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Correlation of Protein Kinase Activation and Testosterone Production after Stimulation of Leydig Cells with Luteinizing Hormone

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The effect of different doses of luteinizing hormone on activation of protein kinases, cyclic AMP and testosterone production was studied in purified rat testis Leydig-cell preparations in the presence of 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor). In addition, the nature of the protein kinases present in these cells and other tissues was investigated. The following results were obtained. 1. With all the amounts of luteinizing hormone used (0.1–1000ng/ml), both activation of protein kinase and stimulation of testosterone production were demonstrated. With the lowest amount of luteinizing hormone (0.1ng/ml), an 8.4±0.9% (S.E.M., $n = 6$) stimulation of protein kinase activation occurred, increasing to 100% with 1000ng/ml, compared with 3.2±1.0% (S.E.M., $n = 7$) and 100% stimulation of testosterone production with 0.1 and 100ng/ml respectively. 2. With amounts of luteinizing hormone up to 1ng/ml (which gave half-maximal stimulation of testosterone production) no detectable increases in net cyclic AMP production were obtained. With higher amounts of luteinizing hormone, cyclic AMP production increased, but maximal production was not reached with 1000ng/ml. 3. Two isoenzymic forms of protein kinase were present in Leydig cells and seminiferous tubules; type I was eluted with 0.075M- and type II with 0.22–0.25M-NaCl from DEAE-cellulose columns. 4. The protein kinase activity was not affected by the presence of erythrocytes in the Leydig-cell preparation, but varied depending on the type of histone used as substrate (histone F2b > mixed > histone F1).

The available evidence indicates that cyclic AMP is an intracellular messenger of luteinizing-hormone (lutropin, LH) action on steroidogenesis in the rat testis (see review by Rommerts *et al.*, 1974). However, the evidence for an obligatory role of this nucleotide is less certain. This is mainly because several investigations have shown that there is a discrepancy between the amounts of luteinizing hormone required to stimulate synthesis of testosterone and cyclic AMP; approximately ten times more luteinizing hormone is required to detect changes in cyclic AMP production compared with the amount required to stimulate testosterone production (Catt & Dufau, 1973; Moyle & Ramachandran, 1973; Rommerts *et al.*, 1973). Further, Catt & Dufau (1973) showed that the production of testosterone was already maximal before changes in cyclic AMP could be detected. This has led to the conclusion that the role of cyclic AMP at physiological concentrations of choriogonadotropin and luteinizing hormone may not be obligatory. However, small changes in cyclic AMP production may occur within the cell which are not detectable by the methods used; that the latter may be true is indicated by the stimulatory effect of theophylline (a phosphodiesterase inhibitor) on testosterone release, but not on detectable cyclic AMP production, by low amounts of choriogonadotropin (Catt & Dufau, 1973).

The only known mechanism by which cyclic AMP acts within eukaryotic cells is by activation of cyclic AMP-dependent protein kinases (see review by Rubin & Rosen, 1975), according to the following scheme:

$$R_2C_2 \text{ (inactive)} + 2 \text{ cyclic AMP} \rightleftharpoons R_2 \text{ cyclic AMP}_2 + 2C \text{ (active)}$$

in which cyclic AMP binds to a regulatory protein (R) affecting the release of the active catalytic subunit (C). On removal of the cyclic nucleotide, the R and C components reassociate to re-form the inactive holoenzyme. Protein kinase activation after incubation of testis Leydig-cell preparations with luteinizing hormone has previously been demonstrated (Cooke & van der Kemp, 1976). The present investigation was undertaken to determine if at low concentrations of luteinizing hormone, protein kinase activation in Leydig cells (which presumably reflects cyclic AMP changes) is a more sensitive parameter than cyclic AMP concentration itself. During the course of this study it was found that the testis contains two isoenzymic forms of protein kinase; various methods

have been investigated to optimize the assay conditions so that the activity of both these enzymes may be determined.

