THE EFFECT OF GONADOTROPINS ON THE MITOCHONDRIAL ADENYLATE CYCLASE OF RAT TESTIS

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SUMMARY

The formation of adenosine 3':5'-cyclic monophosphate from ATP by testicular mitochondria of immature and mature rats was increased to the same extent by addition of either human chorionic gonadotropin or luteinizing hormone. Follicle stimulating hormone was found to be more active in stimulating adenylate cyclase activity in testicular mitochondria of immature rats. The stimulatory effect of gonadotropins were not suppressed by Ca^{++} complexing agent ethylene-glycol-bis-(β-amino-ethyl ether) N, N'-tetra-acetic acid. The detergent Lubrol PX, solubilized 75-80% of the mitochondrial adenylate cyclase. The solubilized enzyme was activated by sodium fluoride but not by gonadotropins. The present results indicate a direct effect of gonadotropins on the adenylate cyclase attached to mitochondrial membranes.

It has been suggested that adenosine 3':5'-cyclic monophosphate (cAMP) functions as an intracellular mediator of the steroidogenic action of gonadotropins on rat testis (1). A selective effect of gonadotropins to stimulate testicular adenylate cyclase has been shown in slices (2) homogenates and particulate preparations (3) of testicular tissue, as well as in the isolated tubules (4) and interstitial cells (5). The adenylate cyclase is considered to be a component of the plasma membrane (6). In some mammalian tissues examined, this enzyme was also found to be associated with multiple membrane systems of the cell (7-9). Little information exists concerning the mitochondrial adenylate cyclase. In the testis, as in other steroidogenic tissues, it is assumed that the mitochondrial cholesterol side-chain cleavage enzymes include the rate limiting step in steroid biosynthesis, which is specifically stimulated by tropic hormones (10). This raises the question whether gonadotropins which bind to testicular tissue (11) act on mitochondrial adenylate cyclase to stimulate the formation of cAMP. In the present study we report the effect of gonadotropic hormones added in vitro, upon the cAMP content of rat testis mitochondria, and some characteristics of the adenylate cyclase system of this organelle.

MATERIALS AND METHODS

Human chorionic gonadotropin (HCG) 3800 IU/mg was obtained from Ikafarm (Israel). Human urinary luteinizing hormone (LH) 100 IU/mg, containing 0.41 IU FSH/mg and human urinary follicle stimulating hormone (FSH) 1055 IU/mg containing 2.3 IU LH/mg were kindly supplied by the Istituto Serono (Italy). The non-ionic detergent Lubrol PX was from ICI (England). Bovine serum albumin (BSA) and ethylene-glycol-bis-(β-amino-ethyl ether) N, N'-tetra-acetic acid (EGTA) were obtained from Sigma (U.S.A.). (^{3}H) adenosine 3':5'-cyclic monophosphate (spec. activ. 27.5 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, England).

Immature male rats (Charles River colony), 20 days old, weighing 30-50 g and mature rats, 60-80 days old, weighing 200-250 g were used in these experiments. The rats were killed by a sharp blow in the head and the testes were removed, decapsulated and homogenized in ice-cold 0.25 M sucrose. Preparation and purification of mitochondria was performed as previously described (12). The incubation mixture consists of 0.1-0.2 ml mitochondrial pellets suspended in 0.05 M Tris. HCl buffer (pH 7.8), 3 mM $MgSO_4$, 5 mM theophylline, 1 mM ATP, 5 mM phosphoenolepyruvate and pyruvate Kinase (60 μg/ml). The total volume was 0.4 ml and incubations were carried out at 37° C for 10 minutes. The reaction was terminated by addition of trichloroacetic acid to give a final concentration of 5%. The mixture was homogenized and brought to a final volume of 1 ml with Tris. HCl buffer (pH 7.8). The homogenate was centrifuged at 3000 rpm for 10 minutes. To the supernatant fraction 0.1 ml of N HCl was added and the trichloroacetic acid was extracted with 5 x 5 volumes of distilled diethyl-ether. The residual ether was evaporated from the aqueous solution by placing the samples in a water bath at 55°C. The aqueous solution was taken to dryness under nitrogen. cAMP was determined according to the method of Gilman (13) modified by using charcoal plus 2% BSA (14) to separate the bound from the free nucleotide. It was confirmed that the amounts of ATP employed in the binding assay (0.02-0.1mM) did not interfere with the binding of cAMP (13). In order to evaluate if other nucleotides present in mitochondria will influence the binding of cAMP, aliquots of mitochondrial extracts were chromatographed on a column containing hydrous aluminium oxide and cAMP was separated from other

nucleotides by eluting the column with 10mM Tris. buffer pH 7.4 (15). The results were found to be identical with those obtained with mitochondrial extracts not chromatographed on aluminium oxide. Protein concentration was measured by the method of Lowry et al. (16).

