J. steroid Biochem. Vol. 22, No. 4, pp. 507–512, 1985
Printed in Great Britain. All rights reserved

0022-4731/85 $3.00 + 0.00
Copyright © 1985 Pergamon Press Ltd

RELATIONSHIPS BETWEEN UNCONJUGATED AND SULPHATED STEROIDS IN PORCINE PRIMARY LEYDIG CELL CULTURE

M. ORAVA, F. HAOUR*, P. LEINONEN, A. RUOKONEN and R. VIHKO
Department of Clinical Chemistry, University of Oulu, SF-90220 Oulu, Finland and *INSERM U. 162, Hopital Debrousse, 29 Rue Soeur Bouvier, 69322 Lyon Cedex 1, France

(*Received* 23 *August* 1984)

Summary—Steroidogenesis in immature porcine Leydig cells was investigated in primary culture at 48–84 h under basal conditions and in the presence of hCG. The basal accumulation of unconjugated steroids was close to linear only during the first 4 h of study, whereas the sulphate-conjugated steroids accumulated essentially linearly over the 36 h experimental period. At the last time point, 95% of the steroids measured were sulphated. Stimulation with hCG (1 ng/ml) led to a still more pronounced sulphate conjugation, and approx 99% of the steroids measured were sulphated at 36 h. Under maximal stimulation with hCG (100 ng/ml) the sulphates accounted for 74% of the total steroids measured at 36 h. Testosterone, androstenedione, dehydroepiandrosterone, 5-androstene-3β,17β-diol and estrone were usually quantitatively the most important unconjugated steroids, and sulphated dehydroepiandrosterone, estrone, testosterone and 5-androstene-3β,17β-diol were the most important steroid sulphates, especially following maximal stimulation of the cultures. These data emphasize the importance of steroid sulphates in porcine testicular steroid metabolism. Under stimulation with hCG, there was a rapid response in testicular steroidogenesis, initially seen as a rapid increase in the secretion of unconjugated and sulphated steroids. At approx 4–12 h, the rate of sulphate conjugation appeared to reach or even to exceed that of steroid biosynthesis, which lead to stabilisation or a decrease in the concentrations of unconjugated steroids. Only high doses of hCG, 10–100 ng/ml, were then able to lead to a net accumulation of unconjugated steroids, at 24–36 h of incubation with hCG.

INTRODUCTION

In contrast to several other species, human and porcine testis tissues contain large concentrations of endogenous steroids [1, 2]. A considerable proportion of these are present as sulpho-conjugates, and in both species some of the conjugates are secreted into the peripheral circulation [3–6] and into the testicular lymph [7]. The biological importance of steroid sulphates in testis tissue is insufficiently known, but there is evidence that they may be precursors of T [8].

The recently described porcine primary Leydig cell culture [9] is an attractive model for studies of the regulation of testicular steroidogenesis. In addition to T, the production of DHEA [10] and DHEA-S [9] are stimulated by hCG in this model, which therefore might be useful for studies of the relationships between unconjugated and sulphated steroids. Using this model, we have investigated hCG-induced changes of T production and their relationships to changes in the concentrations of T precursors and metabolites, some estrogens and their respective sulphated conjugates.

The following trivial names and abbreviations have been used: Androstenediol: 5-androstene-3β,17β-diol (Adiol); Androstenediol sulphate (Adiol-S); Androstenedione: 4-androstene-3,17-dione (Adione); Dehydroepiandrosterone: 3β-hydroxy-5-androsten-17-one (DHEA); Dehydroepiandrosterone sulphate (DHEA-S); Dihydrotestosterone: 17β-hydroxy-5α-androstan-3-one (DHT); Estradiol-17β: 1,3,5(10)-estratriene-3,17β-diol (E_2); Estradiol-17β sulphate (E_2-S); Estrone: 3-hydroxy-1,3,5(10)-estratrien-17-one (E_1); Estrone sulphate (E_1-S); 17-Hydroxypregnenolone: 3β,17α-dihydroxy-5-pregnen-20-one (OH-Preg); 17-Hydroxyprogesterone: 17α-hydroxy-4-pregnene-3,20-dione (OH-Prog); Pregnenolone: 3β-hydroxy-5-pregnen-20-one (Preg); Progesterone: 4-pregnene-3,20-one (Prog); Testosterone: 17β-hydroxy-4-androsten-3-one (T).