Materials and Methods

Materials

Sheep luteinizing hormone (NIH-LH-S18; 1.03 i.u./mg) was a gift from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. ATP and cyclic AMP were purchased from Boehringer, Mannheim, W. Germany. Mixed histone preparation (dialysed, salt-free, freeze-dried) and histones F1 (lysine-rich) and F2b (low lysine content) were obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. and 3-isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Triton X-100 was purchased from BDH Chemicals, Poole, Dorset, U.K., and Whatman 3MM paper and Whatman DEAE-cellulose type DE-23 were from W. and R. Balston Ltd., Maidstone, Kent, U.K. [γ-^{32}P]ATP (ammonium salt; freeze-dried preparation of specific radioactivity 16.10Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Adult male Wistar rats, substrain R-Amsterdam (14–16 weeks old, 200–250g), kept under controlled light (14h light and 10h dark) and temperature (20–22°C) conditions, were used in this study.

Leydig-cell preparation

The method used was as described by Janszen *et al.* (1976) and is briefly as follows. The decapsulated testes were incubated with collagenase and the cell suspension obtained was centrifuged through 13% (w/v) Ficoll/0.2% albumin solutions to increase the percentage of Leydig cells. The resuspended Leydig cells were then incubated (1×10^6–3×10^6 nucleated cells/ml; incubation volume 0.25ml) for 20min (for protein kinase and cyclic AMP assay) and 2h (for testosterone and cyclic AMP assay) at 32°C, with and without various amounts of luteinizing hormone and 0.5mM-3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor).

Protein kinase assay

After incubation the cells were cooled in ice and then centrifuged for 10min at 100*g*. The pellets were resuspended, sonicated (5s), and protein kinase activity was determined as described by Cooke & van der Kemp (1976), except that the reactions were stopped by pipetting 50μl samples on to strips of 3MM Whatman paper and then washed and counted for radioactivity as described by Corbin *et al.* (1975). The protein kinase activity is expressed in pmol of ^{32}P incorporated/10^6 cells or as the protein kinase activity ratio (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP). In Fig. 2 the effect of luteinizing hormone was calculated on the basis of the following formula:

$$100-\left(\frac{A_{LH}-B_{LH}}{A_c-B_c}\times100\right)$$

where A and B are protein kinase activities after and before addition of excess of cyclic AMP to the cell homogenates. The suffixes refer to the values obtained with the controls (c) and after incubation of the intact cells with luteinizing hormone (LH). All protein kinase assays were carried out as rapidly as possible after homogenization of the cells; no change in the recovery or protein kinase activity ratio was observed between control samples assayed at the beginning of the assay and again after the whole series of samples had been assayed (time taken approx. 35min).

Determinations of cyclic AMP and testosterone

These were carried out on the combined cell and incubation extracts by saturation analysis as previously described (Rommerts *et al.*, 1973).

DEAE-cellulose column chromatography

The DEAE-cellulose was regenerated and used in the acetate form. A column (4cm×1cm) was prepared and washed with 5mM-Tris/HCl buffer, pH7.5, containing 1mM-EDTA. The testis tissue extracts were prepared by wet dissection of testis tissue as previously described (Cooke *et al.*, 1972) and then homogenized (1g wet wt/ml) in Tris buffer, pH7.5, in a Teflon-pestle/glass-tube homogenizer at 4°C (3×1100rev./min) and then centrifuged at 27000*g* for 30min. As controls, rat heart and adipose-tissue homogenates were similarly prepared as described by Corbin *et al.* (1975). The tissues were then separately chromatographed (2ml and 1ml of the seminiferous-tubule and interstitial-tissue supernatant fractions were used respectively) as described by Corbin *et al.* (1975) as follows: the column was washed with 50ml of Tris buffer containing 1mM-EDTA, pH7.5, followed by a salt gradient of 100ml of Tris/EDTA buffer, pH7.5, and 100ml of Tris/EDTA buffer, pH7.5, containing 0.4M-NaCl. Fractions (3ml) were collected at approx. 0.5ml/min. The chromatography was carried out at room temperature (20°C).

