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In Vitro Effect of Testosterone and cAMP on Cholesterol Synthesis in Rat Ventral Prostate (40822)

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Testosterone is well known to induce changes in RNA, DNA, and protein synthesis in the rat ventral prostate (1–3). Administration of testosterone to castrated rats was also shown to result in large increases in cholesterol synthesis in the ventral prostate (4). *In vitro* studies also revealed that testosterone significantly increased cAMP formation in the prostate tissues of the mouse (5). Cyclic adenosine 3′,5′-monophosphate (cAMP) produces testosterone-like induction of hexokinase, phosphofructokinase, pyruvate kinase, and glucose 6-phosphate dehydrogenase in the seminal vesicles of castrated rats (6). This effect is enhanced by the simultaneous administration of theophylline, an inhibitor of the phosphodiestrase that is known to inactivate cAMP. Likewise *in vivo* studies showed a significant increase in the prostate weight and comparable increases in the level of glycolytic enzymes when testosterone or cAMP and theophylline were administered parenterally to castrated rats (7). The rate of cholesterol synthesis was shown to decrease when cAMP was added to liver slices or to homogenates (8, 9). In contrast, an increase of *in vitro* cholesterol synthesis in the rat uterus was found when high concentrations of cAMP were added (10).

This paper reports our findings from *in vitro* studies involving the effect of testosterone on cholesterol, protein, RNA, and DNA synthesis in the ventral prostate of castrated rats in the presence of the metabolic inhibitors, actinomycin D, and cycloheximide. The results of other studies on the *in vitro* effect of cAMP on cholesterol, protein, and RNA synthesis in the ventral prostate of castrated rats in the presence of theophylline, actinomycin D, and cycloheximide are also reported.

Materials and methods. Animals. Groups of six adult male Wistar rats (275–325 g) were maintained on Purina rat chow and water *ad libitum* and were kept under an alternating 12-hr light and 12-hr dark schedule. The rats were castrated, and 7 days thereafter they were anaesthetized with intraperitoneal injections of sodium barbital and sacrificed by exsanguination. The two ventral prostate lobes were excised free of their fatty covering. The tissues were minced and weighed immediately in tared Teflon test tubes and kept in ice until further use. The tissues of the prostate lobes were used to study the incorporation of radioactive precursors into cholesterol, protein, RNA, and DNA.

Tissue incubation conditions. The minced ventral prostate tissues were preincubated in 2 ml of Hank's balanced salt solution (HBSS, pregassed with 95% O_2 and 5% CO_2) at 37°C for 0.5 hr on a constant speed shaker. After this period either 145 μM of testosterone propionate or 1 mM N^6, $O^{2'}$-dibutyryladenosine-3′-5′-cyclic phosphate (cAMP) were added to respective tubes. No additions were made to control tubes. The tubes were again incubated at 37°C for 2 hr. At the end of this period the medium was decanted from each tube, and 2 ml of fresh HBSS was added with the appropriate supplement of 145 μM testosterone propionate or 1 mM N^6,$O^{2'}$-dibutyryl cAMP.

In order to study the effect of theophylline on cAMP two additional experimental groups were prepared. As described above supplements of 1 mM theophylline alone and 1 mM N^6, $O^{2'}$-dibutyryl-cAMP with 1 mM theophylline were added to HBSS in these groups.

To study the effect of the metabolic inhibitors, actinomycin D, and cycloheximide on the testosterone- or cAMP-mediated ef-

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fect on cholesterol synthesis in prostate tissue, additional experimental groups were prepared. Tissues in HBSS were preincubated with 8 μM actinomycin D or 1 mM cycloheximide at 37°C for 0.5 hr before the testosterone or cAMP was added to the medium. Proper actinomycin D and cycloheximide controls were also prepared. Thereafter, the same incubation procedure as described above was followed.

In vitro incorporation of radioactive precursors into cholesterol, protein, DNA, and RNA by minced prostate tissues. For the determination of the rate of incorporation of biochemical precursors into cholesterol, protein, DNA, and RNA on a tissue weight basis (rate of synthesis) the tubes containing incubating prostate tissue in each experimental group then received either 1 μCi of [2-^{14}C]acetate (sp act 50.3 mCi/mmole), 1 μCi of [1,2-^{14}C]acetate (sp act 50.0 mCi/mmole), 1 μCi of L-[4,5-^{3}H]leucine (sp act 5 Ci/mmole), 1 μCi of [5-^{3}H]uridine (sp act 7 Ci/mmole) or 2 μCi of [5-*methyl*-^{3}H]-thymidine (sp act 6.7 Ci/mmole), respectively. After the addition of precursor the tubes were further incubated at 37°C for 2 hr before the reaction was terminated by instant freezing of the tubes in a dry ice–acetone bath.

