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ANTI-MASCULINIZING ACTION OF ESTRADIOL AND CYPROTERONE ACETATE: REGULATION OF A PROTEIN FRACTION WITH PHOSPHOLIPASE-A_2 STIMULATORY AND MASCULINIZING ACTIVITIES

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Summary—Recently we have identified a protein fraction (55–63 K) from male and testosterone-exposed female mouse genital tract, which stimulates phospholipase A_2 (PLA_2) and induces masculine differentiation in an undifferentiated mouse genital explant, suggesting a role of this protein in the action of testosterone. In the current study we have further investigated the role of this protein by determining whether anti-masculinizing agents, namely, estradiol and cyproterone acetate, have any effect on the production of this protein. The results described here indicate that a protein fraction containing PLA_2 stimulatory activity was present in both control male and estradiol- or cyproterone acetate-exposed male fetal genital tract. However the specific activity of the PLA_2-stimulatory protein was significantly higher in the control males than in the experimental males. We did not find any major difference in the behavior of this protein fraction in various chromatographic steps except that in CM–sepharose column; the PLA_2-stimulatory activity from the male preparation was eluted in two overlapping peaks with 0.3 and 0.25 M NaCl and that from the treated males was eluted only with 0.25 M NaCl. The SDS–gel analysis of this protein fraction revealed a doublet band (55 and 63 K) in control samples and primarily a 63 K band in experimental samples. The protein fraction from all these sources showed a significant difference in their biological activity. The control male preparation induced Wolffian duct whereas the estradiol sample was completely ineffective and the cyproterone acetate sample was partially effective in inducing Wolffian duct. Thus, it appears that the protein fraction has a role in the masculinizing action of testosterone.

INTRODUCTION

It has been known for a long time that masculine differentiation of genitalia is determined by the functioning embryonic testis or by testicular hormones, namely testosterone and Mullerian inhibiting hormones [1]. Testosterone is responsible for the stabilization of the Wolffian duct leading to the formation of epididymis and seminal vesicle and for masculinizing the urogenital sinus to form prostate and external genitalia whereas Mullerian inhibiting factor causes regression of Mullerian duct. Agents that interfere with the synthesis [2, 3], circulation [4] or the action of testosterone [5, 6] block masculine differentiation as determined by the production of hypospadias (displacement of the urethral orifice down the shaft of the penis with a shortening of the anogenital distance) and regression of internal genital system, such as prostate, Wolffian duct and epididymis.

The mechanism by which testosterone organizes this differentiation is not clear at present. It is known that testosterone is required to bind its receptor to produce this function [5, 6]. However subsequent steps following this function remain to be determined. Recently we identified a protein fraction of approx. 55–63 K molecular weight from the developing male genital tract, which has both phospholipase-A_2 stimulating and masculinizing activity [7]. The protein fraction stimulates both bee venom and genital PLA_2 in a dose-dependent manner and it stabilizes and stimulates the Wolffian duct system of the undifferentiated reproductive tract of male fetuses in the absence of testosterone in organ culture system. In order to analyze further the masculinizing role of this protein fraction we analyzed two situations where testosterone-induced masculinization was inhib-

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ited by two agents namely, estradiol [3] and cyproterone acetate [6]. Estradiol administered to pregnant rats at the critical period of fetal sexual differentiation inhibits the appearance of masculinized genital ducts, epididymis, prostate and masculine external genital structures [8]. It was shown that estradiol inhibits adrenal and testicular synthesis of testosterone and interferes with sexual differentiation of fetal rats [9]. Cyproterone acetate also inhibits the fetal sexual differentiation and prevents the appearance of male structures as described for estradiol, however it works by preventing the binding of testosterone to its receptor [6]. We report that estradiol and cyproterone acetate inhibit the PLA_2-stimulatory activity of the 55–63 K protein fraction and that the protein fraction isolated from these two sources produces partial or no masculine activity when tested *in vitro*.