Results

(I) *Factors affecting protein kinase activity in testis Leydig cells*

(i) *Number of cells and incubation time.* Incubation of the cells with and without luteinizing hormone for

Table 1. *Incorporation of ^{32}P into different histones by Leydig-cell homogenates*

The protein kinase activity was determined in Leydig-cell homogenates in the presence of different histones (0.5mg) from cells incubated with and without luteinizing hormone (100ng/ml) and 3-isobutyl-1-methylxanthine (0.5mM) as described in the Materials and Methods section. Means of duplicate determinations are given. The protein kinase activity ratios (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP) were also calculated.

	Control			+Luteinizing hormone (100ng/ml)		
	Protein kinase activity (pmol of $^{32}P/10^6$ cells)			Protein kinase activity (pmol of $^{32}P/10^6$ cells)		
Histone	−Cyclic AMP	+Cyclic AMP	Protein kinase activity ratio	−Cyclic AMP	+Cyclic AMP	Protein kinase activity ratio
Mixed	5.2	11.7	0.46	7.7	8.3	0.93
Hf1 histone (lysine-rich)	1.4	8.5	0.17	4.1	9.4	0.44
Hf2b histone (lysine-poor)	14.2	50.4	0.27	30.2	46.1	0.65

Table 2. *Effect of luteinizing hormone on protein kinase activity in rat testis Leydig cells*

Results are the means from duplicate incubations of Leydig cells with various amounts of luteinizing hormone with 3-isobutyl-1-methylxanthine (0.5mM). In Expt. 1 the cells were incubated directly with luteinizing hormone immediately after preparation. In Expt. 2 the cells were first preincubated in medium only for 2h before addition of the luteinizing hormone. In both experiments after incubation for 20min with luteinizing hormone the cells were centrifuged (100*g*, 10min) and resuspended and homogenized in a phosphate medium as described in the Materials and Methods section. The incorporation of ^{32}P and the protein kinase activity ratios (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP) were calculated.

	Expt. 1			Expt. 2		
	Protein kinase activity (pmol of $^{32}P/10^6$ cells)			Protein kinase activity (pmol of $^{32}P/10^6$ cells)		
Luteinizing hormone (ng/ml)	−Cyclic AMP	+Cyclic AMP	Protein kinase activity ratio	−Cyclic AMP	+Cyclic AMP	Protein kinase activity ratio
0	2.8	10.2	0.27	10.8	30.3	0.36
0.1	—	—	—	11.9	29.3	0.41
0.5	—	—	—	13.9	30.3	0.46
1.0	4.1	10.4	0.39	15.0	30.1	0.50
10	6.2	7.9	0.78	21.6	28.8	0.75
100	5.7	7.5	0.76	23.9	26.5	0.90
1000	5.1	5.8	0.88	27.4	27.8	0.99

more than 20min did not subsequently further increase the amount of $^{32}P_i$ incorporated into histone and with longer periods of incubation (2h) the total activity decreased, probably owing to the 'instability' of the catalytic subunit (see below).

The amount of $^{32}P_i$ incorporated into histone, without and with added cyclic AMP, increased linearly with increasing number of homogenized cells up to the equivalent of 25000 cells/incubation; 14000 cells were used as a routine. The number of intact nucleated cells incubated varied between 1×10^6 and 3×10^6/ml; the volume of homogenization buffer added after centrifugation of incubated cells was therefore adjusted so that the equivalent of 14000 homogenized cells/20μl was added to the protein kinase assay.

(ii) *Different histones.* Histone Hf2b (low lysine content) incorporated approximately five times as much phosphate as did the mixed histone preparation and histone HF1 (lysine-rich) (Table 1). The activity ratios and the effect of luteinizing hormone also varied, depending on the histone used; with the mixed histone, the protein kinase was completely activated by the luteinizing hormone, whereas the Hf1 and Hf2b histones were less effective. The mixed histone preparation was used in all subsequent studies.

