Biochem. J. (1983) **216**, 747–752
Printed in Great Britain

Stimulation of cholesterol side-chain cleavage by a luteinizing-hormone-releasing hormone (luliberin) agonist (ICI 118630) in rat Leydig cells

Mark H. F. SULLIVAN and Brian A. COOKE
Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, Rowland Hill Street, London NW3 2PF, U.K.

(*Received 25 July 1983/Accepted 30 August 1983*)

The action of a luliberin (luteinizing-hormone-releasing hormone) agonist (ICI 118630) and lutropin (luteinizing hormone) on the activity of the cytochrome *P*-450 cholesterol side-chain cleavage enzyme in rat Leydig cells has been investigated. This has been carried out by studying the metabolism of exogenous (22*R*)-22- and 25-hydroxycholesterol to testosterone. It was found that both hydroxycholesterols increased testosterone production to higher levels than achieved by lutropin alone. Addition of luliberin agonist but not lutropin was found to increase further the metabolism of the hydroxycholesterol to testosterone; this occurred in the presence of saturating and subsaturating levels of the hydroxycholesterols. This effect of luliberin agonist was potentiated in the presence of lutropin. The protein synthesis inhibitor, cycloheximide, inhibited the luliberin agonist-induced stimulation of the hydroxycholesterol metabolism. At low calcium levels (1.1 μM), testosterone production was increased by addition of (22*R*)-22-hydroxycholesterol but the luliberin agonist effect was negated. The calmodulin inhibitor trifluoperazine inhibited (22*R*)-22-hydroxycholesterol-stimulated steroidogenesis and negated the luliberin agonist effect. These results indicate that luliberin agonist specifically increases the synthesis of the cytochrome *P*-450 cholesterol side-chain cleavage enzyme in rat testis Leydig cells.

It has recently been established that luliberin ('luteinizing hormone-releasing hormone') analogues can directly stimulate rat testis Leydig cell steroidogenesis *in vitro* and also potentiate lutropin ('luteinizing hormone') action on steroidogenesis (Hunter *et al.*, 1982; Sharpe & Cooper, 1982). The role of cyclic AMP and Ca^{2+} has been investigated (M. H. F. Sullivan & B. A. Cooke, unpublished work) and it has been found that although cyclic AMP may have a permissive effect the primary second messenger is probably Ca^{2+}. The present study was undertaken to investigate further the locus of action of the luliberin agonist on steroidogenesis. This has been carried out by determining the effect of the luliberin agonist and lutropin on the cholesterol side-chain cleavage enzyme in the presence of exogenous (22*R*)-22- and 25-hydroxycholesterol. The effects of trifluoperazine, a calmodulin inhibitor, and cycloheximide, a protein synthesis inhibitor, have also been investigated.

Materials and methods

Rat Leydig cells were prepared, purified and cultured as reported previously (Aldred & Cooke, 1982; Hunter *et al.*, 1982). Lutropin (in medium; final concentration 100 ng/ml), hydroxycholesterol or pregnenolone (in ethanol; 10 μl/ml of medium) were added as appropriate after 2 h of culture, unless stated otherwise in the text, and incubations were terminated with $HClO_4$ (0.5 M final concentration) after a further 2 h (4 h in total). Luliberin agonist was present, where stated, throughout the culture period (4 h). Samples were stored at −20°C. Immediately before assay the samples were thawed and neutralized with K_3PO_4 (0.23 M-final concentration). Testosterone was measured by the method of Verjans *et al.* (1973), and cyclic AMP was determined by the method of Steiner *et al.* (1972), modified by Harper & Brooker (1975).

The luliberin agonist used was the ICI analogue compound ICI 118630 {[D-Ser(t-butyl)^6,aza-Gly^{10}]luliberin}, and trifluoperazine was a gift from ICI PLC. Pregnenolone, cycloheximide, 25- and (22*R*)-22-hydroxycholesterol were obtained from Sigma Chemical Co. (London).

