Effects of calmodulin and lipoxygenase inhibitors on LH (lutropin)- and LHRH (luliberin)-agonist-stimulated steroidogenesis in rat Leydig cells

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The results of this study, carried out with purified rat Leydig cells, indicate that there are no major differences in the stimulating effects of lutropin (LH) and luliberin (LHRH) agonists on steroidogenesis via mechanisms that are dependent on Ca^{2+} . This was demonstrated by using inhibitors of (1) calmodulin and (2) the lipoxygenase pathways of arachidonic acid metabolism. All three calmodulin inhibitors used (calmidazolium, trifluoperazine and chlorpromazine) were shown to block LH- and LHRH-agonist-stimulated steroidogenesis. This probably occurred at the step of cholesterol transport to the mitochondria. Similarly, three lipoxygenase inhibitors (nordihydroguaiaretic acid, BW755c and benoxaprofen), inhibited both LH- and LHRH-agonist-stimulated steroidogenesis. The amounts of the inhibitors required were similar for LH- and LHRH-agonist-stimulated steroidogenesis. Steroidogenesis stimulated by the Ca^{2+} ionophore A23187 was also inhibited, but higher concentrations of the inhibitors were required. Indomethacin (a cyclo-oxygenase inhibitor) increased LHRH-agonist-stimulated steroidogenesis; this is consistent with the role of the products of arachidonic acid metabolism via the alternative, lipoxygenase, pathway. The potentiation of LH-stimulated testosterone production by LHRH agonist was unaffected by indomethacin or by lipoxygenase inhibitors at concentrations that inhibited LH-stimulated testosterone production by 75–100%. It was not possible to eliminate a role of calmodulin in modulating the potentiation, although higher concentrations of the inhibitors were generally required to negate the potentiation than to inhibit LH- or LHRH-agonist-stimulated testosterone production.

INTRODUCTION

In addition to lutropin (LH), it has been shown that luliberin (LHRH) agonists can directly stimulate steroidogenesis in the gonads [Hunter *et al.*, 1982; Sharpe & Cooper, 1982; Clark, 1982; Cooke & Sullivan, 1985 (review)]. In the testis Leydig cell it is known that cyclic AMP is involved in the action of LH (Cooke *et al.*, 1981), but there is no evidence for the involvement of this nucleotide in LHRH-agonist action (Sullivan & Cooke, 1984a; Molcho *et al.*, 1984a). Ca^{2+} has been shown to be required for both LH (Janszen *et al.*, 1976) and LHRH-agonist action (Sullivan & Cooke, 1984a). The involvement of Ca^{2+} in the potentiation of LH-stimulated testosterone production by LHRH analogues is, however, less clear (Sullivan & Cooke, 1984b). It was the purpose of the present study to investigate further the roles of Ca^{2+} in the actions of LH and LHRH agonists in purified rat testis Leydig cells.

It is known that in other systems the enzymes involved in the release (Craven *et al.*, 1981) and metabolism (Murphy *et al.*, 1979; Jakschik & Lee, 1980; Parker & Aykent, 1982; Rubin *et al.*, 1982) of arachidonic acid from phospholipids are Ca^{2+} -dependent. Recent work in our laboratory has shown that products of the lipoxygenase, but not the cyclo-oxygenase pathway of

arachidonic acid metabolism, are involved in LH-stimulated steroidogenesis (Dix *et al.*, 1984). We have therefore compared the differential effects of inhibitors of lipoxygenase activity on the stimulation of steroidogenesis by LH, LHRH agonist and the Ca^{2+} ionophore A23187. In addition, the effects of various inhibitors of the mediator of Ca^{2+} action, calmodulin, have been determined.

MATERIALS AND METHODS

Purified rat Leydig cells were prepared, purified and cultured as previously described (Aldred & Cooke, 1982; Hunter *et al.*, 1982). The purified Leydig cells were plated out at 50000–100000 cells/ml in Costar wells, and incubated for the times shown in the text. LHRH agonist ICI 118630 (a gift from ICI, Alderley Edge, Macclesfield, Cheshire, U.K.) and LH were present at the appropriate concentrations throughout the experiments.