(iii) *Erythrocytes.* The Leydig-cell preparation obtained by purification through Ficoll contains 6–10 times more erythrocytes than do nucleated cells, which may have contained appreciable protein kinase activity. However, no change in protein kinase activity was found after removal of the erythrocytes by

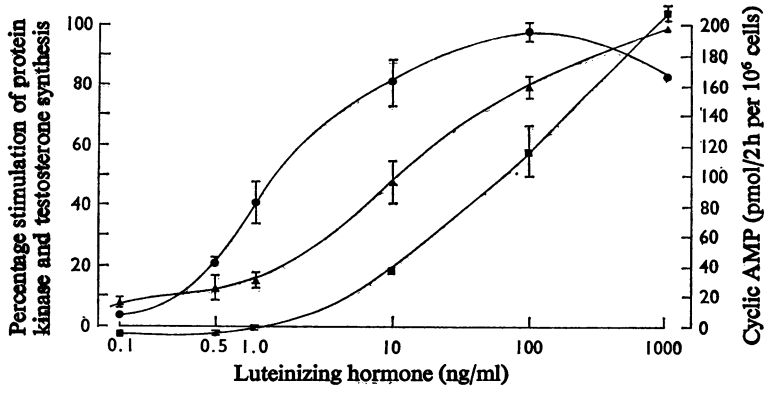

Fig. 1. *Luteinizing hormone dose–response curves for protein kinase activation, production of cyclic AMP and testosterone in Leydig cells*

For testosterone (●) and protein kinase (▲) the results presented are the means±S.E.M. of four separate experiments carried out with Leydig cells prepared from 16 rats (four rats/experiment). The cells were preincubated for 2h at 32°C under CO_2+O_2 (5:95) and then incubated for a further 20min (for protein kinase assay) and 2h (for testosterone and cyclic AMP assays) with different amounts of luteinizing hormone without changing the incubation medium. All incubations were carried out in duplicate for both protein kinase and testosterone determinations. The protein kinase was determined in cell homogenates obtained after centrifugation (100*g*, 10min) of the cells and resuspending in the phosphate medium. Testosterone was determined by radioimmunoassay on the cells plus medium. Results are expressed as percentage of maximum stimulation obtained with luteinizing hormone. Further details are given in the Materials and Methods section. The means±range of duplicates for cyclic AMP production (■) with different amounts of luteinizing hormone are given from one typical experiment.

prior perfusion of the testes *in vivo* with 0.9% NaCl as described by Frederik & van Doorn (1973). In addition, no detectable protein kinase activity was found in diluted rat blood when the same concentration of erythrocytes found in the Leydig-cell preparations were used.

(II) *Luteinizing hormone dose–response curves for protein kinase activation, production of cyclic AMP and testosterone*

The effect of different amounts of luteinizing hormone on the dissociation of the protein kinase holoenzyme was investigated. Increasing the amount of luteinizing hormone increased the dissociation of the holoenzyme as reflected by the increase in protein kinase activity ratio (Table 2). However, decreases in the total amount of protein kinase recovered were observed after addition of 10ng or more of luteinizing hormone/ml. This effect varied from one experiment to another, e.g. in Expt. 1 the recovery was decreased by approx. 50% with 1000ng of luteinizing hormone/ml compared with 9% in Expt. 2. More recent experiments have shown that the loss in activity was minimal and similar to that found in Expt. 2 if the protein kinase assay is carried out as quickly as possible after homogenization of the cells. Addition of albumin (10mg/ml) to the homogenization medium did not prevent this decrease (results not shown). This apparent decrease in activity was probably caused by 'instability' of the catalytic subunit; the undissociated holoenzyme on the other hand is thought to be stable (see the Discussion section). The degree of activation of the protein kinase has therefore been calculated as the percentage change in the amount of the holoenzyme caused by the addition of luteinizing hormone (i.e. zero in the absence of luteinizing hormone) (see the Materials and Methods section for details). The results presented in Fig. 1 (from four different experiments with duplicate incubations) have been calculated in this way. Initial experiments were carried out by incubating the resuspended cells with different amounts of luteinizing hormone immediately after centrifugation with Ficoll solutions (e.g. Expt. 1, Table 2). However, it was found that more consistent results, especially with low amounts of luteinizing hormone, were obtained if the cells were first incubated for 2h at 32°C before addition of the trophic hormone. By using preincubated cells it was found that with all the amounts of luteinizing hormone used (0.1–1000ng/ml) a stimulation of protein kinase activity occurred (Fig. 1). With the lowest amount, 0.1ng/ml, a significant 8.4 (S.E.M.±0.9)% increase ($P<0.001$, $n=6$) was obtained which progressively increased to 100% with 1000ng/ml. The testosterone production (plotted as percentage of maximum luteinizing hormone-stimulated production) was significantly increased