MATERIALS AND METHODS

Animal

CD-1 females from Charles River breeding laboratory (Wilmington, Mass) were bred in our mouse colony. Pregnant mice were injected with cyproterone acetate (20 mg/kg/day) and estradiol (2 mg/kg/day) from day 13 to day 17 of gestation. At day 18, the genital tracts without testes from male fetuses were isolated in saline and kept frozen at −70°C until processing. Approximately 15 genital tracts (60 mg) were combined and homogenized in 0.2 ml 1% Nonidet P-40 in Minimum Essential Medium (MEM, Gibco, Grand Island, N.Y.) using 10-s pulses ($N = 3.5$) from a sonicator (Braun-Sonic 2000; generating 170 W) while keeping the tissue in an ice-water bath. The homogenate was centrifuged at 1000 ***g*** for 10 min and the supernatant was used for further purification.

Purification of PLA_2-stimulatory protein

The tissue-supernatant was purified as described previously [7]. Briefly, the tissue extract was first fractionated by a Bio-Rad P-100 gel filtration column (1 × 30 cm) using 0.05 M Tris buffer, pH 9.0, containing 1.5 mM EDTA as eluting buffer. The fractions containing PLA_2-stimulatory activity were combined and fractionated further by a DEAE–cellulose (Whatman, Clifton, N.J.) column (0.5 × 15) using a 0–1.5 M NaCl gradient in 0.05 M Tris–EDTA buffer, pH 9.0. The PLA_2-stimulatory protein was finally purified by a CM–sepharose (Pharmacia) column (0.05 × 15 cm) using 0–1.0 M NaCl in 0.05 M Tris–EDTA buffer, pH 9.0. The final preparation was concentrated by dialyzing against 2 M sucrose and salts were removed by dialysis against H_2O. One tenth to 1/20th of the dialyzed concentrated protein fraction was used for determining protein content [10], PLA_2-stimulatory activity and for protein profile by SDS-gel analysis. The remainder of the fraction was used in organ culture assay.

Electrophoresis

Approximately 2–3 μg of protein fraction were dissolved in sample buffer (0.125 Tris, pH 6.8, 10% SDS, 50% sucrose and 0.01% bromophenol blue). Samples were denatured at 100°C for 5 min and electrophoresed according to the method of Laemmli [11] through either 7.5% or 5–12.5% gradient SDS–polyacrylamide gel topped with a 3% stacking gel. The gels were fixed and stained with Coomassie blue.

PLA_2 assay

The assay was performed as described previously [7].

Assay of phospholipase A_2-stimulatory protein

The assay was done as described previously [7]. The assay consists of two parts. First, the hydrolysis of 2 nmol [^{14}C]phosphatidylcholin (L-3-phosphatidycholine-1-stearoyl-2-[1-^{14}C]-arachidonoyl SA 52.9 mCi/nmol) (Amersham) with different concentrations of bee venom of PLA_2 (Sigma) was studied to determine the concentrations of PLA_2 that produce a linear increase in [^{14}C]phosphatidylcholine hydrolysis. Next an enzyme concentration, usually 0.005 units of PLA_2, was chosen for assaying PLA_2-stimulatory activity. Ten to 100 μl fractions were tested for their PLA_2-stimulatory activity of 0.005 units of PLA_2. Controls were made using no protein fraction and no enzyme. The increase in phosphatidylcholine hydrolysis over that produced by 0.005 units PLA_2 was presented as units of phospholipase A_2-stimulatory protein based on the standard that the amount of phosphatidylcholine hydrolysed by 1 unit of the protein fraction equals the amount hydrolyzed by 1 unit of PLA_2.

Organ culture

The organ culture was performed as described previously [12]. To test the PLA_2 stimulatory

protein fraction isolated from different sources, the dialyzed and concentrated protein fraction was redialyzed against water and then against MEM containing no glutamine. The protein fraction was then mixed with 1/10th volume of 10 × MEM containing appropriate amount of $NaHCO_3$ (to bring it up to neutral pH), glutamine, essential amino acids, vitamin mixture to obtain the final concentration of normal organ culture medium. Each batch of protein fraction was brought to 6 ml with normal culture medium [96 ml MEM with Earle's salt (Gibco), supplemented with 1 ml each of MEM essential amino acid (100 ×), 1 ml L-glutamine (100 ×), vitamin mixture (100 ×), pyruvate solution (100 ×), 0.5 ml Penstrep solution (100 ×) and 10 ml of fetal bovine serum]. The mixture was adjusted to the final 10% fetal bovine serum by adding an additional amount of serum. On day 5 of the culture, the explants were fixed in Bouin's solution and processed histologically as described earlier [7].

