Protein Kinase Activity in Rat Testis Interstitial Tissue

EFFECT OF LUTEINIZING HORMONE AND OTHER FACTORS

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Protein kinase activity was determined in subcellular fractions of rat testis interstitial tissue after incubation of the intact tissue with LH (luteinizing hormone) *in vitro*. Various factors that might have changed the activity of this enzyme during preparation of the fractions before assay were also investigated. The following results were obtained. 1. LH and 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) added together during incubation of the interstitial tissue caused a twofold increase in the protein kinase activity in the total tissue homogenate and subcellular fractions (12000g×5min pellet and 105000g×60min supernatant and pellet). 2. A decrease of approx. 40% in the total amount of protein kinase recovered in the soluble fraction (105000g supernatant) occurred in tissue incubated with LH and 3-isobutyl-1-methylxanthine when compared with the controls. No change in total activity was found in the other fractions. 3. LH and 3-isobutyl-1-methylxanthine caused an increase in cyclic AMP concentration in the soluble fraction (from 30 ± 6 to 450 ± 40 pmol/mg of protein, means $\pm$ S.E.M., $n=4$), but there was little or no increase in the particulate fractions [from 9 ± 1 to 13 ± 3 pmol/mg of protein ($n=3$) and from 6 ± 2 to 23 ± 11 pmol/mg of protein ($n=3$) in the 12000g and 105000g pellets respectively]. 4. Addition of 3-isobutyl-1-methylxanthine alone had little effect on protein kinase activity or cyclic AMP concentrations. 5. Little or no protein kinase activity could be demonstrated in subcellular particulate fractions unless Triton X-100 was added; the effect of this detergent was shown to be at least partly due to the inhibition of adenosine triphosphatase activity. 6. In the presence of Triton X-100 approx. 57% of the total protein kinase activity in the homogenate was found in the 105000g supernatant compared with 11% in the 105000g pellet and 32% in the 12000g pellet. 7. In contrast with adipose-tissue protein kinase [Corbin *et al.* (1973) *J. Biol. Chem.* 248, 1813-1821] the relative amounts of cyclic AMP-independent and -dependent enzyme were not affected by dilution of the interstitial-tissue fractions. NaCl (0.5M) decreased the estimated total amount of protein kinase activity.

The available evidence suggests that cyclic AMP may be the intracellular mediator of LH* action on steroidogenesis in the testis interstitial Leydig cells (see review by Rommerts *et al.*, 1974). It has also been proposed that a protein regulator(s) may be involved in steroidogenesis in the Leydig cells (Cooke *et al.*, 1974, 1975b; van der Vusse *et al.*, 1975). It is possible that cyclic AMP may stimulate the formation of the protein regulator and that this may occur via mechanisms involving cyclic AMP-dependent protein kinases (ATP-protein phosphotransferase, EC 2.7.1.37). In two other steroidogenic tissues, adrenal gland and ovary, it has been demonstrated that cyclic AMP-dependent protein kinases are acti-

vated by ACTH and LH respectively and that they may also be involved in steroidogenesis (Gill & Garren, 1970; Zor *et al.*, 1972; Menon, 1973; Richardson & Schulster, 1973; Jungmann *et al.*, 1974). The effect of LH on protein kinases in the testis has not been investigated previously, but earlier work on the testis has shown that cyclic AMP-dependent protein kinases are present (Reddi *et al.*, 1971) and that FSH can stimulate protein kinase activity in the seminiferous tubules (Means *et al.*, 1974).

The present investigation was undertaken to determine quantitatively the protein kinase activity in subcellular fractions of rat testis interstitial tissue (which contains the Leydig cells). The particular emphasis has been to determine if the activities of the cyclic AMP-dependent and cyclic AMP-independent protein kinases are altered by the method of

* Abbreviations: LH, luteinizing hormone; ACTH, adrenocorticotrophin; FSH, follicle-stimulating hormone; ATPase, adenosine triphosphatase.

isolation. Protein kinase activity and cyclic AMP concentrations were determined in the subcellular fractions after incubation of the interstitial tissue with LH and 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) *in vitro*. A preliminary communication of part of this work has been presented (Cooke *et al.*, 1975a).