Results

It was found (Fig. 1) that addition of 25-hydroxycholesterol to the rat Leydig cells increased

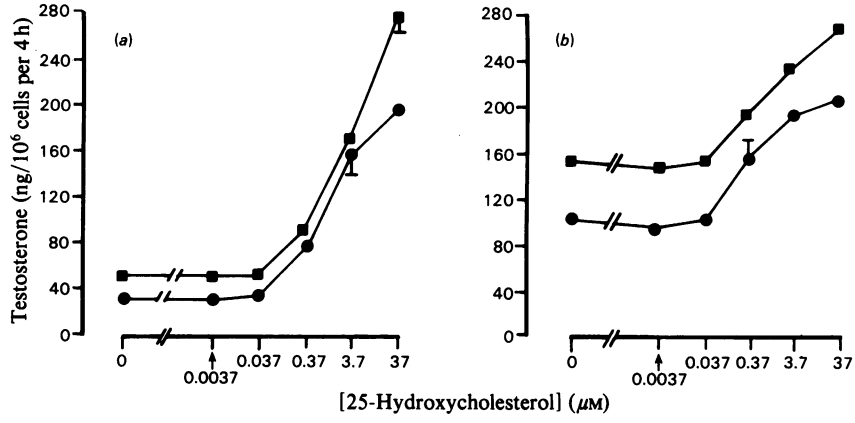

Fig. 1. *Testosterone production in response to various concentrations of (25R)-25-hydroxycholesterol*
Luliberin agonist was present for 4h and lutropin and 25-hydroxycholesterol for the final 2h of incubation as indicated (●, without luliberin; ■, with luliberin). In the absence of lutropin, luliberin agonist increased testosterone production ($P < 0.05$) except in the presence of 0.37 and 3.7 μM-25-hydroxycholesterol. In the presence of lutropin, luliberin agonist increased steroidogenesis ($P < 0.05$) at all 25-hydroxycholesterol concentrations. All points are means ± S.E.M. ($n = 3$); where no error bar is shown, the standard error was within the dimensions of the point. (*a*) In the absence of lutropin; (*b*) in the presence of lutropin.

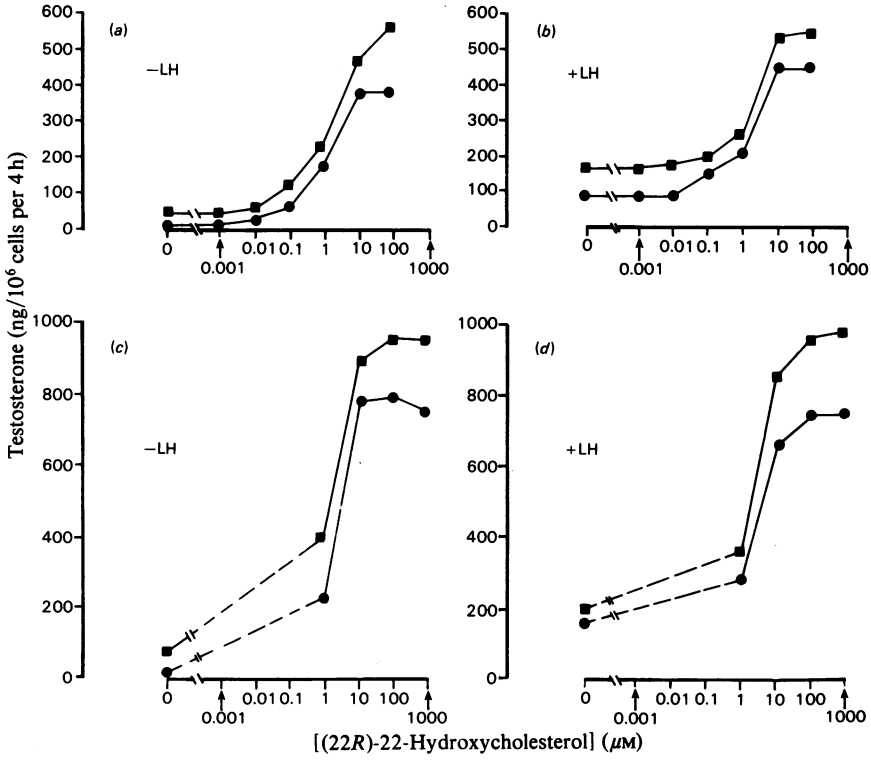

Fig. 2. *Testosterone production in response to various concentrations of (22R)-22-hydroxycholesterol*
Luliberin agonist was present for 4h; (22*R*)-22-hydroxycholesterol and lutropin (LH) were present as appropriate for the final 2h of incubation, where indicated. ●, In the absence of luliberin; ■, in the presence of luliberin. Luliberin agonist significantly increased testosterone production ($P < 0.05$) at all concentrations of (22*R*)-22-hydroxycholesterol except for (*a*) 1 μM and (*b*) 0.1 μM. All points are means of triplicate incubations. All standard errors are within 10% of means. (*a*) + (*b*) and (*c*) + (*d*) are the results from two separate experiments.

