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Extracellular Calcium and Luteinizing Hormone Effects on 22-Hydroxycholesterol Used for Testosterone Production in Mouse Leydig Cells

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ABSTRACT: The effect of extracellular calcium on testosterone synthesis in response to luteinizing hormone (LH) or 22-hydroxycholesterol (22-OH-C) by isolated adult mouse Leydig cells was studied. Leydig cells were isolated by linear density gradient centrifugation. The cells were incubated in minimum essential medium with or without calcium (1.36 mmol/L) in an atmosphere of 95% air and 5% carbon dioxide at 37°C for 3 hours with or without LH (10 ng/sample), or with or without 22-OH-C (10 μmol/L). Testosterone production in response to LH was significantly lower ($P < 0.02$) in the absence of extracellular calcium and in the presence of verapamil (10 μmol/L), a calcium channel blocking agent. Extracellular calcium did not significantly ($P > 0.05$) affect testosterone production in cells incubated with 22-OH-C in either the presence or absence of LH. The results suggest that steps in steroidogenesis from 22-OH-C to testosterone are unaffected by extracellular calcium content and that extracellular calcium affects the use of intracellular cholesterol by the cholesterol side-chain cleavage enzyme.

Key words: Steroidogenesis, androgens.

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Leydig cells are the major site of testosterone synthesis. Luteinizing hormone (LH) elicits the formation of cyclic 3′5′-adenosine monophosphate (cAMP), the main signal system regulating Leydig cell function; intracellular and extracellular calcium also modulate the response to LH (Dufau, 1988; Hall, 1988). Sullivan and Cooke (1986) reported that cytosolic calcium concentrations rise in parallel with the production of testosterone in response to both LH and cAMP. We have observed that extracellular calcium affects the magnitude of the stimulation of testosterone production in response to LH in isolated mouse Leydig cells (Meikle et al, 1989).

Cholesterol is the precursor for the synthesis of steroid hormones secreted by either the adrenal cortex or the gonads (Catt, 1987). The studies of Hall and associates (Iida et al, 1989), Freeman et al (1982), and Freeman (1987) indicate that steroid hormones are synthesized from cholesterol derived from either intracellular stores, endogenous synthesis, or lipoproteins. Hall et al (1981) have observed that intracellular calcium influences testosterone production in response to LH in rat Leydig cells by affecting the transport of cholesterol to the mitochondria. However, no effect on the further metabolism of cholesterol to produce testosterone was observed. Sullivan and Cooke (1983) reported that a calmodulin inhibitor affected the conversion of cholesterol to pregnenolone, suggesting that intracellular calcium and calmodulin may influence the activity of the cholesterol side-chain cleavage enzyme. It was uncertain if the discrepancy in these observations related to possible anaerobic conditions used in experiments with isolated mitochondria by Hall et al (1981).

Extracellular calcium could affect steroidogenesis by acutely modulating either 1) cholesterol synthesis or transport to the mitochondria or 2) the activity of one or more of the mitochondrial or microsomal enzymes that ultimately form testosterone. 22-Hydroxycholesterol (22-OH-C), an intermediate in the synthesis of pregnenolone by the cholesterol side-chain cleavage enzyme, can be added exogenously to isolated Leydig cells and used in testosterone synthesis. Therefore, 22-OH-C treatment was used to discriminate between the two alternative mechanisms of calcium action. In this study, we determined if extracellular calcium affects testosterone production in response to either LH, 22-OH-C, or both.

Materials and Methods

Minimal essential medium (MEM) with Earle's salts (normal Ca^{2+} [1.36 mmol/L] concentration and Ca^{2+} deficient) was purchased from Irvine Scientific (Santa Ana, CA). Highly purified human LH (991 IU/mg, lot #AFP 4360B) was obtained from the National Hormone and Pituitary Program. Bovine serum albumin (BSA), verapamil, and 22-OH-C were purchased from Sigma

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Chemical Corporation (St. Louis, MO). Percoll was obtained from Pharmacia (Piscataway, NJ). All unlabeled steroids were obtained from Steraloids, Inc. (Wilton, NH), and tritiated steroids of high specific activity (50 to 140 Ci/mmol) were purchased from Dupont New England Nuclear Corporation (Boston, MA).