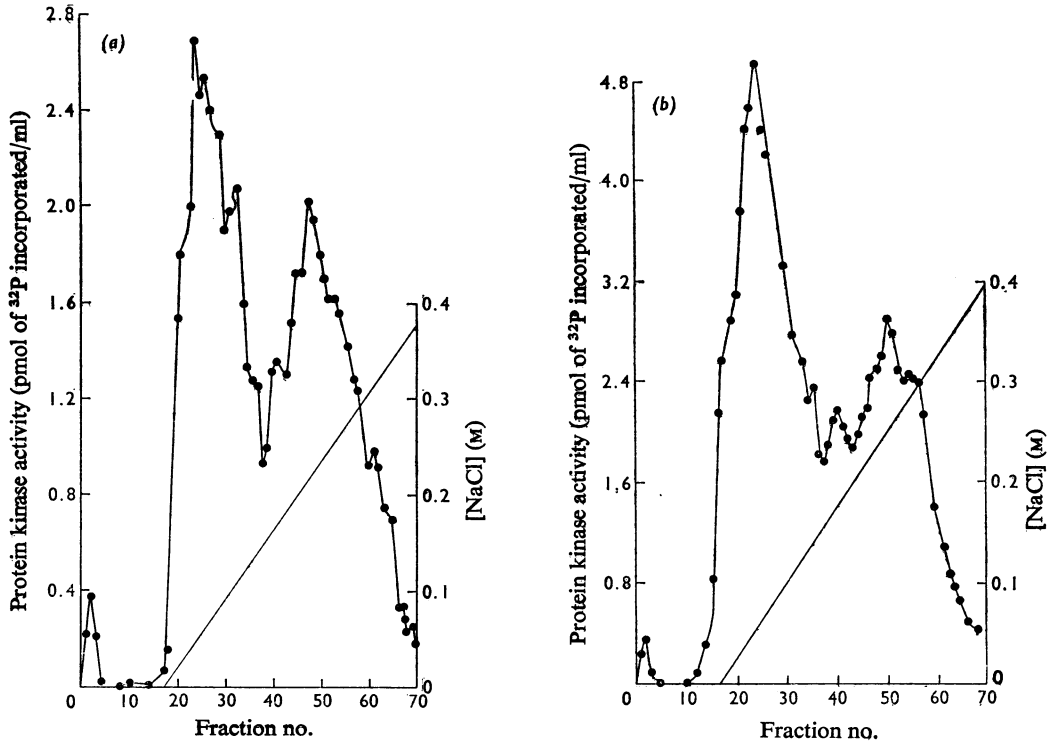

Fig. 2. *DEAE-cellulose column chromatography of testis interstitial tissue (a) and seminiferous tubules (b)*

The interstitial and seminiferous-tubule tissue was homogenized and the supernatant (containing the equivalent of 0.25g and 0.5g wet wt. respectively) obtained after centrifugation at 27000*g* for 30min was chromatographed on DEAE-cellulose columns (4cm×1cm). The columns were eluted with 50ml of Tris buffer/1mM-EDTA, pH7.4, followed by a salt gradient of 100ml of Tris buffer/1mM-EDTA and 100ml of Tris buffer/1mM-EDTA/0.4M-NaCl. Fractions (70) of volume 3ml were collected at a rate of 0.5ml/min at room temperature. The eluates (50μl batches) were assayed for protein kinase activity in the presence of excess of cyclic AMP as described in the Materials and Methods section.

