Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 279–285. Pergamon Press. Printed in Great Britain

A REVIEW

THE ROLE OF CYCLIC AMP IN THE REGULATION OF STEROID BIOSYNTHESIS IN TESTIS TISSUE

F. F. G. ROMMERTS, B. A. COOKE and H. J. VAN DER MOLEN

Department of Biochemistry (Division of Chemical Endocrinology), Medical Faculty, Erasmus University, Rotterdam, The Netherlands

(*Received* 31 *August* 1973)

1. INTRODUCTION

Numerous observations support a role of cyclic adenosine-3′:5′-monophosphate acid (cAMP) as an intracellular mediator of protein hormone actions [1].

For the regulation of steroid production in the adrenals and gonads by trophic hormones, several observations indicate the possible obligatory function of cAMP in the stimulation of steroid production. For the adrenal [2] and ovary [3], ample experimental evidence suggests the second messenger function of cAMP for regulation of steroid production. For the testis such information has become available only recently. In this respect, it appears appropriate to review pertinent results and difficulties related to the possible function of cAMP in testicular steroid production.

For the regulation of steroidogenesis by trophic hormones in testes a working hypothesis may be considered which is derived from the model of Garren *et al.*[2] for the effects of ACTH on adrenal steroid production (Fig. 1). In this model it is considered that the following consecutive stages are involved in the action of ACTH on the adrenal: (i) binding of hormone to the cell, (ii) activation of adenylcyclase, (iii) enhanced production of cAMP, (iv) activation of protein kinase and (v) action of activated protein kinases on protein synthesis, or substrate availability for the cholesterol side-chain cleavage enzyme.

Based on various experimental findings for other endocrine organs, other explanations have also been proposed for the action of trophic hormones on steroid production in the testis [4]: (a) increased production of intracellular NADPH through activation of phosphorylase; (b) increased blood flow through the testis. The many proposed models partially reflect the lack of information on the biochemical regulation of endocrine testis function. For a better understanding of the operating biochemical mechanisms, more detailed information is necessary, particularly with respect to the role of cAMP.

When investigating the biochemical mechanisms of hormone action, it is essential to work with specific methods of analysis and also where possible with specific cells or tissue types. A great deal of work on the testis has been carried out on the whole gland. Results from these investigations in relation to trophic hormone action are therefore difficult to interpret because

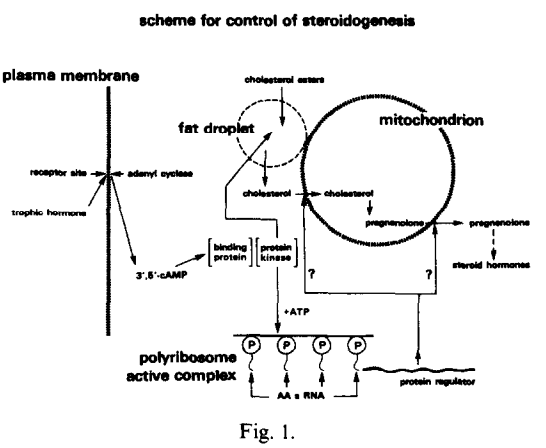

Fig. 1.

The following abbreviations and trivial names have been used: 3α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid: NAD(P) oxidoreductase (EC 1.1.1.50); 3β-hydroxysteroid dehydrogenase, 3β-hydroxysteroid: NAD(P) oxidoreductase (EC 1.1.1.51); 17β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid: NAD(P) oxidoreductase (EC 1.1.1.64); Δ^5, 3β-hydroxysteroid dehydrogenase, Δ^5, 3β-hydroxysteroid: NAD(P) oxidoreductase (EC 1.1.1.51); phosphodiesterase, orthophosphoric diester phosphohydrolase (EC 3.1.4.1); pregnenolone, 3β-hydroxy-5-pregnen-20-one; prostaglandin E_1, 11α, 15(s)-dihydroxy-9-oxo-13-trans-prostenoic acid; Q_{O_2}, rate of oxygen uptake (μl O_2/hr per mg protein); 5α-steroid reductase, 5α-steroid: NAD(P)Δ^4-oxidoreductase (EC 1.3.1.99); 7α-steroid hydroxylase, steroid NAD(P)H oxygen oxidoreductase (7α hydroxylating) (EC 1.14.1.99).

of the many different cell types present. In order to investigate the site of steroid biosynthesis and the biochemical mechanisms of regulation, isolated tissue and cell compartments should be investigated. This type of study should not of course exclude the possibilitites of interaction between the different tissue and cell types.