Analysis of radioactivity in cholesterol, protein, DNA, and RNA. The radioactivity in cholesterol, protein, and DNA was determined by procedures described elsewhere (4). The radioactivity in RNA was determined in the following manner: The thawed frozen tissues were homogenized with a Brinkman polytron. To the homogenates at 0°C were added 3 ml 10% aqueous trichloracetic acid (TCA). The resultant precipitates were washed twice with 5-ml aliquots of cold 6% TCA. The precipitates were then repeatedly washed with 95% ethanol–chloroform (3:1, v/v) in order to remove lipids. The extracted precipitates containing protein, DNA, and RNA were then dissolved in 2 ml 0.3 N KOH at 37°C for 24 hr. A 0.1-ml aliquot of each was counted (count I). To the remaining solutions 8 ml 6% TCA was added and the resultant precipitates were then extracted with 1 ml 16% perchloric acid (PCA) at 70°C for 20 min. A 0.1-ml aliquot of the PCA extracts containing DNA was counted for radioactivity (count II). The total radioactivity (count I) minus the radioactivity found in the PCA extract (count II) gave the radioactivity in RNA, assuming that there was no radioactive label diluted to protein from labeled uridine.

Results. The data presented in Table I clearly reveals that the *in vitro* synthesis of cholesterol, protein, and RNA in the ventral prostates of castrated rats increased by 85, 75, and 92%, respectively, after 4 hr of incubation with testosterone. There was no significant increase in DNA synthesis within the same period of incubation with testosterone in these studies. The results of further *in vitro* studies dealing with the effect of the metabolic inhibitors, actinomycin D, and cycloheximide, on the testosterone-dependent stimulation of cholesterol synthesis in the ventral prostate of castrated rats are also presented in Table I. Actinomycin D at the concentration of 4 μM/ml and cycloheximide at the concentration of 0.5 mM/ml completely inhibited the testosterone-dependent increase in cholesterol, protein, and RNA synthesis in the ventral prostate of castrated rats within 4 hr of incubation with testosterone. Actinomycin D, the known inhibitor of RNA synthesis, in the presence of testosterone reduced the rates of RNA synthesis by 70 and 42% of that exhibited by the controls with (group II) and without (group I) testosterone, respectively. Likewise, cycloheximide, the known inhibitor of protein synthesis, in the presence of testosterone reduced the rates of protein synthesis by 77 and 59% of that exhibited by the controls with (group II) and without (group I) testosterone, respectively. At the same time, actinomycin D and cycloheximide, in the presence of testosterone, reduced the cholesterol synthesis by 49 and 56%, respectively, of that exhibited by controls with testosterone (group II). However, actinomycin D or cycloheximide in these experiments produced no significant changes in DNA synthesis.

The results of *in vitro* studies dealing with

TABLE I. *In Vitro* EFFECT OF ACTINOMYCIN D AND CYCLOHEXIMIDE ON THE TESTOSTERONE STIMULATION OF CHOLESTEROL, PROTEIN, RNA, AND DNA SYNTHESIS IN VENTRAL PROSTATE OF CASTRATED RATS[a]

Group		Rate of synthesis (cpm/g tissue) × 10^4			
		Prostate cholesterol	Prostate protein	Prostate RNA	Prostate DNA
I	Control	5.78 ± 1.27	51.3 ± 7.0	35.1 ± 4.5	3.63 ± 0.57
II	+ Testosterone	10.7 ± 1.3	89.9 ± 9.4	67.3 ± 9.4	4.23 ± 0.92
III	+ Testosterone + Actinomycin D	5.55 ± 1.61	49.0 ± 6.2	20.2 ± 3.2	3.71 ± 1.32
IV	+ Testosterone + Cycheximide	4.67 ± 0.86	21.1 ± 4.9	40.7 ± 7.2	2.89 ± 0.39

[a] All the values are obtained from groups of six rats and expressed as mean ± SE. The radioactive precursors, 1,2-^{14}C]acetate, L-[4,5-^{3}H]leucine, [5-^{3}H]uridine, and [5-*methyl*-^{3}H]thymidine, were employed, respectively, for the cholesterol, protein, RNA, and DNA incorporation studies.

