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Effect of Adenosine 3′,5′-(Cyclic)-Monophosphate on the Synthesis of Progestational Steroids by Rabbit Ovarian Tissue *in vitro*

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1. Adenosine 3′,5′-(cyclic)-monophosphate (3′,5′-AMP) stimulates the synthesis of progestational steroids by rabbit ovarian tissue *in vitro*. 2. Other adenosine phosphates fail to increase steroidogenesis. 3. The ratio of 20α-hydroxypregn-4-en-3-one to progesterone, the maximal response of the tissue, and the responses of separated corpora lutea and interstitial tissue produced by luteinizing hormone are closely paralleled by 3′,5′-AMP. 4. In tissues maximally stimulated by luteinizing hormone, 3′,5′-AMP fails to produce an additional response. 5. The addition of theophylline, an inhibitor of phosphodiesterase, potentiates the effects of 3′,5′-AMP and also luteinizing hormone. 6. The results obtained suggest that 3′,5′-AMP is a mediator of the action of luteinizing hormone on progestational steroid synthesis by rabbit ovarian tissue.

Luteinizing hormone stimulated the synthesis of progestational steroids, 20α-hydroxypregn-4-en-3-one and progesterone, by rabbit ovaries both *in vivo* and *in vitro* (Hilliard, Archibald & Sawyer, 1963; Dorrington & Kilpatrick, 1966*b*). However, the biochemical mechanism involved in the stimulation of ovarian steroidogenesis by luteinizing hormone is not fully understood.

The role of adenosine 3′,5′-(cyclic)-monophosphate (3′,5′-AMP) as an intermediate in hormone action was first suggested by Sutherland & Rall (1957). These workers showed that adrenaline increased the concentration of 3′,5′-AMP in liver and that the 3′,5′-AMP in turn activated liver phosphorylase and thereby increased glycogenolysis (Sutherland & Rall, 1960). Subsequently Haynes (1958) showed that the stimulation of adrenal steroidogenesis by adrenocorticotrophic hormone was associated with an increased adrenal content of 3′,5′-AMP and showed that this nucleotide activated phosphorylase as did adrenocorticotrophic hormone. Corticosteroid synthesis was stimulated by the addition of 3′,5′-AMP to rat adrenal tissue *in vitro* (Haynes, Koritz & Péron, 1959), and on the basis of these findings Haynes, Sutherland & Rall (1960) modified the scheme of adrenocorticotrophic hormone action proposed earlier (Haynes & Berthet, 1957) to involve the stimulation of phosphorylase by 3′,5′-AMP.

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Recently, Savard, Marsh & Rice (1965) have emphasized the possible role of 3′,5′-AMP as an intermediate in the action of luteinizing hormone on the bovine corpus luteum. The present study investigates the effects of 3′,5′-AMP on rabbit ovarian tissue and these effects are compared with those of luteinizing hormone. Preliminary studies have already been described (Dorrington & Kilpatrick, 1966*a*).

MATERIALS AND METHODS

Animals. Sexually mature female New Zealand white rabbits were used. Ovulation and pseudo-pregnancy were induced by 200μg. of luteinizing hormone, given intravenously.

Chemicals. Ovine luteinizing hormone (NIH-LH-S7) was obtained from the National Institutes of Health, Bethesda, Md., U.S.A.

All chemicals used were A.R. grade, and the organic solvents (purchased from British Drug Houses Ltd., Poole, Dorset) with the exception of ethanol (James Burrough Ltd., London, S.E. 11) were redistilled before use.

3′,5′-AMP, 3′-AMP, 5′-AMP, ADP and ATP were purchased from Sigma Chemical Co., London, S.W. 6.

Theophylline was obtained from British Drug Houses Ltd. Details of radioactive progestational steroids were given in Dorrington & Kilpatrick (1966*b*).

Methods. The ovarian tissue was incubated in 5ml. of Krebs–Ringer bicarbonate buffer, pH7·4 (Krebs & Henseleit, 1932), for 3hr. as described by Dorrington & Kilpatrick (1966*b*).

The 3′,5′-AMP was dissolved in Krebs–Ringer bicarbonate buffer, pH7·4, and the pH was checked before addition to the incubation medium.

