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The role of Ca^{2+} in steroidogenesis in Leydig cells

Stimulation of intracellular free Ca^{2+} by lutropin (LH), luliberin (LHRH) agonist and cyclic AMP

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The requirements of purified rat Leydig cells for intra- and extra-cellular Ca^{2+} during steroidogenesis stimulated by LH (lutropin), cyclic AMP analogues and LHRH (luliberin) agonist were investigated. The intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured by using the fluorescent Ca^{2+} chelator quin-2. The basal $[Ca^{2+}]_i$ was found to be 89.4 ± 16.6 nM (mean $\pm$ S.D., $n = 25$). LH, 8-bromo cyclic AMP and dibutyryl cyclic AMP increased $[Ca^{2+}]_i$ by 300–500 nM at the highest concentrations of each stimulator, whereas LHRH agonist only increased $[Ca^{2+}]_i$ by a maximum of approx. 60 nM. Low concentrations of LH (< 1 pg/ml) and all concentrations of LHRH agonist increased testosterone without detectable changes in cyclic AMP. With amounts of LH greater than 1 pg/ml, parallel increases in cyclic AMP and $[Ca^{2+}]_i$ occurred. The steroidogenic effect of the LHRH agonist was highly dependent on extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$), whereas LH effects were only decreased by 35% when $[Ca^{2+}]_e$ was lowered from 2.5 mM to 1.1 μM. No increase in $[Ca^{2+}]_i$ occurred with the LHRH agonist in the low-$[Ca^{2+}]_e$ medium, whereas LH (100 ng/ml) gave an increase of 52 nM. It is concluded that $[Ca^{2+}]_i$ can be modulated in rat Leydig cells by LH via mechanisms that are both independent of and dependent on cyclic AMP, whereas LHRH-agonist action on $[Ca^{2+}]_i$ is independent of cyclic AMP. The evidence obtained suggests that, at sub-maximal rates of testosterone production, Ca^{2+}, rather than cyclic AMP, is the second messenger, whereas for maximum steroidogenesis both Ca^{2+}- and cyclic-AMP-dependent pathways may be involved.

INTRODUCTION

In addition to luteinizing hormone (LH), it is now established that luteinizing-hormone-releasing hormone (LHRH) and its agonists can also directly stimulate steroidogenesis in rat testis Leydig cells [see Cooke & Sullivan (1985) (review) for references]. Cyclic AMP is involved in LH action [see Cooke *et al.* (1981*a*) (review) for references], but not in the action of LHRH (Sullivan & Cooke, 1984*a*). Ca^{2+}, however, is required for LH-dependent (Hall *et al.*, 1981; Janszen *et al.* 1976) and LHRH-agonist-dependent (Sullivan & Cooke, 1984*a*) stimulation of steroidogenesis.

Inhibitors of two Ca^{2+}-dependent processes mediated by calmodulin (Hall *et al.*, 1981; Sullivan & Cooke, 1985*a*) and leukotrienes (Dix *et al.*, 1984; Sullivan & Cooke, 1985*a*) also inhibit LH- and LHRH-agonist-stimulated steroidogenesis. However, these studies on the requirements for Ca^{2+} in the Leydig cell are all indirect. In this present study, the fluorescent intracellular Ca^{2+} chelator quin-2 (Tsien *et al.*, 1982) has been used to measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in purified rat testis Leydig cells; the effects of LH, cyclic AMP analogues and a LHRH agonist on $[Ca^{2+}]_i$ have been determined and compared with those on production of cyclic AMP and testosterone. In addition, the effects of changing the extracellular Ca^{2+} concentration on $[Ca^{2+}]_i$, cyclic AMP and steroidogenesis have been compared in the presence of LH and LHRH analogue. Preliminary reports of parts of this study have been presented (Cooke, 1985; Sullivan & Cooke, 1985*b*).

MATERIALS AND METHODS

The methodology using quin-2 was essentially as described by Tsien *et al.* (1982). Rat Leydig cells were purified by centrifugation on 0–90%-Percoll density gradients as previously described (Cooke *et al.*, 1981*b*; Aldred & Cooke, 1983) and loaded with quin-2 by incubation with quin-2 acetoxymethyl ester (Lancaster Synthesis, Lancaster, U.K.) (final concn. 20 μM, added in dimethyl sulphoxide) for 60–70 min. The cells were washed and then resuspended in MEM (Gibco) (containing 1 mM-Mg^{2+} and 10 mM-Hepes and without Phenol Red) plus bovine serum albumin (0.1%), to a final concentration of 10^6 cells/ml; 1 ml of the cell suspension was used for each fluorimetric measurement. The excitation wavelength was 339 nm (slit width 5 nm), and the emission wavelength was 500 nm (slit width 10 nm). Hydrolysis of quin-2 ester to quin-2 was confirmed by checking that the emission maximum was at 490 nm. The $[Ca^{2+}]_i$ was determined by the following equation:

$$[Ca^{2+}]_i = K_D(F - F_{min.})/F_{max.} - F)$$

where $K_D = 115$ nM, F = fluorimeter reading, $F_{max.}$ = fluorimeter reading at > 1 mM-Ca^{2+} and $F_{min.}$ = fluorimeter reading at < 1 nM-Ca^{2+}. Cells were permeabilized with digitonin (50 μM) or Triton X-100 in 2.5 mM-Ca^{2+} to determine $F_{max.}$, and for $F_{min.}$ by adding excess EGTA (> 4 mM).