IBMX (a phosphodiesterase inhibitor) (Aldrich, Gillingham, Dorset, U.K.), was present where stated at a concentration of 0.5 mM throughout the incubation. The inhibitors were added in maintenance medium (chlorpromazine, trifluoperazine), ethanol (NDGA) or dimethyl sulphoxide (calmidazolium, benoxaprofen and BW755c)

Abbreviations used: indomethacin, 1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-ylacetic acid; NDGA, nordihydroguaiaretic acid, 4,4'-(2,3-dimethylbutan-1,4-diyl)bis(benzene-1,2-diol); benoxaprofen, 2-(2-*p*-chlorophenylbenzoxazol-5-yl)propionic acid; BW755c, 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline hydrochloride; trifluoperazine, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-trifluoromethylphenothiazine; chlorpromazine, 2-chloro-10-(3-dimethylaminopropyl)phenothiazine; calmidazolium, 1-[bis(*p*-chlorophenyl)methyl]-3-[2,4-dichloro- β -(2,4-dichlorobenzyloxy)phenethyl]imidazolium chloride; LHRH, luliberin (luteinizing-hormone-releasing hormone); LH, lutropin (luteinizing hormone); IBMX, 1-isobutyl-3-methylxanthine; ICI 118630, Glp-His-Trp-Ser-Tyr-D-Ser(Bu^t)-Leu-Arg-Pro-azaGly-NH₂ (Glp is pyroglutamate, Bu^t is *t*-butyl).

Fig. 1. Effects of calmodulin inhibitors on LHRH-agonist-stimulated testosterone production

Cells were incubated for 4 h with and without LHRH agonist (100 nM) with the appropriate concentration of calmodulin antagonists. The appropriate basal levels of testosterone production were subtracted from the levels obtained in the presence of LHRH agonist with the various antagonist concentrations. The level obtained in this way with no inhibitor present was taken as the 100% level (left ordinate). The absolute levels of testosterone induced by LHRH agonist as calculated by the above method were 39.2 (calmidazolium, CMZ), 30.7 (trifluoperazine, TFP) and 36.4 (chlorpromazine, CPZ) (ng of testosterone/4 h per 10^6 cells). All data are means $\pm$ S.E.M. ($n = 3$) from three separate experiments.

Fig. 2. Effect of trifluoperazine on LH- or dibutyryl cyclic AMP-stimulated testosterone production in the presence or absence of LHRH agonist

Cells were preincubated for 2 h with (○, ●) and without (△, ▲) LHRH agonist (100 nM). LH (100 ng/ml) (○, △) or dibutyryl cyclic AMP (2 mM) (●, ▲) was added and the incubations were continued for a further 2 h. The appropriate concentrations of trifluoperazine were present throughout the 4 h period. All results are means $\pm$ S.E.M. ($n = 3$). The results obtained with LH relate to the left axis, and the results with dibutyryl cyclic AMP to the right axis.

at 10 μ l/well at 100 times the final concentration. The appropriate solvents were added to control incubations.

Incubations were stopped with HClO_4 (0.5 M final concn.) and frozen at -20°C . Samples were thawed immediately before assay and neutralized with K_3PO_4 (0.27 M final concn.). Testosterone was measured by the method of Verjans *et al.* (1973), and cyclic AMP by the procedure of Steiner *et al.* (1972) as modified by Harper & Brooker (1975).

Statistical analysis was performed by using Student's *t* test.

Trifluoperazine, chlorpromazine, indomethacin and NDGA were obtained from Sigma, and calmidazolium was obtained from Boehringer. Benoxaprofen (Opren) was a gift from Lilly Research Centre (Windlesham, Surrey, U.K.), and BW755c was a gift from Wellcome Research Laboratories (Beckenham, Kent, U.K.).

RESULTS

The effects of different amounts of the calmodulin inhibitors calmidazolium, trifluoperazine and chlorpromazine on LHRH-agonist-stimulated testosterone production by purified Leydig cells were determined (Fig. 1). ID_{50} (50% inhibitory dose) values were respectively 60 ± 12 nM, 10.1 ± 0.8 μ M and 80 ± 18 μ M. These compounds also inhibited LH-stimulated testosterone production; the ID_{50} values were 30 ± 7 nM, 3.3 ± 0.4 μ M and 6.0 ± 0.6 μ M respectively. LH-stimulated steroidogenesis was more sensitive than LHRH-agonist-stimulated steroidogenesis to trifluoperazine and chlorpromazine ($P < 0.01$), but not to calmidazolium ($P > 0.05$). Calmidazolium also inhibited ionophore-A23187-stimulated testosterone production; the ID_{50} was 220 ± 50 nM, which

is significantly ($P < 0.05$) higher than those obtained in the presence of LHRH agonist or LH.