Table 3. *Effect of different concentrations of 3-isobutyl-1-methylxanthine on testosterone production in Leydig cells*

The Leydig cells were preincubated for 2h and then incubated for a further 2h (without changing the incubation medium) with different concentrations of 3-isobutyl-1-methylxanthine in the presence and absence of added luteinizing hormone. Results are means±ranges from duplicate incubations from testosterone determinations in cells plus medium.

Luteinizing hormone (1ng/ml)	3-Isobutyl-1-methylxanthine (mM)	Testosterone production (ng/2h per 10^6 cells)
−	—	2.3±0.3
−	0.1	2.8±0.1
−	0.25	3.1±0.6
−	0.50	4.1±0.3
+	—	4.2±0.2
+	0.1	26.1±4.5
+	0.25	32.2±0.6
+	0.50	26.1±2.1

with 0.1ng of luteinizing hormone/ml ($P<0.01$, $n=7$), but a lower percentage increase [3.2(S.E.M. ±1.0)%] was obtained compared with the stimulation of the protein kinase. Smaller amounts of luteinizing hormone (100ng/ml) were required to reach maximum testosterone production compared with those required for maximum protein kinase activation.

The changes in cyclic AMP production in the cells plus incubation medium were also measured after incubation with different doses of luteinizing hormone. In initial experiments (with non-preincubated cells) large amounts of luteinizing hormone (100ng/ml) were required before significant changes ($P<0.025$, $n=6$) could be detected during 20min incubation. However, significant changes with lower amounts of luteinizing hormone (1.0ng/ml) ($P<0.01$, $n=6$), were detected if the cyclic AMP concentrations were measured after 2h incubation. With both non-preincubated and preincubated cells maximum cyclic

AMP production was not obtained with the highest amount of luteinizing hormone used (1000ng/ml). In Fig. 1 the means and ranges of values for cyclic AMP production are given for preincubated (2h) cells after stimulation with different amounts of luteinizing hormone for 2h.

(III) *Effect of 3-isobutyl-1-methylxanthine on testosterone production in Leydig cells*

All the above studies were carried out in the presence of 0.5mM-3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity in the Leydig cells. The effect of different amounts of this compound was investigated to check if it had any inhibitory effect on testosterone production. The results presented in Table 3 show that the basal testosterone production slightly increased when the amount of 3-isobutyl-1-methylxanthine was increased from zero to 0.5mM. Very little effect of luteinizing hormone (1ng/ml) was seen in the absence of the phosphodiesterase inhibitor, but with 0.1, 0.25 and 0.5mM-3-isobutyl-1-methylxanthine large increases were found. The highest amount used (0.5mM) only slightly decreased the testosterone production compared with 0.25mM, indicating that 3-isobutyl-1-methylxanthine has little inhibitory effect on luteinizing hormone-stimulated testosterone production.

(IV) *Isoenzymic forms of cyclic AMP-dependent protein kinase*

A preparation of rat testis interstitial tissue containing Leydig cells was subjected to DEAE-cellulose column chromatography to determine if the isoenzymic forms of the protein kinases demonstrated in other tissues (Corbin *et al.*, 1975) were also present in this tissue. As controls, rat heart and adipose tissue (containing type I and type II isoenzymes respectively) were also separately chromatographed; in agreement with published data (Corbin *et al.*, 1975), the latter were eluted as single peaks with 0.075M-NaCl and 0.25M-NaCl respectively (results not shown). The interstitial tissue contained two main peaks of activity (Fig. 2*a*) which corresponded to the type I and type II isoenzymes, except that the second peak was eluted with a lower concentration of salt (0.22M). Seminiferous tubules (Fig. 2*b*) and whole testis extracts also contained two main peaks of activity, and these were eluted with 0.075M- and 0.25M-NaCl.