Statistics

Student's *t*-test was applied to evaluate the PLA_2-stimulatory protein content of male, female, estradiol and cyproterone acetate-exposed males.

RESULTS

Identification of PLA_2-stimulatory protein

Estradiol and cyproterone acetate, as expected, produced shorter anogenital distance and disrupted the formation of prostate, seminal vesicle and Wolffian duct (estradiol only) and epididymis (data not shown). PLA_2-stimulatory activity was found in all three different samples (control, estradiol-exposed and cyproterone acetate-exposed male genital tract) at every step of purification. Fractionation of PLA_2-stimulatory activity from all these sources by P-100 gel filtration and DEAE–cellulose revealed no significant difference in the elution pattern of different samples. In the P-100 column, the PLA_2-stimulatory activity from all these sources was eluted in fractions right after the void volume (around 55–63 K fractions, data not shown). The fractions containing PLA_2-stimulatory activity bound DEAE–cellulose with low affinity, and 0.03 M NaCl was able to elute the activity from the column (data not shown). The chromatographic profile of PLA_2-stimulating activity of various samples on the CM–sepharose column is shown in Fig. 1. Again very little difference in their elution pattern was noticed except that the control samples

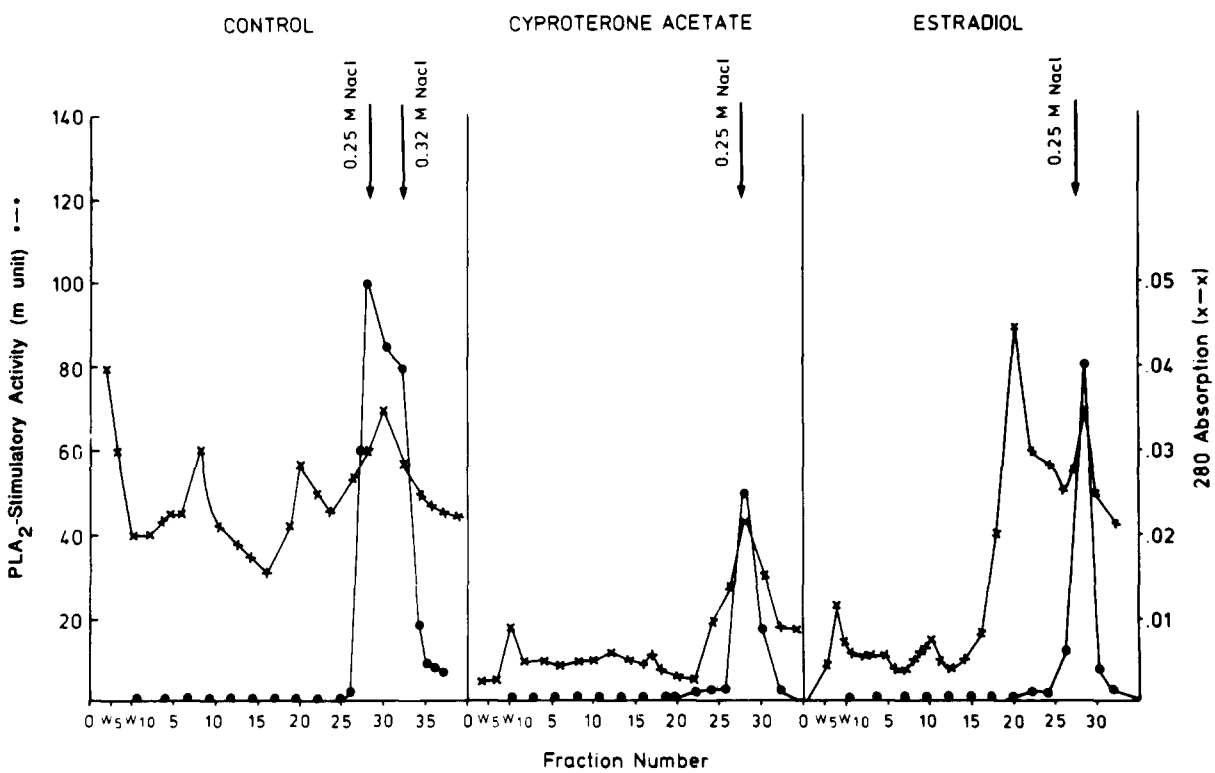

Fig. 1. CM–sepharose chromatography of PLA_2-stimulatory fraction from control, cyproterone acetate- and estradiol-exposed male genital tracts.