RESULTS AND DISCUSSION

The stimulatory effect of HCG, LH and FSH on cAMP accumulation by mitochondria preparations isolated from immature and mature rat testis is shown in Table 1. Similar results were reported for particulate fractions and mitochondria of dog testis (3,17). HCG and LH used at a concentration of 5 IU and 10 IU gave essentially the same increment of cAMP in mitochondria of immature and mature rat testis. On the other hand, 10 IU FSH (contaminated with 0.023 IU LH) increased the adenylate cyclase activity 224% in rat testis

Table 1. Effect of gonadotropins and sodium fluoride on the adenylate cyclase activity of rat testis mitochondria.
The hormones and sodium fluoride were dissolved in 0.05 M Tris. HCl buffer (pH 7.8) and added to the incubation mixture. The incubation procedure and the cAMP assay were carried out as described in the section on Methods. Results are given as mean ± SE of the mean followed by the No. of observations in parentheses.

Additions	cAMP pmoles/mg protein/10 min. incubation			
	Immature rats	% Stimulation	Mature rats	% Stimulation
No addition	3.00 ± 0.05 (4)		2.28 ± 0.07 (5)	
HCG 5 IU	4.49 ± 0.05 (4)	51.0%	3.52 ± 0.04 (4)	54.3%
HCG 10 IU	5.34 ± 0.09 (4)	78.5%	4.00 ± 0.05 (4)	78.9%
LH 1 IU	3.30 ± 0.04 (4)	10.0%	-	
LH 5 IU	4.61 ± 0.09 (4)	53.6%	3.35 ± 0.05 (4)	47.7%
LH 10 IU	5.60 ± 0.11 (4)	86.6%	4.45 ± 0.07 (4)	95.6%
FSH 5 IU	5.00 ± 0.06 (4)	67.2%	3.21 ± 0.10 (4)	41.6%
FSH 10 IU	6.73 ± 0.08 (4)	224.0%	3.96 ± 0.10 (4)	74.3%
NaF 10mM	9.84 ± 0.14 (5)	387.0%	8.81 ± 0.09 (5)	329.0%

mitochondria isolated from immature animals and only 74.3% in mitochondria obtained from mature rat testis. Since 1 IU LH did not significantly increase the concentration of cAMP the effect of FSH could not be attributed to contamination by LH. FSH was previously described to be more active in stimulating adenylate cyclase activity in testicular slices of immature rats (2). Sodium fluoride, a non-selective activator of adenylate cyclase, was found to stimulate the mitochondrial enzyme 3-4 fold (Table 1). The present findings suggest that a gonadotropin-sensitive adenylate cyclase in rat testis is associated with mitochondria.

A requirement for Ca^{++} was postulated for the ACTH stimulation of adenylate cyclase in bovine adrenal membrane fractions (18). Addition of EGTA, a Ca^{++} complexing agent, to rat testis mitochondria did not influence either the adenylate cyclase activity or the stimulatory effect of HCG on the enzyme (Table 2). These results suggest that Ca^{++} is not required for the activation by HCG of mitochondrial adenylate cyclase in rat testis.

The non-ionic detergent Lubrol PX was used to solubilize the adenylate cyclase from heart homogenates (19) and Neurospora crassa membranes (20). Mitochondria isolated from mature rat testis were incubated with Lubrol PX

Table 2. Effect of EGTA on the stimulation of mitochondrial adenylate cyclase by HCG.
Mitochondria isolated from testicular tissue of mature rats were used in these experiments. HCG and EGTA were included in the usual incubation mixture, and the samples were incubated as described under the section on Methods. Results are given as mean $\pm$ SE of the mean followed by the No. of observations in parentheses.

Additions	cAMP pmoles/mg protein/10 min. incubation
No addition	3.80 $\pm$ 0.05 (4)
HCG 10 IU	6.50 $\pm$ 0.08 (4)
HCG 10 IU + EGTA 1 mM	6.20 $\pm$ 0.08 (4)
HCG 10 IU + EGTA 2 mM	6.40 $\pm$ 0.09 (4)
EGTA 2 mM	4.00 $\pm$ 0.04 (4)

at a concentration of 0.3 mg/mg protein. Incubations were carried out at 4°C for 30 minutes. The mitochondria suspension was then centrifuged at 160,000g for 90 minutes. The results illustrated in Table 3 indicate that

Table 3. Effect of Lubrol PX on solubilization of mitochondrial adenylate cyclase.
A Lubrol treated mitochondria was centrifuged at 160,000g for 90 minutes. The resulting precipitate and supernatant were separately assayed for adenylate cyclase activity as described in the section on Methods. Results are given as mean ± SE of the mean followed by the No. of observations in parentheses.