Table 1. *Mobility and specific activity of 20α-hydroxypregn-4-en-3-one from rabbit ovary in various chromatographic systems and after acetylation*

	R_F (reference standard)	Specific activity (mμc/μg.)
Two-dimensional thin-layer chromatography:		
Direction 1 (hexane–ethyl acetate; 3:1, v/v)	0·75 (0·74)	2·3
Direction 2 (dichloromethane–ether; 5:2, v/v)	0·30 (0·30)	2·3
Thin-layer chromatography:		
System 1 (benzene–ethanol; 9:1, v/v)	0·48 (0·48)	2·1
System 2 (chloroform–methanol; 99:1, v/v)	0·32 (0·33)	2·1
Acetylation and thin-layer chromatography:		
System 1 (benzene–ethanol; 9:1, v/v)	0·68 (0·68)	2·0
System 2 (chloroform–methanol; 99:1, v/v)	0·72 (0·75)	1·9

Table 2. *Effect of adenosine phosphates on the synthesis of progestational steroids by rabbit ovarian tissue* in vitro

The ovaries were obtained from animals 6–8 days pseudo-pregnant. Results (means ± S.E.M.) represent the increase in steroid content above control values.

		Steroid content of tissue and medium after incubation for 3 hr. (μg./500 mg. of tissue)	
Addition	No. of expts.	Progesterone	20α-Hydroxypregn-4-en-3-one
3′,5′-AMP (2 μmoles/ml.)	9	2·3 ± 0·5	5·9 ± 1·4
3′,5′-AMP (3 μmoles/ml.)	9	7·9 ± 1·6	30·3 ± 4·2
3′,5′-AMP (5 μmoles/ml.)	9	12·6 ± 1·8	62·6 ± 5·3
3′,5′-AMP (8 μmoles/ml.)	9	15·3 ± 1·6	86·1 ± 4·7
3′,5′-AMP (10 μmoles/ml.)	5	13·9 ± 0·8	82·3 ± 8·8
3′-AMP (5 μmoles/ml.)	3	4·0 ± 1·0	2·5 ± 1·4
5′-AMP (5 μmoles/ml.)	3	3·7 ± 2·1	4·2 ± 1·8
ADP (5 μmoles/ml.)	4	3·6 ± 1·6	1·5 ± 0·9
ATP (5 μmoles/ml.)	4	2·0 ± 0·8	0·8 ± 0·2

The steroids were extracted, purified by thin-layer chromatography and estimated spectrophotometrically (Dorrington & Kilpatrick, 1966*b*). The evidence on the identity of 20α-hydroxypregn-4-en-3-one from mobility in different chromatographic systems, constancy of specific activities in these systems and after acetylation with acetic anhydride is given in Table 1.

RESULTS

In each experiment a portion of the ovarian tissue was incubated without the addition of test substance, and the progestational steroid content estimated at the end of the 3 hr. incubation. The control values were subtracted from the increases produced by the test substance in each experiment. The mean control concentrations of progesterone and 20α-hydroxypregn-4-en-3-one in 30 experiments were 10·9 (S.E.M. ± 1·0) μg./500 mg. of tissue and 21·1 (S.E.M. ± 1·1) μg./500 mg. of tissue respectively.

Effect of 3′,5′-AMP on progestational steroid synthesis by rabbit ovarian tissue in vitro. The mean increases in the synthesis of progesterone and 20α-hydroxypregn-4-en-3-one on the addition of 3′,5′-AMP (2 μmoles/ml.) to the incubation medium are given in Table 2. A striking increase in the synthesis of 20α-hydroxypregn-4-en-3-one, and to a smaller extent of progesterone, was produced by 3′,5′-AMP. The dose–response line was linear between 2 and 5 μmoles of 3′,5′-AMP/ml. and a maximum response was produced by the addition of 8 μmoles of 3′,5′-AMP/ml. For comparison, the mean increases in progestational steroid synthesis in response to luteinizing hormone (0·05–2 μg./ml.) are shown in Table 3.

To show that the stimulation of steroid synthesis was not a general property of adenosine phosphates, 3′-AMP, 5′-AMP, ADP and ATP were also tested in the system *in vitro*. None of these compounds caused a significant stimulation at 5 μmoles/ml. (Table 2), whereas 3′,5′-AMP greatly enhanced progestational steroid synthesis at this concentration (Table 2).

Effects of combinations of 3′,5′-AMP and luteinizing hormone on ovarian steroidogenesis in vitro. The addition of either 0·1 μg. of luteinizing hormone/ml. or 3 μmoles of 3′,5′-AMP/ml. to incubation medium produced a sub-maximal response in the

Table 3. *Effect of ovine luteinizing hormone on the synthesis of progestational steroids by rabbit ovarian tissue* in vitro

The ovaries were obtained from animals 6–8 days pseudo-pregnant. Results (means ±S.E.M.) represent the increase in steroid content above control values.

