The Mode of Action of Carp Gonadotropin on the Stimulation of Androgen Production by Carp Testis *in Vitro*

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Carp gonadotropin (cGTH) can stimulate carp testis to produce androgen *in vitro*. This action can be mimicked by dibutyryl cAMP (Bt₂ cAMP) and prostaglandins (PG). Bt₂ cAMP can enhance the androgen production when submaximal doses of cGTH are used but cannot do so when maximal dose of cGTH is used. PG F_{2α} is more effective than PG E₁ and E₂. Both the inhibitors of RNA and protein synthesis can interfere the stimulatory effect of cGTH. Actinomycin D has 87% while cycloheximide has 100% inhibition. The stimulatory effect of cGTH is much more dependent on *de novo* protein synthesis than on RNA synthesis. In addition, steroidogenesis inhibitors, glutethimide, metyrapone, and spirocyanoketone, can also inhibit the stimulatory effect of cGTH on androgen production.

Luteinizing hormone (LH) can stimulate testis to produce androgen in higher vertebrates either *in vivo* or *in vitro* (Cigorraga *et al.*, 1978; Cooke *et al.*, 1975, 1979; Dufau *et al.*, 1971; Hafiez *et al.*, 1972; Maung and Follett, 1977; Mendelson *et al.*, 1975; Muller, 1977). The available evidences indicate that cyclic adenosine 3',5'-monophosphate (cAMP) is an intracellular messenger of LH on androgen production (Cigorraga *et al.*, 1978; Cooke *et al.*, 1979; Dufau *et al.*, 1971; Rommertz *et al.*, 1974). The stimulatory effect of LH on androgen production is dependent on *de novo* protein synthesis since ribonucleic acid (RNA) and protein synthesis inhibitors can inhibit the stimulatory effect of LH (Cigorraga *et al.*, 1978; Cooke *et al.*, 1975, 1979; Dufau *et al.*, 1974; Mendelson *et al.*, 1975; Moyle *et al.*, 1971; Shin and Sato, 1971). The effect of LH on androgen production is mainly due to its promotion of the conversion of cholesterol to pregnenolone (Bakker *et al.*, 1978; Cigorraga *et al.*, 1978; Hall and Eik-Nes, 1964; Hall and Young, 1968; Purvis *et al.*, 1973; van der Vusse *et al.*, 1975).

Our previous results indicated that fish

gonadotropin (GTH) can stimulate male carp to produce androgen either *in vivo* (Jean, 1980) or *in vitro* (Huang and Chang, 1980). The mode of action of fish GTH has seldom been investigated. Only a few lines of evidence indicate that fish GTH can enhance the fish gonad to produce cAMP (Fontaine *et al.*, 1970; Fontaine-Bertrand *et al.*, 1978; Idler *et al.*, 1975). In this study attempts are made to investigate the mode of action of carp GTH (cGTH) on the stimulation of androgen production by carp testis *in vitro* by the following approaches: (1) whether dibutyryl cAMP (Bt₂ cAMP) and prostaglandins (PG) can mimic the action of cGTH, (2) whether the stimulatory effect of cGTH is dependent on *de novo* RNA and protein synthesis, and (3) whether the various kinds of steroidogenesis inhibitors effective in mammalian systems can inhibit the stimulatory effect of cGTH on androgen production.

MATERIALS AND METHODS

Chemicals. Actinomycin D, bovine serum albumin (BSA), chick ovomucoid, cycloheximide, Bt₂ cAMP, PG E₁, E₂ and F_{2α}, and steroids were purchased from Sigma, St. Louis, Missouri. [³H]Testosterone was from the Radiochemical Center, Amersham, England. Ether was from Wako Pure Chemical Industries,

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Japan. Glutethemide (3-phenyl-3-ethyl-2,6-dioxopiperidin) and metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) were the kind gifts from Dr. J. Y. Chang, Ciba Company, Basel. Spirocyanoketone (2 α -cyano-4,4-dimethyl-20-spirox-5-en-3-one) was the kind gift from Dr. G. H. Rasmusson, Merck, Sharp and Dohme Research Laboratories, New Jersey. The cGTH used in this study was prepared as previously described (Huang and Chang, 1980). For assay, actinomycin D, cycloheximide, Bt₂ cAMP, and glutethemide were dissolved in incubation medium (see below) whereas metyrapone, PGs, and spirocyanoketone were dissolved in absolute alcohol.

