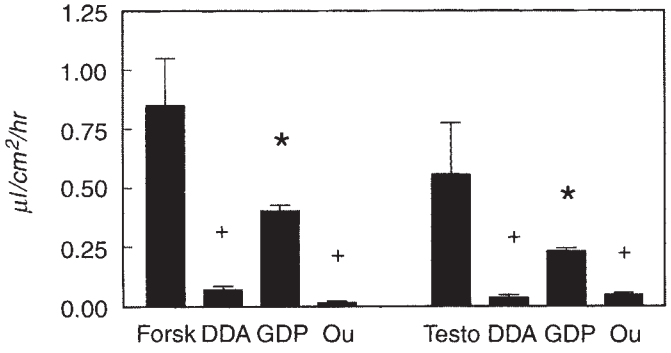

Fig. 3. *Fluid secretion by Madin-Darby canine kidney cells incubated with the agents indicated.* Abbreviations are: DDA, 2,3 dideoxyadenosine; GDPβS, guanosine 5′-O-(2-thiodiphosphate); Ouab, ouabain. $^{*}P < 0.05$, $^{+}P < 0.001$.

0.05). At a concentration of 1 μM, testosterone increased chloride transport 22-fold compared to untreated cells (5.19 ± 0.10 vs. 114.22 ± 51.04 nEq/cm²/hr, $P < 0.001$). Sodium transport paralleled chloride transport (data not shown).

Fluid secretion and chloride transport were lower in all three experimental groups at 72 and 96 hours compared to the values obtained at 48 hours. Nevertheless, the stimulatory effects of testosterone on fluid secretion and chloride transport were sustained (Figs. 1B and 2B). Estradiol had no significant effect on fluid secretion or chloride and sodium transport at any concentration or time period studied (Figs. 1 and 2).

The stimulatory effects of forskolin and testosterone on fluid secretion by MDCK cells were not additive [forskolin (10 μM) 0.85 ± 0.20 μl/cm²/hr; testosterone (1 μM) 0.56 ± 0.22 μl/cm³/hr; forskolin + testosterone 0.93 ± 0.26 μl/cm²/hr, P = NS]. The inhibitor profiles of forskolin and testosterone are shown in Figure 3. DDA reversed the increase in fluid secretion elicited by forskolin (0.07 ± 0.01 vs. 0.85 ± 0.20 μl/cm²/hr, $P < 0.001$) and by testosterone (0.04 ± 0.01 vs. 0.56 ± 0.22 μl/cm³/hr, $P < 0.001$). GDPβS was less effective in suppressing the increase in fluid secretion elicited by forskolin (0.40 ± 0.02 vs. 0.85 ± 0.20 μl/cm³/hr, $P < 0.05$) and by testosterone (0.23 ± 0.01 vs. 0.56 ± 0.22 μl/cm³/hr, $P < 0.05$). Ouabain completely reversed forskolin-stimulated and testosterone-stimulated fluid secretion (Fig. 3).

Testosterone increased cellular cyclic AMP levels 3.2-fold at a testosterone concentration of 1 nM (25.31 ± 8.02 vs. 8.02 ± 4.03 pmol/mg protein, $P < 0.05$) and 12.3-fold at a testosterone concentration of 1 μM (81.34 ± 30.73 vs. 6.61 ± 3.32 pmol/mg, $P < 0.001$). Microsomal membrane Na,K-ATPase activity was unaffected by exposure to sex hormones at concentrations ranging from 1 nM–1 μM (Fig. 4). Neither estradiol nor testosterone had any effect on cellular protein content or on cellular proliferation in MDCK cells (Fig. 5).

Discussion

Gender influences both the renal and extrarenal manifestations of autosomal dominant polycystic kidney disease [1–3]. Nearly all studies have found that the rate at which renal failure progresses is more rapid in affected men than in women [1–3]. The Modification of Diet in Renal Disease study recently showed that in patients with ADPKD and a GFR above 25 ml/min, women had a slower rate of decline in renal function than did men [3]. Accordingly, men require maintenance hemodialysis on average 5.5 years earlier than do women [1]. Although the prevalence of hypertension is greater in affected men, male gender and hypertension each independently contribute to the more aggressive renal course observed in men with ADPKD [2].

