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The Effect of Calcium Ions on Testosterone Production in Leydig Cells from Rat Testis

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Leydig-cell suspensions, prepared from rat testes, were incubated with different amounts of Ca^{2+} with and without added luteinizing hormone. The basal testosterone production in the absence of luteinizing hormone was unaffected by the Ca^{2+} concentration in the incubation medium. The luteinizing hormone-stimulated testosterone production, however, was progressively decreased in the absence of Ca^{2+} to one-third of that with 2.50mM-Ca^{2+}. This decrease in luteinizing hormone-stimulated testosterone production was independent of the different concentrations of luteinizing hormone (0–10μg/ml) used and could be restored by the addition of Ca^{2+} to the incubation medium. The restoration of the stimulation was achieved within 30min after the addition of Ca^{2+} to the medium. Activation of cyclic AMP-dependent protein kinase by luteinizing hormone was not decreased by omission of Ca^{2+} from the incubation medium, suggesting that Ca^{2+} may be involved in steroidogenesis at a stage beyond the luteinizing hormone receptor–adenylate cyclase–protein kinase system.

The regulation of steroidogenesis by trophic hormones such as adrenocorticotropin (corticotropin, ACTH) and luteinizing hormone (lutropin, LH) most probably involves stimulation of cyclic AMP production and protein kinase activation in their respective target organs (Schulster, 1974; Cooke & van der Kemp, 1976). The continuous synthesis of a protein (or proteins) is also necessary for this regulation (Garren *et al.*, 1965; Cooke *et al.*, 1975). However, the involvement of other processes cannot be excluded. For example, it has been established that the presence of extracellular Ca^{2+} is a prerequisite for full stimulation of corticosteroid synthesis by adrenocorticotropin in the adrenal gland (Birmingham *et al.*, 1953; Sayers *et al.*, 1972; Rubin *et al.*, 1972). Much less work has been carried out on the effect of extracellular Ca^{2+} in other steroidogenic tissues. Mendelson *et al.* (1975) reported that omission of Ca^{2+} from the incubation medium resulted in only a small decrease in the stimulation by human choriogonadotropin of testosterone production in rat testis Leydig cells. However, Van der Vusse *et al.* (1976) have reported that the stimulation of pregnenolone production by rat testis mitochondria after administration of luteinizing hormone *in vivo* could be completely mimicked by the addition of Ca^{2+} to the incubation medium of mitochondria. This apparent discrepancy between the effect of Ca^{2+} on steroid production by testis Leydig cells and by isolated mitochondria made us decide to investigate further the role of Ca^{2+} in the stimulation of testosterone production by luteinizing hormone in the rat testis by using a purified Leydig-cell preparation.

Materials and Methods

Sheep luteinizing hormone (NIH S18) was a gift from the NIAM, Bethesda, MD, U.S.A.

Crude collagenase was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A.

EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] and bovine serum albumin (fraction V) were obtained from Fluka A.G., Buchs, Switzerland, and the Ficoll 400 was from Pharmacia Fine Chemicals A.B., Uppsala, Sweden.

Adult male Wistar rats substrain R-Amsterdam (3–5months old) were used in this study.

Isolation and incubation of Leydig cells

The Leydig-cell suspensions were prepared and purified as described by Janszen *et al.* (1976). Briefly this method includes incubation of the testes with collagenase and centrifugation of the cell suspension through 13% Ficoll/0.2% bovine serum albumin in Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1964) containing 0.2% glucose, pH6.5, for 10min at 1500*g* followed by centrifugation of the sedimented cells through 6% Dextran T 250 in 0.9% NaCl solution for 2min at 100*g*.

After purification, the sedimented cells containing about 60% Leydig cells were resuspended in Krebs–Ringer bicarbonate buffer, pH7.4, from which the Ca^{2+} was omitted, but which contained 0.1% bovine serum albumin and 2mM-EGTA, and then pre-incubated for 30min at 32°C under an atmosphere of O_2+CO_2 (95:5) to remove all the bound Ca^{2+}. After preincubation the cells were sedimented by centrifugation for 5min at 100*g* and resuspended in Krebs–Ringer glucose buffer containing different concentrations of Ca^{2+} in 0.1mM-EGTA and 0.1% bovine serum albumin. The cells were then incubated as described in the Results section at 32°C under an atmosphere of O_2+CO_2 (95:5). Cell densities were about 1×10^6 cells/ml and were determined by counting the nucleated cells in a haemocytometer.

Testosterone production

After incubation, the testosterone was extracted from the cells plus medium with ethyl acetate (2×2 ml) and determined by radioimmunoassay as described by Verjans *et al.* (1973).