Fluorescence was measured at 37 °C in a Perkin–Elmer MPF 44B fluorescence spectrophotometer, which was equipped with a magnetic stirrer to prevent cell

Abbreviations used: LH, luteinizing hormone (lutropin); LHRH, luteinizing-hormone-releasing hormone (luliberin); $[Ca^{2+}]_i$, $[Ca^{2+}]_e$, intracellular and extracellular concentration of Ca^{2+} ions respectively; MEM, modified Eagle's medium; DMEM, Dulbecco's MEM.

sedimentation. Measurements were terminated when one of two criteria were met when the compounds added (in 10 μl of MEM) either (1) did not change the fluorescence over 8–10 min, or (2) caused no further change for over 2 min after a change in fluorimetric output.

To test the functional activity of the Leydig cells, the latter were incubated in Dulbecco's MEM (DMEM) (Hunter *et al.*, 1982), MEM or a phosphate buffer (see below), all containing 0.1% albumin, with various concentrations of sheep LH (NIH-S24; 2.3 IU NIH-SI units/mg), cyclic AMP analogues or LHRH agonist (ICI.118630; a gift from ICI) for the times stated in the Figure legends. The incubations were stopped with 3.0 M-$HClO_4$ (final concn. 0.5 M) and frozen at −20 °C until assayed for testosterone (Verjans *et al.*, 1973) and cyclic AMP (Steiner *et al.*, 1972; Harper & Brooker, 1975). Samples were neutralized with 1.08 M-K_3PO_4 (final concn. 0.27 M) after thawing and immediately before assay.

In early experiments, instead of the MEM buffer, a phosphate buffer containing NaCl (145 mM), KCl (5 mM), glucose (5 mM), Hepes (10 mM), Na_2HPO_4 (1 mM), $CaCl_2$ (2.5 mM) and $MgSO_4$ (1 mM) was used for quin-2 loading and fluorescence measurements. The fluorescence data obtained were similar whether the phosphate buffer or MEM was used. LH-stimulated testosterone production was similar in DMEM and MEM, but was decreased approx. 10-fold in the phosphate buffer. LH-stimulated cyclic AMP production was, in contrast, similar in all three media used. The reason for the deleterious effect of the phosphate buffer on Leydig-cell steroidogenesis is unknown. When testosterone and cyclic AMP were measured in the same batch of cells used for $[Ca^{2+}]_i$ determination, MEM was used. DMEM was used for the other experiments when testosterone and cyclic AMP were measured. The dose-dependent LH-stimulated testosterone production was not affected when the Leydig cells were loaded with quin-2. DMEM could not be used for $[Ca^{2+}]_i$ determinations, because it interfered with the fluorescence measurements. The calcium ionophore A23187 (but not ionomycin) also interfered with the fluorescence measurements. Both of these compounds were obtained from Sigma.

DMEM depleted of Ca^{2+} was prepared by adding 3.0 mM-EGTA (Sigma). Extracellular Ca^{2+} ($[Ca^{2+}]_e$) was estimated to be 1.1 μM from the K_D of 1.3×10^{-7} M for Ca^{2+}–EGTA.

RESULTS

The Leydig cells were loaded with quin-2 and the effects of various compounds on $[Ca^{2+}]_i$ were investigated. A typical fluorescence trace for LH is shown in Fig. 1. There was generally no increase above basal (which was 89.4 ± 16.6 nM-Ca^{2+}; mean ± S.D., $n = 25$) during the first 1–2 min, although more rapid responses were occasionally found (e.g. the effect of LHRH agonist in Fig. 1 was detectable after a lag of approx. 45 s). This was followed by a linear increase in $[Ca^{2+}]_i$ to reach a maximum after 6–8 min, which was subsequently maintained for at least 2 min. There was a similar lag time for all the compounds investigated that increased $[Ca^{2+}]_i$, the only exception being the Ca^{2+} ionophore ionomycin, which produced a very rapid increase in $[Ca^{2+}]_i$. The specificity of the hormonal effects was examined by incubating quin-2-loaded cells with corticotropin (0.1 nM) and follitropin (100 ng/ml). The former had no effect on $[Ca^{2+}]_i$ (Fig. 1) or testosterone production (results not shown), whereas the latter stimulated small increases in both $[Ca^{2+}]_i$ and testosterone production (results not shown), consistent with a small contamination of LH in the follitropin preparation. The Ca^{2+} ionophore ionomycin stimulated major increases in $[Ca^{2+}]_i$ to 1.3 μM, indicating a large Ca^{2+} influx.

