The role of calcium in luteinizing hormone-releasing hormone agonist (ICI 118630)-stimulated steroidogenesis in rat Leydig cells

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The luteinizing hormone-releasing hormone (LHRH) agonist ICI 118630 was found to increase testosterone production in purified rat testis Leydig cells in a concentration- and time-dependent manner, but no consistent changes in cyclic AMP levels were detectable. The stimulation of steroidogenesis by LHRH agonist was found to be dependent on the concentration of Ca^{2+} in the incubation medium; at least 1 mM was required. The calcium ionophore A23187 mimicked the effects of the LHRH agonist on steroidogenesis, and addition of both compounds together did not further increase testosterone production. The calcium ionophore caused a small increase in cyclic AMP which was independent of the concentration of the ionophore and of the calcium concentrations. The evidence obtained in this study indicates that LHRH agonist-stimulated steroidogenesis in rat testis Leydig cells is primarily mediated by calcium and not cyclic AMP.

In addition to the established action of LHRH and its agonists on LH production by the anterior pituitary (Conn & Hazum, 1981; Conn *et al.*, 1981, 1982; Snyder & Bleasdale, 1982; Naor & Catt, 1981; Benoist *et al.*, 1981), these compounds have been shown to be capable of exerting direct effects on gonadal function (Humphrey *et al.*, 1977; Hsueh, 1979; reviewed by Bex & Corbin, 1981). Short-term (less than 24 h) stimulatory effects of LHRH agonists in female (Corbin & Bex, 1981; Clark, 1982; Jones & Hsueh, 1982) and male (Sharpe & Cooper, 1982; Hunter *et al.*, 1982; Sharpe *et al.*, 1982; Sullivan & Cooke, 1983, 1984) rat gonadal tissue have been found, but long-term (in excess of 24 h) effects are inhibitory on LH-stimulated steroidogenesis (Hunter *et al.*, 1982; Massicotte *et al.*, 1981).

Studies on the mechanism of action of LHRH and LHRH agonist on the pituitary indicate that the effects of these compounds are mediated by calcium and calmodulin (Conn *et al.*, 1981), but not by cyclic AMP (Benoist *et al.*, 1981). The release of LH is accompanied by changes in phospholipid (Snyder & Bleasdale, 1982) and arachidonic acid (Naor & Catt, 1981) metabolism. Little is known about the mechanisms of the stimulatory and inhibitory actions of LHRH agonists on gonadal steroidogenesis. The present study was, therefore,

undertaken to examine in more detail the role of calcium in testosterone production stimulated by LHRH agonist. The effects of the calcium ionophore A23187 have also been investigated.

Materials and methods

Rat Leydig cells were prepared and purified as described previously (Hunter *et al.*, 1982; Aldred & Cooke, 1982). All preparative procedures were carried out in media containing 2.0–2.5 mM-Ca^{2+}. Media depleted in Ca^{2+} were prepared by adding 2.8 mM-EGTA to complex the calcium. For Ca^{2+}– EGTA, the K_D (dissociation constant) is 1.3×10^{-7} M so the residual calcium in the media is, therefore, calculated to be 1.1 μM. $CaCl_2$ was added where stated to give the appropriate final calcium concentration.

Purified rat Leydig cells were plated out in Costar culture wells (10^5 cells/well unless otherwise stated) and the medium (Dulbecco's modified Eagles medium) (Gibco Europe) was added. LHRH agonist (ICI 118630) (dissolved in medium) and LH (LH-NIH-S20; 2.3 i.u. NIH-SI/mg) (dissolved in medium) were added as stated in the text. The calcium ionophore A23187 (Sigma) was dissolved in dimethyl sulphoxide at 100 times the final concentration and 10 μl/ml of medium was added. The same amount of dimethyl sulphoxide was added to the controls. 1-Methylisobutylxanthine (Sigma) was dissolved in medium at the final

concentration stated in the text. The cells were then incubated at 32°C. After 4 h, incubations were stopped with $HClO_4$ (final concentration 0.5 M) and frozen at $-20°C$ until neutralized with K_3PO_4 (final concentration 0.23 M) and assayed for testosterone (Verjans et al., 1973) and cyclic AMP (Steiner et al., 1972, as modified by Harper & Brooker, 1975). ICI 118630 [< Glu-His-Trp-Ser-Tyr-D-Ser(Buᵗ)-Leu-Arg-Pro-azaGly-NH₂] was a gift from ICI. All data are total (intracellular plus extracellular) levels of testosterone and cyclic AMP, unless stated otherwise. Initial levels were not subtracted.