produced two PLA_2-stimulatory peaks, one next to the other, eluting with 0.25 and 0.3 M NaCl whereas estradiol- and cyproterone acetate-exposed samples produced only one symmetrical PLA_2-stimulatory peak eluting with 0.25 M NaCl.

PAGE–electrophoretic pattern of PLA_2-stimulatory fraction

Figure 2 and 3 show the protein profile of the partially purified PLA_2-stimulatory fraction (CM–sepharose fraction) isolated from control, estradiol- and cyproterone acetate-exposed male genital tract. Control samples produced a doublet of protein bands of mol. wt 55–63 K whereas the estradiol (Fig. 3) and cyproterone acetate (Fig. 3) samples produced a protein band of mol. wt close to that of the upper band of the doublet present in the control samples. In the experimental samples the lower band is either barely visible or not visible at all. No other bands were visible even with a sensitive method of staining, namely silver staining. In the presence of β-mercaptoethanol, the 63 K band appeared with a second band at around 43 K instead of 55 K in control samples whereas the experimental samples showed no such 43 K band with the exclusion of β-mercaptoethanol (data not shown). In the absence of SDS the protein bands of both control and experimental samples disappeared from the gel but the standards remained visible inside the gel.

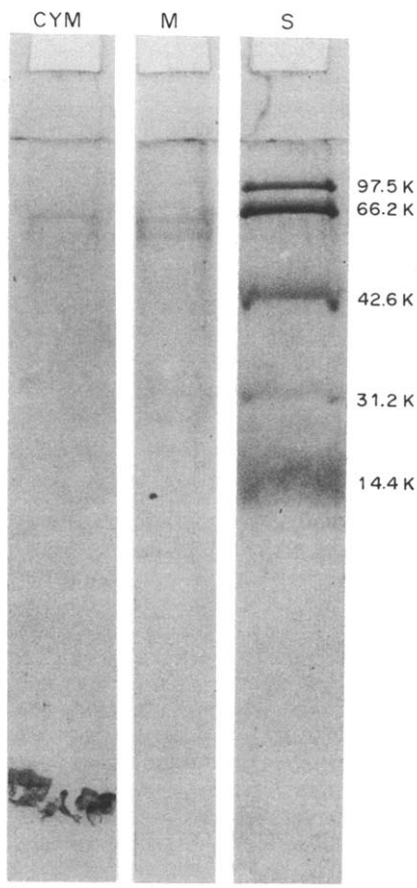

Fig. 2. SDS–gel (12%) electrophoresis of a cyproterone acetate-exposed PLA_2-stimulatory fraction from a CM–sepharose column. CYPM, cyproterone acetate-treated male; M, control male.