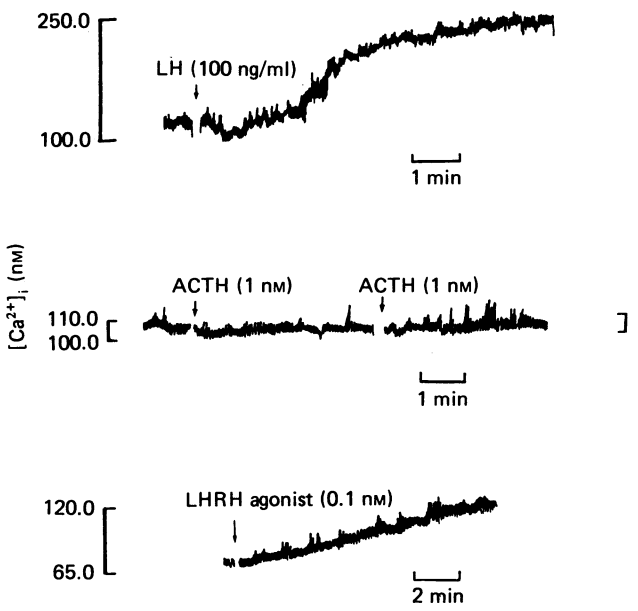

Fig. 1. Typical fluorescence traces after stimulation with three different hormones of rat Leydig cells loaded with quin-2

Purified rat Leydig cells were loaded with quin-2 as described in the Materials and methods section. Fluorescence was measured on a Perkin–Elmer MPF 44B fluorescence spectrophotometer equipped with a magnetic stirrer. The results shown are typical of at least three experiments. Abbreviation: ACTH, corticotropin.

In Fig. 2 the effects of different amounts of LH on $[Ca^{2+}]_i$, cyclic AMP and testosterone concentrations are shown. Small but significant ($P < 0.05$) increases in $[Ca^{2+}]_i$ and testosterone production were detectable with 0.001 pg of LH/ml and in cyclic AMP with 1.0 pg of LH/ml (Fig. 2 and Table 1). Maximum steroidogenesis was reached with 100 pg of LH/ml, whereas both $[Ca^{2+}]_i$ and cyclic AMP continued to increase in parallel with amounts of LH up to 1 ng/ml (Fig. 2). Even with LH concentrations up to 1000 ng/ml, maximum $[Ca^{2+}]_i$ and cyclic AMP concentrations did not reach a plateau (Table 2). LHRH agonist also increased $[Ca^{2+}]_i$ (Fig. 3); a linear increase was obtained with concentrations of 0.1 pM–0.1 μM. The highest concentration reached was 60 nM, which was much lower than that with 1 μg of LH/ml (510 nM) (Table 2). Testosterone increased in parallel, to reach a maximum with 10 nM-LHRH agonist. In agreement with previous results, no increase in cyclic AMP was detected.

The parallel increase in LH-stimulated $[Ca^{2+}]_i$ and cyclic AMP production (in the presence of > 1 pg of LH/ml) suggested that they might be inter-related, and therefore the effects of cyclic AMP and cyclic AMP analogues on $[Ca^{2+}]_i$ were investigated. It was found that

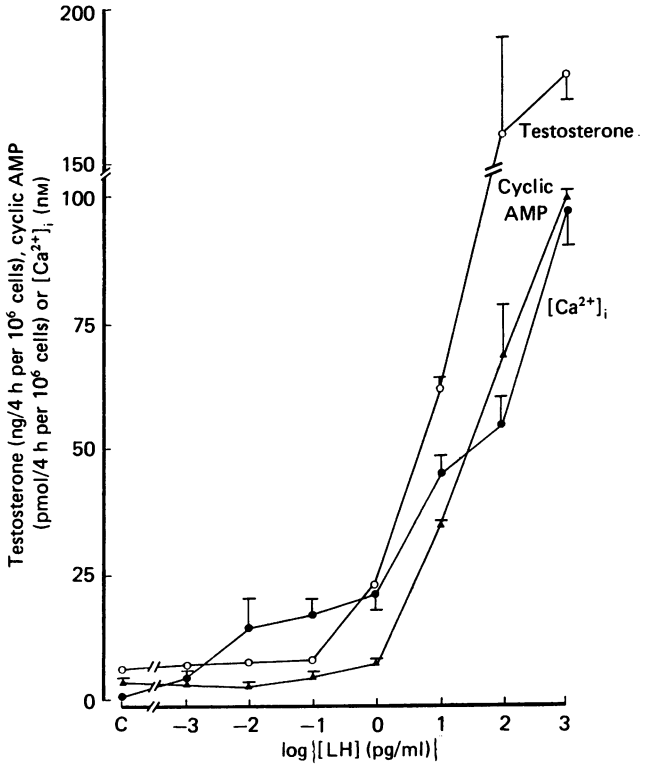

Fig. 2. Effects of LH on $[Ca^{2+}]_i$ and on cyclic AMP and testosterone production

Purified rat Leydig cells were loaded with quin-2 as described in the Materials and methods section. $[Ca^{2+}]_i$ (●) represents the increase above basal $[Ca^{2+}]_i$ induced by hormone treatment. Cyclic AMP (▲) and testosterone (○) production in the Leydig cells in MEM + 0.1% albumin was determined after 4 h in the presence of various concentrations of LH. All data are means ± S.E.M. ($n = 3$), and are representative of two experiments. The $[Ca^{2+}]_i$ measurements and other incubations were carried out with the same batch of cells. Key: C, control (no LH present).

