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Activation of Phospholipases during Masculine Differentiation of Embryonic Genitalia (42766)

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Abstract. We have investigated whether the androgen-induced masculine differentiation of the sex organs involves an induction of phospholipases. We have measured phosphatidylinositol-specific phospholipase C and phosphatidylcholine-specific phospholipase A_2 in the reproductive tract of male and female mouse (CD-I) fetuses at the 18th day of gestation. We report here that (1) the activity of these two enzymes is higher in the male genitalia than in the female genitalia; (2) exogenous testosterone at the 13th to 17th day of pregnancy induces both phospholipase A_2 and phospholipase C in the female fetal genitalia; and (3) prenatal administration of cyproterone acetate, an antiandrogen, known to produce feminized males, completely prevents the stimulation of phospholipase A_2 and C by testosterone in the female fetuses. In the male fetuses, however, cyproterone acetate inhibits the PLC activity but is unable to alter phospholipase A_2 activity. These findings provide evidence that the mechanism by which testosterone organizes the genitalia may involve a modification of phospholipases A_2 and C.

Since 1940, it has been known that masculine differentiation of genitalia is determined by the functioning embryonic testis, or by testosterone secreted by the testis; otherwise, the inborn female program is expressed (1). Agents that interfere with the synthesis, circulation, or the action of testosterone block masculine differentiation of the genitalia of genetically male embryos, as manifested by the production of hypospadias (2, 3). Hypospadias results from a displacement of the urethral orifice down the shaft of the penis from the tip with a shortening of the anogenital distance (2). The means by which testosterone organizes masculine differentiation of the genitalia in the embryo depend on classical androgen receptor mechanisms (3). However, the precise steps involved in this organizing action of testosterone in the embryo are not known. Recently, we have reported (4) that arachidonic acid administered during embryonic development masculinized the external genitalia of gonadal females as well as androgen-deprived genetic males (cyproterone acetate (5) and estradiol-17β-exposed males (6)). Moreover, the agents that block the arachidonic acid cascade (7–9) at the level of phospholipase A_2 (cortisone), or at the level of cyclooxygenase (aspirin and indomethacin), inhibited masculine differentiation. Thus, it appears that the mechanism by which testosterone masculinizes the external genitalia involves the arachidonic acid cascade leading to prostaglandin synthesis.

There are two major pathways for arachidonic acid release (10–13): (1) the induction of phospholipase A_2 (PLA_2), which hydrolyzes membrane-bound phosphatidylcholine and liberates free fatty acids such as arachidonic acid (10, 11); and (2) the induction of phospholipase C (PLC) followed by the action of diacylglycerol lipase (12, 13). PLC hydrolyzes phosphatidylinositol resulting in the production of diacylglycerol, which is subsequently converted into fatty acids by the action of a second enzyme, namely, lipase. Testosterone may activate either or both of the pathways to produce an increased release of arachidonic acid during masculine differentiation of embryonic genitalia. We have tested this hypothesis by examining (1) whether PLA_2 or PLC activity of embryonic genitalia is higher in the male than in female genitalia, (2) whether exogenous testosterone which is known to masculinize genetic females (2) stimulates the phospholipase(s) during embryonic differentiation, and lastly, (3) whether cyproterone acetate, known to feminize male fetuses by blocking the action of androgen at the level of receptor binding

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(5, 14), inhibits the phospholipase(s) during embryonic differentiation. The results described here show that testosterone modifies both PLA_2 and PLC in female embryonic genitalia, and cyproterone acetate completely prevents this modification of enzyme activity.

Materials and Methods. *Animal.* CD-1 females from the Charles River were bred in our mouse colony. Pregnant mice were injected subcutaneously with testosterone (10 mg/kg) with or without cyproterone acetate (20 mg/kg) in 50–100 μl of 10% ethanol and 90% corn oil during the 13th to 17th day of pregnancy. Controls received vehicle only. The fetuses were removed by caesarean section on Day 18 of gestation and whole genital tracts without the gonads along with the tissues surrounding the external genitalia were dissected out by means of a dissecting microscope. The sex was determined by inspection of the gonads. Approximately 40–50 mg of tissue from 8 to 10 fetuses of each sex was used to prepare the enzyme for one assay.

Assay of phospholipase A_2 (PLA_2). The tissue homogenate (1:10) in 0.1 *M* Tris–HCl, pH 9.0, was centrifuged for 15 min at 3600*g* and the resulting supernatant was frozen at −80°C until used for PLA_2 assay, described previously (15, 16). Protein was determined by the method of Lowry *et al.* (17) using bovine serum albumin as a standard.