Protein kinase assay

Protein kinase activity was determined as described by Cooke & van der Kemp (1976) in the presence and absence of 0.6μM-cyclic AMP and the protein kinase activity ratio (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP) was calculated.

Results

Effect of extracellular Ca^{2+} on the testosterone production of isolated Leydig cells

Preincubated Leydig cells were incubated in Krebs–Ringer bicarbonate buffer, pH7.4, containing either 0, 1.25 or 2.5mM-Ca^{2+} with or without added luteinizing hormone, at a dose that gave maximum stimulation of testosterone production (100ng/ml).

The basal testosterone production without added luteinizing hormone was not significantly changed in the absence of Ca^{2+} (Table 1). However, in the absence of extracellular Ca^{2+} the stimulation of the testosterone production by luteinizing hormone was only one-third of the control values.

Influence of dose of added luteinizing hormone on the testosterone production in the absence or presence (2.5mM) of extracellular Ca^{2+}

Preincubated Leydig cells were incubated in the absence or presence of Ca^{2+} with different doses of luteinizing hormone. The absence of Ca^{2+} lowered the testosterone response in the presence of the different doses of luteinizing hormone tested (Fig. 1).

Table 1. *Influence of the concentration of Ca^{2+} in the medium on the testosterone production in isolated Leydig cells in the absence or presence of 100ng of added luteinizing hormone/ml*

Values are means±S.D. for the numbers of experiments in parentheses and are percentage syntheses compared with the control incubation containing 2.5mM-Ca^{2+}.

Ca^{2+} in incubation medium (mM)	Testosterone synthesis (%)	
	−Luteinizing hormone	+Luteinizing hormone
0	125±56% (10)	36±14% (11)*
1.25	129±41% (3)	79±15% (4)*
2.50	100 (10)	100 (11)

* Significantly different from control.

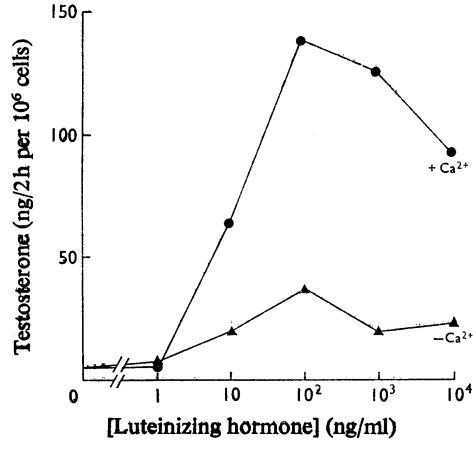

Fig. 1. *Luteinizing hormone dose–response curve of testosterone synthesis by isolated Leydig cells in the absence or presence of Ca^{2+}*

Leydig cells were incubated for 2h in the absence (▲) or presence (●) of Ca^{2+} (2.5mM) in the presence of different doses of luteinizing hormone (0–10μg/ml). Means of duplicate incubations are given.

Time-course of testosterone synthesis in isolated Leydig cells in the absence or presence of extracellular Ca^{2+}

Preincubated Leydig cells were incubated in the absence or presence of Ca^{2+} and in the absence or presence of added luteinizing hormone (100ng/ml) for different time-periods. No difference was detected in amounts of testosterone in all four types of incubation after 15min (Fig. 2). After 30min a stimulation

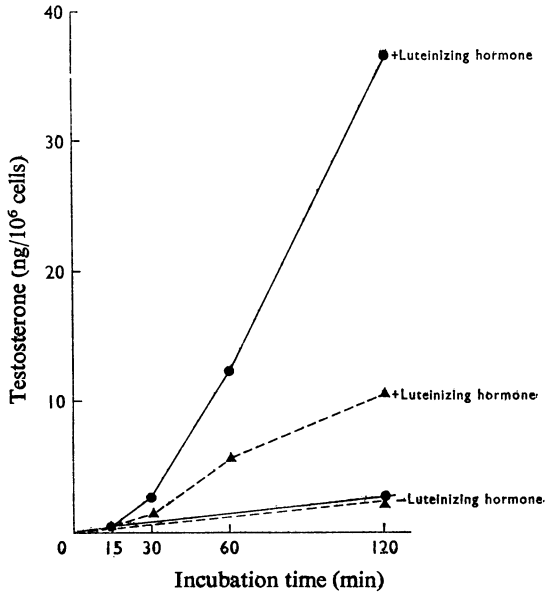

Fig. 2. *Time-course of testosterone production by Leydig cells incubated with or without Ca^{2+} with or without added luteinizing hormone*

Leydig cells were incubated for different time-periods up to 120min without Ca^{2+} (▲), with and without luteinizing hormone (100ng/ml) and with Ca^{2+} (2.5mM) (●) with and without luteinizing hormone. Means of duplicate incubations are given.