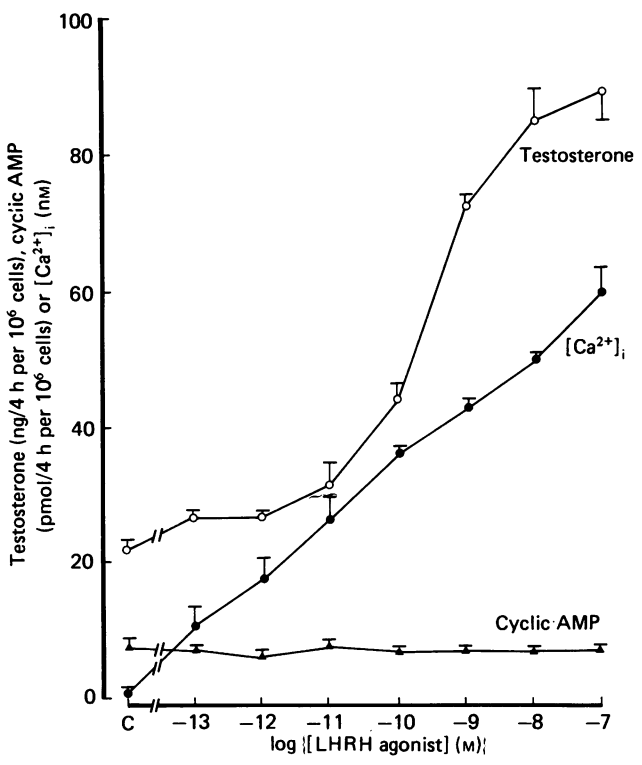

Fig. 3. Effects of the LHRH agonist on $[Ca^{2+}]_i$ and on cyclic AMP and testosterone production

Purified rat Leydig cells were loaded with quin-2 as described in the Materials and methods section. $[Ca^{2+}]_i$ (●) represents the increase above basal $[Ca^{2+}]_i$ induced by hormone treatment. Cyclic AMP (▲) and testosterone (○) production in the Leydig cells in MEM + 0.1% albumin was determined after 4 h in the presence of various concentrations of LHRH agonist. All data are means ± S.E.M. ($n = 3$), and are representative of two experiments. The $[Ca^{2+}]_i$ measurements and other incubations were carried out with the same batch of cells.

Table 1. Effects of LH and LHRH agonist on testosterone production by rat Leydig cells in DMEM containing 2.5 mM- or 1.1 μM-Ca^{2+}

Cells were prepared and incubated as described in the Materials and methods section. All data are means ± S.E.M. ($n = 3$): *$P < 0.05$ versus no hormone; †$P < 0.05$ versus 2.5 mM-Ca^{2+}.

	Testosterone production (ng/4 h per 10^6 cells)	
Concn. of LH (pg/ml)	2.5 mM-Ca^{2+}	1.1 μM-Ca^{2+}
0	10.0 ± 0.3	10.3 ± 0.7
0.001	14.6 ± 1.2*	14.8 ± 0.8*
0.01	14.5 ± 0.5*	14.7 ± 1.3*
0.1	12.7 ± 0.9*	14.3 ± 1.1*
1.0	16.2 ± 0.9*	18.0 ± 1.5*
10	74.7 ± 2.5*	42.6 ± 1.7*†
100	357.6 ± 39.1*	239.3 ± 22.2*†
1000	479.8 ± 21.7*	378.9 ± 4.55*†
Concn. of LHRH agonist (M)		
0	10.0 ± 0.3	10.3 ± 0.7
10^{-12}	10.6 ± 0.2	16.7 ± 1.0*
10^{-11}	12.1 ± 0.2*	14.6 ± 0.6*
10^{-10}	16.0 ± 1.6*	15.9 ± 0.8*
10^{-9}	52.7 ± 2.8*	18.5 ± 0.9*†
10^{-8}	89.8 ± 4.5*	16.4 ± 1.1*†
10^{-7}	106.0 ± 4.4*	27.2 ± 2.2*†