The assay system for PLA_2 consisted of 0.1 *M* Tris–HCl, pH 9.0, 6 m*M* $CaCl_2$, and 2 nmole of [^{14}C]phosphatidylcholine (L-3-phosphatidylcholine 1-stearoyl-2-[1-^{14}C] arachidonyl, sp act 52.9 mCi/mmole, Amersham) in a total volume of 300 μl. The radio-labeled substrate was evaporated under N_2 and the residue was dissolved in water, with the aid of a sonicator-ice bath. An aliquot of this mixture was counted to assure completeness of suspension. The incubation at 37°C was started by adding 20 μl (2 nmole) of the substrate and carried on for 10 min unless specified. The activity was measured by the release of ^{14}C fatty acid from phosphatidylcholine. Free fatty acid was separated from phosphatidylcholine (18) and an aliquot of the fatty acid layer was counted in a scintillation counter.

Assay of phospholipase C (PLC). The enzyme for the PLC assay was prepared as described by Rittenhouse (19). The tissue was homogenized (1:10) in 60 m*M* Tris acetate buffer, pH 6.5, containing 2 m*M* EDTA. The homogenate was then spun at 105,000*g* for 60 min at 4°C. The supernatant was removed carefully so that the upper fatty layer (if any) was excluded. The supernatant was kept at −80°C until ready for assay.

A simple and rapid assay for the detection of phosphatidylinositol-specific PLC was used. The final concentrations of assay components were as follows: 200 μ*M* [^{3}H]-phosphatidylinositol (Amersham, approximately 0.02 μCi); 5 m*M* $CaCl_2$; 0.6 mg deoxycholate–Na-salt and 25 to 100 μg supernatant protein in a total volume of 0.3 ml of 60 m*M* Tris–HCl buffer, pH 6.5. The chloroform solution of radiolabeled phosphatidylinositol (PTI) was evaporated under N_2. The residue was dissolved in H_2O at −4°C using a sonicator. The final suspension should be opalescent and aliquots of this suspension were counted to confirm the completeness of suspension. The reactions were started by adding the substrate and continued up to 30 min. At the end of incubation, the reactions were terminated by the addition of 1.5 ml of ice-cold chloroform–*n*-butanol–concd HCl (10:10:0.06) and 0.45 ml of 1 *M* HCl. The mixtures were vortexed and then spun at room temperature at 1000 rpm for 5 min and 250 μl of the upper aqueous phase was transferred to scintillation vials for counting. The activity of the PLC was estimated from the radioactivity released in the aqueous layer and the results were expressed as pmole of radioactive phosphatidylinositol hydrolyzed/μg protein.

Results. *Characterization of PLA_2 and PLC in fetal genitalia.* Since no information is available regarding the phospholipase activity in fetal genitalia, we first examined the male and female genital tracts for the presence of these enzymes and standardized the assays in terms of protein concentration and time of incubation. Figures 1A and 1B show that hydrolysis of phospholipids (PTC for PLA_2 and PTI for PLC) increased with time of incubation. The PLC reaction was linear for 10 min at a protein concentration of 25