the effect of N^6, $O^{2'}$-dibutyryl-cAMP on the rates of cholesterol, protein, and RNA synthesis in the ventral prostate of castrated rats are given in Table II. The results of the effects of the metabolic inhibitors, actinomycin D, cycloheximide, and theophylline on these cAMP dependent syntheses in the prostate of castrated rats are also shown in Table II. When actinomycin D at 4 μM/ml was added to prostate tissues of castrated rats, RNA, protein, and cholesterol syntheses were reduced by 28, 33, and 41%, respectively. The addition of cycloheximide at 0.5 mM/ml reduced the syntheses of protein and cholesterol by 86 and 59%, respectively. Although there was a slight reduction in the synthesis of RNA, this change could not be considered as significant. As compared to controls the cAMP derivative at a concentration of 0.5 mM/ml increased RNA and protein synthesis by 31 and 29%, respectively, whereas it decreased cholesterol synthesis by 73%. The addition of theophylline at 0.5 mM/ml to the cAMP group further increased RNA and protein synthesis to 36 and 43% of control values, respectively, whereas cholesterol synthesis further declined to 78% of that of the controls. Theophylline itself exhibited no sig-

TABLE II. EFFECT OF N^6, $O^{2'}$-DIBUTYRYL cAMP, ACTINOMYCIN D, CYCLOHEXIMIDE AND THEOPHYLLINE ON THE RATE OF SYNTHESIS OF PROSTATE CHOLESTEROL, PROTEIN, AND RNA IN CASTRATED RATS[a]

Group		Rate of synthesis (cpm/g tissue) × 10^4		
		Prostate cholesterol	Prostate protein	Prostate RNA
I	Control	16.3 ± 2.1	32.7 ± 4.2	20.6 ± 1.6
II	+ Actinomycin D	9.61 ± 0.48	22.0 ± 3.5	14.8 ± 0.9
III	+ Cycloheximide	6.66 ± 0.35	4.46 ± 0.57	17.8 ± 1.6
IV	+ Theophylline	18.8 ± 1.2	35.2 ± 3.4	21.4 ± 2.3
V	+ cAMP	4.48 ± 0.38	42.2 ± 1.6	26.9 ± 2.2
VI	+ cAMP + actinomycin D	6.28 ± 0.43	24.1 ± 3.0	16.5 ± 1.1
VII	+ cAMP + cycloheximide	10.2 ± 1.8	4.95 ± 0.69	22.6 ± 1.6
VIII	+ cAMP + theophylline	3.62 ± 0.31	46.9 ± 2.0	28.1 ± 2.0

[a] All the values are obtained from groups of six rats and expressed as mean ± SE. The radioactive precursors, [2-^{14}C]acetate, L-[4,5-^{3}H]leucine, and [5-^{3}H]uridine, were employed, respectively, for the cholesterol, protein, and RNA incorporation studies.

nificant effect on cholesterol, protein or RNA synthesis. When actinomycin D was added to the cAMP group at 4 μM/ml, both protein and RNA synthesis were decreased by 43 and 39% of the cAMP control (group V), respectively, whereas cholesterol synthesis was increased by 40% of the cAMP control. When cycloheximide at 0.5 mM/ml was added to the cAMP group both protein and RNA syntheses declined by 88 and 16% of the cAMP control values, respectively. Cholesterol synthesis increased most significantly by 128% of cAMP control levels.

Discussion. It is well recognized today that the condition and function of the prostate gland is dependent on testicular function and in particular, the production of the male androgenic hormone, testosterone. Castration, resulting in a decline in serum testosterone levels, is immediately associated with a decline in prostate weight and function. In particular, the glandular epithelial cells rather than the stroma shrink and atrophy with the decline in testosterone levels. Likewise RNA, DNA, and protein synthesis are also adversely affected.

In our earlier *in vivo* studies (4) it was also clearly shown that testosterone is of major importance in the synthesis of cholesterol by the prostate gland. In the current studies the revelation of the effective stimulation of an *in vitro* system by testosterone is certainly of fundamental importance. Here the rate of cholesterol synthesis in minced ventral prostate tissues obtained from rats 7 days after castration increased significantly after 4 hr from the addition of testosterone to this *in vitro* system. In parallel with this increase of cholesterol synthesis there was also the expected increase in protein and RNA synthesis. However, within this short interval after the addition of testosterone there was no net increase in DNA synthesis, indicating that the early increase in cholesterol synthesis is independent of DNA synthesis.

Histopathological examination of the rat ventral prostate tissues used in these studies and taken 7 days after castration clearly revealed the expected reduction and atrophy of the epithelial cells associated with the decline in prostate weight. Likewise a histopathological examination of the tissues only 4 hr after the addition of testosterone did not reveal any significant change in the epithelial cell structure.