Androgen production by carp testis *in vitro*. The method for the assay of the effect of cGTH and other substances on androgen production by carp testis *in vitro* was the same as previously described (Huang and Chang, 1980). Mature male carps (*Cyprinus carpio* L.) were collected during breeding season (December to March). Their body weights and gonadosomatic indexes (testis wt/body wt $\times$ 100) were from 300 to 500 g and 15 to 25, respectively. Testes from 3 to 4 fishes were pooled and finely chopped. The chopped tissues were washed with washing medium (8/9 strength of Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 0.1% BSA and 0.03% chick ovomucoid) until no whitish material was left in the supernatant. The washed tissues were preincubated in washing medium under 95% O₂-5% CO₂ for 15 min and then centrifuged at 5000g for 10 min to remove excess medium. For assay, 200 mg of preincubated tissue were suspended in 2 ml of incubation medium (same as washing medium except 1 mM theophylline was supplemented). An aliquot containing a given quantity of cGTH or other substances was also added to the incubation medium. For those alcohol-soluble substances, a given quantity in 10 μ l of absolute alcohol was added. Vehicle alone was served as control. In this study, samples for all assays in a set of experiment (i.e., all assays included in a given figure) were derived from a same batch of tissue preparation unless otherwise indicated. Triplicate determinations were made for each assay.

Incubation was performed at 25° under 95% O₂-5% CO₂ with slow constant shaking for 4 hr and terminated by centrifugation (5000g for 10 min). The supernatant was extracted with ether and then the androgen content was measured by radioimmunoassay as previously described. The antiserum used was raised against testosterone 17-hemisuccinate BSA conjugate. By using testosterone as reference (100%), the antiserum had 153% and 6% cross-reactivity toward androstenedione and 11-ketotestosterone, respectively.

Evaluation of the effect of Bt₂ cAMP, PGs, and inhibitors on androgen production. The dose-response curve of the stimulatory effect of cGTH on carp testis to produce androgen is nonlinear (see Results). For

evaluation of the stimulatory effect of Bt₂ cAMP and PGs, the amount of androgen produced was intrapolated into the dose-response curve of cGTH to get the equivalent amount of cGTH by which similar amount of androgen was produced. For evaluation of the inhibitory effect of inhibitor on the action of cGTH, carp testis was incubated with 0.8 μ g cGTH in the absence or presence of inhibitor. Similar intrapolation as that mentioned above was also made to get the equivalent amount of cGTH (in the unit of μ g) in the presence of inhibitor and denoted as eq-cGTH_{inh}. The percentage of inhibition was calculated as $(1 - \text{eq-cGTH}_{\text{inh}}/0.8) \times 100\%$.

RESULTS

The Stimulatory Effect of cGTH

The cGTH can stimulate carp testis to produce androgen *in vitro*. The range of dose-response curve is from 0.1 to 1.6 μ g, and in certain cases to 3.2 μ g (Figs. 1 and 3). The time course of androgen production under the stimulation of cGTH is shown in Fig. 4. The rate of androgen production is slower at the earlier than at the later period of incubation.

The Effect of Bt₂ cAMP

The Bt₂ cAMP can stimulate carp testis to produce androgen *in vitro*. The effect of 1, 5, 10, and 20 mM Bt₂ cAMP are equivalent to 0.10, 0.20, 0.64, and 0.90 μ g of

FIG. 1. The dose-response curve of cGTH on the stimulation of androgen production by carp testis *in vitro*. Each point and line indicate the mean $\pm$ SD of triplicate determinations.

cGTH on the aspect of the stimulation of androgen production (Fig. 2).

When Bt_2 cAMP and cGTH are added together to the incubation medium, the effect is always higher than that when either of them is present alone in the incubation medium. These results indicate that Bt_2 cAMP can express its effect in the presence of cGTH. However, the extent of the effect of Bt_2 cAMP is dependent on the concentration of coexisting cGTH. At the lower concentration of cGTH (0.1–0.4 μ g), the effect of Bt_2 cAMP and cGTH are additive. When the concentration of cGTH is increased up to 0.8 μ g and more, the effect of Bt_2 cAMP becomes progressively less and finally disappears (Fig. 3). Under the stimulation of maximal dose of cGTH, the amount of androgen produced is the same whether Bt_2 cAMP is present or not. Thus the effect of Bt_2 cAMP is completely masked by maximal dose of cGTH.

The Effect of PG

As shown in Fig. 2, PG E_1 , E_2 , and $F_{2\alpha}$ have stimulatory effect on androgen production. At the concentration of 20 μ g/ml,

FIG. 2. The stimulatory effects of Bt_2 cAMP, PG E_1 , E_2 , and $F_{2\alpha}$ on androgen production by carp testis *in vitro*, and the inhibitory effects of actinomycin D (AD) and cycloheximide (CH) on Bt_2 cAMP. The doses of cGTH, Bt_2 cAMP, and PGs are shown in the figure whereas those of actinomycin D and cycloheximide are 0.1 mM. Each bar and line indicate the mean and standard deviation of triplicate determinations.