Luteinizing hormone added (μg./ml.)	No. of expts.	Steroid content of tissue and medium after incubation for 3hr. (μg./500mg. of tissue)	
		Progesterone	20α-Hydroxypregn-4-en-3-one
0·05	5	6·3±2·0	16·0±4·3
0·1	7	7·6±2·3	41·2±5·3
0·2	7	10·4±1·5	58·9±7·9
0·5	5	13·8±1·7	84·8±6·5
2·0	7	14·0±3·2	81·9±6·9

Table 4. *Effect of combinations of 3′,5′-AMP and luteinizing hormone on steroid synthesis by rabbit ovarian tissue* in vitro

The ovaries were obtained from animals 6–8 days pseudo-pregnant. Results (means ±S.E.M.) represent the increase in steroid content above control values.

Addition	No. of expts.	Steroid content of tissue and medium after incubation for 3hr. (μg./500mg. of tissue)	
		Progesterone	20α-Hydroxypregn-4-en-3-one
Luteinizing hormone (0·1μg./ml.)	4	5·6±3·0	38·0±6·4
3′,5′-AMP (3μmoles/ml.)	4	7·5±1·8	32·8±7·2
Luteinizing hormone (0·1μg./ml.)+3′,5′-AMP (3μmoles/ml.)	4	15·7±4·5	73·0±9·0
Luteinizing hormone (2μg./ml.)	7	13·5±1·4	80·9±6·3
3′,5′-AMP (8μmoles/ml.)	7	13·6±1·1	85·1±6·8
Luteinizing hormone (2μg./ml.)+3′,5′-AMP (8μmoles/ml.)	7	15·0±0·9	88·5±6·9

tissue (Tables 2 and 3). However, the addition of 0·1μg. of luteinizing hormone/ml. and 3μmoles of 3′,5′-AMP/ml. simultaneously produced an additive effect both on the 20α-hydroxypregn-4-en-3-one and progesterone concentrations (Table 4).

The addition of either 2μg. of luteinizing hormone/ml. or 8μmoles of 3′,5′-AMP/ml. maximally stimulated the tissue, as shown in Tables 3 and 2 respectively.

Incubation with 8μmoles of 3′,5′-AMP/ml. and 2μg. of luteinizing hormone/ml. added simultaneously did not cause a significant additional increase in progestational steroid content, compared with the effect produced by either substance alone.

Effect of 3′,5′-AMP on the synthesis of progestational steroids by separated corpora lutea and interstitial tissue. As previously described (Dorrington & Kilpatrick, 1966*b*) luteinizing hormone *in vitro* stimulated the synthesis of both progesterone and 20α-hydroxypregn-4-en-3-one by interstitial tissue, but only slightly stimulated corpora lutea. A comparison of the effects of 2μg. of luteinizing hormone/ml. and 5μmoles of 3′,5′-AMP/ml. on the mean increases in steroid content of isolated corpora lutea and interstitial tissue are shown in Table 5. Both luteinizing hormone and 3′,5′-AMP stimulated interstitial tissue to produce increased amounts of progestational hormones, but only stimulated corpora lutea to a slight extent.

Effect of theophylline on the synthesis of progestational steroids in vitro. It is possible that some of the 3′,5′-AMP was converted into inactive 5′-AMP (Table 2) by cyclic nucleotide phosphodiesterase (Sutherland & Rall, 1958) in the above experiments. This possibility has been investigated by using theophylline, a methyl xanthine, which is an inhibitor of phosphodiesterase (Butcher & Sutherland, 1959). The mean increases in progestational steroid content on the addition of 10μmoles of theophylline/ml. are given in Table 6. The addition of 1μmole of 3′,5′-AMP/ml. to the incubation medium failed to stimulate progestational steroid synthesis in each experiment. When 10μmoles of theophylline/ml. were added together

Table 5. *Effect of ovine luteinizing hormone and 3′,5′-AMP on separated corpora lutea and interstitial tissue* in vitro

The ovaries were obtained from rabbits 6 days pseudo-pregnant. Results (means ± S.E.M. of five experiments) represent the increase in steroid content above control values.