EXPERIMENTAL

Culture medium

Ham's F12 Nutrient Mixture and Dulbecco's Modified Eagle's medium (1:1) [Grand Island Biological Co.] with the addition of 1.2 g/l sodium bicarbonate, 15 mmol/l Hepes (Sigma), 20 mg/l gentamycin (Sigma) and 2.5 mg/l Fungizone (Grand Island Biological Co.) was used for cell isolation (= DME/F12-medium). For cell culture, DME/F12-medium was further supplemented with porcine insulin (5 mg/l, Sigma), human transferrin (5 mg/l, Sigma), vitamin E (5 mg/l, Sigma) and 0.1% FCS (Grand Island Biological Co.) (= hormone supplemented DME/F12-medium).

Cell culture

Testes (12–16 per preparation) were from 3–4 week old piglets, and cells were prepared for culture essentially as described by Mather *et al.*[9]. In short, the testes were decapsulated, minced with scissors and pieces were washed twice in DME/F12-medium. Tes-

ticular pieces were enzymatically dispersed at 35°C for 1.5 h with gentle shaking in 400 ml of DME/F12-medium containing 0.5 g/l collagenase (Collagenase/Dispase, Boehringer Mannheim) and 0.05 g/l of soy trypsin inhibitor (Sigma). The undigested tubular fragments and cell clumps were removed by two successive filtrations through nylon mesh (pore size 150 and 40 μm, respectively). The interstitial cell fraction was washed 4 times in DME/F12-medium before plating. The cell number was determined by using a Bürker cell counting chamber. Leydig cells routinely represented 60–70% of the total cell population, as determined by histochemical staining for 3β-hydroxysteroid dehydrogenase [11]. The cells were plated at a density of 1.5×10^5 cells/cm^2 in hormone supplemented DME/F12-medium. The cultures were maintained in a humidified atmosphere of 95% air–5% CO_2 at 35°C. The medium was changed every 24 h. After 48 h pre-incubation, the cells were treated with hCG (Batch No. CR119, 11600 IU/mg, prepared by Dr R. Canfield, Columbia Univ., New York and provided by the Center for Population Research of the NICHD) and at the appropriate time points the medium was collected for assay of the concentrations of steroids and cAMP.

Assays of unconjugated steroids, steroid sulphates and cyclic AMP

Methodologies for the quantification of unconjugated and sulphated steroids have been described previously [12–15]. Briefly, unconjugated steroids were extracted from the media and then fractionated on Lipidex-5000™ microcolumns (Packard-Becker, B.V., Chemical Operations, Netherlands) followed by radioimmunoassay of each steroid from the appropriate fraction using antisera of defined specificity [12]. The chromatography for unconjugated E_1 and E_2 was slightly modified by using a more polar solvent system [13]. The appropriate fractions were analysed for E_1 and E_2 using commercial RIA kits with highly specific antisera (E_1: Wien Laboratories Inc., Succasunna, U.S.A. and E_2: Farmos Diagnostica, Oulunsalo, Finland) according to the instructions given by the manufacturers.

Steroid sulphates were analysed from the water phases of the above mentioned extracts [14]. These were dried under nitrogen, the residue was dissolved in 3 ml of absolute ethanol and centrifuged to remove traces of proteins. The ethanol extract was dried and solvolysed in 3 ml of ethyl acetate, previously equilibrated with H_2SO_4 (2 mol/l). Before overnight incubation at 37°C, an additional 50 μl of H_2SO_4 (2 mol/l) was added to the tubes to ensure that the medium remained acidic during the incubation. After solvolysis the mixture was neutralised and the ethyl acetate phase was transferred into another tube, dried and fractionated on Lipidex-5000™ microcolumns. Chromatography was followed by radioimmunoassays of steroids as described above. Water blanks and serum controls monitored assay quality. The intra- and interassay coefficients of variation were < 16% for all steroids measured.