2. TROPHIC HORMONE RECEPTORS AND ADENYLCYCLASE IN TESTIS TISSUE

A first event in the action of trophic hormones on target organs is in many cases an interaction of the hormone with a receptor. For testis it has been reported that radioactive FSH is preferentially bound to seminiferous tubules [5, 6] and that radioactive LH and HCG are predominantly bound to interstitial tissue [7, 8]. There is little information on the subcellular localization of the receptors in testis. A few studies have demonstrated, however, an association with membrane fractions [9, 10]. Recently Dufau and Catt reported solubilization of receptors for LH and HCG [11].

It has been found that binding of protein hormones such as glucagon, insulin and ACTH occurs in membrane fractions. In addition, a correlation was shown between the binding and adenylcyclase activity for glucagon [12] and ACTH [13]. It has been postulated therefore that the receptor and the adenylcyclase form together a specific hormone sensitive system. The localization of this system in cell membranes fits with the concept that hormones on the outside of the cytoplasmic membrane affect the conformation—and thus the activity—of the adenylcyclase on the inside of the cell membrane. It has also been reported that adenylcyclase may be associated with the microsomal fractions of fat cells [14] and with mitochondrial fractions of dog testis [15] and rat testis [16] and with nuclei of the prostate [17]. The exact localization and the hormone specificity of these adenylcyclases obviously require further study especially with respect to the characterization of the subcellular fractions, because the presence of an intracellular localized trophic hormone dependent adenylcyclase would be contrary to the proposed theory. With the presence of intracellular hormone dependent adenylcyclases, the role of cAMP as the intracellular messenger of hormone action would have to be revised.

It has been shown that FSH and LH stimulate cAMP production in testis tissues [15, 18–22]. It appears that LH or HCG specifically stimulate cAMP production in interstitial tissue [21] whereas FSH specifically stimulates cAMP production in seminiferous tubules [20]. Also testosterone production could be stimulated with prostaglandin E_2 during perfusion experiments with dog testis *in situ* [24]. In rat testis tissue prostaglandin synthetase activity has been reported [25] and in swine testes prostaglandin contents have been measured [26]. Kuehl *et al.*[27] have presented evidence for an intermediate function of prostaglandins in the action of LH on the production of cAMP and steroidogenesis in the mouse ovary. Whether the trophic hormone increases cAMP formation through the intermediate formation of prostaglandins can only be concluded after more information is available. No other hormones have been shown to stimulate cAMP production in testis tissues.

3. ASSESSMENT OF STEROID AND cAMP PRODUCTION

The ability of cAMP to stimulate testicular steroid production was first shown by Sandler and Hall[28] in 1966 and by Connell and Eik-Nes[29] in 1968. Hence, at that time, independent effects of trophic hormones on cAMP production and effects of cAMP on steroid production were known, but there was no evidence for a causal relationship between cAMP and steroid production. Recent studies have concentrated on the effect of trophic hormones *in vitro* on the endogenous production of cAMP and testosterone in different testis tissues of the rat. Testosterone has been used as a parameter for steroid production because it is the quantitatively most important steroid secreted by the rat testis [30] and because its production can be stimulated by small doses of HCG *in vivo* [31]. However factors such as metabolism of testosterone and other pathways of steroid synthesis should be taken into consideration.

Conversions of testosterone and androstenedione to androstanediols have been demonstrated and from the structure of the main metabolites isolated it can be concluded that in rat testis tissue the following enzymes are present: 17β-hydroxysteroid dehydrogenase [32, 34, 35], 5α-steroid reductase [33–35], 3α-hydroxysteroid dehydrogenase [34], 3β-hydroxysteroid dehydrogenase [36] and 7α-steroid hydroxylase [37]. In isolated interstitial tissue, 5α-steroid reductase and 17β-hydroxysteroid dehydrogenase are present, whereas in isolated tubules 3α- and 17β-hydroxysteroid dehydrogenases and 5α-steroid reductase have been detected [35]. The measurement of testosterone during *in vitro* incubation may therefore give an underestimation of the production rate of testosterone if testosterone is further metabolized. This would be a small error, however, because it has been reported that after 2 h incubations of tritiated testosterone with isolated tubules and interstitial tissue 85% of the substrate was unconverted [38].

After the formation of pregnenolone from cholesterol, two pathways may result in the biosynthesis of androgens [39]. Based on the steroid structure of the intermediates in the two pathways a "Δ^4-route" and a "Δ^5-route" have been distinguished. It may be possible therefore that pregnenolone is converted via Δ^5-compounds to androstanediols and may not be converted to testosterone.