In order to determine whether the site of action of the inhibitors was at the level of the plasma membrane and/or the pathways of steroidogenesis, the effects of calmidazolium, trifluoperazine and chlorpromazine on LH-stimulated cyclic AMP production were investigated. It was found that, in the presence of IBMX (0.5 mM), a dose-related inhibition was obtained; the ID_{50} values were 1.7 μ M, 8 μ M and 300 μ M respectively. However, it was also found that dibutyryl cyclic AMP-stimulated steroidogenesis was inhibited by the three inhibitors, with ID_{50} values of 32 nM, 3 μ M and 2 μ M respectively. Thus trifluoperazine inhibits both cyclic AMP production and the pathways of steroidogenesis stimulated by cyclic AMP with similar potency, but different amounts of calmidazolium and chlorpromazine were required.

It has been shown that LHRH agonist potentiates LH- or dibutyryl cyclic AMP-stimulated testosterone production by a mechanism independent of cyclic AMP (Hunter *et al.*, 1982). This potentiation was also negated by trifluoperazine (Fig. 2). Recalculation of the potentiation gave the following ID_{50} values: 13 ± 1.5 μ M (LH) and 17.0 ± 1.7 μ M (dibutyryl cyclic AMP). These are significantly ($P < 0.01$) higher than those obtained with LH and dibutyryl cyclic AMP alone (3 ± 1 μ M). Similar results were obtained for the effect of calmidazolium on LH $\pm$ LHRH-agonist-stimulated steroidogenesis (Table 1). The ID_{50} values were 30 ± 7 nM (LH), 63 ± 14 nM (LH + LHRH agonist) and 53 ± 5 nM (LHRH-agonist-induced potentiation).

Table 1. Effects of calmidazolium on LH ± LHRH-agonist-stimulated testosterone production

All results are means ± s.e.m. (n = 3). *P < 0.05 versus no calmidazolium.

[Calmidazolium] (M)	Testosterone (ng/4 h per 10 ⁶ cells)		
	(a) LH	(b) LH + LHRH agonist	(b-a)
0	206.1 ± 14.9	287.6 ± 7.1	81.5 ± 7.1
10 ⁻¹²	210.7 ± 14.7	262.1 ± 13.5	51.4 ± 13.5
10 ⁻¹¹	213.1 ± 15.4	265.8 ± 6.0	52.7 ± 6.0
10 ⁻¹⁰	202.8 ± 2.3	248.5 ± 16.5	45.7 ± 16.5
10 ⁻⁹	210.0 ± 21.9	275.1 ± 14.0	65.1 ± 14.0
10 ⁻⁸	143.1 ± 5.5*	190.9 ± 19.0*	47.8 ± 19.0
10 ⁻⁷	74.9 ± 4.4*	107.4 ± 2.2*	32.5 ± 2.2*
10 ⁻⁶	23.6 ± 4.6*	28.1 ± 5.0*	4.5 ± 5.0*
10 ⁻⁵	0*	0*	0*

Fig. 3. Effects of lipoxygenase inhibitors on LHRH-agonist-stimulated testosterone production

Cells were incubated with (●, ▲, ■) or without (○, △, □) LHRH agonist (100 nM) for 4 h. The inhibitors were present throughout the incubation at the concentrations shown. All results are means ± s.e.m. (n = 3). ●, ○, NDGA; ▲, △, BW755c; ■, □, benoxapofen.

Evidence has been obtained that the site of action of calmodulin in controlling steroidogenesis is the transport of cholesterol to the mitochondria (Hall *et al.*, 1981). It was therefore of interest to determine the effect of the calmodulin inhibitors on the metabolism of a hydroxy-cholesterol that is thought to by-pass this rate-limiting step (Bakker *et al.*, 1979). It was found that amounts of calmidazolium which inhibited LH-stimulated steroidogenesis had little or no effect on 22(R)-hydroxycholesterol metabolism (results not shown). Inhibition occurred at higher concentrations, and the ID₅₀ values for inhibition of 22(R)-hydroxycholesterol alone and with LHRH agonist were 310 ± 40 nM and 370 ± 50 nM respectively. The potentiation due to LHRH agonist also required high levels of calmidazolium (ID₅₀ = 540 ± 40 nM), as did the conversion of pregnenolone into testosterone (ID₅₀ = 460 ± 40 nM).