Samples of incubation media for cAMP analysis were taken in 4 vol of absolute ethanol and cAMP was assayed using RIA kits provided by New England Nuclear (U.S.A.) with prior acetylation, following the instructions of the manufacturer.

RESULTS

Primary cultures of porcine testicular interstitial cells (60–70% Leydig cells) were pre-incubated for 2 days with medium changes every 24 h. Following this, cultures were treated with increasing doses of hCG and terminated at timed intervals up to 36 h.

Basal conditions

The basal accumulation of unconjugated steroids was close to linear only during the first 4 h, whereas the sulphoconjugated steroids accumulated essentially linearly over the 36 h experimental period (Figs 1–3; observe the logarithmic scale in Figs 2 and 3). Concentrations of unconjugated and sulphated steroids at 4 h incubation are given in Table 1 and those at 36 h in Figs 2 and 3. In control incubations without hCG, approx 26 and 5% of the steroids measured in the media were in an unconjugated form at 4 and 36 h, respectively, the majority being sulphated. Among the unconjugated steroids, Adiol, DHEA, T and Adione were present in highest concentrations. Of the sulphated steroids, DHEA-S alone comprised 45 and 69% at 4 and 36 h, respectively. In addition to DHEA-S, the concentrations of E_1-S and T-S were greatly increased between 4 and 36 h of incubation.

Effects of hCG on unconjugated steroids

Addition of hCG resulted in major time- and dose-dependent changes in the concentrations of the unconjugated steroids measured in the culture media (Figs 1 and 2, Table 1). At 4 h of incubation in the presence of a maximally stimulating hCG-dose (100 ng/ml), the total concentration of unconjugated steroids increased 18.7-fold, by far the most marked relative increases taking place in the concentrations of T and Adione, followed by those of DHEA and E_1. The relative increases in the concentrations of C21 steroids and DHT were smaller, in most cases clearly less than 10-fold.

The kinetics of hCG-induced T-accumulation are shown in Fig. 1A and the corresponding response patterns for other unconjugated steroids are presented in Fig. 2. For clarity, the data from experiments using 0.1 and 10 ng of hCG per ml have not been incorporated into the latter figure. With lower doses of hCG (0.1–1 ng/ml, Fig. 1A) T first accumulated linearly up to 4–8 h, after which it gradually decreased towards the control levels. Higher doses of hCG used (10–100 ng/ml) first induced a rapid and