Mature Swiss-Webster mice from Simonsen Inc. (Gilroy, CA) were fed chow (RMH-3000, Agway Inc., Syracuse, NY). The mice were sacrificed by cervical dislocation as described previously (Meikle et al, 1989). After removal, the testes were decapsulated and shaken for 10 minutes at room temperature in MEM (10 ml/testis) containing 0.07% serum albumin, 100 U penicillin/ml, 50 μg streptomycin/ml, and 25 mmol/L Hepes, pH 7.4 (Kerr et al, 1985). The mixture was then filtered through one layer of gauze and centrifuged at 80g for 10 minutes at 20°C. The pellet was resuspended in MEM (1 to 2 testes/ml), layered over a continuous gradient of Percoll and Earle's salts (0 to 90%), and centrifuged for 20 minutes at 800g at 20°C. Purified Leydig cells (>85% as evaluated by light and electron microscopy) were found in a band corresponding to a Percoll concentration of 38% to 52% (v/v). Cells were washed twice before use in the assay; trypan blue exclusion tests indicated more than 90% viability.

Cells were incubated in 300 μL of MEM with 3% BSA, and various substrates were included. Luteinizing hormone was added at a concentration of 10 ng/sample unless otherwise stated. Each culture tube was gassed with 95% air/5% carbon dioxide and incubated at 37°C for 3 hours. Typical cell numbers ranged from 1 to 4 × 10^5/well. Incubation was stopped by adding 1 ml of phosphate-buffered saline and placing culture tubes in a 60°C water bath for 30 minutes.

Three 10-μl samples from each well were assayed in triplicate for testosterone by radioimmunoassay as described previously (Meikle et al, 1989). There was inter-experimental variation in the production of testosterone by the Leydig cell preparations that could not be accounted for by differences in methods. Each experiment was repeated two or three times to be certain that observed responses were reproducible, and data from typical experiments are presented in Results. Testosterone production in each well ranged from 5 to 500 ng/10^6 cells, depending on the agonists added. Testosterone was quantitated by specific double antibody radioimmunoassay as detailed elsewhere (Meikle et al, 1979). Blank values were less than 5 pg/tube, and inter- and intra-assay coefficients of variation for the assay were 10% or less.

The 22-OHC was prepared as follows. One gram of 22-OH-C was dissolved in 5 ml dimethyl sulfoxide, and 100-μl portions of this solution were then added slowly to a warm solution of 50 ml MEM with 3% BSA while stirring. This solution was added to the incubation mixture in appropriate amounts. Verapamil was dissolved in MEM at final concentrations of 0.1 μmol/L to 10 μmol/L.

Statistical Analyses

The data are reported as mean ± SE. Analysis of variance and Student's t test were used to determine statistical significance; $P < 0.05$ was considered significant.

Results

The effect of calcium concentration on testosterone production in response to LH (10 ng/well) in Leydig cells was investigated in preliminary experiments by incubating the cells in calcium-free Hank's balanced salt solution with concentrations of calcium from 0.01 to 100 mmol/L (Table 1). Testosterone production was markedly reduced at both higher (100 mmol/L) and lower concentrations (0.1 to 0.01 mmol/L compared with 1 and 10 mmol/L). The influence of calcium on the dose–response effect of LH (0 to 1 ng/well) on testosterone production was then investigated as shown in Figure 1. Testosterone production for doses of LH between 0.1 and 1 ng/well were significantly higher ($P < 0.02$) in the presence of calcium (1.36 mmol/L) than in its absence.

Verapamil (0.1 to 10 μmol/L), which affects the slow calcium channel, was shown to significantly ($P < 0.05$ to 0.02) decrease testosterone production in response to LH (10 ng/well) from 45% to 73% of the control (Fig 2). The effect of 22-OH-C (10 μmol/L) without LH and with calcium on testosterone production is shown in Figure 3. The 22-OH-C without LH resulted in testosterone production of 508 ng/10^6 cells per 3 hours. Removal of extracellular calcium had an insignificant ($P > 0.05$) effect on steroidogenic response to 22-OH-C. Figure 4 shows the production of testosterone in response to a combination of LH and 22-OH-C either with or without extracellular calcium at a concentration of 1.36 mmol/L. Testosterone production was unaffected ($P < 0.05$) by the concentration of extracellular calcium in response to LH and 22-OH-C in combination.