Sexual dimorphism is also a feature of renal cystic disease in the HAN:SPRD *cy* rat [4, 5]. Heterozygous males develop renal cysts and progressive azotemia leading to death in uremia at approximately six months of age [5]. In contrast, severe azotemia is absent and renal cystic transformation is more slowly progressive in female heterozygotes [4]. Castration reduces renal enlargement and the degree of cystic changes in male heterozygotes while exogenous testosterone is renotropic and worsens BUN in female heterozygotes [5]. In contrast, ovariectomy was found to have no effect on the course of renal disease [5].

Gender may also influence the development of acquired renal cystic disease in patients with end-stage renal disease [27, 28]. Although not a universal observation, most studies have demonstrated a preponderance of males with this disorder [28]. Renal cyst size and the extent of cyst formation is nearly twice as great in men on hemodialysis compared to women, despite a similar duration of end-stage renal disease [27]. Despite reduced circulating levels of testosterone in men with end-stage renal failure, testosterone may play a role in stimulating cystic transformation in patients with acquired renal cystic disease.

The C57BL/6J *cpk:cpk* murine model of autosomal recessive renal cystic disease does not show sexual dimorphism [29]. Homozygous *cpk* mice rapidly progress to uremia over several

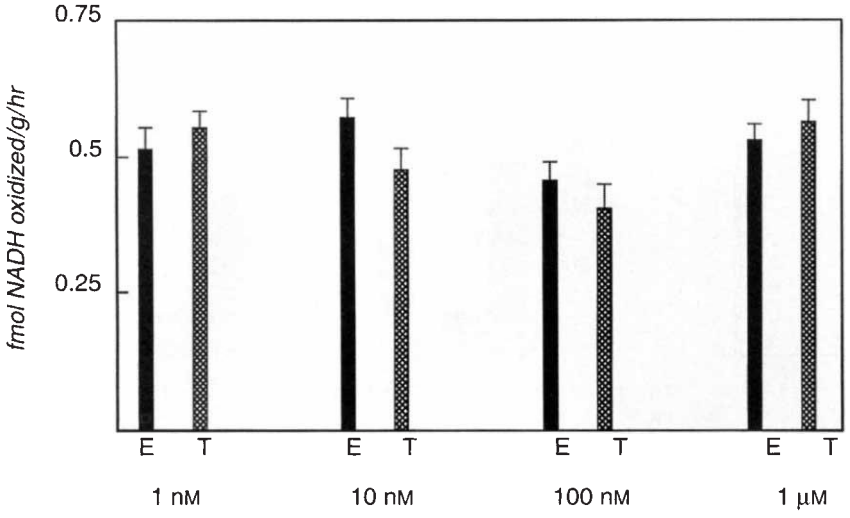

Fig. 4. *Na,K-ATPase activity in microsomal membranes of estradiol and testosterone-treated Madin-Darby canine kidney cells.* Abbreviations are: E, estradiol; T, testosterone.

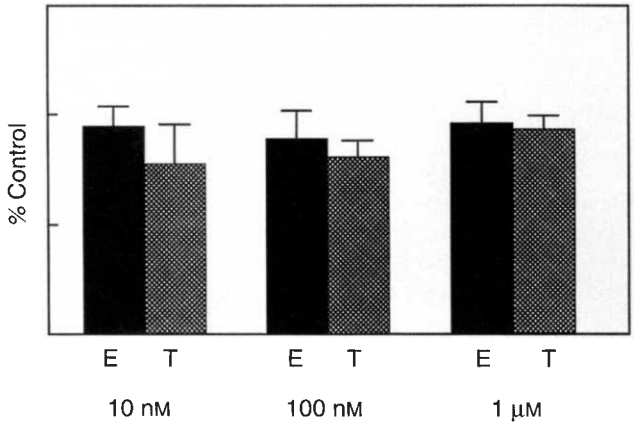

Fig. 5. *Proliferation of Madin-Darby canine kidney cells incubated with estradiol or testosterone expressed as a percent of untreated, control cells.* Abbreviations are: E, estradiol; T, testosterone.

weeks [29]. In contrast, renal disease in heterozygous Han:SPRD *cy* rats progresses more slowly and does not lead to uremia in males until six months of age [4, 5]. The genetically programmed aggressive course of the renal cystic enlargement in *cpk* mice may overshadow any effects of testosterone on disease progression and explain the failure of gender to influence the course. DBA/2FG *pcy:pcy* mice develop a slowly progressive form of autosomal recessive renal cystic disease. Female *pcy* mice develop uremia and die sooner than males despite a similar rate of renal enlargement [30]. The adverse effect of female gender in *pcy* mice contrasts with the ameliorative effect of female gender observed in most experimental models of progressive renal injury [8] and may reflect inherited sex-linked factors unique to this model.