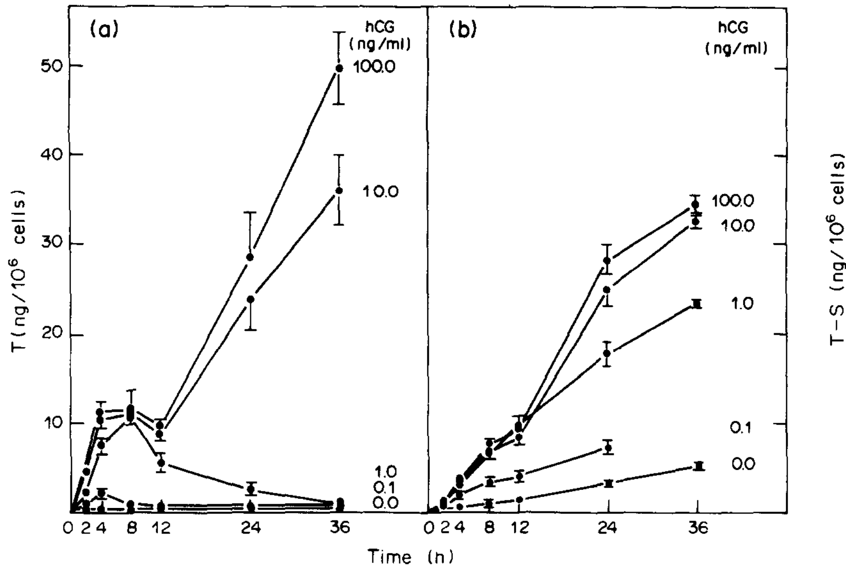

Fig. 1. Effect of treatment with hCG on T and T-S accumulation in primary culture of pig testicular cells. After isolation, testicular cells were pre-incubated for 48 h in hormone-supplemented DME/F12-medium with a medium change at 24 h. After pre-incubation, the medium was changed and the cells were treated with increasing concentrations of hCG (0–100 ng/ml) and at the time points indicated media were collected for steroid analysis. Each point represents the mean ± SEM of triplicate incubations of two independent cell cultures.

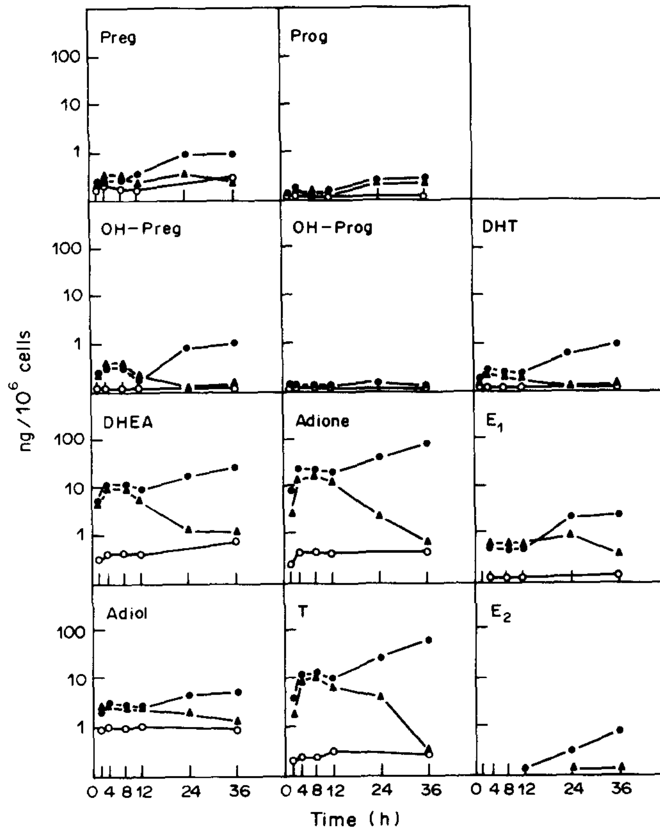

Fig. 2. Time course of Preg, Prog, OH-Preg, OH-Prog, DHEA, Adione, Adiol, T, DHT, E_1 and E_2 accumulation under basal conditions (○——○) and after treatment with 1 ng/ml (▲——▲) and 100 ng/ml (●——●) of hCG in piglet primary testicular cell culture. Testicular cells were pre-incubated for 48 h in hormone supplemented DME/F12-medium with a medium change at 24 h. The medium was then changed and the cells were treated with or without hCG. At the time points indicated, media were collected for steroid analysis. Each point represents the mean of three incubations.