The lipoxygenase inhibitors NDGA, BW755c and benoxapofen were found to inhibit LHRH-agonist-

Fig. 4. Effects of indomethacin on LHRH-agonist-stimulated testosterone production

Cells were incubated with (●) or without (○) LHRH agonist (100 nM) for 4 h. Indomethacin was present at the concentrations shown throughout the incubation. All results are means ± s.e.m. (n = 3). *P < 0.05 versus no indomethacin; **P < 0.01 versus no indomethacin.

stimulated testosterone production, with ID₅₀ values of 2.4 ± 0.2 μM, 25 ± 5 μM and 30 ± 6 μM respectively (Fig. 3). These are similar to the ID₅₀ values obtained for LH-stimulated steroidogenesis (Dix *et al.*, 1984) (2.5 μM, 25 μM and 30 μM respectively). In contrast, higher concentrations of all three compounds were required to inhibit steroidogenesis stimulated by the Ca²⁺ ionophore A23187. The ID₅₀ values were 9.5 ± 0.3 μM, 120 ± 11 μM and 160 ± 15 μM respectively, all of which are significantly (P < 0.01) higher than the results obtained with LHRH agonist.

It was found that the cyclo-oxygenase inhibitor indomethacin enhanced LHRH-agonist-stimulated testosterone production (Fig. 4).

The potentiation of LH-stimulated steroidogenesis by LHRH agonist was not affected by BW755c and benoxapofen unless 40–50 μM concentrations of the inhibitors were used (Fig. 5). We have shown elsewhere (Sullivan & Cooke, 1983) that NDGA does not affect this

Fig. 5. Effects of BW755c or benoxaprofen on LH-stimulated testosterone production in the presence or absence of LHRH agonist

Cells were pre-incubated with (●, □) or without (■) LHRH agonist (100 nM) for 2 h. LH (100 ng/ml) was added, and the incubation continued for a further 2 h. BW755c (a) or benoxaprofen (b) was present at the concentrations shown throughout the 4 h incubation period. All results are means $\pm$ S.E.M. ($n = 3$).

potentiation. Furthermore, indomethacin had no effect on the potentiation of LH-stimulated steroidogenesis by LHRH agonist (Table 2).

Table 2. Effect of indomethacin on testosterone production

All results are means $\pm$ S.E.M. ($n = 3$). * $P < 0.05$ for LHRH agonist versus control.

	Testosterone (ng/4 h per 10 ⁶ cells)		
	Control	Indomethacin	
		30 μ M	300 μ M
LH	327 $\pm$ 11	298 $\pm$ 6	274 $\pm$ 13
LH + LHRH agonist	381 $\pm$ 10*	371 $\pm$ 4*	387 $\pm$ 15*

DISCUSSION

One of the main objects of carrying out this study was to determine if there were any essential differences between LH and LHRH agonist in the requirements of Ca^{2+} for the control of steroidogenesis. From the results obtained, all three inhibitors of one of the mediators of Ca^{2+} effects, calmodulin, inhibit LH- and LHRH-agonist-stimulated steroidogenesis, and the most specific calmodulin inhibitor, calmidazolium, had the same ID_{50} for both hormones. Similarly, the results obtained with the inhibitors of the lipoxygenase pathway of arachidonic acid metabolism on LHRH-agonist-stimulated steroidogenesis are similar to those previously obtained for LH (Dix *et al.*, 1984). Furthermore, the calmodulin/lipoxygenase-dependent steps are mainly before cholesterol side-chain cleavage for LHRH agonist; this agrees with results of previous studies on LH (Hall *et al.*, 1981; Dix *et al.*, 1984). These results indicate, therefore, that LHRH agonists are able to mimic the effects of LH that depend on Ca^{2+} . These agonists are, however, unable to stimulate the adenylate cyclase system (Clayton *et al.*, 1978; Benoist *et al.*, 1981; Sullivan & Cooke, 1984a).

The potentiation of LH-stimulated steroidogenesis by LHRH agonists indicates that these agonists also have an additional effect that is independent of LH. This is supported by the results of the present study, which showed that this potentiation was much less sensitive to calmodulin inhibitors and inhibitors of lipoxygenases compared with their effects on LH and LHRH agonist alone. We have also previously found that LHRH agonist, but not LH, will stimulate cholesterol-side-chain-cleavage enzyme activity (Sullivan & Cooke, 1983). The potentiation of LH- or 22(R)-hydroxycholesterol-stimulated testosterone production by LHRH agonist was also unaffected by NDGA and indomethacin, and relatively insensitive to benoxaprofen and BW755c.