Ovarian tissue	Addition	Steroid content of tissue and medium after incubation for 3 hr. (μg./500 mg. of tissue): Progesterone	20α-Hydroxypregn-4-en-3-one
Corpora lutea	Luteinizing hormone (2 μg./ml.)	12·1 ± 3·1	5·6 ± 1·9
Corpora lutea	3′,5′-AMP (5 μmoles/ml.)	9·4 ± 3·0	3·5 ± 1·1
Interstitial tissue	Luteinizing hormone (2 μg./ml.)	7·6 ± 3·9	53·6 ± 6·6
Interstitial tissue	3′,5′-AMP (5 μmoles/ml.)	7·1 ± 2·9	48·4 ± 7·7

Table 6. *Effect of 3′,5′-AMP and theophylline on the synthesis of progestational steroids* in vitro *by rabbit ovarian tissue*

The ovaries were obtained from animals 6–8 days pseudo-pregnant. Results (means ± S.E.M. of five experiments) represent the increase in steroid content above control values.

Addition	Steroid content of tissue and medium after incubation for 3 hr. (μg./500 mg. of tissue): Progesterone	20α-Hydroxypregn-4-en-3-one
Theophylline (10 μmoles/ml.)	6·9 ± 2·8	10·4 ± 2·5
3′,5′-AMP (1 μmole/ml.)	0·5 ± 0·9	1·1 ± 1·3
3′,5′-AMP (2 μmoles/ml.)	2·3 ± 1·6	7·6 ± 3·2
3′,5′-AMP (3 μmoles/ml.)	6·3 ± 2·4	31·4 ± 6·2
3′,5′-AMP (1 μmole/ml.) + theophylline (10 μmoles/ml.)	10·7 ± 4·9	55·6 ± 10·5
3′,5′-AMP (2 μmoles/ml.) + theophylline (10 μmoles/ml.)	11·9 ± 4·5	56·0 ± 4·7
3′,5′-AMP (3 μmoles/ml.) + theophylline (10 μmoles/ml.)	10·8 ± 4·0	71·0 ± 11·3

with 1 μmole of 3′,5′-AMP/ml. steroid synthesis was greatly enhanced compared with the effect of theophylline alone. Similarly, when 10 μmoles of theophylline/ml. were added together with 2 or 3 μmoles of 3′,5′-AMP/ml. the stimulation produced was greater than the addition of effects of theophylline and 3′,5′-AMP incubated separately.

Effect of theophylline and luteinizing hormone on the synthesis of progestational steroids in vitro. Theophylline together with a range of concentrations of luteinizing hormone was also added to ovarian tissue *in vitro* to determine whether the inhibition of 3′,5′-AMP breakdown had any effect on the stimulatory action of luteinizing hormone (Table 7).

The addition of 0·2 μg. of luteinizing hormone/ml. to the ovarian tissue *in vitro* failed to stimulate steroid synthesis in each of the four experiments. However, when 10 μmoles of theophylline/ml. were added together with 0·02 μg. of luteinizing hormone/ml. steroidogenesis was considerably enhanced, as shown in Table 7. Similarly, the addition of 10 μmoles of theophylline/ml. together with 0·05 μg. of luteinizing hormone/ml. or 0·10 μg. of luteinizing hormone/ml. potentiated progestational steroid synthesis, compared with the addition of effects produced by luteinizing hormone and theophylline incubated separately (Table 7).

DISCUSSION

The results clearly indicate that 3′,5′-AMP mimics the action of luteinizing hormone on progestational steroid synthesis by rabbit ovarian tissue *in vitro*. Stimulation of the tissue by luteinizing hormone or 3′,5′-AMP greatly enhances the synthesis of 20α-hydroxypregn-4-en-3-one, the progesterone content being increased to a smaller extent.

The capacity of ovarian tissue to synthesize progestational hormones when maximally stimulated by luteinizing hormone or 3′,5′-AMP is the same, and suggests that both substances increase steroidogenesis by the same metabolic pathway, and that the stimulation of progestational steroidogenesis by luteinizing hormone may be mediated by 3′,5′-AMP. Further support of the latter hypothesis is provided by the observation that the addition of 3′,5′-AMP to ovarian tissue maximally stimulated

Table 7. *Effect of ovine luteinizing hormone and theophylline on the synthesis of progestational steroids* in vitro *by rabbit ovarian tissue*

The ovaries were obtained from animals 6–8 days pseudo-pregnant. Results (means ± S.E.M. of four experiments) represent the increase in steroid content above control values.