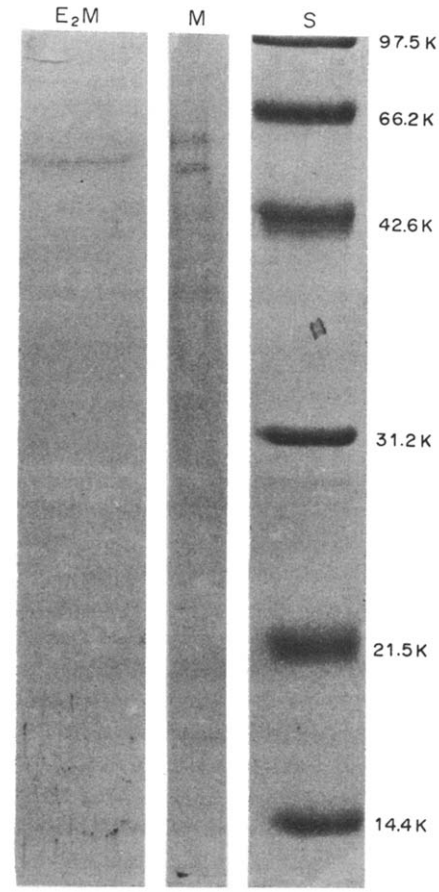

Fig. 3. SDS–gel (5–12% gradient) electrophoresis of an estradiol-exposed PLA_2-stimulatory fraction from a CM–sepharose column. E_2M, estradiol-exposed male; M, control male.

PLA_2-stimulatory activity

The PLA_2-stimulatory activity of various samples was measured at the last step of purification (CM–sepharose chromatography). The results as shown in Table 1 indicate that the specific activity of PLA_2-stimulatory CM–sepharose preparation was significantly higher in males than in estradiol- and cyproterone acetate-exposed males. Estradiol and cyproterone acetate produced 2–3-fold reduction in PLA_2-stimulatory activity of this preparation. The reduction of PLA_2-stimulatory

Table 1. Effect of estradiol and cyproterone acetate on PLA_2-stimulatory activity of the male fetal genital tract protein fraction (CM–Sepharose fraction)

Treatment	Number of observation	PLA_2-stimulatory activity (mU/mg protein)	
Vehicle	5	4469 ± 583	
Estradiol	4	1302 ± 1207	$P = 0.0020$
Cyproterone	4	1774 ± 702	$P = 0.0005$

Data present mean ± SD. The results were compared with vehicle-treated control samples using Student's t-test.

activity was evident even when calculated based on the total protein content of the genital tract, i.e. per genital tract. Estradiol and cyproterone acetate reduced total protein content (10–15%), but this was too small to account for 2–3-fold reduction in PLA_2-stimulatory activity per mg of protein.

Masculine activity

The masculine activity of various PLA_2-stimulatory preparations isolated from control (5 samples), estradiol- (4 samples) and cyproterone acetate- (3 samples) treated males was assessed by determining their effect on the development and stimulation of Wolffian duct of male explants in the absence of testes and the results are shown in Fig. 4. Control male explant, as expected, was composed of ducts with proliferative epithelial layer (Mullerian duct) containing columnar cells [Fig. 4(A)]. Wolffian duct is not visible in most of the specimens except in a few spots (see the area enclosing "W") where the regressing duct appeared. The PLA_2-stimulatory fraction from control males stabilized the Wolffian duct (see proliferated lumen "W") while the Mullerian duct (see "M" area) was present [Fig. 4(B)]. On the contrary, the PLA_2-stimulatory fraction from estradiol-exposed males was unable to stabilize or stimulate the Wolffian duct in the specimens indicating its absence [Fig. 4(C)]. Mullerian ducts were visible in all instances. The PLA_2-stimulatory fraction from cyproterone acetate-exposed males, on the other hand, partially prevented the appearance of Wolffian duct [Fig. 4(D)]. Mullerian duct was visible through-