Previously it has been demonstrated that NaCl dissociates the type I holoenzyme (Corbin *et al.*, 1975), whereas it prevents reassociation of the R and C subunits of the type II enzyme (Corbin *et al.*, 1973). The ionic strength of the homogenization medium used will therefore influence the apparent endogenous protein kinase activity in homogenized cells which contain both isoenzymic types, as in the Leydig cells.

Table 4. *Effect of 0.15M-KCl and albumin on protein kinase activity in Leydig-cell homogenates*

Cells obtained from incubations without (A) and with (B) luteinizing hormone (100ng/ml) and 3-isobutyl-1-methylxanthine (0.5mM) were centrifuged and resuspended and homogenized in a phosphate medium (see the Materials and Methods section) containing 0.15M-KCl and albumin (10mg/ml) and incubated for various times at 0–4°C before assay of protein kinase activity in the presence and absence of 0.6μM-cyclic AMP. The incorporation of ^{32}P/10^6 cells and protein kinase activity ratios (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP) were calculated.

	Incubation time (min)	Protein kinase activity (pmol of ^{32}P/10^6 cells) −Cyclic AMP	Protein kinase activity (pmol of ^{32}P/10^6 cells) +Cyclic AMP	Protein kinase activity ratio
A	0	19.4	56.5	0.34
	15	10.6	30.3	0.35
	30	13.2	34.2	0.39
	60	23.0	42.7	0.53
B	0	44.4	53.4	0.83
	15	26.6	28.2	0.94
	30	27.6	34.0	0.81
	60	46.8	50.0	0.94

Previous studies with Leydig-cell preparations (Cooke & van der Kemp, 1976) showed that 0.25–0.5M-NaCl caused dissociation and inhibition of the protein kinases and therefore the present experiments were carried out in the absence of NaCl in the homogenization medium. When it was found that the type II enzyme was present in the Leydig-cell preparation, it was decided to re-investigate the effect of salt at concentrations which caused minimum dissociation of the type I enzyme but possibly prevented reassociation of the type II enzyme. Another reason for doing this was that Keely *et al.* (1975) have shown that 0.15M-KCl prevents loss of protein kinase activity because of adsorption on subcellular particles (see the Discussion section).

Addition of 0.15M-KCl and albumin to the homogenization medium was found to have little effect on the protein kinase activity ratio if the protein kinase assays were carried out immediately after homogenization. However, if the homogenates were left at 0–4°C a loss of activity occurred (Table 4).

Good recoveries of the protein kinase activity were obtained in homogenates from luteinizing hormone-stimulated cells (Table 4), but again decreases occurred if the homogenates were left at 0–4°C. After 60min most of the initial activity was recovered, but the protein kinase activity ratio had increased, indicating increased dissociation of the type I holoenzyme. These changes in activity did not occur in the absence

of added KCl and albumin or with albumin alone. Several incubations were carried out with Leydig cells stimulated with different doses of luteinizing hormone, followed by homogenization in 0.15M-KCl and albumin (10mg/ml) and immediate assay of protein kinase activity. The minimal amount of luteinizing hormone required to detect protein kinase activation under these conditions was 1–10ng/ml, whereas the testosterone production was stimulated with 0.1–1.0ng/ml.

It may be concluded that although 0.15M-KCl prevents 'losses' of protein kinase activity if the assays are carried out immediately after homogenization, the apparent total endogenous protein kinase activity is decreased, as reflected by an apparent loss in sensitivity to luteinizing hormone compared with results obtained after homogenization in low-ionic-strength media [see Paragraph (II), above].