the amounts of testosterone formed above those obtained with maximally stimulatory amounts of lutropin. The amounts of 25-hydroxycholesterol metabolized to testosterone were not increased by lutropin. In fact, with concentrations greater than 0.37 μM-25-hydroxycholesterol the lutropin-stimulated testosterone production in the presence of the hydroxycholesterol decreased compared with 25-hydroxycholesterol alone; the lutropin-induced stimulation of testosterone production above basal decreased from 69.5 ± 3.8 ng/10^6 cells per 4h over a range of 0–0.37 μM-25-hydroxycholesterol to 38.4 ± 6.2 ng with 3.7 μM-25-hydroxycholesterol and to 13.2 ± 5.4 ng with 37 μM-25-hydroxycholesterol. In contrast, luliberin agonist markedly stimulated more 25-hydroxycholesterol metabolism to testosterone with concentrations of the former greater than 0.37 μM; the increase due to luliberin agonist was 16.6 ± 1.2 ng of testosterone/10^6 cells per 4h with no hydroxycholesterol present, and increased to 83.5 ± 7.9 ng of testosterone/10^6 cells per 4h in the presence of 37 μM-25-hydroxycholesterol. The metabolizing enzymes were apparently not saturated even in the presence of 37 μM-25-hydroxycholesterol. In agreement with previous results (Hunter *et al.*, 1982; Sharpe & Cooper, 1982) luliberin agonist enhanced the lutropin-stimulated testosterone production (Fig. 1).

Because more testosterone is formed from (22*R*)-22-hydroxycholesterol than the 25-hydroxy compound the metabolism of the former was also investigated. Higher concentrations were also used so that the metabolizing enzyme became saturated. The results presented in Figs. 2(*a*) and 2(*c*) demonstrate that in the absence of lutropin, the luliberin agonist significantly (see the legend to Fig. 2) increased the amounts of (22*R*)-22-hydroxycholesterol metabolized to testosterone, especially with concentrations greater than 1 μM. Saturation of the enzyme with substrate was reached in the presence of luliberin agonist with 1000 μM-(22*R*)-22-hydroxycholesterol. When lutropin and luliberin agonist were added to the Leydig cells there was also an enhancement of the hydroxycholesterol metabolism, especially in the experiment shown in Fig. 2(*d*) with amounts greater than 1 μM. Again, saturation of the enzyme was reached. In the latter experiment cyclic AMP levels were measured. No effect of the (22*R*)-22-hydroxy-steroid could be detected on the amounts of cyclic AMP formed (Table 1).

In these experiments the Leydig cells were incubated with the luliberin agonist for a total of 4h and the hydroxycholesterol and lutropin were present during the last 2h of incubation. This was done because the luliberin agonist has a slow action on steroidogenesis compared with the rapid effect of lutropin (M. H. F. Sullivan & B. A. Cooke, unpublished work). In order to check that the apparent lack of effect of lutropin on (22*R*)-22-hydroxycholesterol metabolism was not due to the shorter incubation time an additional experiment was carried out in which lutropin and/or luliberin agonist was present for 4h. The (22*R*)-22-hydroxycholesterol (0.1, 1.0 and 10.0 μM) was added after 2h as before. Luliberin agonist increased the production of testosterone as the concentration of (22*R*)-22-hydroxycholesterol was increased (from 23.4ng of testosterone/10^6 cells per 4h with no hydroxy-steroid to 101.2ng of testosterone/10^6 cells per 4h with 10 μM-hydroxy-steroid) ($P < 0.05$) (results taken from Table 2). In contrast, and in agreement with the previous results, the stimulation by lutropin compared with hydroxy-steroid alone remained constant (61.9 ± 5.2 ng/10^6 cells per 4h over the whole concentration range). Lutropin plus luliberin agonist potentiated steroidogenesis further ($P < 0.05$) compared with the effects of lutropin and luliberin agonist when present separately, e.g. with 10 μM-(22*R*)-22-hydroxycholesterol the testosterone production due to lutropin plus luliberin agonist was 326.4ng compared with 101.2 and 52.5ng/10^6 cells per 4h for luliberin agonist and lutropin respectively alone (Table 2).