The cyclo-oxygenase inhibitor indomethacin was shown to potentiate LHRH agonist effects on steroidogenesis. This may be done to a Mass Action effect on the diversion of the arachidonic acid from the cyclo-oxygenase pathway to the lipoxygenase pathway. The marked inhibition of LHRH-agonist-stimulated steroidogenesis by the lipoxygenase inhibitors and their relative potencies are very similar to the results obtained with LH (Dix *et al.*, 1984). In the latter study, no effect of the cyclo-oxygenase inhibitors was found, and Molcho *et al.* (1984a) have also reported that indomethacin inhibited LH- and LHRH-agonist-stimulated prostaglandin production in crude Leydig-cell preparations without affecting steroidogenesis.

In view of the Ca^{2+} -dependency of the lipoxygenase enzymes (Jakschik & Lee, 1980; Parker & Aykent, 1982; Rubin *et al.*, 1982), it is possible that LHRH agonist stimulates a Ca^{2+} flux, possibly by changes in phospholipid metabolism (Michell, 1975; Molcho *et al.*, 1984b), which then stimulates lipoxygenase activity. The lower sensitivity of ionophore-A23187-stimulated testosterone production to lipoxygenase inhibitors is consistent with a higher Ca^{2+} flux stimulating a higher level of lipoxygenase enzyme activity, and hence a higher level of testosterone production than with LHRH agonist under identical conditions (Sullivan & Cooke, 1984a).

In conclusion, the Ca^{2+} -dependent effects of LHRH agonist and LH on testosterone production appear to be modulated both by calmodulin and by lipoxygenase

products. However, the potentiation of LH-stimulated testosterone production by LHRH agonist is relatively insensitive to changes in Ca^{2+} and arachidonic acid metabolism.

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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
- Benoist, L., Le Dafniet, M., Rotsztein, W. H., Besson, J. & Duval, J. (1981) *Acta Endocrinol.* **97**, 329–337
- Clark, M. R. (1982) *Endocrinology* **110**, 146–152
- Clayton, R. N., Shakespear, R. A. & Marshall, J. C. (1978) *Mol. Cell. Endocrinol.* **11**, 63–78
- 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
- Cooke, B. A. & Sullivan, M. H. F. (1985) *Mol. Cell. Endocrinol.* **41**, 115–122
- Craven, P. A., Struder, R. K. & Derubertis, F. R. (1981) *J. Clin. Invest.* **68**, 722–732
- Dix, C. J., Habberfield, A. D., Sullivan, M. H. F. & Cooke, B. A. (1984) *Biochem. J.* **219**, 529–537
- Hall, P. F., Osawa, S. & Mrotek, J. (1981) *Endocrinology* **109**, 1677–1682
- Harper, J. 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
- Jakschik, B. A. & Lee, L. H. (1980) *Nature (London)* **287**, 51–52
- Janszen, F. H. A., Cooke, B. A., van Driel, M. J. A. & van der Molen, H. J. (1976) *Biochem. J.* **160**, 433–437
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Molcho, J., Eli, Y., Zakut, H. & Naor, Z. (1984a) *Endocrinology* **114**, 2382–2387
- Molcho, J., Zakut, H. & Naor, Z. (1984b) *Endocrinology* **114**, 1048–1050
- Murphy, R. C., Hammarstrom, S. & Samuelsson, B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4275–4279
- Parker, C. W. & Aykent, S. (1982) *Biochem. Biophys. Res. Commun.* **109**, 1011–1016
- Rubin, R. P., Kelly, K. L., Halenda, S. P. & Laychock, S. C. (1982) *Prostaglandins* **24**, 179–193
- Sharpe, R. M. & Cooper, I. (1982) *Mol. Cell. Endocrinol.* **26**, 141–150
- Steiner, A. L., Parker, C. W. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1106–1113
- Sullivan, M. H. F. & Cooke, B. A. (1983) *Biochem. J.* **216**, 747–752
- Sullivan, M. H. F. & Cooke, B. A. (1984a) *Biochem. J.* **218**, 621–624
- Sullivan, M. H. F. & Cooke, B. A. (1984b) *Mol. Cell. Endocrinol.* **34**, 17–22
- Verjans, H. L., Cooke, B. A., de Jong, F. H., de Jong, C. M. M. & van der Molen, H. J. (1973) *J. Steroid Biochem.* **4**, 665–676