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STEROID PRODUCTION BY THE ISOLATED RABBIT OVARIAN FOLLICLE—IV: EFFECTS OF CYCLIC NUCLEOTIDES

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SUMMARY

Ovarian follicles were isolated from virgin New Zealand White rabbits and incubated in medium containing Hanks balanced salt solution, Medium 199 and normal rabbit serum. Medium was changed every 15 min and stored at −15°C until assayed for progestin and androgen by established radioimmunoassay procedures. At fixed time intervals after the start of the incubations various cyclic nucleotides, theophylline or sodium fluoride were added. Of the nucleotides tested only cyclic AMP, dibutyryl cyclic AMP and cyclic CMP influenced steroid secretion; androgen production increased from negligible levels to about 10 ng/ml and progestin from 0·5 mg/ml to approximately 3 ng/ml. Sodium fluoride had no effect at any of the doses tested. Adenosine 5′-monophosphate also stimulated steroid secretion. These data suggest that in the intact rabbit follicle cyclic AMP probably mediates the response of the follicles to tropic hormone stimulation.

INTRODUCTION

Adenosine cyclic 3′5′-monophosphate (cyclic AMP) has been accepted as the second messenger which mediates the actions of a number of hormones including luteinizing hormone (LH) [1, 2]. Steroidogenesis by the ovarian follicle of the rabbit is known to be very sensitive to LH [3] which can specifically stimulate the synthesis of cyclic AMP in this tissue [4]. More progesterone than 20α-hydroxy-4-pregnen-3-one is produced by homogenates of rabbit ovaries when stimulated by cyclic AMP [5]. However, other data suggest that LH binds to the plasma membrane and probably does not enter the cell [6, 7]. In previous studies we have observed that the rabbit follicle *in vitro* is sensitive to LH but the dominant steroid produced is an androgen [8]. *In vivo* studies also indicate that the rabbit ovary is able to produce testosterone [9, 10].

In view of the similarity of the effects of LH and cyclic nucleotides on steroidogenesis the present study was designed to determine to what extent the steroidogenic effect of cyclic nucleotides on intact rabbit ovarian follicles is similar to LH effects. The secretion of progestin and androgen was examined since these steroids showed the most marked changes under these experimental conditions [8]. Sodium fluoride was also investigated since fluoride ions are reported to enhance adenylate cyclase activity [2].

MATERIALS AND METHODS

All rabbits used in this study were virgin New Zealand White, 3–9 months old and weighing 2–3·5 kg. They were individually housed in stainless steel cages on a 12 h light–12 h dark schedule with water and rabbit pellet type chow *ad libitum*.

The methods of isolating follicles and incubations were essentially the same as previously described [8, 11]. Maturity of rabbits was judged by weights of animals and their uteri. Follicles were dissected from the ovaries within minutes of sacrifice. The intact follicles were then randomly divided into groups of 10–20 and incubated at 37°C in 1·0 ml portions of medium consisting of Hanks balanced salt solution–Medium 199–normal rabbit serum (55:30:15), with medium being changed every 15 min. At different times after the start of the incubations media containing test substances were added. A National CO_2 incubator with air as the gas phase was used. The duration of each experiment was 4–8 h. Media were stored at −15°C until assayed within a week of storage.

Radioimmunoassays were carried out as previously described [8]. The limits of sensitivity of the progestin and androgen assays were 25 and 400 pg respectively. The coefficients of variation of replicate analyses were less than 10%. Immunoreactivity of medium alone was approximately 20 pg progestin and 200 pg androgen per sample. Values were generally more than twice that of the medium. For the androgen assay the method probably measures only testosterone and for progestin only progesterone. Results are therefore expressed as total immunoreactive progestin and androgen.

Nucleotides were obtained from Sigma and sodium fluoride (NaF) from Fisher Scientific. These stimulants were dissolved directly in the incubation medium to the required concentration.

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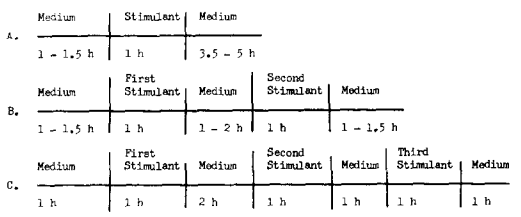

Fig. 1. Methods of incubations.