Discussion

It has been presumed that the major action of LH was stimulating the cholesterol side-chain cleavage enzyme that was thought to be the rate limiting step in steroidogenesis. Luteinizing hormone results in the formation of cAMP, which stimulates a rise in cytosolic calcium derived, at least in part, from extracellular calcium (Dufau, 1988; Hall, 1988; Sullivan and Cooke, 1986). Cytosolic calcium may then act as a second messenger in the stimulation of steroidogenesis by affecting both cholesterol transport to the

Table 1. *Effects of extracellular calcium concentration on testosterone production*

Calcium concentration (mmol/L)	Testosterone production (ng/10^6 cells per 3 hours)
100	39 ± 3
10	211 ± 12
1	105 ± 7
0.01	72 ± 6
0.001	59 ± 5

Values are presented as the mean ± SE, n = 9. The concentrations of calcium listed above were added to calcium-free Hank's balanced salt solution. Leydig cells were incubated for 3 hours at 37°C in 95% air/5% carbon dioxide with 10 ng/well of LH. Testosterone production in the absence of LH was <3 ng/10^6 cells per 3 hours.

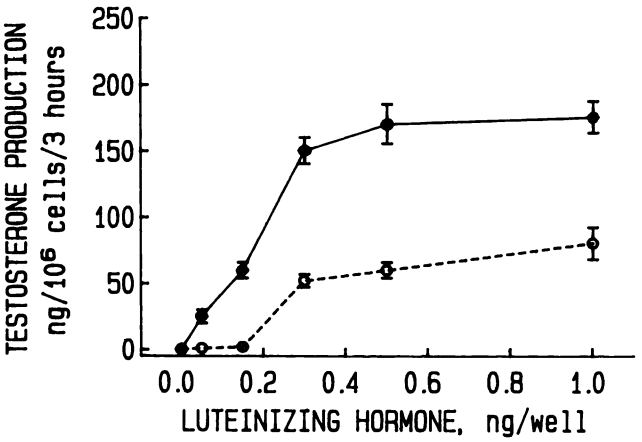

FIG. 1. Effect of extracellular calcium (1.36 mmol/L) on testosterone production by Leydig cells in response to LH (0-1 ng/sample). Values are presented as the mean ± SE, n = 9 replicates. Solid circles = testosterone values for LH and normal calcium; open circles = values for LH without calcium. $P < 0.02$ for LH concentrations greater than 0.1 ng/well for testosterone production at the respective dose with and without calcium.

mitochondria and the activity of the cholesterol side-chain cleavage enzyme.

Steps affected by LH-stimulated steroidogenesis:

LH → cellular cholesterol →

mitochondria[-22-OH, 20-OH cholesterol → pregnenolone]→

microsomes → testosterone

The effect of LH on stimulating testosterone production requires more than the elevation of intracellular calcium concentrations. We have shown that a calcium ionophore increases testosterone production in the absence of LH, but the degree of stimulation is much less than that observed with LH alone (Patterson et al, 1990).

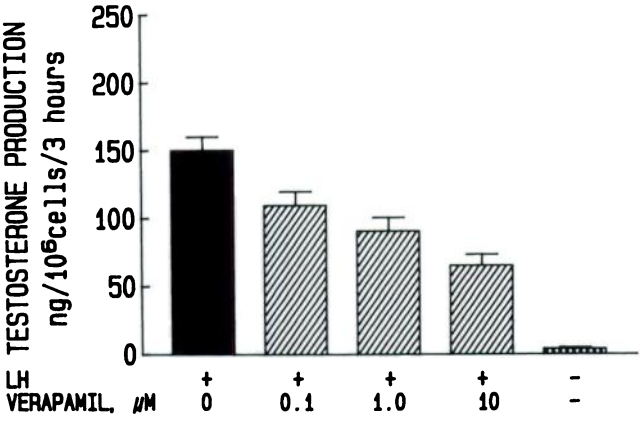

FIG. 2. Effect of verapamil (0.1 to 10 μmol/L) on testosterone production by Leydig cells incubated with normal calcium (1.36/mmol/L) and LH (10 ng/sample). Values are presented as the mean ± SE, n = 9. Solid column = LH and normal calcium; hatched column = LH, normal calcium, and 0.1 to 10 μmol/L verapamil; dotted column = no LH or normal calcium.

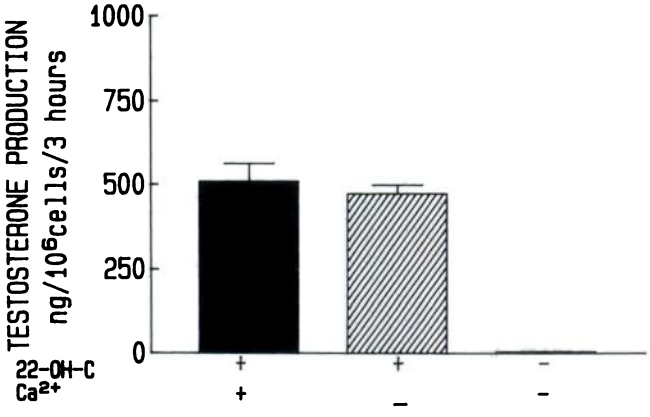

FIG. 3. Effect of extracellular calcium (1.36 mmol/L) on testosterone production by Leydig cells in response to 10 μmol/L 22-OH-C. Values are presented as the mean ± SE, n = 9. Solid column = 22-OH-C and normal calcium; hatched column = 22-OH-C without calcium; third column = no LH, 22-OH-C, or normal calcium.