Addition	Steroid content of tissue and medium after incubation for 3 hr. (μg./500 mg. of tissue)	
	Progesterone	20α-Hydroxypregn-4-en-3-one
Theophylline (10 μmoles/ml.)	4·8 ± 1·1	7·9 ± 2·0
Luteinizing hormone (0·02 μg./ml.)	1·2 ± 0·8	2·2 ± 1·0
Luteinizing hormone (0·05 μg./ml.)	4·2 ± 0·6	11·9 ± 3·2
Luteinizing hormone (0·1 μg./ml.)	10·4 ± 2·0	27·0 ± 4·1
Luteinizing hormone (0·02 μg./ml.) + theophylline (10 μmoles/ml.)	8·3 ± 2·3	25·9 ± 4·9
Luteinizing hormone (0·05 μg./ml.) + theophylline (10 μmoles/ml.)	12·7 ± 0·9	43·1 ± 1·3
Luteinizing hormone (0·1 μg./ml.) + theophylline (10 μmoles/ml.)	13·4 ± 1·5	64·6 ± 6·5

by luteinizing hormone causes no further increase in progestational steroid production over that found with luteinizing hormone alone. An analogous effect has been described by Koritz (1962) in rat adrenal tissue, where 3′,5′-AMP failed to elicit an additional increase in corticosteroidogenesis when the tissue was maximally stimulated by adrenocorticotrophic hormone.

Of the adenosine monophosphates 3′,5′-AMP alone activated phosphorylase in the liver (Rall & Sutherland, 1958) and the adrenal (Haynes, 1958), thus mimicking the action of adrenaline and adrenocorticotrophic hormone respectively. Similarly, adenosine phosphates other than 3′,5′-AMP did not influence steroid synthesis in rabbit ovarian tissue, indicating that luteinizing hormone may stimulate steroidogenesis by selectively increasing the synthesis of 3′,5′-AMP in its target organ.

The concentration of 3′,5′-AMP required to stimulate rabbit ovarian tissue *in vitro* is higher than the endogenous concentrations of the nucleotide found in the bovine corpus luteum (Savard *et al.* 1965) and in the adrenal (Haynes, 1958). The response of rabbit ovarian tissue to combinations of theophylline and 3′,5′-AMP suggested that phosphodiesterase may inactivate some of the 3′,5′-AMP by conversion into 5′-AMP either in the tissue or on diffusion into the medium from damaged cells. However, it is probable that poor penetration of this nucleotide into intact cells (Rall & Sutherland, 1961) is the major factor contributing to the requirement of a high concentration of exogenous 3′,5′-AMP in the present studies.

Incubation of luteinized rabbit ovarian tissue in the presence of theophylline alone elicits a small but significant increase in progestational hormone content, and indicates that in control tissue endogenous 3′,5′-AMP is inactivated by phosphodiesterase. The stimulation of steroidogenesis by theophylline is evidence in favour of the concept that 3′,5′-AMP is a mediator of luteinizing hormone action, and suggests that the conversion of endogenous 3′,5′-AMP into 5′-AMP by phosphodiesterase may be a regulatory mechanism in the control of ovarian steroid synthesis. The potentiating effect of theophylline on the ovarian response to luteinizing hormone is analogous to the effect of theophylline on the response to 3′,5′-AMP, and provides further evidence that luteinizing hormone stimulates steroidogenesis by increasing the concentrations of 3′,5′-AMP in the ovary. Theophylline has also been found to simulate and enhance the action of vasopressin on the toad bladder (Orloff & Handler, 1962) and the action of thyroid-stimulating hormone on the thyroid (Gilman & Rall, 1966), implicating 3′,5′-AMP as an intermediate in these tissues.

The differences in response to luteinizing hormone displayed by separated corpora lutea and interstitial tissue (Dorrington & Kilpatrick, 1966*b*) were paralleled closely by the effects of 3′,5′-AMP.

The present results indicate that the stimulation of progestational steroid hormones by luteinizing hormone involves the formation and action of 3′,5′-AMP: however, the steps that follow the formation of 3′,5′-AMP and result in increased steroidogenesis are not known. Addition of 3′,5′-AMP has been shown to activate phosphorylase and hence stimulate the breakdown of glycogen both in the adrenal (Haynes, 1958) and the liver (Rall & Sutherland, 1958). However, it seems unlikely that 3′,5′-AMP acts in a similar manner in the ovary, since luteinized rat ovarian tissue (Armstrong, 1963) and rabbit ovarian tissue (J. H. Dorrington & R. Kilpatrick, unpublished work) contain negligible amounts of glycogen. Also, Savard *et al.* (1965) demonstrated that 3′,5′-AMP did not activate phosphorylase in the bovine corpus luteum even though steroid synthesis was enhanced by this nucleotide.

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