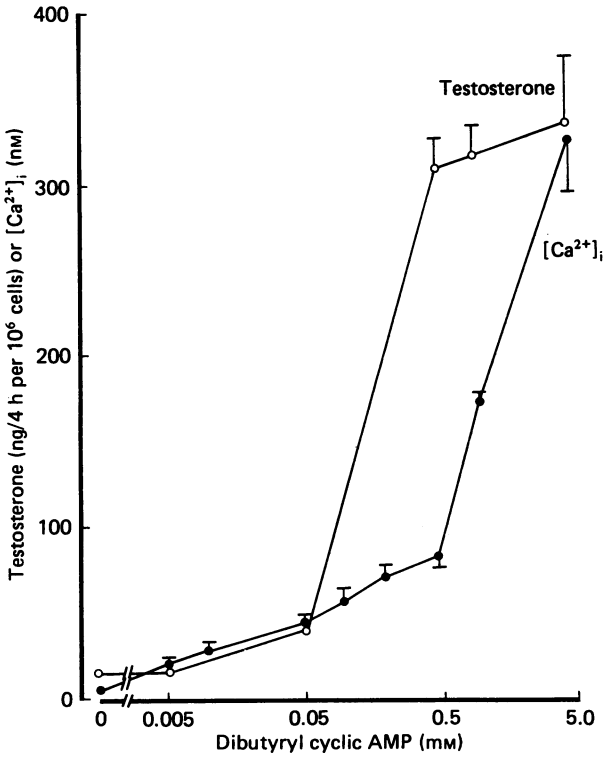

Fig. 4. Effect of dibutyryl cyclic AMP on $[Ca^{2+}]_i$ and testosterone production

Purified rat Leydig cells were loaded with quin-2 as described in the Materials and methods section. $[Ca^{2+}]_i$ (●) represents the increase above basal $[Ca^{2+}]_i$ induced by dibutyryl cyclic AMP. Testosterone production (○) in the Leydig cells in MEM + 0.1% albumin was determined after 4 h in the presence of various concentrations of dibutyryl cyclic AMP. All data are means ± S.E.M. ($n = 3$), and are representative of two experiments. The $[Ca^{2+}]_i$ measurements and other incubations were carried out with the same batch of cells.

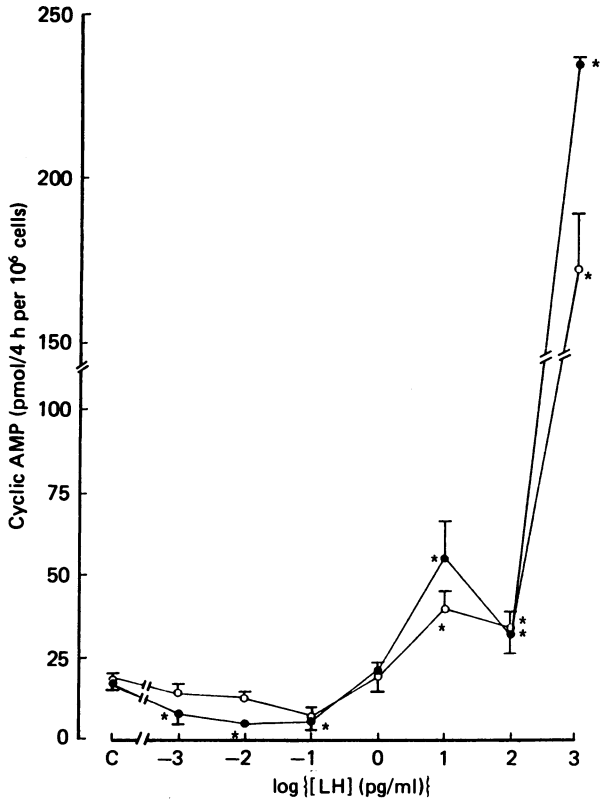

Fig. 5. Effects of lowering extracellular $[Ca^{2+}]$ on LH-stimulated cyclic AMP production

Cyclic AMP production by purified rat Leydig cells was stimulated in DMEM + 0.1% albumin for 4 h with various concentrations of LH in (●) 2.5 mM- or (○) 1.1 μM-Ca^{2+}. All data are means ± S.E.M. ($n = 3$): *$P < 0.05$ versus no LH.

dibutyryl cyclic AMP increased $[Ca^{2+}]_i$ (Fig. 4). At the minimum concentrations of dibutyryl cyclic AMP and LH required to give maximum steroidogenesis (Figs. 4 and 2 respectively), similar increases in $[Ca^{2+}]_i$ were obtained (70–80 nM and 60–90 nM respectively). 8-Bromo cyclic AMP also gave similar increases in $[Ca^{2+}]_i$; with 0.01, 0.5 and 5.0 mM the increases in $[Ca^{2+}]_i$ were 29.7 ± 8.3, 107.9 ± 6.3 and 265.3 ± 13.3 nM respectively (means ± range from two separate experiments). Cyclic AMP (5.0 mM) itself gave a very small increase in $[Ca^{2+}]_i$ (26.4 ± 5.2 nM). None of the other compounds tested (butyrate, AMP, ADP, adenosine and ATP) gave a detectable increase in $[Ca^{2+}]_i$. All the compounds tested gave essentially similar results with respect to testosterone production (Table 3); 8-bromo and dibutyryl cyclic AMP gave dose-related increases in testosterone production, and the other compounds had no effect, except for cyclic AMP (5 mM) and adenosine (250 μM), which caused small increases in testosterone production.