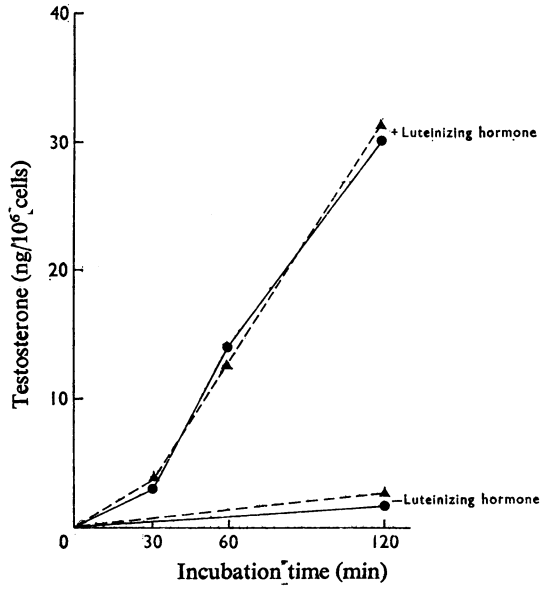

Fig. 3. *Time-course of testosterone production by Leydig cells in the presence of Ca^{2+} with or without luteinizing hormone, preincubated without or with Ca^{2+}*

Preincubated Leydig cells were incubated for 2h without (▲) or with Ca^{2+} (2.5mM) (●). After this incubation period Ca^{2+} was added (final concentration 2.5mM) to the cells incubated without Ca^{2+} and the Leydig cells were incubated for a further 2h without or with luteinizing hormone (100ng/ml). The amount of testosterone in cells plus medium was determined at different times after the addition of Ca^{2+} and luteinizing hormone. Means of duplicate incubations are given.

of the testosterone production was found in the presence of luteinizing hormone. The stimulation was less in the absence of Ca^{2+}. This decrease in the stimulation of the testosterone production remained during the rest of the incubation period.

Influence of re-addition of Ca^{2+} on the luteinizing hormone-stimulated testosterone synthesis in isolated Leydig cells after incubation without Ca^{2+}

Preincubated Leydig cells were incubated for 2h with or without Ca^{2+} in the absence of luteinizing hormone. After this incubation period, Ca^{2+} was added to the incubation medium without Ca^{2+} to give a final concentration of 2.5mM. The cells were then incubated with or without luteinizing hormone (100ng/ml) for 2h. Testosterone production was virtually the same after adjustment of the Ca^{2+} concentration to the control value (Fig. 3). This effect was already seen at 30min (the first time-interval studied) after the addition of the Ca^{2+} and luteinizing hormone. However, stimulated testosterone production did not reach the control value in all experiments after the addition of Ca^{2+} to the incubation medium. In six experiments the percentage of stimulated testosterone production during the first 2h incubation period in the absence of Ca^{2+} was 39 (S.E.M. $\pm$7)% of the control value, and after the addition of Ca^{2+} this value became 71 (S.E.M. $\pm$12) % of the control value during the second 2h incubation period. These values were significantly ($P<0.005$) different from each other.

Influence of extracellular Ca^{2+} on the activation of protein kinase activity in isolated Leydig cells

Leydig cells were prepared as described in the Materials and Methods section, except that the centrifugation through Dextran was omitted in order to obtain enough cells for the determination of testosterone production and protein kinase activity in the same cell preparation. The preincubated cells were incubated in Krebs–Ringer glucose buffer with or without 2.5mM-Ca^{2+} and with 0.1mM-EGTA. For the determination of protein kinase activity the cells were incubated with or

Table 2. *Influence of extracellular Ca^{2+} on the activation of protein kinase activity in isolated Leydig cells*

Values are means±S.D. with numbers of incubations in two separate experiments in parentheses. Luteinizing hormone when present was at a concentration of 100ng/ml.

Ca^{2+} in incubation medium (mM)	Testosterone production (ng/2h per 10^6cells)		Protein kinase activity ratio	
	−Luteinizing hormone	+Luteinizing hormone	−Luteinizing hormone	+Luteinizing hormone
2.50	1.4±0.4*(5)	135±15.4 (6)	0.17±0.09 (4)	0.76±0.10 (6)
0	2.4±1.8 (5)	71.4± 8.9 (6)	0.21±0.05 (4)	0.72±0.10 (6)

without luteinizing hormone for 20min, and for the determination of testosterone production cells were incubated for 120min. In the absence of Ca^{2+} the luteinizing hormone-stimulated testosterone production decreased to about one-half of that in the presence of 2.5mM-Ca^{2+}. However, omission of Ca^{2+} did not affect luteinizing hormone-stimulated protein kinase activity; in both the presence and the absence of Ca^{2+}, luteinizing hormone increased the protein kinase activity ratio from 0.2 to 0.75 (Table 2). The total protein kinase activity determined in the presence of excess of cyclic AMP was the same in all incubations [39.6 (S.D.±12.4, $n = 20$) pmol of ^{32}P incorporated/10^6cells].