To measure intra- and extracellular cyclic AMP and testosterone levels, the medium was removed from the cells, acidified with $HClO_4$ as above and frozen. $HClO_4$ (150 μl) was added to the cells, and this was followed by freezing. Before assay, the cellular extract was neutralized with 300 μl of K_3PO_4; the medium was treated as above.

Results

The rat Leydig cells responded to LHRH agonist in a concentration-dependent manner during a 4 h incubation (Table 1). Maximum steroidogenesis was achieved with 5×10^{-8} M–1×10^{-7} M agonist and represents a 5-fold increase. The ED_{50} (50% effective dose) is 8.3×10^{-8} M.

Investigation of the initial 2 h of incubation showed (Table 2) that cyclic AMP levels (intracellular and extracellular) are unaffected by LHRH agonist in the presence of the phosphodiesterase

inhibitor methylisobutylxanthine (added to prevent cyclic AMP metabolism). A significant extracellular increase $(P < 0.05)$ in testosterone production was detected after 40 min incubation, and the major increase occurred in the period from 120 min to 240 min incubation.

To determine the effect of the calcium ionophore A23187 on LHRH agonist-stimulated testosterone production, cells were incubated with different concentrations of the ionophore in the presence and absence of the agonist $(10^{-7}$ M) for 4 h (in 2.5 mM-calcium). The results obtained (Fig. 1a) show that the ionophore itself stimulated testosterone production and that this occurred in a concentration-dependent manner. LHRH agonist had little or no additional effect in the presence of

Table 1. *Response of rat Leydig cells to various concentrations of LHRH agonist*

LHRH agonist was present at the concentrations shown. All data are means ± s.e.m. $(n = 3)$: $^*P < 0.05$ compared with '0'.

[LHRH agonist] (M)	Testosterone (ng/10^6 cells per 4 h)
0	9.5 ± 0.8
10^{-11}	9.9 ± 0.4
10^{-10}	12.5 ± 0.2*
10^{-9}	32.6 ± 1.0*
5×10^{-9}	43.9 ± 1.8*
10^{-8}	48.4 ± 1.0*
5×10^{-8}	45.0 ± 1.0*
10^{-7}	44.0 ± 0.9*
10^{-6}	37.1 ± 0.9*

Table 2. *Time response of testosterone and cyclic AMP levels to LHRH agonist*

All results are means ± s.e.m. $(n = 3)$. LHRH agonist $(10^{-7}$ M) was present throughout the incubations as indicated. Methylisobutylxanthine (0.5 mM) was present throughout. Cell concentrations were 240 000 cells/0.5 ml for 5–40 min, 240 000 cells/ml for 60–120 min, and 120 000 cells/ml for 240 min. $^*P < 0.05$ of LHRH agonist over control.

Time (min)	Source Cellular	Testosterone (ng/10^6 cells)		Cyclic AMP (pmol/10^6 cells)	
0	+ medium ...	0.75 ± 0.02		4.0 ± 0.3	
		Control	+ LHRH agonist	Control	+ LHRH agonist
5	Cellular	0.26 ± 0.01	0.25 ± 0.01	1.6 ± 0.2	1.7 ± 0.2
	Medium	0.94 ± 0.03	0.88 ± 0.02	2.1 ± 0.1	2.1 ± 0.1
10	Cellular	0.26 ± 0.01	0.27 ± 0.01	2.6 ± 0.4	3.2 ± 0.2
	Medium	0.94 ± 0.02	0.92 ± 0.02	2.0 ± 0.1	2.0 ± 0.3
20	Cellular	0.28 ± 0.02	0.29 ± 0.01	3.2 ± 0.2	3.3 ± 0.1
	Medium	1.06 ± 0.05	1.10 ± 0.02	1.9 ± 0.2	1.6 ± 0.1
40	Cellular	0.34 ± 0.01	0.38 ± 0.02	4.1 ± 0.5	3.8 ± 0.2
	Medium	1.64 ± 0.02	1.85 ± 0.03*	1.6 ± 0.2	1.7 ± 0.1
60	Cellular	0.29 ± 0.01	0.33 ± 0.02	4.0 ± 0.3	4.0 ± 0.2
	Medium	2.30 ± 0.05	2.66 ± 0.07*	1.9 ± 0.1	1.9 ± 0.1
90	Cellular	0.36 ± 0.02	0.57 ± 0.02*	3.6 ± 0.2	3.9 ± 0.2
	Medium	2.93 ± 0.06	4.32 ± 0.06*	2.6 ± 0.2	2.0 ± 0.1
120	Cellular	0.37 ± 0.02	0.72 ± 0.04*	3.8 ± 0.3	4.4 ± 0.4
	Medium	4.03 ± 0.10	7.96 ± 0.24*	2.4 ± 0.3	2.3 ± 0.2
240	Cellular	0.63 ± 0.05	1.69 ± 0.12*	3.4 ± 0.2	4.2 ± 0.4
	Medium	21.97 ± 0.79	87.64 ± 5.22*	5.8 ± 0.4	4.6 ± 0.4