Actinomycin D, which binds to DNA blocking the DNA-directed synthesis of nuclear RNA (11) and cycloheximide, which inhibits protein synthesis by blocking the transfer of amino acids from tRNA to protein (12), inhibited the testosterone-stimulated synthesis of cholesterol. Since these inhibitors of transcription and translation completely inhibited the testosterone-dependent increase in cholesterol synthesis, this indicated that the *de novo* synthesis of enzymes of the cholesterol biosynthetic pathway is essential for this increase in cholesterol synthesis. The inhibition of prostate RNA and protein synthesis by actinomycin D and cycloheximide, respectively, was particularly evident since the observed levels of synthesis were far less than those of non-testosterone-treated controls.

Evidence supporting the involvement of cAMP in the action of testosterone on the seminal vesicles of immature and orchidectomized rats has been presented (6). Singhal *et al.* (7) showed that when testosterone or cAMP and theophylline were administered parenterally to castrated rats, a significant increase in prostate weight and comparable increases in prostatic hexokinase, phosphofructokinase, pruvate kinase, and glyceraldehyde phosphate dehydrogenase activity were noted. These responses to cAMP and theophylline administration were also clearly inhibited by actinomycin D or cycloheximide. However, when cAMP was added to liver slices or homogenates, it was found (8, 9) that the rate of cholesterol synthesis declined. When cAMP was added to rat uterus in high concentration, other investigators (10) found an increase in cholesterol synthesis which was inhibited by actinomycin D.

In the present studies it was immediately apparent that cAMP at the concentration of 0.5 mM/ml increased protein and RNA synthesis. The simultaneous addition of theophylline at the concentration of 0.5 mM/ml further increased this stimulating effect. Both actinomycin D and cycloheximide inhibited the cAMP-stimulated

increases in the rates of RNA and protein synthesis in the prostate tissues. The inhibition of RNA or protein synthesis was expectedly more pronounced with the addition of actinomycin D or cycloheximide, respectively. In sharp contrast, the synthesis of cholesterol was significantly inhibited by the addition of cAMP. This inhibitory effect was enhanced by the simultaneous addition of theophylline. It is interesting to note that actinomycin D and particularly cycloheximide somewhat reversed the inhibitory effect of cAMP on cholesterol synthesis. This might suggest that the effect of cAMP on cholesterol synthesis, in part, depends on the *de novo* synthesis of one or more proteins that would block cholesterol synthesis in some manner. Another possibility exists that cAMP also acts directly on some enzyme of the cholesterol synthetic pathway without involving any *de novo* synthesis of proteins.

The decrease in *in vitro* cholesterol synthesis in the prostate by cAMP agrees with the results (8, 9) of similar studies involving slices and homogenates of the liver. The observation (10) that cAMP increases the *in vitro* synthesis of cholesterol in the rat uterus is of interest since it contrasts with the results of the current studies.

It has been suggested (6) that cAMP may play the role of a second messenger in the action of testosterone on rat secondary sex organs. In the current studies the N^6, $O^{2'}$-dibutyryl derivative of cAMP was employed since it is absorbed by cells more readily being more lipid soluble and more resistant to enzymatic breakdown. In earlier *in vivo* studies (7) the stimulating effects of cAMP on the prostate gland of castrated animals manifest by increases in prostate weight and levels of glycolytic enzymes clearly mimics that of testosterone. This is further seen in the stimulating effect of cAMP like that of testosterone on the *in vitro* synthesis of protein and RNA in prostate tissues of castrated rats. In is interesting to speculate on the direct opposite effect of cAMP to that of testosterone on the *in vitro* synthesis of cholesterol.

Summary. The effect of testosterone and cAMP on the *in vitro* synthesis of cholesterol in minced tissues of ventral prostate gland from castrated rats was studied. The synthesis of cholesterol was found to increase by 85% after incubation with testosterone for 4 hr. The increase in cholesterol synthesis was completely inhibited by blocking RNA or protein synthesis using actinomycin D or cycloheximide, respectively. Whereas cAMP, like testosterone, increased the *in vitro* synthesis of prostatic protein and RNA in tissues obtained from castrated rats, unlike testosterone, it inhibited the *in vitro* synthesis of cholesterol by 73% after 4 hr of incubation. All of these cAMP-stimulated effects on the synthesis of protein, RNA, and cholesterol were enhanced by the simultaneous addition of theophylline. The inhibitory effect of cAMP on cholesterol synthesis was partially reversed by the simultaneous addition of either actinomycin D or cycloheximide.

Thus, although both testosterone and cAMP increased the synthesis of protein and RNA, they showed an opposite effect on cholesterol synthesis, indicating that cAMP, if at all, may be only partially mediating testosterone action.

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