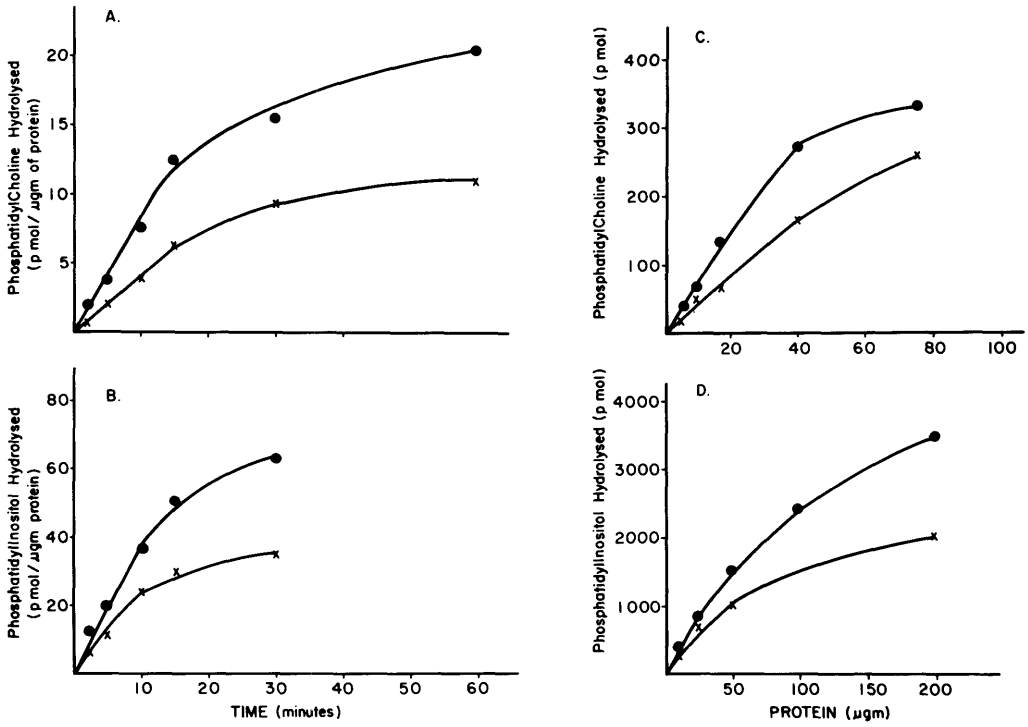

FIG. 1. PLA_2 and PLC activity of fetal genitalia as functions of time of incubation and added protein concentration. PLA_2: Figs. A and C; and PLC: Figs. B and D. (●) Male genitalia, and (×) female genitalia. The data represent a mean from three determinations.

µg/0.3 ml whereas the PLA_2 reaction was linear for 15 min at a protein concentration of 40 µg/0.3 ml. Both PLC and PLA_2 activities were higher in the male reproductive tract than that in the female reproductive tract at all times.

Figures 1C and 1D show the effect of the enzyme protein concentration on the hydrolysis of the PLA_2-specific phosphatidylcholine and PLC-specific phosphatidylinositol. The incubations were carried out for 10 min. The hydrolysis of PTC progressed linearly up to 40 µg of protein/0.3 ml incubation in both sexes, whereas the hydrolysis of PI was linear up to 25 µg of protein/0.3 ml incubation. Again the enzyme activity was much higher in the male genitalia than in the female genitalia at all protein concentrations tested here.

Effect of testosterone and cyproterone acetate on PLA_2. Using an enzyme concentration in the linear range (<40 µg/0.3 ml) and using 10 min for time at incubation, we next examined whether testosterone induced PLA_2 in the fetal genital tract and whether an anti-androgen, cyproterone acetate blocked the stimulation. Table I shows that testosterone significantly increased (more than double) the PLA_2 activity in the fetal female genitalia. The activity, however, was not altered by testosterone in the fetal male reproductive tract. When cyproterone acetate was coadministered with testosterone, the induction of PLA_2 by testosterone in the female was blocked. Cyproterone acetate, however, did not alter the PLA_2 activity of male genitalia.

Effect of testosterone and cyproterone acetate on phospholipase C. PLC was assayed in various groups using a protein concentration of less than 25 µg/0.3 ml and using 15 min of incubation time. The results in Table II show that testosterone administration during the prenatal period significantly induced PLC activity in the females only. Testosterone had no effect on the PLC activity of the male

TABLE I. EFFECT OF TESTOSTERONE AND CYPROTERONE ACETATE ON PHOSPHOLIPASE A_2 ACTIVITY

Treatment	Dosage (mg/kg)	Phospholipase A_2 (pmole/μg protein) Male	Female
Vehicle	—	6.92 ± 0.90	4.00 ± 0.62[a]
Testosterone	10	7.37 ± 1.38	8.35 ± 1.67[b]
Testosterone plus cyproterone acetate	10 20	6.24 ± 1.12	5.23 ± 1.16[c]
Cyproterone acetate	20	7.26 ± 0.74	4.73 ± 0.87

Note. Data represent means ± SD, $n = 5$.

[a] $P < 0.002$, vehicle-treated males vs vehicle-treated females by Student's t test.

[b] $P < 0.005$, vehicle-treated females vs testosterone-treated females.

[c] $P < 0.01$, testosterone-treated females vs testosterone plus cyproterone acetate-treated females.

genital tract. Cyproterone acetate coadministered with testosterone completely prevented the induction of PLC by testosterone in the females. Further it decreased the PLC activity in the males to a value of an untreated female. Cyproterone acetate alone also inhibited the enzyme in male genitalia and had no effect on the enzyme activity of female genitalia.