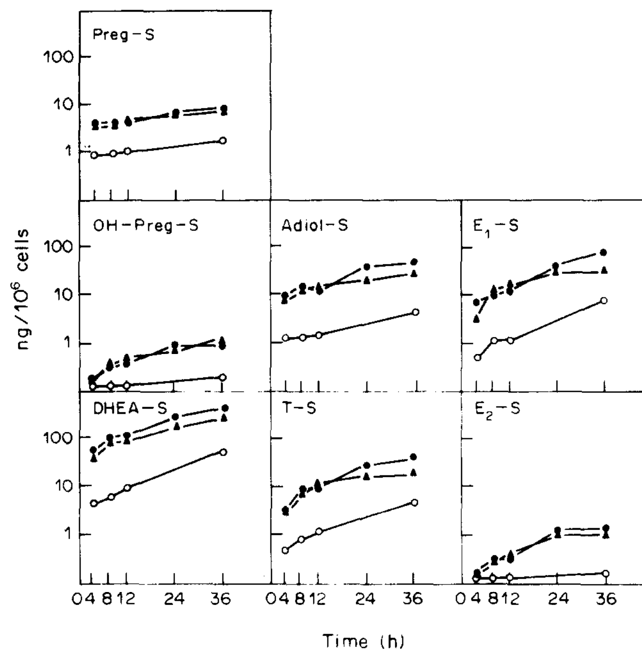

Fig. 3. Time course of Preg-S, OH-Preg-S, DHEA-S, Adiol-S, T-S, E_1-S and E_2-S accumulation under basal conditions (○——○) and after treatment with 1 ng/ml (▲——▲) and 100 ng/ml (●——●) of hCG in piglet primary testicular cell culture. Testicular cells were pre-incubated for 48 h in hormone-supplemented DME/F12-medium with a medium change at 24 h. The medium was then changed and the cells were treated with or without hCG. At the time points indicated, media were collected for steroid analysis. Each point represents mean of triplicate incubations.

linear response up to 4 h. After 4 h, there was a lag period between 4–12 h followed by a second linear stimulation up to 36 h. Similar non-linearity was also clearly evident in the response patterns of most of the other unconjugated steroids measured (Fig. 2). To investigate the mechanisms behind this non-linearity in the response of T (Fig. 1A), the cells were exposed to another hCG treatment during the lag period. In Fig. 4A, the cells were first stimulated with a high dose of hCG (100 ng/ml). At 8 h, when the T concentration was stable, the medium was changed and the cells were again treated with 100 ng/ml of hCG. In a second experiment (Fig. 4B), the cells originally stimulated with a low dose of hCG (1 ng/ml), were restimulated with hCG (1 and 100 ng/ml) at 24 h without changing the medium. In both cases (Figs 4A and B, dotted lines), there was a major response in T concentration, which indicates that the changes in T levels were not due to decreased steroidogenic capacity of the cells, nor to a major loss of LH/hCG receptors.

Effects of hCG on sulphated steroids

The formation of steroid sulphates was very active both in unstimulated (see above) and stimulated cultures (Fig. 3). As was the case in unstimulated cultures, sulphate-conjugated steroid concentrations reached markedly higher than those of the respective unconjugated steroids. A most obvious exception was T, which reached higher concentrations than did T-S following hCG-stimulation (Fig. 1). DHEA-S maintained its position as the quantitatively most important steroid measured in porcine Leydig cell culture (Table 1). E_1-S was the predominant sulfated estrogen, whereas the concentration of E_2-S remained relatively low.

Table 1. Steroidogenic responsiveness of porcine testicular cells in primary culture

	Basal (B) (ng/10^6 cells × 4 h)	hCG-stimulated (S) (ng/10^6 cells × 4 h)	S/B
Preg	0.20	0.22	1.1
Prog	0.12	0.15	1.3
OH-Preg	0.03	0.39	13.0
OH-Prog	0.02	0.05	2.5
DHEA	0.52	13.90	26.7
Adione	0.47	21.56	45.9
Adiol	1.09	3.55	3.3
T	0.20	11.49	57.5
DHT	0.10	0.21	2.1
E_1	0.04	0.66	16.5
E_2	<0.01	<0.01	
Total	2.79	52.18	18.7
Preg-S	0.90	2.94	3.3
OH-Preg-S	0.04	0.18	4.5
DHEA-S	4.91	72.69	14.8
Adiol-S	1.22	9.93	8.1
T-S	0.50	3.22	6.4
E_1-S	0.44	6.99	15.9
E_2-S	0.02	0.15	7.5
Total	8.03	96.10	12.0

Accumulation of unconjugated and sulphated steroids after 4 h incubation in basal conditions and after stimulation with 100 ng/ml of hCG. Results are from experiments shown in Figs 2 and 3. Each point represents the mean of triplicate incubations.