The effects of lowering $[Ca^{2+}]_e$ on LH-stimulated cyclic AMP production are shown in Fig. 5. Only small effects of depleting $[Ca^{2+}]_e$ from 2.5 mM to 1.1 μM were found; with the highest amounts of LH added, approx. 25%

Table 2. Effects of LH on $[Ca^{2+}]_i$ and on testosterone and cyclic AMP production

All data are means ± S.E.M. ($n = 3$). *The basal $[Ca^{2+}]_i$ value has been subtracted. Cells were prepared, incubated and loaded with quin-2 as described in the Materials and methods section.

Concn. of LH (ng/ml)	Testosterone produced (ng/4 h per 10^6 cells)	Cyclic AMP produced (pmol/4 h per 10^6 cells)	$[Ca^{2+}]_i$* (nM)
0	7.7 ± 0.3	4.2 ± 0.7	—
1	189.1 ± 27.6	61.4 ± 4.2	97.1 ± 7.3
10	240.6 ± 16.4	177.0 ± 16.5	151.4 ± 10.8
100	288.5 ± 5.5	286.4 ± 28.9	239.5 ± 9.0
1000	240.3 ± 26.2	434.6 ± 33.3	510.9 ± 29.4

Table 3. Effects of various adenosine derivatives and butyrate on rat Leydig-cell testosterone production

All data are means ± S.E.M. ($n = 3$): *$P < 0.05$ versus no addition. LH (100 ng/ml) increased testosterone production to 389.4 ± 9.7 ng/4 h per 10^6 cells in the same experiment.

	Testosterone production (ng/4 h per 10^6 cells)			
Concn. of compound added...	0	50 μM	500 μM	5000 μM
8-Bromo cyclic AMP	9.2 ± 0.4	143.3 ± 5.2*	311.6 ± 15.7*	324.4 ± 12.6*
Dibutyryl cyclic AMP	7.8 ± 0.3	132.5 ± 2.1*	178.0 ± 12.6*	369.6 ± 17.9*
Butyrate	10.0 ± 0.5	10.2 ± 0.2	9.2 ± 0.4	9.8 ± 0.2
Cyclic AMP	13.0 ± 0.5	13.0 ± 0.2	15.1 ± 1.2	83.6 ± 2.4*
AMP	7.9 ± 0.6	10.2 ± 0.3	10.5 ± 0.8	10.7 ± 0.7
ADP	9.7 ± 0.7	11.6 ± 0.6	11.4 ± 0.8	11.6 ± 0.9
ATP	10.0 ± 0.7	10.8 ± 0.8	11.3 ± 0.3	18.2 ± 0.6*
Concn. of adenosine added...	0	2.5 μM	25 μM	250 μM
Adenosine	11.0 ± 1.3	10.2 ± 0.9	10.2 ± 0.7	17.7 ± 1.4*

Table 4. Effects of decreasing $[Ca^{2+}]_e$ on LH- and LHRH-agonist-induced changes in $[Ca^{2+}]_i$

Data for $[Ca^{2+}]_e = 2.5$ mM are taken from Figs. 2 and 3 and Table 1. All data are means ± S.E.M. from three different experiments. The basal $[Ca^{2+}]_i$ concentrations have been subtracted.

		Increase in $[Ca^{2+}]_i$ (nM)	
	$[Ca^{2+}]_e$...	2.5 mM	1.1 μM
LH	0.10 pg/ml	16.8 ± 3.5	6.2 ± 1.0
	100 pg/ml	54.6 ± 5.6	46.8 ± 1.2
	100 ng/ml	239.5 ± 9.0	52.2 ± 3.5
LHRH agonist	10^{-12} M	17.3 ± 3.8	6.7 ± 1.0
	10^{-9} M	42.2 ± 1.5	7.1 ± 1.0
	10^{-7} M	60.6 ± 3.1	9.1 ± 1.5

lower cyclic AMP production occurred during a 4 h incubation.

The effects of lowering $[Ca^{2+}]_e$ on $[Ca^{2+}]_i$ during stimulation with LH and LHRH agonist are given in Table 4. Basal $[Ca^{2+}]_i$ was decreased to 61.3 ± 5.7 nM (mean ± S.E.M., $n = 20$) from 89.4 ± 16 nM, although basal testosterone production was not affected (see Table 1). With 100 pg of LH/ml a substantial increase in $[Ca^{2+}]_i$ was still obtained with 1.1 μM-$[Ca^{2+}]_e$ and was similar to that obtained with 2.5 mM. No further increase occurred with 100 ng of LH/ml in the low-Ca^{2+} medium. With 0.001–1.0 pg/ml, lowering $[Ca^{2+}]_e$ had no effect on testosterone production (Table 1). With 10–1000 pg of LH/ml, testosterone production was decreased by an average of 35.2 ± 6.7% (Table 1). In contrast, LHRH-agonist effects on $[Ca^{2+}]_i$ were almost completely negated (Table 4), and testosterone production was inhibited by 85.2 ± 2.9% (Table 1).