Discussion. This study demonstrates that prenatal testosterone, the organizer of male genitalia (1), appears to regulate phospholipases A_2 and C in fetal genitalia. The activities of these two enzymes are not only higher in the fetal male tract than in the female tract, but are also induced by exogenous testosterone in female embryonic genitalia. In an earlier study (4), we have suggested that an induction of arachidonic acid leading to PG synthesis mediates testosterone-induced masculine differentiation of fetal external genitalia. The present findings provide further evidence in support of this hypothesis as the activities of phospholipase A_2 and phospholipase C, the major mediators of arachidonic acid release, appear to be altered by fetal androgen. We have tested this hypothesis further using an anti-androgen, namely, cyproterone acetate which has been shown to prevent the action of testosterone at the level of receptor binding leading to the formation of feminine external genitalia (5, 14). We have found that cyproterone acetate completely blocked the testosterone-induced PLC and PLA_2 activity in the female genitalia. Thus, the induction of the phospholipase activity by testosterone correlates well with the masculinizing effects of testosterone on fetal genitalia.

Although we have shown here that prenatal testosterone (testicular or exogenous) increases the phospholipase activity in the genital tract, we have no information regarding the biochemical mechanism of the enzyme induction by testosterone. Testosterone may alter the enzymes by affecting directly the structures of the enzymes or by the synthesis of these enzymes. Alternatively, testosterone may alter some other biochemical reactions which consequently lead to the induction of phospholipase activity. Further studies are needed to explore these possibilities.

Arachidonic acid, after its release from the phospholipid pool, is quickly metabolized into various biologically active products, namely, prostaglandins, thromboxanes, and leukotrienes (20, 21). It remains to be deter-

TABLE II. EFFECT OF TESTOSTERONE AND CYPROTERONE ACETATE ON PHOSPHOLIPASE C

Treatment	Dosage (mg/kg)	Phospholipase C (pmole/μg protein) Male	Female
Vehicle	—	35.06 ± 3.13	23.95 ± 3.63[a]
Testosterone	10	38.57 ± 2.08	32.73 ± 2.75[b]
Testosterone plus cyproterone acetate	10 20	19.06 ± 1.92[d]	19.06 ± 0.49[c]
Cyproterone acetate	20	21.07 ± 1.7[e]	22.03 ± 1.52

Note. Data represent means ± SD, $n = 4$.

[a] $P < 0.02$, vehicle-treated males vs vehicle-treated females by Student's t test.

[b] $P < 0.03$, testosterone-treated females vs vehicle-treated females.

[c] $P < 0.005$, testosterone treated-females vs testosterone plus cyproterone acetate-treated females.

[d] $P < 0.005$, testosterone plus cyproterone acetate treated-males vs vehicle-treated males.

[e] $P < 0.005$, cyproterone acetate-treated males vs vehicle-treated males.

mined whether these products mediate the masculine action of testosterone. Our earlier works suggest the importance of the arachidonic acid prostaglandin pathway in this process. We have shown that prenatal injection of two inhibitors of cyclooxygenase and indomethacin produced males with feminine external genitalia, suggesting a role of prostaglandins in this process. Other possibilities, however, cannot be ruled out since aspirin and indomethacin have other effects in addition to inhibiting cyclooxygenase. Experiments are in progress to elucidate the specific role of prostaglandins in this process.

It is interesting to note that cyproterone acetate did not inhibit PLA_2 in the male genitalia. However, it prevented the rise in PLA_2 by testosterone in female. Exogenous testosterone at the critical period of differentiation is known to produce masculine genital structures in genetic females (22). Thus the present results suggest that the effect of cyproterone acetate on PLA_2 varies, depending on the source of testosterone (exogenous vs endogenous). Alternatively, it may be that the time course of endogenous secretion of testosterone in males is not well correlated with the time course of exogenously administered cyproterone acetate concentration. At the present time, we have no explanation for the variable effects.

The breakdown of phosphatidylinositol, an important phospholipid, is thought to play a key role in the mechanism of growth stimulation induced by a wide variety of hormones, growth factors, and other mitogenic agents (23–25). It is believed that these agents first bind to and activate their plasma membrane receptor (26), and the activated receptor then stimulates a PI-specific PLC, which degrades the phospholipid to inositol phosphates and 1,2-diacylglycerol (27). The activation of the PI cycle is thought to stimulate calcium entry into the cell and/or an increase in prostaglandin synthesis (28). Thus, testosterone-induced growth stimulation and organization may involve an activation of PI cycle leading to an induction of second messengers. Further experiments are necessary to determine the specificity of second messenger systems mediating androgen-induced masculine differentiation.

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