Cyclic AMP. (cAMP) and adenosine cyclic monophosphoric acid were used at a concentration of 7·5 mM; no stimulation of steroidogenesis was obtained with concentrations up to 60 μM. All other nucleotides were used at the following concentrations: inosine cyclic monophosphate (cIMP), 2·5 mM; $N^6O^{2'}$-dibutyryl adenosine cyclic monophosphate (dbcAMP), 2·5 mM; guanosine cyclic monophosphate (cGMP), 3 mM; uridine cyclic monophosphate (cUMP), 10 mM; cytidine cyclic monophosphate (cCMP) or the monophosphoric acid. 10 mM; adenosine-5'-monophosphate (5'AMP), 10 mM; 8-bromo-cGMP, 12 μM; $N^2O^{2'}$-dbcGMP, 0·3 mM. Theophylline, an inhibitor of phosphodiesterase, was used at a concentration of 5 mM. All of the nucleotides were used as the sodium salt except for cAMP and cCMP which were used both as the acid and as the sodium salt. Sodium fluoride was used at concentrations of 20 mM, 10 mM and 20 μM in a total of four experiments.

In most incubations the effects of two cyclic nucleotides were tested within the total duration of each incubation. Thus, one nucleotide could be added for four media changes between a specified period of incubation followed by a 2 h interval when medium alone was used. The second nucleotide was then added for four media changes in the usual manner. In all incubations a 1–1·5 h preincubation with medium alone was carried out. The various procedures carried out are outlined in Fig. 1. In one additional experiment cAMP was added at every medium change for 4 h.

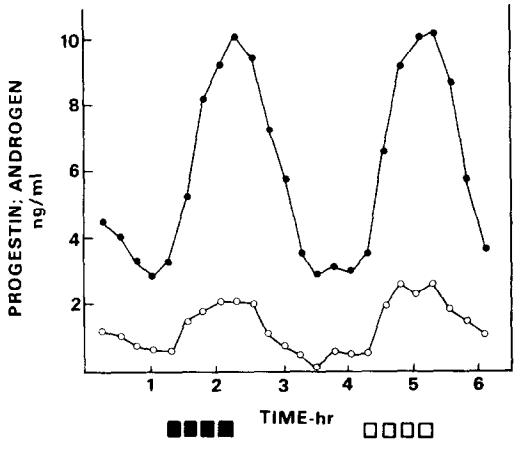

Fig. 2. Effects of cCMP (10 mM) and cAMP (7·5 mM) on follicular steroidogenesis. Solid bars refer to cCMP added between 1 and 2 h of incubation and open bars refer to cAMP added between 4 and 5 h of incubation. Twenty follicles from two rabbits with uterine weights of 0·8 and 0·4 g were used. ○—○ Progestin; ●—● Androgen.

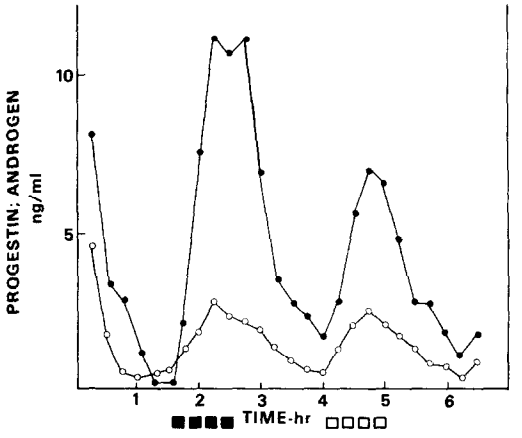

Fig. 3. Effects of adenosine cyclic 3'5' monophosphoric acid and theophylline (5 mM) on follicular steroidogenesis. Solid bars refer to nucleotide added between 1·5 and 2·5 h of incubation and open bars to theophylline added between 4 and 5 h. Symbols are the same as for Fig. 2. Twelve follicles were obtained from three rabbits with uterine weights 3·7, 13·2 and 15·6 g respectively.

Experiments with cAMP, dbcAMP, cCMP and theophylline were done at least in duplicate: cAMP—12; dbcAMP—4; cCMP—2; theophylline—6. In some, a mixture of theophylline and cAMP was used to see if a synergistic effect could be observed. In one other experiment, shown as C in Fig. 1, the first stimulant was a mixture of cAMP and theophylline, the second cAMP and the third theophylline. The order of addition of these three stimulants were also changed to theophylline, cAMP and then the mixture. In none of the experiments was it observed that pH changes occurred during the incubations. However, on repetitive freezing and thawing of the samples the phenol red in the media appeared more basic.