Materials and Methods

Materials

Sheep LH (NIH-LH-S18, 1.03 units/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, Germany. Histone (dialysed; salt-free; freeze-dried) was obtained from Worthington Biochemical Co., Freehold, N.J., U.S.A., and 3-isobutyl-1-methylxanthine from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Triton X-100 was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and PPO (2,5-diphenyloxazole) and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] from Packard Instruments Co., La Grange, Ill., U.S.A. [γ - 32 P]ATP (NH_4^+ salt; freeze-dried; sp. radioactivity 16.10 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. T.l.c. on polyethyleneimine-cellulose F (E. Merck A.-G., Darmstadt, Germany) with 1.0M- KH_2PO_4 as the solvent was used to check the radiochemical purity of the [γ - 32 P]ATP and to determine the amount of [γ - 32 P]ATP metabolized during the protein kinase assay.

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.

Incubation of tissue and subcellular fractionation

The interstitial tissue (approx. 300mg wet wt.; obtained by wet dissection of rat testis) and total testis tissue (approx. 600mg wet wt.) were pre-incubated for 1 h at 32°C in Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1964), pH 7.4 (2ml), containing 0.2% glucose (Krebs–Ringer glucose buffer) as described by Cooke *et al.* (1972). In some experiments the tissues were incubated with LH (1 $\mu\text{g}/\text{ml}$) and/or 3-isobutyl-1-methylxanthine (0.5 mM) for 20 min at 32°C in fresh Krebs–Ringer glucose buffer. The tissues were removed from the Krebs–Ringer glucose buffer incubation medium and then homogenized by ultrasonication for 15 s at 20 kHz with an amplitude of 5 μm at 0°C in 10 mM- KH_2PO_4 /NaOH buffer, pH 6.5, containing 10 mM-EDTA and 2 mM-theophylline (1,3-dimethylxanthine). The final concentration of tissue was 2.5% (w/v). The homogenates were then centrifuged at 12000g at 0°C for 5 min and the supernatant obtained was centrifuged

at 105000g at 0°C for 1 h. The 12000g and 105000g pellets were resuspended, by sonication, in the original volume of the homogenate in the above KH_2PO_4 medium.

Protein kinase assay

The protein kinase activity was determined, essentially as described by Corbin *et al.* (1973), in the subcellular fractions by using two or three different samples (between 5 and 20 μg of protein). Triplicate assays were carried out on each sample. In most experiments (except where indicated) the subcellular fractions were sonicated for 15 s in 0.1% Triton X-100 at 4°C before being incubated for 5 min at 32°C in the presence of [γ - 32 P]ATP (0.23 μM , 0.8×10^6 d.p.m.), histone (0.5 mg), ATP (1.65 μM), NaF (20 mM), KH_2PO_4 buffer, pH 6.8 (17 mM), magnesium acetate (6 mM) and with and without cyclic AMP (0.6 μM). The total incubation volume was 0.1 ml. The reaction was stopped by the addition of trichloroacetic acid [0.2 ml; 10% (w/v), containing ATP (0.01%)] followed by bovine serum albumin (20 μl ; 1.0%, w/v). The samples were mixed and left for 10 min at 0°C, then centrifuged and the protein precipitated was washed four times with trichloroacetic acid [0.2 ml; 5% (w/v), containing 0.005% ATP]. The protein was dissolved in NaOH (0.5 ml; 0.2 M) and the amount of ^{32}P was counted for radioactivity in a Nuclear–Chicago liquid-scintillation counter. The scintillant contained 80 g of naphthalene, 4.0 g of PPO and 40 mg of dimethyl-POPOP per litre of toluene/methoxyethanol (10:9, v/v). Protein was determined in the subcellular fractions by the method of Lowry *et al.* (1951), with albumin fraction V as a standard.

Parallel incubations were carried out without the addition of the subcellular fractions; the values obtained (less than 10% of the ^{32}P incorporations in enzyme incubations) were subtracted from results of incubations containing subcellular fractions. Cyclic AMP was determined in the subcellular fractions by saturation analysis as described previously (Cooke *et al.*, 1972). The protein kinase activity is expressed, in the text and the Figures, as pmol of ^{32}P incorporated/mg of protein or as the protein kinase activity ratio (ratio of protein kinase activity without added cyclic AMP: activity with added cyclic AMP).