0.2–1.0 µM-A23187. At the two highest concentrations of ionophore, the LHRH agonist decreased testosterone production compared with the respective controls ($P < 0.01$). The ionophore A23187 alone (0.8–1.9 µM) was more potent than LHRH agonist alone (10^{-7}M) in stimulating testosterone production.

The addition of the phosphodiesterase inhibitor methylisobutylxanthine was found to negate the effects of ionophore A23187: control 17.0 ± 0.2; + methylisobutylxanthine 17.4 ± 0.5; + A23187 37.2 ± 0.8; + A23187 + methylisobutylxanthine 17.5 ± 1.0 [all results are ng of testosterone/10^6 cells per 4 h and are means $\pm$ s.e.m. ($n = 3$)].

The ionophore (0.1 µM) with and without the LHRH agonist gave a small increase in cyclic AMP levels which decreased with higher concentrations of the ionophore. These changes in cyclic AMP did not correlate with the changes in testosterone, particularly in the absence or with small amounts of the ionophore (Fig. 1b), as determined by regression analysis (−LHRH $r = 0.51$, +LHRH $r = 0.20$).

The effect of changing the calcium concentration in the incubation medium in the presence and absence of the ionophore and LHRH agonist was investigated (Fig. 2). LHRH agonist and ionophore A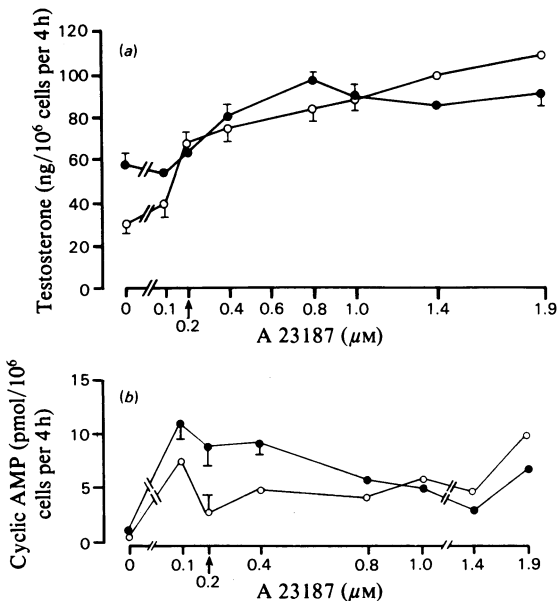23187 were added as before at the beginning of the 4 h incubation period. Basal testosterone production was unaffected by different calcium concentrations (Fig. 2a). LHRH agonist (10^{-7}M) stimulated testosterone production 2–3-fold ($P < 0.05$) except at the lowest (1.1 µM) calcium concentration. The calcium ionophore A23187 significantly increased ($P < 0.01$) testosterone production in 1–10 mM-calcium; maximum testosterone production was obtained with 2.5 mM-calcium. A small further stimulation was caused by the combination of LHRH agonist and ionophore A23187 at 1.1 µM- and 1 mM-calcium ($P < 0.05$) but not at higher calcium levels compared with the ionophore alone. With 10 mM-calcium, the ionophore effect (in the presence or absence of LHRH agonist) was the same as the effect of LHRH agonist alone.