This study was designed to determine if the action of extracellular calcium affects either the delivery of cholesterol to the mitochondria in preparation for metabolism by the cholesterol side-chain cleavage enzyme or the steroidogenic enzyme activity involved in the conversion of cholesterol to testosterone. If extracellular calcium were required to enhance the activity of steroidogenic enzymes in converting 22-OH-C to testosterone, removal of the calcium from the incubation medium should have reduced steroidogenesis. This was not observed, indicating that extracellular calcium has an effect on steroidogenesis at a site independent of these enzymes. Cholesterol is transported to the mitochondria and serves as a substrate for the cholesterol side-chain cleavage enzyme to form pregnenolone. Preg-

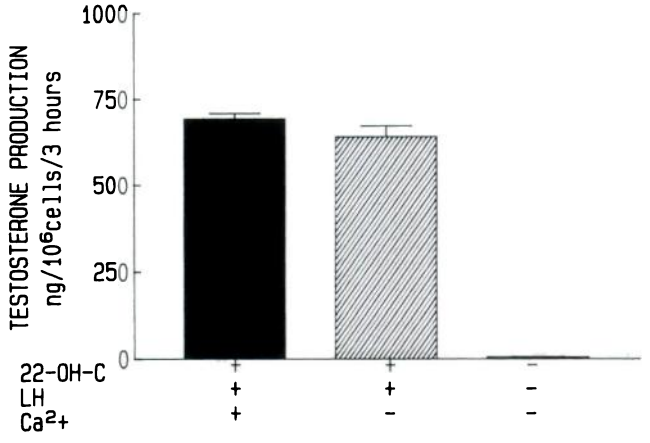

FIG. 4. Effect of extracellular calcium (1.36 mmol/L) on testosterone production by Leydig cells in response to 10 μmol/L 22-OH-C and LH (10 ng/sample). Values are presented as the mean ± SE, n = 9. Solid column = 22-OH-C, LH, and normal calcium; hatched column = 22-OH-C and LH without calcium; third column = no 22-OH-C, LH, or calcium.

nenolone then leaves the mitochondria and is converted to testosterone by a series of enzymes. Our results suggest that LH and extracellular calcium make cholesterol available to the cholesterol side-chain cleavage enzyme by enhancing its transport to the mitochondria or from the outer to the inner portion of the mitochondrial membrane.

Affected by extracellular calcium:

LH → cellular cholesterol → mitochondria

Not affected by extracellular calcium:

mitochondria[-22-OH, 20-OH cholesterol-pregnenolone] →

microsomes → testosterone

Our results agree with those reported by Hall et al (1981) who studied the effects of calmodulin and cytosolic calcium on steroidogenesis in rat Leydig cells. They reported that calmodulin is involved in testosterone production by regulating the transport of cholesterol to the mitochondria. Once cholesterol reaches the mitochondria, it is beyond the action of calmodulin, suggesting that the conversion of cholesterol to pregnenolone by the cholesterol side-chain cleavage enzyme is unaffected by calmodulin. These steps are presumed to be affected by both LH and cAMP. The studies were conducted using isolated mitochondria. Cholesterol side-chain cleavage is inhibited if the mitochondria become anaerobic because oxygen is required for normal enzyme activity. Our studies were conducted on intact cells with oxygen. Our results also indicate that calcium affects cholesterol use or transport to the cholesterol side-chain cleavage enzyme in the mitochondria. An effect of LH and extracellular calcium on the further metabolism of cholesterol to pregnenolone is not supported by our results or those of Hall et al (1981).