The addition of the protein synthesis inhibitor cycloheximide (0.1 mM) did not inhibit the metabolism of (22*R*)-22-hydroxycholesterol but the effect of the luliberin agonist was inhibited (Table

Table 1. *Cyclic AMP levels in the presence of lutropin, luliberin agonist, luliberin agonist plus lutropin and various concentrations of (22R)-22-hydroxycholesterol*

Cells were treated as in Figs. 2(*c*) and 2(*d*). Results are means ± S.E.M. ($n = 3$). Abbreviation used: b.d., below detection.

(22*R*)-22-Hydroxycholesterol (μM) ...	Cyclic AMP (pmol/10^6 cells per 4h)				
	0*	1	10	100	1000
Control	0.9±0.2	0.6±0.5	0.6±0.2	2.8±0.4	0.4±0.3
Luliberin agonist	b.d.	b.d.	b.d.	b.d.	b.d.
Lutropin	638±40	689±19	606±31	579±12	537±68
Lutropin + luliberin agonist	512±28	519±33	485±18	466±37	394±30

* Contained 10 μl of ethanol; (22*R*)-22-hydroxycholesterol was added in the same amount of this solvent.

Table 2. *Testosterone production in the presence of luliberin agonist, lutropin, lutropin plus luliberin agonist and various concentrations of (22R)-22-hydroxycholesterol*

Cells were incubated with luliberin agonist (0.1 μM) and lutropin (100 ng/ml) as stated in the text for 4 h. (22*R*)-22-Hydroxycholesterol was present for the final 2 h of incubation.

	Testosterone (ng/10^6 cells per 4 h)			
(22*R*)-22-Hydroxycholesterol (μM) ...	0**	0.1	1.0	10.0
Control	20.1 ± 0.6	75.2 ± 6.4	166.8 ± 7.2	340.8 ± 17.6
Luliberin agonist	43.5 ± 2.3	104.0 ± 6.2	208.0 ± 12.0	442.0 ± 18.4
Lutropin	69.4 ± 8.8*	140.4 ± 10.0*	240.8 ± 6.0	400.0 ± 6.4
Lutropin + luliberin agonist	189.1 ± 13.6	219.2 ± 9.6	523.2 ± 6.4	667.2 ± 31.2

* $P < 0.05$ for lutropin versus luliberin agonist; all luliberin agonist and lutropin results are higher ($P < 0.05$) than controls. All lutropin + luliberin agonist results are higher ($P < 0.001$) than controls. All results are means ± S.E.M. ($n = 3$).

** Contained 10 μl of ethanol; (22*R*)-22-hydroxycholesterol was added in the same amount of solvent.

Table 3. *Effect of various factors on (22R)-22-hydroxycholesterol metabolism*

Cells were incubated with cycloheximide, low Ca^{2+} or trifluoperazine (TFP) for the complete incubation period. All results are means ± S.E.M. ($n = 3$).

	Testosterone (ng/10^6 cells per 4 h)			
	Control	Cycloheximide (0.1 mM)	Ca^{2+} (1.1 μM)	TFP (50 μM)
(22*R*)-22-Hydroxycholesterol	415 ± 28	458 ± 23	685 ± 58	77 ± 6
(22*R*)-22-Hydroxycholesterol + luliberin agonist	578 ± 41*	513 ± 43	734 ± 54	75 ± 4

* $P < 0.01$ (+luliberin versus − luliberin).

Table 4. *Effect of trifluoperazine on (22R)-22-hydroxycholesterol metabolism in the presence and in the absence of luliberin analogue*

Trifluoperazine (TFP) was present throughout the incubation period. Results are means ± S.E.M. ($n = 3$). The basal solution contained no (22*R*)-22-hydroxy-steroid.