FIG. 3. The effects of Bt_2 cAMP and cGTH on the stimulation of androgen production by carp testis *in vitro*. The doses of cGTH used are shown in the figure whereas that of Bt_2 cAMP is 10 mM. $\circ$, cGTH is present alone; $\bullet$, cGTH and Bt_2 cAMP are coexisting. Each point and line indicate the mean $\pm$ SD of triplicate determinations.

the effect of PG E_1 , E_2 , and $F_{2\alpha}$ are equivalent to 0.33, 0.62, and 0.73 μ g of cGTH, respectively.

The Effect of Actinomycin D and Cycloheximide

When the concentration of actinomycin D is kept at 0.01 mM, the stimulatory effect of cGTH on androgen production is inhibited by 87% (Fig. 6). The inhibitory effect of actinomycin D becomes progressively less marked as the interval between the addition of cGTH and actinomycin D is increased. When the time interval is increased up to 3 hr, actinomycin D has no more effect (Fig. 4). In addition, actinomycin D can also inhibit the stimulatory effect of Bt_2 cAMP on androgen production (Fig. 2).

When added simultaneously with cGTH, 0.1 mM of cycloheximide can completely abolish the stimulatory effect of cGTH. Furthermore, the amount of androgen produced by the tissue in the coexistence of cycloheximide and cGTH is even lower

FIG. 4. The effect of actinomycin D added at various time intervals of incubation on the androgen production by carp testis *in vitro* under the stimulation of cGTH. The doses used for cGTH and actinomycin D are $0.8 \mu\text{g}$ and 0.01 mM , respectively. ● and lines, means $\pm$ SD of triplicate determinations of control; ○ and lines, means and SD of triplicate determinations in which actinomycin D is added at various time intervals and incubation is allowed to continue up to 4 hr. ** $P < 0.05$ and * $P < 0.10$ versus control of 4-hr incubation, respectively.

than that produced by the control tissue (significant at 10% but not at 5% level) (Fig. 6). In contrast with actinomycin D, the inhibitory effect of cycloheximide on the cGTH does not become lessened as the time interval between the addition of cGTH and cycloheximide is increased. Whenever the cycloheximide is added, the effect of cGTH is abolished (Fig. 5). The statistical analysis indicates that the amount of androgen produced by control group within a given time interval is not different significantly from that produced by the group in which cycloheximide is added at a given time interval and incubation is allowed to continue for another time interval up to 4 hr. In addition, cycloheximide can also abolish the stimulatory effect of Bt_2 cAMP on androgen production (Fig. 2).

The Effect of Steroidogenesis Inhibitors

As shown in Fig. 6, the three types of inhibitors effective in mammalian systems are also effective in carp testis. At the concentration of 2, 2, and 0.02 mM , glutethimide, metyrapone, and spirocyanoketone have 95.8, 84.0, and 73.0% of inhibition

on the stimulatory effect of cGTH on androgen production, respectively.

DISCUSSION

The stimulation of testis to produce androgen is one of the main effects of LH in higher vertebrates (Cigorrage *et al.*, 1978; Cooke *et al.*, 1979; Dufau *et al.*, 1971; Hafiez *et al.*, 1972; Maung and Follett, 1977; Mendelson *et al.*, 1975; Muller, 1977). The testis or its dispersed Leydig cells has been used by many investigators as a model system to probe the mode of action of LH on steroidogenesis (Bakker *et al.*, 1978; Cigorrage *et al.*, 1978; Cooke *et al.*, 1975, 1979; Hall and Eik-Nes, 1964; Hall and Young, 1968; Mendelson *et al.*, 1975; Purvis *et al.*, 1973; van der Vusse *et al.*, 1975). Recently, we have demonstrated that fish GTH can stimulate carp testis to produce androgen *in vitro* (Huang and Chang, 1980), therefore attempts have been made to use this newly developed system as a model to investigate the mode of action of fish GTH.

The action of LH on steroidogenesis is proposed to be via the cAMP as cellular

FIG. 5. The effect of cycloheximide added at various time intervals of incubation on the androgen production by carp testis *in vitro* under the stimulation of cGTH. The doses used for cGTH and cycloheximide are $0.8 \mu\text{g}$ and 0.1 mM , respectively. ● and lines, means $\pm$ SD deviations of triplicate determinations of control; ○ and lines, means and SD of triplicate determinations in which cycloheximide is added at various time intervals and incubation is allowed to continue up to 4 hr. The same batch of tissue is used for assays of Figs. 4 and 5.