Discussion

In agreement with previous studies with testis Leydig-cell preparations (Catt & Dufau, 1973; Moyle & Ramachandran, 1973; Rommerts *et al.*, 1973; Mendelson *et al.*, 1975), it has been found in the present study that testosterone production is stimulated by amounts of luteinizing hormone that do not result in detectable stimulation of cyclic AMP production; approximately half-maximum testosterone production was reached before changes in cyclic AMP concentrations could be detected. However, the activation of protein kinase by luteinizing hormone was found to be very closely linked to the changes in testosterone production; at all doses of luteinizing hormone used, changes in both protein kinase activation and testosterone production could be demonstrated. The only difference found was that maximum testosterone production was reached with lower amounts of luteinizing hormone than those required to stimulate protein kinase activity maximally. However, it is highly likely that cyclic AMP-dependent protein kinases are utilized for processes in the cell in addition to those involved in steroidogenesis. In this respect the type I and type II protein kinases found in the Leydig cells (and seminiferous tubules) may have different functions. Activation of cyclic AMP-dependent protein kinases in intact cells has now been reported for several hormones and tissues (see review by Rubin & Rosen, 1975), although relatively few dose–response studies have been carried out in which the protein kinase activation has been correlated with physiological effects of the hormone.

Field *et al.* (1975) have reported that the minimum and maximum amounts of thyrotropin required to activate protein kinases in intact thyroid cells correlated well with other effects of this hormone (including cyclic AMP production). For the adrenal cell, Richardson & Schulster (1973) have also reported a correlation between protein kinase activation and steroidogenesis in intact cells, although with low amounts of adrenocorticotropin, which gave a submaximum stimulation of steroidogenesis, no detectable increase in protein kinase activation was detected.

The R and C subunits of the type II protein kinase present in Leydig cells, as demonstrated by DEAE-cellulose chromatography, would have been expected to reassociate under the homogenization of the cells (Corbin *et al.*, 1973), yet full activation of the protein kinases was achieved. Perhaps the Leydig-cell type II protein kinase is different from that found in adipose tissue and also seminiferous tubules (slightly different concentrations of salt were required to elute it from the DEAE-cellulose column). In Sertoli-cell preparations from seminiferous tubules Means *et al.* (1976) have reported that follicle-stimulating hormone did not cause full activation of cyclic AMP-dependent protein kinases and that this was due to a specific effect on the type I but not the type II enzyme present in this cell type.

A decrease in the total amount of protein kinase in Leydig-cell homogenates was found in the present experiments after stimulation with large amounts (over 10ng/ml) of luteinizing hormone. There have been other reports of similar findings in various tissues (Walaas *et al.*, 1973; Korenman *et al.*, 1974; Jungmann *et al.*, 1974; Palmer *et al.*, 1974; Keely *et al.*, 1975; Cooke & van der Kemp, 1976) and this has been attributed to the instability of the catalytic subunit and/or the binding of the subunit to subcellular particles. It has been reported that the catalytic subunit, but not the holoenzyme, exhibits a high isoelectric point (Chen & Walsh, 1971), so that it binds to proteins or other compounds of opposite charge in crude systems. This is particularly so if the studies are carried out in media of low ionic strength. Keely *et al.* (1975) found that the binding of the catalytic subunit to 12000*g* heart particulate fractions was inversely related to ionic strength up to 150mM. In the present study, good recoveries of the protein kinase were obtained if the homogenization medium contained 150mM-KCl, provided that there was no delay in assaying the protein kinase. However, this method is difficult to carry out with a large series of samples and the apparent sensitivity to luteinizing hormone for protein kinase activation was lowered. It was decided therefore that it was more accurate to omit salt from the homogenization medium and express the results obtained in terms of changes in the stable holoenzyme (see also Walaas *et al.*, 1973). The amount of holoenzyme present in the Leydig-cell homogenate was determined by adding excess of cyclic AMP to convert it into the active catalytic subunit; under these circumstances the latter presumably quickly and preferentially binds to the excess of histone substrate added.

To conclude, a close correlation between protein kinase activation and testosterone production has been demonstrated. It is possible therefore that cyclic AMP is an obligatory intermediate in the action of luteinizing hormone and that small but undetectable changes in cyclic AMP production occur with submaximal amounts of luteinizing hormone. However, it cannot be excluded that there are other factor(s) involved which also stimulate protein kinase activation.

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