Cyclic AMP levels were increased by the ionophore in the presence of 1.1 µM–10 mM-Ca^{2+} (Fig. 2b) but the LHRH agonist with and without the ionophore with different concentrations of Ca^{2+} had no detectable effect. The levels of cyclic AMP, particularly with low and high amounts of Ca^{2+}, did not correlate with the changes in testosterone production (Fig. 2).
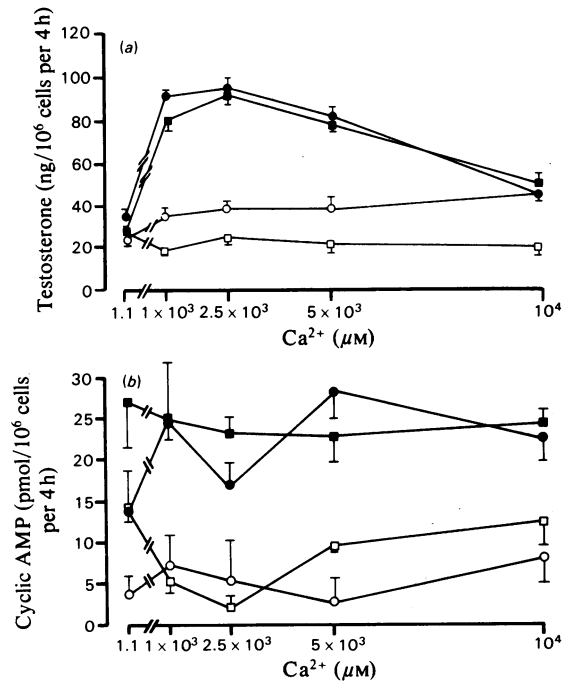

Fig. 1. *Response curves to ionophore A23187 (0–1.9 µM) for testosterone (a) and cyclic AMP (b) production in the presence (●) and absence (○) of LHRH agonist (10^{-7}M) during 4 h incubation*

All values are means $\pm$ s.e.m. for triplicate cultures. Ionophore was added in dimethyl sulphoxide at 10 µl/ml of medium.

Fig. 2. *Response curves to calcium (1.1 µM–10 mM) for testosterone (a) and cyclic AMP (b) production during a 4 h incubation period*

□, Basal; ○, LHRH agonist (10^{-6}M) present; ■, A23187 (1.9 µM) present; ●, A23187 and LHRH agonist present. All points are means $\pm$ s.e.m. for triplicate cultures.

The effect of A23187 on the viability of the Leydig cells was assessed by diaphorase histochemistry (Aldred & Cooke, 1983); only damaged cells which take up NADH from the medium are stained by this technique. LHRH agonist had no effect on the viability of the cells during 4h incubations (control $89.7 \pm 1.6\%$, LHRH agonist $90.6 \pm 1.6\%$ diaphorase negative). Dimethyl sulphoxide and the ionophore also had no significant effect (dimethyl sulphoxide $85.0 \pm 1.7\%$, A23187 $85.5 \pm 3.0\%$ diaphorase negative) [all data are means $\pm$ S.E.M. ($n = 5$)].

Discussion

The results obtained in this study are consistent with a primary role for Ca^{2+} rather than cyclic AMP in the stimulation of testosterone production by the LHRH agonist. Similarly the stimulation of LH release from the rat pituitary by LHRH agonists has been found to be dependent on Ca^{2+} (Samli & Geschwind, 1968) and independent of cyclic AMP (Benoist et al., 1981).

In the present study at least 1 mM-Ca^{2+} was required for the steroidogenic effect of the LHRH agonist. The Ca^{2+} ionophore A23187 in the presence of 2.5 mM-Ca^{2+} was found to mimic the effect of LHRH on steroidogenesis and no additional effect of the latter was found when both compounds were added together. Although a small increase in cyclic AMP did occur when the ionophore was added it was not time- or dose-related or correlated with Ca^{2+} levels on the effect of A23187 on testosterone production. These results are similar to those obtained in another steroidogenic system, the rat glomerulosa cell, where A23187 was shown to stimulate steroidogenesis and to have no effect on cyclic AMP levels (Fakunding & Catt, 1982). They differ, however, from those obtained by Lin et al. (1980) with rat testicular cells; these workers showed that A23187 increased cyclic AMP levels but not steroidogenesis. The lack of effect of A23187 on steroidogenesis obtained by these authors may have been due to either the low percentage of Leydig cells in their preparation, which would lower the 'sensitivity' of detecting testosterone production changes, and/or to the presence of the phosphodiesterase inhibitor methylisobutylxanthine. In our experiments it was found that methylisobutylxanthine inhibited the effect of A23187. In addition, A23187 plus methyl-isobutylxanthine, but not A23187 alone, also inhibited LH-stimulated steroidogenesis (Sullivan & Cooke, 1984). The effects of A23187 on cyclic AMP found by Lin et al. (1980) may have been due to the stimulation of testicular cells other than Leydig cells; their preparation probably only contained about 5% steroidogenically active Leydig cells (Aldred & Cooke, 1982) compared with 90% pure Leydig cells in our preparation (Aldred & Cooke, 1982).

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