Discussion

The results obtained in the present investigation clearly show that maximum luteinizing hormone stimulation of testosterone production in rat testis Leydig cells can only be obtained in the presence of Ca^{2+}. This requirement for Ca^{2+} was demonstrated at all doses of luteinizing hormone used; in the absence of Ca^{2+} the luteinizing hormone-stimulated testosterone production was decreased to about one-third of that obtained in the presence of 2.5mM-Ca^{2+}. These results are in contrast with those of Mendelson *et al.* (1975), who have reported that omission of Ca^{2+} from the incubation medium decreased the testosterone response only slightly at high doses of human choriogonadotropin and not at lower doses. In the adrenal for maximum stimulation of steroidogenesis by adrenocorticotropin the presence of Ca^{2+} in the medium is also necessary (Birmingham *et al.*, 1953; Farese, 1971; Rubin *et al.*, 1972; Sayers *et al.*, 1972; Haksar & Peron, 1973; Bowyer & Kitabchi, 1974; Kowal *et al.*, 1974; Wishnow & Feist, 1974). However, the decrease in the adrenal steroid production by omission of Ca^{2+} in the medium could be overcome at least partly by using higher amounts of adrenocorticotropin (Sayers *et al.*, 1972; Haksar & Peron, 1973; Bowyer & Kitabchi, 1974; Kowal *et al.*, 1974). From these latter results it was concluded that Ca^{2+} may be involved in the transmission of the signal arising with the adrenocorticotropin–receptor–adenylate cyclase system. Bowyer & Kitabchi (1974) reported for adrenal cells that lowering the Ca^{2+} concentration in the incubation medium also decreased the corticosteroid response to dibutyrylcyclic AMP, indicating that in adrenal cells Ca^{2+} may also be important after the elaboration of the second messenger. In their experiments the concentration of dibutyryl cyclic AMP necessary to achieve half-maximum steroid response did not change with decreasing Ca^{2+} concentrations; the corticosterone production in the absence of Ca^{2+} decreased to the same extent with different concentrations of dibutyryl cyclic AMP. In this respect the effect of Ca^{2+} concentration on the luteinizing hormone dose–response curve in rat Leydig cells may be compared with the effect of Ca^{2+} on the dibutyryl cyclic AMP dose–response curve in rat adrenal cells. Further, the present study has shown that luteinizing-hormone activation of protein kinase in the Leydig cells is not affected by omission of Ca^{2+} from the medium, so the effect of Ca^{2+} is then most probably after the activation of protein kinase. One objection to the results for the protein kinase activity could be that the enzyme activity was measured only 20min after the addition of luteinizing hormone. However, as shown in Figs. 2 and 3, the effect of Ca^{2+} on the stimulation of steroidogenesis is obtained within 30min of incubation. It is not possible to conclude which of the processes involved in the regulation of testosterone synthesis, after the activation of protein kinase, have been affected by the omission of Ca^{2+} from the medium; it may be protein synthesis, as suggested by Farese (1971), or at the mitochondrial level, as suggested by Van der Vusse *et al.* (1976). Another possible explanation is that Ca^{2+} is necessary for the production of certain substrates for testosterone synthesis, which become limited when higher amounts of testosterone are produced. If this were true, one would expect that omission of Ca^{2+} had a greater effect on high testosterone production rates rather than on lower ones. This was shown not to be the case; even 30min after addition of luteinizing hormone, when testosterone production was still low, a smaller production in the absence

of Ca^{2+} compared with the control was already apparent. Also with submaximum doses of luteinizing hormone, testosterone production was markedly decreased compared with the control.

Another explanation that has to be considered is that the decrease in luteinizing-hormone stimulation of testosterone synthesis could have been caused by damage to the cells through the absence of Ca^{2+} in the incubation medium. This possibility was investigated by measuring testosterone synthesis with and without luteinizing hormone after the addition of Ca^{2+} to the Leydig-cell suspension, which had been preincubated for 2h in the absence of Ca^{2+}. In all experiments the addition of Ca^{2+} considerably increased the testosterone production and in some of the experiments testosterone production was restored to control values within 30min after the addition of Ca^{2+} to the cells. This indicates that damage to the cells did not take place or only to a limited extent.

In conclusion, it is apparent from the present study that Ca^{2+} is necessary for luteinizing-hormone stimulation of testosterone biosynthesis in testis Leydig cells. Its site of action is probably after the activation of protein kinase by luteinizing hormone. However, its precise mode of action remains to be elucidated.

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