In various *in vitro* models of renal cystic epithelia, cyclic AMP agonists play an integral role in the induction and/or enlargement of renal cysts [11, 13–15]. Although MDCK cysts absorb fluid in the absence of secretagogues, they secrete chloride-rich fluid in response to cAMP [14]. In MDCK cells grown in collagen gel matrix, cAMP agonists stimulate fluid secretion and promote cyst formation [11]. cAMP-stimulated fluid secretion by MDCK monolayers is suppressed by inhibitors of the apical chloride channel and by inhibitors of basolateral Na,K-2Cl cotransport [11, 15]. On the basis of these observations, it has been suggested that fluid secretion in cystic epithelia may be driven by cAMP-stimulated chloride transport [15]. In this context, testosterone has been shown to stimulate adenylate cyclase activity in all cell types and tissues studied to date, including adipose tissue, hippocampal slices, striatal neurons and rat hepatocytes [16–19]. In rat adipose tissue, testosterone increases adenylate cyclase activity by activating the catalytic subunit [18]. In hamster epididymal adipose tissue, testosterone increases adenylate cyclase activity via increased Gs density as well as via direct stimulatory effects on the catalytic subunit [16].

Na,K-ATPase has also been shown to play an important role in cyst formation [14, 25, 31–33]. In this context, testosterone has been shown to stimulate Na,K-ATPase in numerous tissues and cell types [20–22]. Na,K-ATPase activity is increased in testosterone-treated cultured brain astrocytes and in tissue isolated from the liver or from the hypothalamic and preoptic regions of the brain of male rats [20–22].

Renal volume in ADPKD, which reflects the number and size of renal parenchymal cysts, is correlated with the rapidity of renal functional deterioration [2]. Renal volume is greater in normotensive men than in normotensive women with ADPKD (390 ± 43 vs. 338 ± 24 cm^3, statistical comparison not reported), which parallels the faster rate of progression to end-stage renal failure in men [1–3, 10]. Sex hormones may influence renal cyst volume by modulating fluid secretion by cystic epithelia. Since testosterone has been shown to stimulate cAMP generation and Na,K-ATPase activity in numerous cell types, we tested the hypothesis that sexual dimorphism in ADPKD is due in part to the ability of testosterone to stimulate fluid secretion by cystic epithelia and that this stimulatory effect is mediated by enhanced cAMP generation and/or Na,K-ATPase activity.

Our studies demonstrate that testosterone stimulates fluid secretion and solute transport by MDCK cells by increasing cAMP generation. We failed to find any effect of sex hormones on microsomal Na,K-ATPase activity. Our inhibitor studies suggest that testosterone increases cellular cAMP levels by directly stimulating the catalytic subunit of adenylate cyclase and via activation of Gs. Forskolin stimulates cAMP production in two ways: by directly activating the catalytic subunit of adenylate cyclase and by activating Gs [34, 35]. Approximately one third of the ability of

forskolin to stimulate cAMP production is mediated via activation of Gs [34, 35]. These conclusions are consistent with our observation that DDA, an inhibitor of the catalytic subunit of adenylate cyclase, almost completely reverses forskolin-stimulated fluid secretion whereas GDPβS, an inhibitor of Gs activation, causes only a partial reversal. An identical inhibitor profile was observed with testosterone. Moreover, the effects of forskolin and testosterone were not additive. These data suggest that forskolin and testosterone increase cAMP by similar mechanisms involving both direct stimulation of the catalytic subunit of adenylate cyclase as well as activation of Gs. Basolateral ouabain also reversed testosterone-stimulated solute transport and fluid secretion. These observations are consistent with the conclusion that testosterone-stimulated fluid secretion by MDCK cells is driven by cAMP-stimulated chloride transport which in turn is dependent on the sodium gradient established by Na,K-ATPase.

Our data suggest that in ADPKD, testosterone may contribute to cyst expansion by enhancing fluid secretion. This observation may help explain the worse prognosis of polycystic kidney disease observed in men.

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