DISCUSSION

In this study we have demonstrated that LH, cyclic AMP analogues and a LHRH agonist stimulate increases in intracellular Ca^{2+} concentrations in rat Leydig cells. Both LH and cyclic AMP analogues increased $[Ca^{2+}]_i$ to higher values than did the LHRH agonist. With amounts of LH greater than 1 pg/ml, parallel increases in cyclic AMP and $[Ca^{2+}]_i$ were obtained, whereas the LHRH agonist did not cause any detectable changes in cyclic AMP concentrations. These data indicate that cyclic AMP itself increases $[Ca^{2+}]_i$ in rat Leydig cells.

Previous studies have repeatedly demonstrated a discrepancy between the amounts of LH required to stimulate steroidogenesis and increase cyclic AMP production [see Cooke *et al.* (1981*a*) for references], more LH being required to give detectable changes in cyclic AMP production than to stimulate testosterone synthesis. In the present study, with less than 0.1 pg of LH/ml significant changes in testosterone and $[Ca^{2+}]_i$ occurred, whereas there was no detectable increase in cyclic AMP. Thus, at low concentrations of LH, $[Ca^{2+}]_i$ is probably the modulator of steroidogenesis, rather than cyclic AMP. This is supported by the data with the LHRH agonist, which increases $[Ca^{2+}]_i$ and testosterone without altering cyclic AMP concentrations. In the present study the amounts of LH required to give minimum and maximum changes in steroidogenesis and cyclic AMP are two orders of magnitude less than those previously found. This is in part due to the longer incubation times used (4 h instead of 2 h), which increases the sensitivity and capacity (M. H. F. Sullivan & B. A. Cooke, unpublished work; Sharpe & Harmer, 1983) and probably also to the different buffers used. In preliminary experiments it was found, by using a simple phosphate buffer for quin-2 experiments (see the Materials and methods section), that

the capacity for steroidogenesis in this buffer was approx. 10% of that obtained with the MEM buffer.

Maximum steroidogenesis in the presence of the LHRH agonist was approx. 85 ng of testosterone/4 h per 10^6 cells. The corresponding increase in $[Ca^{2+}]_i$ was approx. 50 nM. With the same increase in $[Ca^{2+}]_i$, similar extents of steroidogenesis were obtained for LH (80 ng/4 h per 10^6 cells) and dibutyryl cyclic AMP (120 ng/4 h per 10^6 cells). It could be argued from these data that $[Ca^{2+}]_i$ alone is sufficient to control steroidogenesis and that cyclic AMP simply stimulates an increase in $[Ca^{2+}]_i$. However, in view of the fact that the Ca^{2+} ionophore A23187 is unable to stimulate maximal steroidogenesis (Sullivan & Cooke, 1984*b*), an additional pathway involving cyclic AMP cannot be excluded.

The steroidogenic effect of LHRH agonist was found to be highly dependent on $[Ca^{2+}]_e$, whereas the LH effects were only decreased by approx. 35%. The latter is essentially in agreement with earlier studies (Janszen *et al.*, 1976; Sullivan & Cooke, 1984*a*). Lowering $[Ca^{2+}]_e$ almost completely negated LHRH-agonist effects on $[Ca^{2+}]_i$ and also caused a marked decrease in the LH-stimulated increase in $[Ca^{2+}]_i$; the latter was decreased to 52 nM above basal, which, however, still corresponds to the amounts needed to give near-maximum steroidogenesis. These results illustrate that the increases in $[Ca^{2+}]_i$ are mainly derived from the extracellular medium. Whether there is also a mobilization of intracellular stores via the Ca^{2+} mobilizer inositol 1,4,5-trisphosphate, as has been shown in other tissues (Berridge, 1984), remains to be determined. Both LH and LHRH agonist have been shown to stimulate phosphoinositide metabolism in rat Leydig cells (Lowitt *et al.*, 1982; Molcho *et al.*, 1984). However, the time required to increase the Leydig-cell $[Ca^{2+}]_i$ by the addition of LH, LHRH agonist and cyclic AMP analogues (2–3 min) is much slower than in other tissues (within seconds) where inositol trisphosphate has been shown to be involved. This possibly indicates another mechanism. The direct action of cyclic AMP on $[Ca^{2+}]_i$ may be via phosphorylation of a plasma-membrane Ca^{2+} channel by a cyclic AMP-dependent protein kinase. This has been suggested for heart cells (Cachelin *et al.*, 1983).

In studies with adrenal glomerulosa cells (Capponi *et al.*, 1984; Braley *et al.*, 1984), it was shown that angiotensin II increases $[Ca^{2+}]_i$. This hormone stimulates steroidogenesis independently of cyclic AMP, and thus has similar characteristics to LHRH-agonist-stimulated steroidogenesis in Leydig cells. The changes in $[Ca^{2+}]_i$ were similar with LHRH agonist and angiotensin II (up to approx. 100 nM $[Ca^{2+}]_i$). The Ca^{2+}-dependency of the effects of LHRH on pituitary cells are well documented (Samli & Geschwind, 1968; Borges *et al.*, 1983), and a more recent paper (Clapper & Conn, 1985) indicated that LHRH may increase $[Ca^{2+}]_i$ in an enriched gonadotroph population to similar concentrations (20–40 nM) to those that we have shown in purified rat Leydig cells (10–60 nM).