Exp.	Fraction	cAMP pmoles/mg protein/ 10 min. incubation	% Solubilization
1	160,000g precipitate	0.75 ± 0.02 (3)	
	160,000g supernatant	2.30 ± 0.05 (3)	75.4%
2	160,000g precipitate	0.90 ± 0.03 (4)	
	160,000g supernatant	3.80 ± 0.07 (4)	81.0%
3	160,000g precipitate	1.20 ± 0.05 (3)	
	160,000g supernatant	4.40 ± 0.09 (3)	78.5%

75-80% of the adenylate cyclase activity was found in the 160,000g supernatant. The solubilized enzyme was activated by sodium fluoride but not by gonadotropic hormones which stimulate the mitochondrial adenylate cyclase. The activation of the solubilized adenylate cyclase by sodium fluoride is shown in Fig. 1. These results are in good agreement with those obtained with myocardial adenylate cyclase, where the solubilized enzyme was activated by sodium fluoride but not by hormones (19). The results reported here suggest that gonadotropic hormones activate the adenylate cyclase attached to mitochondrial membranes and that the enzyme might have some similarities with the adenylate cyclase found to be associated with other membrane systems of the cell.

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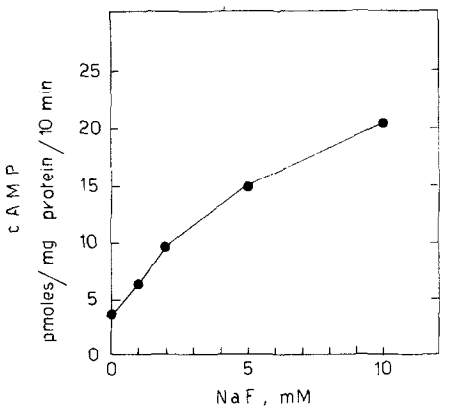

Figure 1. Effect of sodium fluoride on cAMP accumulation in a 160,000g supernatant of a Lubrol treated mitochondria. The data represent the means of 4 determinations.

REFERENCES

1. Eik-Nes, K.B. (1971) Rec. Prog. Horm. Res. 27, 517-535.
2. Kuehl, F.A., Jr., Patanelli, D.J., Tarnoff, J., and Humes, J.L. (1970) Biol. Reprod. 2, 154-163.
3. Murad, F., Strauch, B.S., and Vaughan, M. (1969) Biochim. Biophys. Acta. 177, 591-598.
4. Dorrington, J.H., Vernon, R.G., and Fritz, I.B. (1972) Biochem. Biophys. Res. Commun. 46, 1523-1528.
5. Cooke, B.A., van Beurden, W.M.O., Rommerts, F.F.G., and van der Molen, H.J. (1972) FEBS Letters 25, 83-86.
6. Robison, G.A., Butcher, R.W., and Sutherland, E.W., Jr. (1968) Ann. Rev. Biochem. 37, 149-174.
7. Hechter, O., Bär, H.P., Matsuba, M., and Soifer, D. (1969) Life Sciences 8, 935-942.
8. Hollinger, M.A. (1970) Life Sciences 9, 533-540.
9. De Robertis, E., De Lores Arnaiz, C.G., Alberici, M., Butcher, R.W., and Sutherland, E.W., Jr. (1967) J. Biol. Chem. 242, 3487-3493.
10. Menon, K.M.J., Dorfman, R.I., and Forchielli, E. (1967) Biochim. Biophys. Acta. 148, 486-494.
11. Catt, K.J., Dufau, M., and Tsuruhara, T. (1972) J. Clin. Endocrinol. Metab. 34, 123-132.
12. Sulimovici, S., Bartoov, B., and Lunenfeld, B. (1973) Biochim. Biophys. Acta. 321, 27-40.
13. Gilman, A.G. (1970) Proc. Nat. Acad. Sci. U.S.A. 67, 305-312.
14. Brown, B.L., Albano, J.D.M., Ekins, R.P., and Sgherzi, A.M. (1971) Biochem. J. 121, 561-562.

15. Ramachandran, J. (1971) Anal. Biochem. 43, 227-239.
16. Lowry, O.H., Rosebrough, N.J., Farr, A.L.,and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
17. Pulsinelli, W.A. and Eik-Nes, K.B. (1970) Fed. Proc. 29, 918.
18. Bär, H.P. and Hechter, O. (1969) Biochem. Biophys. Res. Commun. 35, 681-686.
19. Levey, G.S. (1970) Biochem. Biophys. Res. Commun. 38, 86-92.
20. Flawia, M.M. and Torres, H.N. (1972) Biochim. Biophys. Acta. 289, 428-432.