Bell *et al.*[32] have presented evidence, however, that the main route for biosynthesis of testosterone in the rat is via Δ^4-compounds. This preference for the Δ^4-route may be explained by a very active Δ^5 3β-hydroxysteroid dehydrogenase and Δ^5–Δ^4 isomerase in rat testis when compared with testis tissues from other species [40].

Increased production of cAMP from endogenous precursors by hormonal activation of adenylcyclase in testis has been shown [19–22]. cAMP, however, can also be metabolized by cyclic nucleotide phosphodiesterase which is present in many tissues [41] including the testes [42, 43]. Thus the degradation of cAMP in testis tissue will influence the net production of cAMP. Inhibitors of phosphodiesterase, e.g. the methyl xanthines, can be added but they may inhibit trophic hormone stimulation of testis steroidogenesis especially with the high concentrations required to achieve complete inhibition [50, 71].

4. GONADOTROPHIC STIMULATION OF TESTICULAR TESTOSTERONE PRODUCTION *IN VITRO*

Stimulation of *in vitro* testosterone production has been reported for mouse interstitial cell cultures with cAMP [44], mouse Leydig cell tumours and rat Leydig cell preparations with LH [45, 46], testis tissue from 20-day-old rats with cAMP and LH [28, 47] and for rabbit testis slices with cAMP and LH [29]. Dufau *et al.*[48, 49] reported the gonadotrophic stimulation of testosterone production of total testis of adult rats using very small amounts of LH and HCG. Teased testes, however, were almost unresponsive to trophic stimulation. In the initial experiments on the relationship between cAMP and testosterone production by Rommerts *et al.*[50], it was also found that no reproducible effects of HCG could be obtained on testosterone produced by teased testis. In many experiments testicular testosterone production could not even be stimulated with high doses of HCG (10 I.U.). Hypophysectomy and pre-treatment of rats with HCG, or the addition of albumin to the incubation medium did not improve these results. When testes from 20-day-old rats were used, testosterone production could be stimulated with HCG which was in agreement with results published by Sandler and Hall [47]. The latter authors also reported that the rate of incorporation of radioactivity from cholesterol into testosterone was most sensitive to stimulation with HCG in 20-day-old rats [51]. Further work with tissue from normal adult rats showed that by pre-incubating the teased whole testis tissue or interstitial tissue for 1 h before the addition of fresh medium and HCG or LH, a consistent stimulation of cAMP and testosterone production could be obtained [21, 50, 68]. Similar observations have been reported for corpus luteum tissue [52] and for quartered adrenals [53]. These effects of preincubation may theoretically be explained by removal of an endogenous effector. In rat adipocytes the release of a hormone antagonist has been shown [54]. For testis tissue, however, there is no evidence for such an antagonist.

5. cAMP AS SECOND MESSENGER FOR TROPHIC HORMONE ACTION ON TOTAL TESTIS TISSUE

Criteria for cAMP as a second messenger in trophic hormone action on testosterone production by total testis tissue are supported by the following evidence obtained from *in vitro* experiments [48–50, 55, 56].

(1) HCG and LH increase the levels of testosterone and cAMP in total testis tissue;

(2) the increase in cAMP levels precedes the increase in testosterone levels;

(3) dibutyryl-cAMP increases testosterone levels in total testis tissue.

In incubation experiments no effects on the *in vitro* testosterone production were observed with FSH alone or together with LH [55]. Clearcut synergistic effects of FSH were shown, however, on testosterone secretion by rabbit testes perfused *in vitro* with LH [57].

It thus appears that in total testis tissue cAMP is an intermediate in the action of LH or HCG on steroidogenesis. However, in testis many different cell types are present and from experiments with whole testis tissue no conclusions can be drawn on the specificity of cAMP and steroid production. Therefore it is necessary to investigate this relationship in a more homogeneous cell system and to study the *in vitro* steroid production in isolated interstitial tissue and seminiferous tubules.

6. TESTOSTERONE PRODUCTION IN ISOLATED INTERSTITIAL TISSUE AND SEMINIFEROUS TUBULES

The dissection technique as described by Christensen and Mason [58] for the isolation of interstitial tissue and seminiferous tubules from rat testis has now been used by many workers. Recently Rommerts *et al.*[59] described some characteristics of the dissection of wet tissue and of freeze-dried cryostat sections.