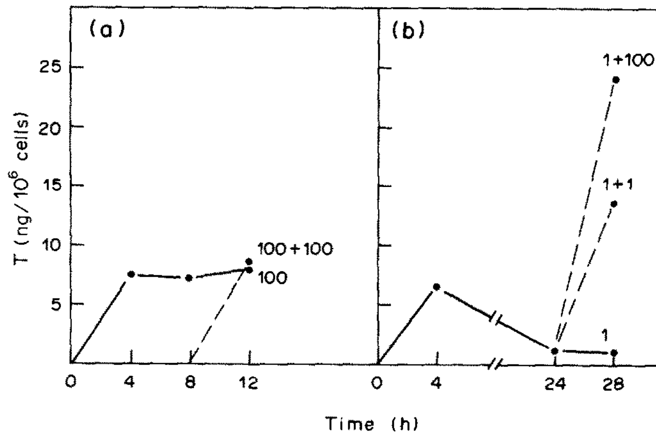

Fig. 4. Restimulation of T production with hCG. Porcine testicular cells in culture were pre-incubated for 48 h in hormone-supplemented DME/F12-medium with a medium change at 24 h. At 48 h the medium was changed and the cells were treated with 100 ng/ml (A) or 1 ng/ml (B) of hCG. The accumulation of T was measured at the time points indicated (●——●). In parallel incubations the cells were first stimulated with 100 ng/ml (A) or 1 ng/ml (B) of hCG and then restimulated with 100 ng/ml of hCG after 8 h after changing the medium (A) or with 1 and 100 ng/ml of hCG at 24 h without changing the medium (B), and the accumulation of T was assayed after an additional 4 h (●--●). Each point represents the mean of triplicate incubations.

Dose-response to hCG-stimulation

The sensitivities of the responses of T and DHEA, their respective sulphoconjugates and cAMP to hCG-stimulation were measured (Fig. 5). The concentrations of hCG required to obtain half-maximal stimulation of the accumulation of T (0.3 ng/ml), T-S (0.2 ng/ml), DHEA (0.2 ng/ml) and DHEA-S (0.2 ng/ml) were similar, whereas a much higher concentration of hCG (3.0 ng/ml) was needed to stimulate cAMP production, which is consistent with the findings described in rat cells [11, 16].

DISCUSSION

In this study we have characterized the effects of hCG on unconjugated and sulphated steroid production in primary cell cultures (60–70% Leydig cells) from immature porcine testes. The concentrations of C21 steroids and their sulphoconjugates were low compared to those of C19 steroids under both basal and hCG-stimulated conditions (Table 1, Figs 2 and 3). Quantitatively the most important steroid produced by piglet testicular cells was DHEA-S (Table 1). The major estrogen produced was E_1-S, a finding which is consistent with previous studies *in vivo* [6, 7] and *in vitro* [17]. The concentrations of E_2-S were much lower. We did not measure 16-androstenes or their sulphate conjugates, which comprise the main testicular steroids in the boar [2]. Because 16-androstanes are clearly less polar compounds compared to the steroids measured in this study, they