FIG. 6. The effects of inhibitors of RNA and protein synthesis, and steroidogenesis on androgen production by carp testis *in vitro* under the stimulation of cGTH. The doses of cGTH used for dose-response curve are shown inside the bar whereas those for assay of the inhibitory effect of various inhibitors are 0.8 μ g. The doses of inhibitors used are as follows: actinomycin D (AD), 0.01 mM; cycloheximide (CH), 0.1 mM; glutethimide (GT), 2 mM; metyrapone (MR), 2 mM; spirocyanoketone (SC), 0.02 mM. Each bar and line indicate the mean and SD of triplicate determinations.

messenger (Cigorraga *et al.*, 1978; Cooke *et al.*, 1979; Dufau *et al.*, 1971; Rommertz *et al.*, 1974). It is also demonstrated that Bt₂ cAMP can stimulate androgen production by carp testis in the present study. This finding seems to suggest that the action of cGTH may also be through cAMP as the second messenger.

The effects of PG E₁, E₂, and F_{2 α} on steroidogenesis are variable depending on the type of steroid, type of tissue and species of animal investigated (Behrman and Hichens, 1976; Erickson and Ryan, 1975, 1976; Labhsetwar, 1974; Lindner *et al.*, 1974; McNatty *et al.*, 1975; Neal *et al.*, 1975; Patwardhan and Lanthier, 1977). The present results provide additional evidence that PG E₁, E₂, and F_{2 α} can also stimulate carp testis to produce androgen. Whether the stimulation of PGs on androgen production in carp testis is through the stimulation of cAMP formation as found in mouse ovary (Kuehl *et al.*, 1970) remains to be elucidated.

The present results indicate that the

stimulatory effect of cGTH on androgen production by carp testis is dependent on *de novo* RNA and protein synthesis because actinomycin D and cycloheximide can inhibit or abolish this effect. A similar situation is also found in the mammalian system (Cigorraga *et al.*, 1978; Cooke *et al.*, 1979; Dufau *et al.*, 1974; Mendelson *et al.*, 1975; Moyle *et al.*, 1971; Shin and Sato, 1971). In rat the dependence of stimulatory effect of LH on *de novo* protein synthesis is more than that on *de novo* RNA synthesis. The inhibitory effect of actinomycin D becomes progressively less marked as the time interval between the addition of LH and actinomycin D is extended and finally disappears if the interval is increased up to 2.5 hr. On the other hand, even when cycloheximide is added 2.5 hr following LH, it can still affect the testosterone production although the extent is lessened relatively (Mendelson *et al.*, 1975). In carp, the dependence of stimulatory effect of cGTH on *de novo* RNA synthesis becomes progressively less marked and finally becomes independent if the time interval between the addition of cGTH and actinomycin D is long enough. On the contrary, the stimulatory effect of cGTH is stringently dependent on the *de novo* protein synthesis. Cycloheximide can abolish the effect of cGTH whenever it is added to the incubation medium. Our data suggest that cGTH can induce the synthesis of regulatory proteins including enzymes for steroidogenesis by which androgen production in carp testis is stimulated. These regulatory proteins are very labile, therefore the stimulatory effect of cGTH is dependent on their continuous synthesis. The finding that the inhibitory effect of actinomycin D becomes less marked or even totally insignificant may suggest that the messenger RNAs for these regulatory proteins are comparatively more stable and can be accumulated. Other possibilities are not excluded, such as the presence of lag period for actinomycin D to penetrate into the testicular tissue.

The present results indicate that glutethimide, an inhibitor of cholesterol 20 α -hydroxylase (Kahnt and Neher, 1966), metyrapone, an inhibitor of cytochrome P-450-linked reactions (Satre and Vignais, 1974) and spirocyanoketone, an inhibitor of C₁₉- and C₂₁-3 β -hydroxysteroid dehydrogenase (Goldman and Sheth, 1973) can inhibit the stimulatory effect of cGTH on androgen production by carp testis. The fact that the stimulatory effect of cGTH can be almost completely abolished by glutethimide indicates that the conversion of cholesterol to pregnenolone is necessary for expression of cGTH effect on androgen synthesis. The regulatory role of LH on the conversion of cholesterol to pregnenolone has been well demonstrated in mammals (Bakker *et al.*, 1978; Cigorraga *et al.*, 1978; Hall and Eik-Nes, 1964; Hall and Young, 1968; Purvis *et al.*, 1973; van der Vusse *et al.*, 1975). Whether the conversion of cholesterol to pregnenolone in carp testis is also under the control of cGTH is currently being investigated in this laboratory.

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