ATPase determination

The amount of [^{32}P]ATP hydrolysed to P_i during incubation was determined (as described by Siegel & Albers, 1967) in the first trichloroacetic acid extract obtained after protein assay of the testis subcellular fractions (this contained approx. 95% of the added ^{32}P). The extracts (10 μl) were added to 50 μl of ammonium molybdate in 2M- H_2SO_4 . The phosphomolybdate formed was extracted with 100 μl of

2-methylpropan-1-ol by mixing on a Vortex mixer for 15 s. After centrifugation at 4°C at 1000g for 5 min, 50 μ l of the 2-methylpropan-1-ol extract was removed for radioactivity counting. In extracts from incubations containing no tissue, approx. 3–4% of the added radioactivity ($[^{32}\text{P}]\text{ATP}$) was extracted by the 2-methopropan-1-ol.

Results

Conditions for protein kinase activity

The subcellular fractions of the interstitial tissue were obtained by centrifugation and then incubated without and with cyclic AMP to determine the cyclic AMP-independent and cyclic AMP-dependent protein kinase activity.

Protein kinase activity was first determined in the 105000g supernatant (by using 5 μ g of protein) with different amounts of histone as substrate (0.1–25 mg). A constant amount of cyclic AMP-dependent and -independent protein kinase activity was found, thus indicating that an excess of substrate was present; 0.5 mg of histone was used in subsequent experiments. The assays were carried out for 5 min at 32°C; incubations for longer times did not increase the incorporation of ^{32}P . The concentration of cyclic AMP was varied between 2.7 nM and 2.3 μ M. A maximum incorporation of ^{32}P was found with 0.6 μ M-cyclic AMP; this concentration was used in subsequent experiments to determine the cyclic AMP-dependent protein kinase.

A linear relationship between incorporation of ^{32}P into histone and the apparent protein kinase activity in the 105000g supernatant could only be obtained if a sample of the latter containing less than 6 μ g of protein was used. However, when Triton X-100 was added (see below) the relationship was linear with amounts up to 20 μ g of protein from all the subcellular fractions. In all subsequent experiments at least two different concentrations of the tissue fractions were used to check the linearity of the response.

Effect of NaCl on protein kinase activity

Corbin *et al.* (1973) showed the necessity of adding NaCl to the dilution medium to prevent changes in the activity of adipose-tissue protein kinase (possibly by reassociation of the subunits) caused by dilution of the enzyme. To investigate similar effects on dilution of the interstitial-tissue protein kinase, 0.5M-NaCl was added before homogenization. The enzyme subsequently assayed in the 105000g supernatant gave a high protein kinase activity ratio (Table 1), indicating a high cyclic AMP-independent protein kinase activity in this tissue. Similar results were found with the 105000g supernatant from total testis tissue (Table 1). A low ratio was found, however, in both tissue preparations if the NaCl was omitted (Table 1). Further, the activity ratio remained

Table 1. Effect of adding NaCl before homogenization of rat testis tissues on protein kinase activity ratio

The protein kinase activity ratio (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP) was measured on the 105000g supernatant (5 μ g of protein) prepared from whole rat testis and interstitial-tissue homogenates. Both extracts were prepared in the absence and presence of 0.5M-NaCl. The means $\pm$ s.e.m. are given. See the Materials and Methods section for further details of the protein kinase assay.

Tissue	Protein kinase activity ratio		No. of experiments
	Control	+NaCl	
Interstitial	0.30 $\pm$ 0.02	0.75 $\pm$ 0.03	16
Total testis	0.35 $\pm$ 0.04	0.65 $\pm$ 0.05	5

Fig. 1. Effect of varying the amount of interstitial-tissue protein on the protein kinase activity ratio

The protein kinase activity ratio (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP) was measured with different amounts of the 105000g supernatant prepared from rat testis interstitial tissue that had been homogenized in the presence (●) and in the absence (■) of added NaCl (0.5M). See the Materials and Methods section for further details of the protein kinase assay.

constant in the absence (and the presence) of NaCl with different dilutions of the enzyme (Fig. 1). The high activity ratio in the presence of NaCl was due mainly to a decrease in the total protein kinase activity caused by an apparent decrease in the cyclic AMP-dependent protein kinase form (see Table 2 and the Discussion section). The effect of NaCl was dependent on the concentration used; with 0.25M-NaCl the decrease in total activity was less than with 0.5M [with a lower concentration (0.05M) very little effect was found]. When NaCl was added directly