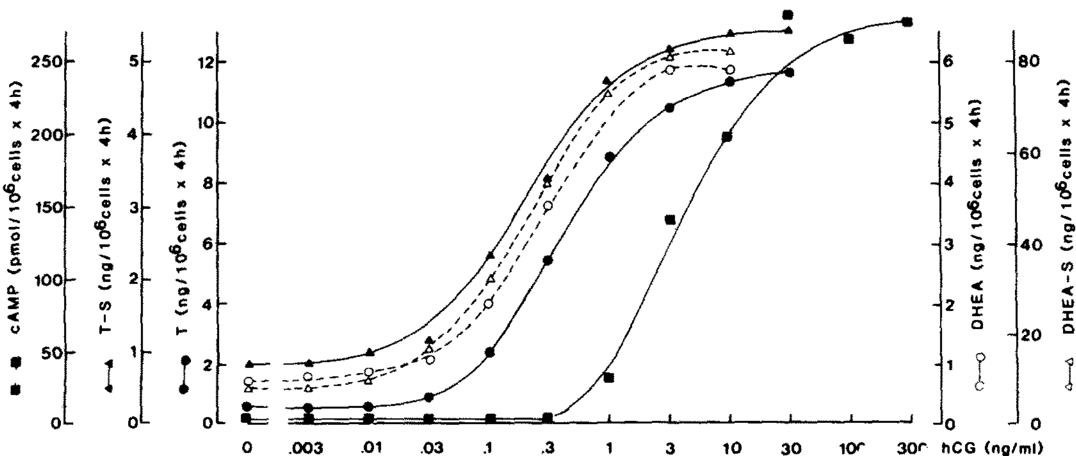

Fig. 5. hCG dose–response curves for T, T-S, DHEA, DHEA-S and cAMP production. Porcine testicular cells in culture were pre-incubated for 48 h in hormone-supplemented DME/F12-medium with a medium change at 24 h. The medium was then changed and the cells were treated with the hCG-concentrations indicated for 4 h. Each point represents the mean of duplicate incubations.

were excluded from the chromatographic fractions measured [2] and therefore did not disturb the quantitative measurements performed.

The dominance of sulphated steroid production compared to that of unconjugated steroids was already clearly evident at 4 h and was still more pronounced at 36 h of incubation. It can be concluded that at the time points when the concentrations of the unconjugated steroids were stable or decreasing, the rate of sulphate conjugation reached or even exceeded that of steroid biosynthesis. Only the highest concentrations of hCG used (10–100 ng/ml) were able to lead to a net accumulation of unconjugated C19 and C18 steroids in the incubation medium at 36 h.

The possible role of the hCG-induced desensitization phenomenon in the non-linearity of the early accumulation of the unconjugated steroids at 4–12 h was also studied. This non-linearity was not due to impairment in steroidogenic capacity nor to a major loss of LH/hCG receptors in the cells, because after 8 h incubation with hCG at 100 ng/ml, restimulation with hCG induced rapid T accumulation (Fig. 4A), which is in agreement with a previous study [18]. In addition, after 24 h incubation with hCG at 1 ng/ml, when T levels were decreasing (Fig. 4B), restimulation with hCG induced a rapid T response. It appears that a relatively long exposure (24–48 h) to high concentrations of hCG (1 ng/ml) is needed to effect a major decrease in hCG-receptor levels in porcine Leydig cell cultures [19].

Although no direct measurements of the enzyme activities of T synthetic pathways were made to reveal possible steroidogenic lesions following pregnenolone formation, the essentially temporally similar response patterns to hCG of all the unconjugated steroids measured suggests that the non-linearity in T-accumulation was not due to selective inhibition of any of these activities.

Taken together, our data show that accumulation of T and other unconjugated steroids in porcine testicular cell culture is largely dependent on concomitant sulphoconjugation.

Acknowledgements—We wish to thank Mrs Kaarina Malila, Mrs Liisa Ollanketo, Mrs Lea Sarvanko and Mrs Airi Vesala for their skillful technical assistance. This investigation was supported by the Research Council for Medicine of The Academy of Finland.