The present documentation of a positive effect of a hormone (LH) on both $[Ca^{2+}]_i$ and cyclic AMP and the possible involvement of both these second messengers in steroidogenesis is unusual; in most other systems either one or the other is increased, and they usually have opposite effects (Tsuruta *et al.*, 1982; Feinstein *et al.*, 1983), although dibutyryl cyclic AMP has been shown to stimulate Ca^{2+} influx in pancreatic β-cells (Henquin & Meissner, 1983). There is also evidence, however, that Ca^{2+} can have a negative as well as a positive effect in the Leydig cell, because the Ca^{2+} ionophore A23187 and LHRH agonist both decrease LH-stimulated cyclic AMP production, probably via activation of a phosphodiesterase and also possibly by uncoupling of the LH receptor from the adenylate cyclase (Sullivan & Cooke, 1984*b*,*c*; Cooke *et al.*, 1986).

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REFERENCES

Aldred, L. F. & Cooke, B. A. (1983) J. Steroid Biochem. **18**, 411–444
Berridge, M. J. (1984) Biochem. J. **220**, 345–360
Borges, J. L. C., Scott, D., Kaiser, D. L., Evans, W. S. & Thorner, M. O. (1983) Endocrinology (Baltimore) **113**, 557–562
Braley, L., Menacheny, A., Brown, E. & Williams, G. (1984) Biochem. Biophys. Res. Commun. **123**, 810–815
Cachelin, A. B., de Peyer, J. E., Kokubun, S. & Reuter, H. (1983) Nature (London) **304**, 462–464
Capponi, A. M., Lew, P. D., Jornot, L. & Vallotton, M. B. (1984) J. Biol. Chem. **259**, 8863–8869
Clapper, D. L. & Conn, P. M. (1985) Biol. Reprod. **32**, 269–278
Cooke, B. A. (1985) Symp. 'Hormonal Control of Testicular Function', Int. Congr. Androl. 3rd
Cooke, B. A. & Sullivan, M. H. F. (1985) Mol. Cell. Endocrinol. **41**, 115–122
Cooke, B. A., Dix, C. J., Magee-Brown, R., Janszen, F. H. A. & van der Molen, H. J. (1981*a*) Adv. Cyclic Nucleotide Res. **14**, 593–609
Cooke, B. A., Magee-Brown, R., Golding, M. & Dix, C. J. (1981*b*) Int. J. Androl. **4**, 355–366
Cooke, B. A., Dix, C. J., Habberfield, A. D. & Sullivan, M. H. F. (1986) Horm. Cell Regul. **10**, in the press
Dix, C. J., Habberfield, A. D., Sullivan, M. H. F. & Cooke, B. A. (1984) Biochem. J. **219**, 529–537
Feinstein, M. B., Egan, J. J., Sha'afi, R. I. & White, J. (1983) Biochem. Biophys. Res. Commun. **113**, 598–604
Hall, P. F., Osawa, S. & Mrotek, J. (1981) Endocrinology (Baltimore) **109**, 1677–1682
Harper, T. F. & Brooker, G. (1975) J. Cyclic Nucleotide Res. **1**, 207–218
Henquin, J.-C. & Meissner, H. P. (1983) Biochem. Biophys. Res. Commun. **112**, 614–620
Hunter, M. G., Sullivan, M. H. F., Dix, C. J., Aldred, L. F. & Cooke, B. A. (1982) Mol. Cell. Endocrinol. **27**, 31–44
Janszen, F. H. A., Cooke, B. A., van Driel, M. J. A. & van der Molen, H. J. (1976) Biochem. J. **160**, 433–437
Lowitt, S., Farese, R. V., Sabir, M. A. & Root, A. W. (1982) Endocrinology (Baltimore) **111**, 1415–1417
Molcho, J., Zakut, H. & Naor, Z. (1984) Endocrinology (Baltimore) **114**, 1048–1050
Samli, M. H. & Geschwind, I. I. (1968) Endocrinology (Baltimore) **82**, 225–231
Sharpe, R. M. & Harmer, T. J. (1983) Horm. Cell Regul. **7**, 217–230
Steiner, A. L. Parker, C. W. & Kipnis, D. M. (1972) J. Biol. Chem. **247**, 1106–1113
Sullivan, M. H. F. & Cooke, B. A. (1984*a*) Biochem. J. **218**, 621–624
Sullivan, M. H. F. & Cooke, B. A. (1984*b*) Mol. Cell. Endocrinol. **34**, 17–22

Sullivan, M. H. F. & Cooke, B. A. (1984*c*) Mol. Cell. Endocrinol. **36**, 115–122
Sullivan, M. H. F. & Cooke, B. A. (1985*a*) Biochem. J. **232**, 55–59
Sullivan, M. H. F. & Cooke, B. A. (1985*b*) J. Endocrinol. **107**, Suppl., abstr. no. 125
Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) J. Cell Biol. **94**, 325–334
Tsuruta, K., Greive, C. W., Cote, T. E., Eskay, R. L. & Kabian, J. W. (1982) Endocrinology (Baltimore) **110**, 1133–1140
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

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