Table 2. *Effect of NaCl on protein kinase activity in rat testis interstitial tissue*

The protein kinase activity was measured in the 105000g supernatant (5 µg of protein) obtained from interstitial tissue homogenized in the absence of NaCl and in the presence of 0.25 and 0.5 M-NaCl. In addition the protein kinase activity was measured in the 105000g supernatant after the addition of 0.5 M-NaCl. The protein kinase activities were determined in the absence and presence of added 0.6 µM-cyclic AMP. To compare experiments with tissue obtained from different rats all results were calculated as a fraction of the protein kinase activity in the 105000g supernatant isolated in the absence of NaCl. The means ± S.E.M. are given. See the Materials and Methods section for further details of the protein kinase assay.

	NaCl concn. (M)	Relative protein kinase activity (pmol of ³² P/mg of protein)		No. of experiments
		-cyclic AMP	+cyclic AMP	
Control	0	0.33 ± 0.04	1.0	6
NaCl added before homogenization	0.25	0.47 ± 0.06	0.75 ± 0.07	4
	0.5	0.43 ± 0.04	0.66 ± 0.04	6
NaCl added directly to 105000g supernatant	0.5	0.30 ± 0.06	0.60 ± 0.08	3

Table 3. *Effect of Triton X-100 on protein kinase activity in interstitial tissue*

The protein kinase activity was measured in different subcellular fractions obtained from interstitial-tissue homogenates in the absence and presence of added 0.6 µM-cyclic AMP. Similar experiments were carried out after prior sonication (15s) of the subcellular fractions in 0.1% Triton X-100. Protein kinase activity was also measured in the absence and presence of Triton X-100 after recombining the 105000g pellet and supernatant obtained after centrifuging the 12000g supernatant. The means and ranges for the protein kinase activities determined in the absence and presence of added 0.6 µM-cyclic AMP are given for two or three different protein concentrations with tissue from the same rat. See the Materials and Methods section for further details of the protein kinase assay.

Subcellular fraction	Cyclic AMP (0.6 µM)	Protein kinase activity (pmol of ³² P/mg of protein)	
		Control	+Triton X-100
12000g Supernatant	-	2.3 ± 0.9	24.8
	+	6.6 ± 2.3	78.7 ± 2.7
105000g Supernatant	-	13.1 ± 5.3	32.9 ± 1.2
	+	47.8 ± 5.6	93.1 ± 7.5
105000g Pellet	-	0	27.6 ± 4.4
	+	0	40.9 ± 4.7
105000g Supernatant + 105000g pellet	-	0.7 ± 0.4	29.4 ± 3.4
	+	3.8 ± 1.7	66.5 ± 5.1

to the 105000g supernatant, it also decreased the cyclic AMP-dependent protein kinase activity (Table 2).

Effect of Triton X-100 on apparent protein kinase activity

Little or no protein kinase activity could be demonstrated in homogenates of interstitial tissue and in particulate-containing subcellular fractions

(12000g pellet, 12000g supernatant and 105000g pellet); apparently only the 105000g supernatant fraction contained active protein kinase. This higher activity in the 105000g supernatant was not due to activation of the protein kinase caused by the high-speed centrifugation, because reconstitution of the 12000g supernatant by combining the 105000g supernatant and pellet again resulted in a low activity (Table 3). To overcome possible effects of inactivation of this enzyme by binding to particulate fractions, 0.1% Triton X-100 was added to the incubations; this resulted in a large increase in ³²P incorporation (Table 3) in all fractions, including the 105000g supernatant. Increasing the Triton X-100 concentration to 0.5% did not further increase the ³²P incorporation. Little or no incorporation of ³²P was found in the presence of 0.01% Triton X-100.

To investigate the effect of Triton X-100 at higher temperatures the 12000g supernatant and homogenate (500 µl fractions) were incubated at 32°C for 10 min after sonication in 0.1 and 0.4% Triton X-100; the protein kinase activity was not different from similar fractions incubated at 4°C for 10 min with the same concentrations of Triton X-100.

Effect of Triton X-100 on metabolism of ATP and ATPase activity

The trichloroacetic acid extracts obtained after protein kinase assay were subjected to t.l.c. and the resulting chromatograms were scanned in a thin-layer radiochromatogram scanner. In the extracts from incubations carried out with the 12000g supernatant and 105000g pellets in the absence of Triton X-100 little or no radioactivity corresponding to ATP (R_f 0.5) was detectable; the main radioactive band moved with the solvent front. In contrast, in the extracts from similar incubations carried out in the presence of Triton X-100 the main radioactive band corresponded to ATP.