REFERENCES

1. Ruokonen A., Laatikainen T., Laitinen E. A. and Vihko R.: Free and sulfate-conjugated neutral steroids in human testis tissue. *Biochemistry* **11** (1972) 1411–1415.
2. Ruokonen A. and Vihko R.: Steroid metabolism in testis tissue: concentrations of unconjugated and sulfated neutral steroids in boar testis. *J. steroid Biochem.* **5** (1974) 33–38.
3. Baulieu E. E., Fabre-Jung I. and Huis in't Veld L. G.: Dehydroepiandrosterone sulfate: a secretory product of the boar testis. *Endocrinology* **81** (1967) 34–38.
4. Raeside J. I. and Howells G. A.: The isolation and identification of androstenediol sulfate from spermatic vein blood and testes of the boar. *Can. J. Biochem.* **49** (1971) 80–84.
5. Laatikainen T., Laitinen E. A. and Vihko R.: Secretion of neutral steroid sulfates by the human testis. *J. clin. Endocr. Metab.* **29** (1969) 219–224.
6. Claus R. and Hoffman B.: Oestrogens, compared to other steroids of testicular origin, in blood plasma of boars. *Acta endocr., Copenh.* **94** (1980) 404–411.
7. Setchell B. P., Laurie M. S., Flint A. P. F. and Heap R. P.: Transport of free and conjugated steroids from the boar testis in lymph, venous blood and rete testis fluid. *J. Endocr.* **96** (1983) 127–136.
8. Ruokonen A.: Steroid metabolism in testis tissue: the metabolism of pregnenolone, pregnenolone sulfate, dehydroepiandrosterone and dehydroepiandrosterone sulfate in human and boar testis *in vitro*. *J. steroid Biochem.* **9** (1978) 939–946.
9. Mather J. P., Saez J. M. and Haour F.: Primary cultures of Leydig cells from rat, mouse and pig: advantages of porcine cells for the studies of gonadotropin regulation of Leydig cell function. *Steroids* **38** (1981) 35–44.
10. Bernier M., Gibb W., Haour F., Collu R., Saez J. M. and Ducharme J.: Studies with purified immature porcine Leydig cells in primary culture. *Biol. Reprod.* **29** (1983) 1172–1178.
11. Mendelson C., Dufau M. and Catt K.: Gonadotropin binding and stimulation of cyclic adenosine 3′:5′-monophosphate and testosterone production in isolated Leydig cells. *J. biol. Chem.* **250** (1975) 8818–8823.
12. Hammond G. L., Ahonen V. and Vihko R.: The radioimmunoassay of testosterone, 5α-dihydrotestosterone and their precursors in human testis, before and after estrogen treatment. *Int. J. Androl.* (Suppl 2) (1978) 391–399.
13. Leinonen P.: Estrone and estradiol concentrations in the testes and spermatic and peripheral venous blood of elderly men: the influence of estrogen treatment. *J. steroid Biochem.* **13** (1980) 737–742.
14. Ruokonen A., Lukkarinen O. and Vihko R.: Secretion of steroid sulfates from human testis and their response to a single intramuscular injection of 5000 IU hCG. *J. steroid Biochem.* **14** (1981) 1357–1360.
15. Bolton N. J., Ruokonen A. O. and Vihko R. K.: Stimulation of the synthesis of steroids and steroid sulphates in human testicular tissue *in vitro* by hCG and by 8-bromo-cyclic AMP. *J. steroid Biochem.* **22** (1985) In press.
16. Catt K. J. and Dufau M. L.: Spare gonadotropin receptors in rat Leydig cells. *Nature* **244** (1973) 219–221.
17. Raeside J. I. and Renaud R. L.: Estrogen and androgen production by purified Leydig cells of mature boars. *Biol. Reprod.* **28** (1983) 727–734.
18. Saez J. M., Benahmed M., Reventos J., Bommelaer M. C., Mombrial C. and Haour F.: Hormonal regulation of pig Leydig cells in culture. *J. steroid Biochem.* **19** (1983) 375–384.
19. Mombrial F. C., Bommelaer M. C., Sanchez P. and Haour F.: Relationship between hCG receptors disappearance and steroid accumulation during long term hCG stimulation in porcine Leydig cell cultures. *J. Receptor Res.* (1984). In press.