Using this wet dissection technique, Cooke *et al.*[60] have found that the interstitial tissue is the main site of testosterone production in the testis. During 3 h incubations with rat interstitial tissue, testosterone levels increased (from 17 ± 10 ng to 56 ± 15 ng testosterone/100 mg tissue, wet wt.), whereas with tubules an apparent decrease in testosterone levels was observed (from 2·3 ± 1·3 ng to 1·4 ± 0·6 ng testosterone/100 mg tissue, wet wt.). The decrease in testosterone levels during the incubation of tubules suggests the absence of steroid production in tubules. Production in tubules might have been undetected, if the synthesis of testosterone was balanced by its degradation, so that the net production was zero. However experiments with [^{3}H] testosterone indicate that this steroid is only slowly metabolized by seminiferous tubules (90% unconverted in 2 h [73]). *De novo* steroid production can also be studied with isolated mitochondria which has the advantage that catabolic enzymes which are mainly microsomally bound or present in the cytoplasm [61] are absent. When the endogenous steroid production (expressed as the production of Δ^5 pregnenolone and testosterone) in isolated mitochondria from interstitial tissue, tubules and total testis tissue are compared, it appears that 92–97% of the steroid production in testis is produced by mitochondria from the interstitial tissue [62]. These results strongly suggest the absence of *de novo* testosterone synthesis in tubules which is in agreement with the above observations [60] and Hall *et al.*[63]. This is in contradiction, however, with the conclusions of others [64–67], that steroid biosynthesis from cholesterol may take place in the seminiferous tubules of rat or human testes.

7. STIMULATION OF cAMP AND TESTOSTERONE IN INTERSTITIAL TISSUE WITH DIFFERENT DOSES OF LH

It has been shown that endogenous testosterone production occurs in the interstitial tissue and therefore this tissue has also been used to study the effects of LH on cAMP and steroid production [68]. cAMP and testosterone production could be stimulated by LH. Stimulation of steroid production could be obtained with 20 ng LH/ml, whereas stimulation of cAMP formation in interstitial tissue required five to ten times more LH [46, 68]. Comparable observations for the amounts of ACTH required to stimulate cAMP and steroid production have been made with adrenal tissue by Beall and Sayers [69] although Mackie *et al.*[70] found very little difference in the amounts required. The stimulation of testicular steroidogenesis with 20 ng LH/ml without a simultaneous effect on cAMP production could be interpreted as a direct hormonal effect on steroidogenesis without the involvement of cAMP, although it may also reflect the inadequacy of the analytical method for cAMP, to detect small differences. Significant effects on cAMP may occur in a particular intracellular compartment which cannot be measured if the total system is analysed. On the other hand, it may be possible that all measurable effects on cAMP levels are caused by overstimulation of the adenylcyclase by unphysiological doses of trophic hormone. The simplest explanation, however, would be that the cAMP is not an obligatory mediator of LH action on testosterone production. Clearly more work is required to clarify this problem.

8. EFFECTS OF ISOLATION OF TESTICULAR TISSUES ON TESTOSTERONE PRODUCTION

Of the total protein in testis tissue 17% is estimated to be interstitial tissue protein [59]. Thus in isolated interstitium the number of Leydig cells per unit of protein is six times higher compared to the total testis tissue. It may be expected therefore that the steroid production in isolated interstitial tissue is six times higher than in total testis tissue. In practice, however, lower values have been found [68]. In contrast, the amount of cAMP produced during 10 min in LH stimulated tissue was about 6 times higher in interstitial tissue than in total testis [21]. Hence effects of the tissue isolation procedure were apparently not detectable at the level of the adenylcyclase system, but were somewhere else in the sequence of reactions which regulate steroidogenesis. Dufau *et al.*[49] also found that the stimulation of testosterone production with dibutyryl-cAMP or LH was less in teased testis tissue than in unteased tissue. They were not able to stimulate testosterone production in interstitial tissue. Recently the same group reported, however, stimulated testosterone synthesis in isolated interstitial cells [71].

One possible reason for lower testosterone production in interstitial tissue is a possible destruction of cellular integrity during the dissection procedure. Light microscopical and electron microscopical examinations have shown the presence of some tubular cells in the isolated interstitial tissue and deteriorations of the normal Leydig cell structure [72, 73]. Also, a limitation of nutritional factors in isolated interstitium may have caused the relatively low production. The addition of glucose stimulates testosterone production in interstitial tissue and teased testis [68] in the presence of LH. The dependency of isolated interstitial tissue on added glucose was larger than in teased testis tissue. This may possibly be caused by more easily removed essential factors from isolated interstitial tissue than from intact tissue, where leakage may be

smaller and supply by the lymphatic system surrounding the cells may still be intact. The decrease of cAMP levels after 30 min observed in total testis when incubated without glucose [50] may also be explained by a lack of nutritional factors especially after a certain incubation period. It also raises the question as to whether the uptake of glucose by Leydig cells is hormonally regulated.