To determine quantitatively the apparent hydrolysis of the [γ - 32 P]ATP by the particulate fractions in the absence of Triton X-100 the amount of ATP hydrolysed to P_i was determined in the extracts (see the Materials and Methods section). In the incubations with the 12000g supernatant (containing 20 μ g of protein) in the absence of Triton X-100, $73.5 \pm 2.6\%$ (mean $\pm$ S.E.M., $n = 8$) of the ATP was hydrolysed to P_i compared with $28.5 \pm 1.9\%$ ($n = 8$) in the presence of 0.1% Triton X-100. Similar results were obtained with the 105000g pellet and whole tissue homogenate.

Subcellular distribution of protein kinase activity in interstitial tissue

The distribution of the cyclic AMP-dependent and cyclic AMP-independent protein kinase was determined (in the presence of Triton X-100) in the subcellular fractions of interstitial tissue. The relative specific activity of cyclic AMP-dependent protein kinase was highest in the 105000g supernatant (Fig. 2). The smallest amounts of both forms of the enzyme were found in the 105000g pellets. The remaining activity (approximately half of the cyclic AMP-independent protein kinase) was in the 12000g pellet, which probably contained unfractionated nuclei, plasma membranes and mitochondria. The total activity recovered in the subcellular fractions was greater than that found in the whole tissue homogenate (Fig. 2).

Effect of LH/3-isobutyl-1-methylxanthine on protein kinase activity and cyclic AMP production in subcellular fractions of interstitial tissue

Incubations with intact interstitial tissue were carried out in the presence and absence of added LH (1 μ g/ml) and 3-isobutyl-1-methylxanthine (0.5 mM) for 20 min at 32°C. At the end of the incubation time the tissue was homogenized and fractionated by centrifugation. Protein kinase activity and the content of cyclic AMP was determined in each fraction.

In the homogenate and all the subcellular fractions obtained from tissue incubated with LH and 3-isobutyl-1-methylxanthine, the amount of cyclic AMP-independent protein kinase was increased (Table 4). The mean change in the protein kinase activity ratios in the absence and presence of LH and 3-isobutyl-1-methylxanthine were as follows: homogenate, from 0.41 to 0.80; 12000g supernatant, from 0.35 to 0.82; 12000g pellet, from 0.59 to 0.83; 105000g supernatant, from 0.29 to 0.72; 105000g pellet, from 0.72 to 0.91.

After stimulation with LH/3-isobutyl-1-methylxanthine the total protein kinase activity recovered in the 105000g supernatant was markedly decreased; there were no changes in the other fractions, although there was a small increase in the 105000g pellet ($P < 0.05$). The highest concentration of cyclic AMP was in the 105000g supernatant (Table 5), and it was in this fraction that the largest increase in cyclic AMP

Fig. 2. Subcellular distribution of protein kinase activity in interstitial tissue

The protein kinase activity was measured in subcellular fractions and homogenates from interstitial tissue in the absence and presence of added 0.6 μ M-cyclic AMP. Before assay all fractions were sonicated (15s) in 0.1% Triton X-100. The results are expressed as the relative specific activity [ratio of specific activity of the subcellular fraction (12000g pellet): specific activity of the total fraction (105000g pellet+supernatant)]. The total 32 P incorporated in the subcellular fractions and homogenate are also given. The mean results obtained in the absence and presence of added cyclic AMP are given with two different concentrations of protein. See the Materials and Methods section for further details of the protein kinase assay. Homogenate, ▨; 12000g pellet, ▩; 105000g pellet, ■; 105000g supernatant, □.

Table 4. *Effect of LH and 3-isobutyl-1-methylxanthine on protein kinase activity in subcellular fractions of interstitial tissue*

Interstitial tissue was incubated in the presence and the absence of LH (1 μ g/ml) and 3-isobutyl-1-methylxanthine (0.5 mM) for 20 min at 32°C. The tissue was then homogenized and subcellular fractions were obtained. After sonication (15 s) the homogenates and fractions in 0.1% Triton X-100 the protein kinase activity was measured in the absence and the presence of added 0.6 μ M-cyclic AMP. The means $\pm$ s.e.m. of results obtained from different rats with two different concentrations of protein are given. See the Materials and Methods section for further details of the protein kinase assay.