	Testosterone (ng/10^6 cells per 4 h)					
TFP (μM) ...	0	0.1	1.0	10	100	Basal
(22*R*)-22-Hydroxycholesterol	518 ± 32	568 ± 24	549 ± 6	296 ± 15	0	15.7 ± 0.9
(22*R*)-22-Hydroxycholesterol + luliberin agonist	787 ± 51	772 ± 27	731 ± 70	390 ± 27	0	41.2 ± 0.7

3). Lowering the Ca^{2+} concentration from 2.5 mM to 1.1 μM in the medium also inhibited the effect of luliberin agonist, although an increase in (22*R*)-22-hydroxycholesterol-stimulated steroidogenesis was found (Table 3). The addition of the calmodulin inhibitor trifluoperazine had the same effect and also inhibited (22*R*)-22-hydroxycholesterol metabolism (Table 4). The ED_{50} (concentration required for 50% inhibition) for the latter was found to be 11 μM for both inhibition of luliberin agonist effects and metabolism of (22*R*)-22-hydroxycholesterol alone. This compares with 3 μM for inhibition of lutropin-stimulated testosterone production by trifluoperazine.

Substitution of 1.6 μM-pregnenolone (added in 10 μl of ethanol) for (22*R*)-22-hydroxycholesterol in the incubations also resulted in high testosterone production (562 ± 24 ng/10^6 cells per 4 h), but the addition of luliberin agonist had no effect on testosterone production (pregnenolone + luliberin agonist: 558 ± 16 ng/10^6 cells per 4 h).

Discussion

In the present study it has been demonstrated that the luliberin agonist increases (22*R*)-22- and 25-hydroxycholesterol metabolism to testosterone. At high concentrations of (22*R*)-22-hydroxycholes-

terol, luliberin agonist plus lutropin also increased testosterone production to more than that obtained with the hydroxy-steroid alone. In contrast lutropin did not increase metabolism of the hydroxysteroids; this is in agreement with the results of Taoff *et al.* (1982), who found that the metabolism of 25-hydroxycholesterol by dispersed rat luteal cells was not influenced by addition of lutropin. The present results therefore indicate a specific action of the luliberin agonist on steroidogenesis that is different from that of lutropin. Our previous work also indicated different modes of action, i.e. luliberin agonist stimulated steroidogenesis primarily via changes in Ca^{2+} flux (M. H. F. Sullivan & B. A. Cooke, unpublished work), whereas cyclic AMP is a second messenger for lutropin (for review, see Cooke *et al.*, 1981).

The hydroxy-steroids were used in the present study because it was previously shown that they could increase steroidogenesis both in the ovary and in the testis in dispersed cells and isolated mitochondria and that more steroid was formed than from added cholesterol (Bakker *et al.*, 1979; Taoff *et al.*, 1982). Also, in agreement with these studies, it was found that the amount of steroid formed in the presence of the hydroxy-steroids was higher than could be achieved with lutropin alone. As Taoff *et al.* (1982) pointed out, these results suggest that not enough endogenous substrate is available and/or cannot be mobilized to achieve the highest rates of steroidogenesis possible. Taoff *et al.* (1982) found that there was a direct relationship between cytochrome *P*-450 content and 25-hydroxycholesterol-supported steroid production in the ovary. The evidence obtained by Bakker *et al.* (1979) using [^{3}H]hydroxycholesterol suggests that added hydroxycholesterols are directly metabolized by testis mitochondria rather than these compounds increasing metabolism of endogenous cholesterol. The metabolism of the hydroxysteroids with saturating levels of substrate in the present study is, therefore, probably a reflection of the amounts of the cytochrome *P*-450 cholesterol side-chain cleavage enzyme present in the Leydig cell mitochondria and the results indicate that luliberin analogues increase the synthesis of the cytochrome *P*-450 enzyme. Support for this was obtained by the inhibition obtained of the luliberin agonist effect on (22*R*)-22-hydroxycholesterol metabolism by the protein synthesis inhibitor cycloheximide. This compound had, as expected, no effect on the metabolism of (22*R*)-22-hydroxycholesterol alone (Taoff *et al.*, 1982). Further evidence for the specific effect of luliberin agonist on the cholesterol side-chain-cleavage enzyme in the present study was obtained by the finding that luberin agonist had no effect on the conversion of exogenous pregnenolone into testosterone. The findings with cycloheximide indicated that lutropin-stimulated steroidogenesis is sensitive to protein synthesis inhibitors at a point in the pathway between cyclic AMP and the mitochondrion (Cooke *et al.*, 1975).