Glucose may possibly act as a substrate for interstitial tissue. Effects of glucose on oxygen uptake of interstitial tissue have however not been found [74]. In contrast, glucose can serve as a substrate for oxidative processes in tubules [74] and for protein synthesis in total testis [75] as well as for maintaining ATP levels [76]. Other obligatory endogenous substrates for the interstitial tissue have been suggested: lipids have been mentioned [74, 77], but no conclusive evidence was presented, and Hamberger *et al.*[78] reported an increase of the Q_{O_2} of interstitial tissue with succinate. Effects of these substrates on steroid production have not been measured. It is not known to which extent under *in vitro* conditions incubations in a gas phase containing unphysiological (95%) oxygen tension may affect the steroid production of testis tissue.

The time lag between the addition of the trophic hormone and the response of the steroid production also seems to be dependent on manipulation of the tissue. In studies with total testis tissue [50] and interstitial tisse [68] a time lag of approximately 30 min was found before significant increases in testosterone production could be observed. Dufau *et al.*[55] observed a marked stimulation of the testosterone production after 15 min (the earliest time of sampling in unteased testis tissue). Clear stimulation of the *in vivo* testosterone production in the rat has been reported after 15 min (earliest time of sampling) [31]. In perfusion experiments with dog testis a time lag of less than 10 min was reported [79]. A comparison between the observations of Rommerts *et al.*[50, 68] and those by others is however not completely valid, because experiments under conditions which do not reflect possible release mechanisms are different from experiments where release of steroids may be dependent on trophic hormones. Regulation of release has been postulated by Eik-Nes for steroids in the testis [79] and has been demonstrated for the release of free fatty acids from adipocytes under the influence of cAMP [80].

9. BIOCHEMICAL MECHANISM OF ACTION OF cAMP IN TESTIS TISSUE

Several mechanisms have been proposed for the next steps in the biochemical action of trophic hormones after the production of cAMP. In the hypothetical model presented in Fig. 1, effects on protein kinase and subsequently on protein synthesis and on the availability of cholesterol have been indicated. In testis tissue a cAMP-dependent protein kinase has been detected [81]. After administration of LH or dibutyryl-cAMP *in vivo*, stimulation of protein synthesis has been shown to occur in Leydig cells *in vitro* [82]. In various total testis preparations, comparable effects were observed with FSH [83–85]. Direct inhibiting effects of cAMP on testicular enzyme activities such as NAD^+-dependent 17β-hydroxysteroid dehydrogenase, isocitrate dehydrogenase and malate dehydrogenase have been measured [86, 87]. The physiological meaning of these effects is doubtful, because cAMP was only effective if added in millimolar concentrations whereas intracellularly micromolar concentrations are present.

Also stimulation of RNA synthesis by FSH in total testis has been described [88]. Effects of trophic hormones *in vivo* on different cholesterol pools in testis have been described by various investigators [89–91]. Trophic hormones have also been reported to stimulate enzyme activities, such as lactate dehydrogenase [92] and alcohol dehydrogenase [93] which are not directly related to steroidogenic processes. It is difficult to establish the significance of these results in relation to the role of cAMP because many results have been obtained from experiments with total testis tissue and also many effects have not been related with cAMP or steroid production. Also some effects may be a consequence of regulation of growth under influence of the trophic hormone. This may be an effect distinct from direct effects on steroidogenesis. At least for the adrenal, different mechanisms have been proposed for the effect of ACTH on steroidogenesis and on growth [94].

10. SUMMARY AND CONCLUSIONS

Recently there has been a rapid increase in knowledge about the control of steroidogenesis in the testes. This has come about because of the relatively simple and sensitive methods that are now available for measuring steroids and cAMP and because of the *in vitro* tissue systems which have been developed. It is now possible to use intact testis tissue and testis separated into interstitial tissue and seminiferous tubules to study the effects of physiological amounts of trophic hormones on cAMP and steroidogenesis.

The data obtained indicate that cAMP may be a second messenger of LH on steroidogenesis in the testis because LH specifically stimulates cAMP and steroidogenesis in interstitial tissue and whole testis tissue and the increase in cAMP occurs before the increase in steroidogenesis. In addition dibutyryl-cAMP

stimulates testosterone production. Not in accordance with cAMP being the second messenger is the finding that low levels of LH stimulate steroidogenesis without there being detectable changes in cAMP levels. FSH on the other hand specifically stimulates cAMP production only in the seminiferous tubules and apparently has no effect on steroidogenesis.

Very little is known about the mechanism of cAMP steroidogenic action in the testis. It has yet to be determined if similar mechanisms to those proposed for the adrenal gland and ovary i.e. via protein kinase and protein synthesis also exist in the testis.

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