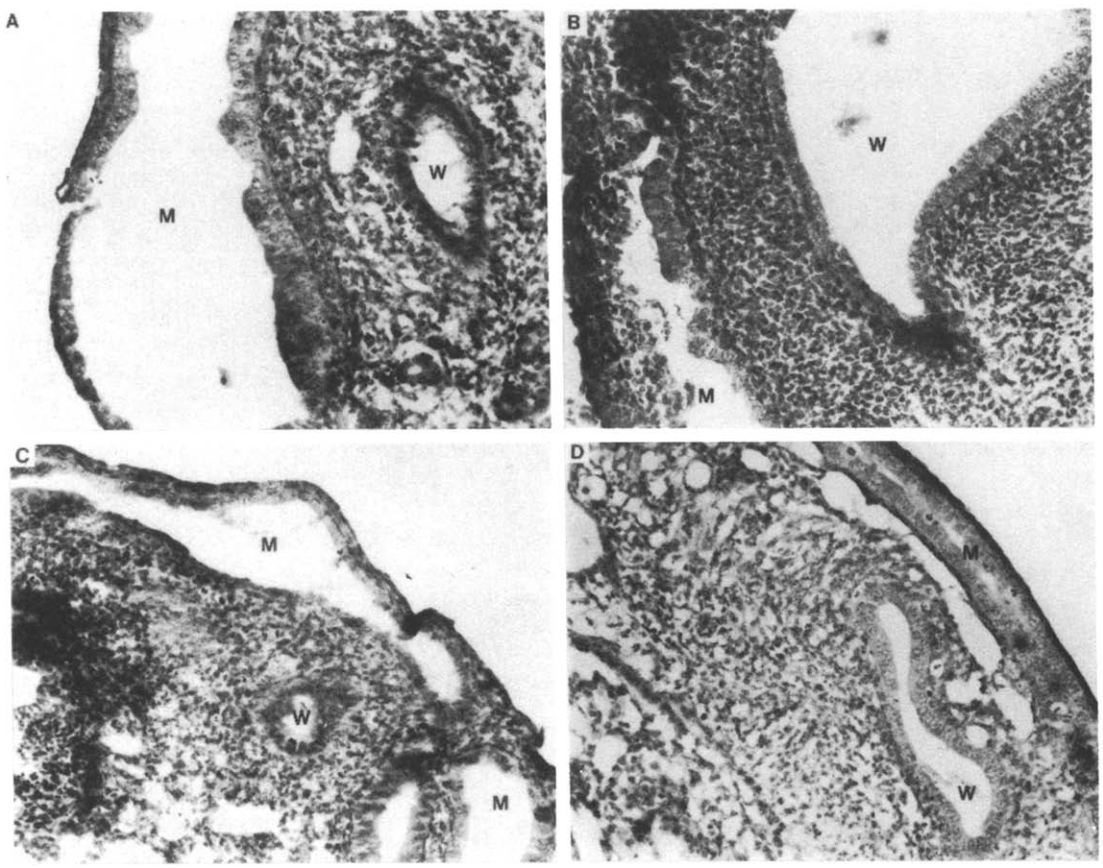

Fig. 4. Organ culture assay of masculinizing activity of PLA_2-stimulatory fractions from different sources. (A) No PLA_2-stimulatory fraction; (B) PLA_2-stimulatory fraction from control male; (C) PLA_2-stimulatory fraction from estradiol male; (D) PLA_2-stimulatory fraction from cyproterone acetate male. Thirteen-day old male fetal genital tracts containing no testes were used in the organ culture bioassay of masculinization. M, Mullerian duct; W, Wolffian duct. Magnification 250×.

out the entire specimen, but the Wolffian duct was found only in the mesonephric or anterior segment (See "W" area). The PLA_2-stimulatory fraction from estradiol-exposed males also produced some degeneration throughout the specimens. However, this did not affect the development of the ductal system as demonstrated by the appearance of a reasonably healthy Mullerian duct [Fig. 4(C)].

DISCUSSION

We have shown previously that fetal testicular testosterone induces the level of a PLA_2-stimulatory protein fraction in mouse genital tract, which can mimic the masculinizing action of fetal testosterone *in vitro* [7]. In order to understand further the role of this protein in masculine differentiation, we investigated the effect of two compounds with anti-masculinizing effects on the production of this protein fraction. The results described here show that estradiol [3] and cyproterone acetate [6], the anti-masculinizing agents, inhibit the PLA_2-stimulatory activity of this protein fraction and secondly, the protein fraction isolated from the estradiol- or cyproterone acetate-exposed males has reduced masculinizing activity compared to that from control males. The protein fraction isolated from estradiol-exposed males completely lacks the ability to masculinize a male genital tract in the absence of testosterone and the protein fraction from cyproterone acetate-exposed males has a low level of masculinizing activity.