Subcellular fraction	Cyclic AMP (0.6 μ M)	Protein kinase activity (pmol of 32 P/mg of protein)		No. of experiments
		Control	LH + 3-isobutyl-1-methylxanthine	
Whole tissue homogenate	—	10.1 $\pm$ 1.5	20.8 $\pm$ 0.8	3
	+	23.9 $\pm$ 1.3	25.8 $\pm$ 0.3	3
12000g Supernatant	—	11.5 $\pm$ 1.5	26.2 $\pm$ 5.6	4
	+	32.2 $\pm$ 4.2	31.9 $\pm$ 6.3	4
12000g Pellet	—	9.5 $\pm$ 2.9	17.4 $\pm$ 1.9	3
	+	20.5 $\pm$ 2.0	21.0 $\pm$ 2.2	3
105000g Supernatant	—	14.9 $\pm$ 1.7	25.3 $\pm$ 3.2	7
	+	50.4 $\pm$ 5.2	30.2 $\pm$ 5.7	7
105000g Pellet	—	12.8 $\pm$ 1.8	19.6 $\pm$ 5.8	4
	+	17.4 $\pm$ 0.4	21.1 $\pm$ 2.5	4

Table 5. *Effect of LH and 3-isobutyl-1-methylxanthine on cyclic AMP concentrations in subcellular fractions of interstitial tissue*

Whole interstitial tissue was incubated with and without LH and 3-isobutyl-1-methylxanthine as described in the legend for Table 4. After incubation of the tissue, cyclic AMP was determined in the homogenate and subcellular fractions. The means $\pm$ s.e.m. of results obtained with tissues from different rats are given.

Subcellular fraction	Cyclic AMP concn. (pmol/mg of protein)		No. of experiments
	Control	LH + 3-isobutyl-1-methylxanthine	
Whole tissue homogenate	11.0 $\pm$ 5.0	103 $\pm$ 31.7	5
12000g Supernatant	65.7 $\pm$ 46.5	402 $\pm$ 122	5
12000g Pellet	9.1 $\pm$ 2.7	12.9 $\pm$ 4.8	3
105000g Supernatant	30.5 $\pm$ 6.6	449 $\pm$ 37.2	4
105000g Pellet	6.5 $\pm$ 2.3	23.6 $\pm$ 11.4	3

was found after LH/3-isobutyl-1-methylxanthine stimulation. The cyclic AMP content was very low in the 12000g and 105000g pellets and little or no increase occurred in these fractions after LH/3-isobutyl-1-methylxanthine stimulation.

Table 6. *Effect of LH and 3-isobutyl-1-methylxanthine on protein kinase activity and cyclic AMP production in interstitial-tissue homogenates*

Interstitial tissue was incubated for 20 min at 32°C in the control media, in the presence of LH (1 μ g/ml) or 3-isobutyl-1-methylxanthine (0.5 mM) or LH + 3-isobutyl-1-methylxanthine. Protein kinase activity and cyclic AMP concentrations were determined in the tissue homogenates. The latter were sonicated in 0.1% Triton X-100 before protein kinase activities were determined in the absence and presence of 0.6 μ M-cyclic AMP. The means and ranges of results with two or three different amounts of homogenate protein are given. See the Materials and Methods section for further details of the assays used.

	Protein kinase activity (pmol of 32 P/mg of protein)		Cyclic AMP production (pmol/mg of protein)
	—cyclic AMP	+cyclic AMP	
Control	10 $\pm$ 2.3	26.4 $\pm$ 4.0	8.0
LH	15.7 $\pm$ 1.3	20.6 $\pm$ 0.7	133
3-Isobutyl-1-methylxanthine	10.4 $\pm$ 0.5	23.8 $\pm$ 1.7	13.0
LH + 3-isobutyl-1-methylxanthine	21.0 $\pm$ 3.0	25.5 $\pm$ 5.7	228

The effects of LH and 3-isobutyl-1-methylxanthine added separately during incubation of interstitial tissue are shown for the homogenate in Table 6. 3-Isobutyl-1-methylxanthine alone had little or no effect on cyclic AMP concentrations and on the

protein kinase activity ratio, thus indicating that inhibition of the metabolism of basal production of cyclic AMP has little effect on the activation of the protein kinase. LH and 3-isobutyl-1-methylxanthine added together had a more marked effect on cyclic AMP concentrations than LH alone; in both cases large increases in cyclic AMP-independent protein kinase activity were found.