The observations of Sullivan and Cooke (1983), in contrast to those of Hall et al (1981) and the current results, suggest that intracellular calcium affects side-chain cleavage of cholesterol to produce pregnenolone. Trifluoperazine was used to inhibit calmodulin, and it also inhibited steroidogenesis from 22-OH-C by isolated rat Leydig cells. There are several possible explanations for the apparent discrepancies between our observations and theirs. In contrast to isolated Leydig cell mitochondria, intact Leydig cells may have adequate levels of intracellular calcium to support the side-chain cleavage enzyme. Moreover, the studies of Hall et al (1981) suggest that isolated mitochondria do not require calcium to support side-chain cleavage activity. Finally, trifluoperazine may have an effect on the cholesterol side-chain cleavage enzyme that is independent of its inhibition of calmodulin. It is clear from our observations that extracellular calcium affects the steps involved in the delivery of cholesterol to the mitochondria for metabolism by the cholesterol side-chain cleavage enzyme. Our observations and those of Hall et al (1981) do not support an effect of intracellular calcium on side-chain cleavage of cholesterol in Leydig cell mitochondria.

Verapamil is a calcium channel blocking agent that affects "slow" and "high threshold" or L channels (Bean, 1989). Our results coincide with those of Moger (1983) who also reported inhibition of Leydig cell testosterone production by verapamil. Winters et al (1983) reported that calcium modulates membrane phosphorylation and adenylate cyclase activity in Leydig cells. These results suggest that the slow channel is involved in LH stimulation of calcium entry and adenylate cyclase activity, which then affect cholesterol movement from the mitochondria and thereby affect steroidogenesis. Further studies are required to determine if verapamil and the absence of extracellular calcium have similar mechanisms of action in affecting LH-stimulated testosterone production.

References

Bean BP. Classes of calcium channels in vertebrate cells. *Ann Rev Physiol.* 1989;51:367–384.

Catt KJ. Molecular mechanisms of hormone action: control of target cell function by peptide, steroid, and thyroid hormones. In: Felig P, Baxter JD, Broadus AE, Frohman LA, eds. *Endocrinology and Metabolism,* 2nd ed. New York: McGraw-Hill; 1987;82–164.

Dufau ML. Endocrine regulation and communicating functions of the Leydig cell. *Ann Rev Physiol.* 1988;50:483–508.

Freeman DA. Cyclic AMP mediated modification of cholesterol traffic in Leydig tumor cells. *J Biol Chem.* 1987;262:13061–13068.

Freeman DA, Ascoli M. Studies on the source of cholesterol used for steroid biosynthesis in cultured Leydig cells. *J Biol Chem.* 1982;257: 14231–14238.

Hall PF. Testicular steroid synthesis: organization and regulation. In: Knobil E, Neill J, eds. *The Physiology of Reproduction.* New York: Raven Press; 1988;975–998.

Hall PF, Osawa S, Mortek J. The influence of calmodulin on steroid synthesis in Leydig cells from rat testes. *Endocrinology.* 1981;109: 1677–1682.

Iida S, Papadopoulos V, Hall PF. The influence of exogenous free cholesterol on steroid synthesis in cultured adrenal cells. *Endocrinology.* 1989;124:2619–2624.

Kerr JB, Robertson DM, DeKretser DM. Morphological and functional characterization of interstitial cells from mouse testes fractionated on Percoll density gradients. *Endocrinology.* 1985;116:1030–1043.

Meikle AW, Stringham JD, Wilson DE, Dolman LI. Plasma 5 alpha-reduced androgens in men and hirsute women: role of adrenals and gonads. *J Clin Endocrinol Metab.* 1979;48:969–975.

Meikle AW, Benson SJ, Liu X-H, Boam WD, Stringham JD. Nonesterified fatty acids modulate steroidogenesis in mouse Leydig cells. *Am J Physiol.* 1989;257:E937–E942.

Moger WH. Effects of calcium channel blockers cobalt, verapamil, and D600 on Leydig cell steroidogenesis. *Biol Reprod.* 1983;28:528–535.

Patterson TR, Stringham JD, Meikle AW. Nicotine and cotinine inhibit steroidogenesis in mouse Leydig cells. *Life Sci.* 1990;46:265–272.

Sullivan MHF, Cooke BA. Stimulation of cholesterol side-chain cleavage by luteinizing-hormone releasing (luliberin) agonist (ICI 118630) in rat Leydig cells. *Biochem J.* 1983;216:747–752.

Sullivan MHF, Cooke BA. The role of Ca2+ in steroidogenesis in Leydig cells: stimulation of intracellular free Ca2+ by lutropin (LH), luliberin (LHRH) agonist and cyclic AMP. *Biochem J.* 1986;236:45–51.

Winters CA, Baukal AJ, Dufau ML. Calcium modulates membrane phosphorylation and adenylate cyclase activity in the Leydig cell. *67th Prog Endocrine Society.* 1983; 843.