Although there is a significant difference in the masculinizing action of various PLA_2-stimulatory fractions isolated from different sources (control vs estradiol- and cyproterone acetate-exposed males) the protein profiles of these preparations, as indicated by different protein purification procedures and by SDS gel electrophoresis, were not vastly different. The PLA_2-stimulatory protein from male genital tract was eluted in two peaks with a small difference in NaCl (0.25 and 0.3 M) whereas that from estradiol- and cyproterone acetate-exposed males was eluted only with 0.2 M NaCl. Gel electrophoresis revealed the presence of a doublet-band in the control sample whereas in the experimental samples the intensity of the second band was either greatly reduced or the second band was entirely absent. It is not known whether these small differences can account for their differences in masculine activity.

It appears from the results presented in Table 1 and Fig. 4, that masculinizing activity of a genital preparation is closely related to its PLA_2-stimulatory activity. However, it is not clear at present whether these two activities reside on the same protein or on different proteins. Although electrophoretic analysis of genital preparations from control and experimental samples indicates the role of 55 K protein in both of the activities of this preparation, the possibility that the activities are associated with different proteins of mol. wt close to 55 K or of entirely different mol. wt cannot be excluded until the 55 K protein is better characterized.

The presence of a small amount of masculinizing activity in cyproterone acetate-exposed samples, as demonstrated by the induction of a small segment of Wolffian duct, was not surprising. It has been shown previously that although cyproterone acetate acts at the level of androgen-receptor, it is not effective in preventing testosterone-dependent Wolffian duct development in mouse [13]. However, it prevents other androgen-dependent developments such as prostate and other urogenital systems [13]. Thus, Wolffian duct specific information, present in the genital tract and required for its development, appears not to be destroyed completely by cyproterone acetate and this may explain the partial maintenance of the Wolffian duct by the protein fraction isolated from cyproterone acetate-exposed males. Estradiol, on the other hand, prevents the appearance of entire male tract [8] and thus, as expected, the protein fraction isolated from estradiol-exposed males completely prevented the development of the Wolffian system.

The results described here and reported earlier [7] suggest that PLA_2-stimulatory activity of the protein fraction plays a role in masculinizing the fetal genital tract. However, it is not clear what role is played by the PLA_2-stimulatory activity. One possibility exists that the PLA_2-stimulatory protein activates genital PLA_2 resulting in increased PGE_2 synthesis and this, in turn, induces masculinization. The results reported previously [12, 14–16] support such a possibility, as we found that the PLA_2-stimulatory protein was indeed able to stimulate genital PLA_2 [7] and that PGE_2 was able to masculinize a female genital tract [12]. PGs are known to be implicated in the regulation of cell proliferation [17], differentiation [18] and organogenesis of several systems [19–22].

Kosher and Walker [19] reported a stimulatory effect of PGE_2 on chick limb bud chondrogenesis, while Chepenik *et al.* [20] reported inhibition of chondrogenesis in limb cell cultures treated with inhibitors of PGs, implicating these lipids in the regulation of cartilage differentiation. PGs have also been implicated in the differentiation of palatal tissues [21] and neural crest cells [22]. We showed that inhibitors of PG synthesis, namely aspirin [23], indomethacin [24] and cortisol [25], during the critical period of sexual differentiation, inhibited masculine organization of external genitalia [14]. Recently we demonstrated [12] that PGE_2 is able to masculinize not only the external genitalia but also internal genitalia of genetic females and androgen receptor deficient and estradiol-exposed genetic males. In that case, question remains about the role of the PLA_2-stimulatory activity of the feminized genital tract and how it differs from the male PLA_2-stimulatory activity. Is this protein structurally different from the PLA_2-stimulatory protein present in the masculine tract? Do they differ in their cellular localization resulting in the difference in actions? Further work is necessary to elucidate the role of this protein in masculine differentiation.

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