Discussion

In the present study a cyclic AMP-dependent protein kinase has been found in rat testis interstitial tissue, localized mainly in the 105000g supernatant. Reddi *et al.* (1971) have shown that the soluble fraction of whole testis homogenates contained the protein kinase enzyme and that cyclic AMP markedly stimulated the enzyme with histone as substrate. Bernard & Wasserman (1974) also reported cyclic AMP-dependent protein kinases in rat testis interstitial tissue and seminiferous tubules. It has been demonstrated in many other tissues that the cyclic AMP activation of protein kinase activity involves the dissociation of the active kinase (C) from a regulatory subunit (R), followed by binding of the cyclic AMP to R according to the following equation (Brostrom *et al.*, 1971):

It is essential when studying the cyclic AMP-mediated hormonal dependence of the protein kinase that changes in the equilibrium of this system do not occur during preparation of the tissue for assay. Corbin *et al.* (1973) showed that in adipose tissue rapid reassociation of the R and C units occurred on homogenization and dilution of this tissue but that reassociation could be prevented if 0.5M-NaCl was added to the homogenization medium. In the present study no changes in the activity of the protein kinase have been observed on dilution of the interstitial-tissue homogenate preparations. In addition NaCl itself was found to alter the activity of the protein kinase. This indicates that the protein kinase in this tissue has different properties from that in adipose tissue. Corbin *et al.* (1975) have shown that there are two different types of protein kinases; the type I enzyme (e.g. from heart tissue) is rapidly dissociated into the R and C subunits by NaCl and slowly reassociates on removal of cyclic AMP, whereas the type II enzyme (e.g. from adipose tissue) is only slowly dissociated by NaCl and rapidly reassociates on removal of cyclic AMP. The two forms of the enzyme can also be separated by DEAE-cellulose column chromatography. The results from the present study indicate that interstitial tissue contains the type I enzyme. Corbin *et al.* (1975) also showed that extracts of whole rat testis contained predominantly the type I enzyme.

An additional property of the interstitial protein kinase is that in the presence of NaCl the total activity of the protein kinase is decreased. Also in the tissue stimulated with LH, the total activity recovered in the 105000g supernatant was lower than in the controls. A possible explanation is that both LH (via cyclic AMP) and NaCl cause dissociation of the RC unit and that the cyclic AMP-independent protein kinase (C) formed is inactivated (e.g. by a protein kinase inhibitor). Similar results have been reported by Walaas *et al.* (1973), who found that cyclic AMP-independent protein kinase formed during incubation of muscle tissue was mainly converted into an inactive complex in muscle extracts. The cyclic AMP-dependent protein kinase, on the other hand, was stable and could be quantitatively recovered after fractionation and purification procedures. An alternative explanation for the lower recovery of the soluble protein kinase after LH stimulation is that the enzyme was 'translocated' to another subcellular compartment, as demonstrated by Korenman *et al.* (1974) for the rat uterus after stimulation with adrenaline. However, in the present study the 'lost' activity was not recovered in any of the subcellular compartments.

The apparent inhibition of ATPase by Triton X-100 in the rat testis interstitial tissue was somewhat unexpected; this detergent has been shown to activate Na⁺+K⁺-dependent ATPases (Jorgensen & Skou, 1971). The increased activity of the protein kinase found in the presence of Triton X-100 may not be entirely due to inhibition of the ATPase. It is quite possible that partial solubilization and/or activation of membrane-bound protein kinase took place. This has been demonstrated in other tissues (Lemay *et al.*, 1974).

In conclusion it has been clearly demonstrated in the present study that incubation of interstitial tissue with LH activates the protein kinase in all the subcellular fractions isolated. This activation was accompanied by increases in cyclic AMP concentrations, especially in the soluble fraction of the interstitial tissue. Means *et al.* (1974) have shown that FSH specifically stimulates protein kinase activity in seminiferous tubules in the rat testis and that the stimulation was also accompanied by corresponding increases in cyclic AMP production. These results are in accordance with the specific sites of action of LH and FSH in the testis (see review by Cooke, 1975).

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