Sharpe & Fraser (1981) have found that human choriogonadotropin increases the formation of a 'luliberin-like' substance in the testes, possibly from the Sertoli cells. The results reported in the present paper suggest that lutropin does not only have direct stimulatory effects on steroidogenesis (Cooke *et al.*, 1981), but also indirect effects via the locally produced factor which increases the levels of cytochrome *P*450 in the Leydig cells. Purvis *et al.* (1973) have reported that human choriogonadotropin increased cytochrome *P*-450 in rat testis mitochondria from intact rats; the present paper indicates a possible mechanism for this effect.

Hall *et al.* (1981) have demonstrated that the calmodulin inhibitor trifluoperazine prevented the stimulation of testosterone synthesis in rat testis Leydig cells. They showed that increased transport of cholesterol to mitochondria produced by lutropin and the stimulation of cholesterol side-chain cleavage was inhibited by this drug. In the present study both the luliberin agonist-induced and the unstimulated metabolism of (22*R*)-22-hydroxycholesterol was inhibited by trifluoperazine with amounts similar to those used by Hall *et al.* (1981). This is in accordance with the proposed role of Ca^{2+} in cholesterol side-chain cleavage.

In conclusion, the results of the present study indicate that the luliberin agonist, in contrast with lutropin, increases the metabolism of (22*R*)-22- and 25-hydroxycholesterol in rat testis Leydig cells to testosterone. This may well result from an increased synthesis of the mitochondrial cytochrome *P*-450 enzyme.

We acknowledge the financial support of the S.E.R.C. and ICI p.l.c. We thank Dr. B. J. A. Furr (ICI p.l.c.) for the gift of the luliberin agonist. We are grateful to the National Pituitary Agency, Bethesda, MD, U.S.A., for the gift of ovine lutropin. M. H. F. S. is a C.A.S.E. Student with the S.E.R.C. and ICI p.l.c.

References

Aldred, L. F. & Cooke, B. A. (1982) *Int. J. Androl.* **5**, 191–195

Bakker, C. P., van der Plank/van Winsen, M. P. I. & van der Molen, H. J. (1979) *Biochim. Biophys. Acta* **584**, 94–103

Cooke, B. A., Janszen, F. H. A., Clotscher, W. F. & van der Molen, H. J. (1975) *Biochem. J.* **150**, 413–418

Cooke, B. A., Dix, C. J., Magee-Brown, R., Janszen, F. H. A. & van der Molen, H. J. (1981) *Adv. Cyclic Nucleotide Res.* **14**, 593–609

Hall, P. F., Osawa, S. & Mrotek, J. (1981) *Endocrinology* **109**, 1677–1682
Harper, T. F. & Brooker, G. (1975) *J. Cyclic Nucleotide Res.* **1**, 207–218
Hunter, M. G., Sullivan, M. H. F., Dix, C. J., Aldred, L. F. & Cooke, B. A. (1982) *Mol. Cell. Endocrinol.* **27**, 31–44
Purvis, J. L., Canick, J. A., Rosenbaum, J. H., Hologgitas, J. & Latif, S. A. (1973) *Arch. Biochem. Biophys.* **159**, 32–38
Sharpe, R. M. & Cooper, I. (1982) *Mol. Cell Endocrinol.* **26**, 141–150
Sharpe, R. M. & Fraser, H. M. (1981) *Nature (London)* **287**, 642–643
Steiner, A. L., Parker, C. W. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1106–1113
Taoff, M. E., Schleyer, H. & Strauss, J. F. (1982) *Endocrinology* **111**, 1785–1790
Verjans, M. L., Cooke, B. A., De Jong, C. M. M. & van der Molen, H. J. (1973) *J. Steroid Biochem.* **4**, 665–676