RESULTS

At the dose levels used cIMP, cGMP, cUMP, 8-bromo cGMP and cytidine cyclic monophosphoric

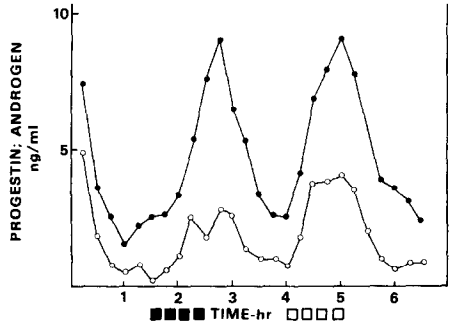

Fig. 4. Effects of dbcAMP (2·5 mM) and theophylline (5 mM) on follicular steroidogenesis. Solid bars refer to dbcAMP added between 1·5 and 2·5 h of incubation and open bars to theophylline added between 4 and 5 h. Symbols are the same as for Fig. 2. Twelve follicles from the same rabbits as the data of Fig. 3 were used.

Table 1. Secretion of androgen and progestin after cAMP stimulation (n = 12)

Preincubation	Progestin ng/ml/15 min	Androgen ng/ml/15 min
1	0.8±0.1	2.1±0.4
2	0.6±0.1	1.8±0.3
cAMP		
3	0.9±0.1	2.5±0.3
4	2.0±0.3	6.0±0.6
5	2.5±0.5	8.8±1.0
6	2.9±0.5	10.8±1.1
Postincubation		
7	2.9±0.5	10.5±0.9
8	2.0±0.3	8.2±1.0
9	1.2±0.1	5.1±0.5
10	0.9±0.1	3.4±0.4

Results are expressed as mean±SEM of samples prior to, during and after cAMP stimulation.

acid had no significant effects on steroidogenesis by rabbit follicles. Dibutyryl cGMP had a slight effect. However, cCMP had similar effects on steriodogenesis as cAMP (Fig. 2). An increase in androgen production (3–10 ng/ml) and progestin from 0·5 to 2 ng/ml was observed in the presence of these two nucleotides.

When cAMP and theophylline were added to the medium separately the effects on steriodogenesis observed are shown in Fig. 3. Cyclic AMP caused a dramatic increase in androgen production from negligible levels to more than 10 ng/ml/15 min, whereas progestin increased from about 0·5 ng/ml/15 min to about 3 ng/ml/15 min. The changes in androgen production were similar when cAMP or the monophosphoric acid was used. This is in contrast to the results with cytidine cyclic monophosphoric acid. When theophylline was added later the effects on androgen production were not as pronounced whereas progestin secretion was similar to that in the presence of cAMP.

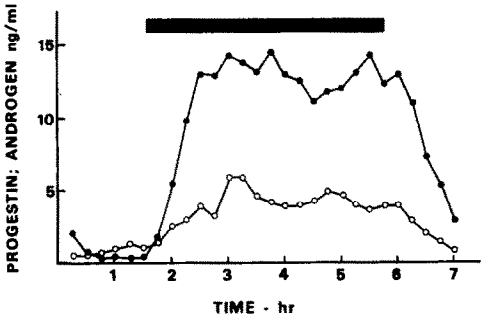

Fig. 5. Effects of prolonged presence of cAMP in medium on steroidogenesis. Solid bar refers to cAMP. Twenty follicles were used from two rabbits with uterine weights of 4·1 and 3·8 g. Symbols are the same as for Fig. 2.

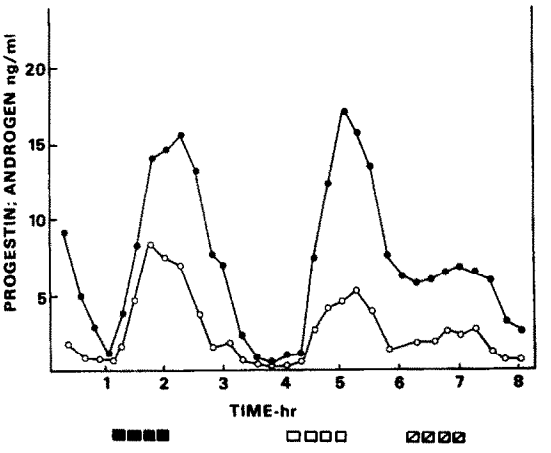

Fig. 6. Effects of cAMP and theophylline on steroidogenesis. ■ cAMP + theophylline, □ cAMP, ▨ cAMP + theophylline + theophylline. Other symbols are the same as for Fig. 2. Twenty follicles from two rabbits with uterine weights 4·1 and 3·8 g were used.

Dibutyryl cAMP at a lower concentration than cAMP had effects similar to those of cAMP (Fig. 4). However, the response of the follicles to theophylline appeared to be greater than for the previous experiment.

In order to assess how much stimulation was initiated by cAMP, the combined results of the twelve separate experiments were analysed statistically as shown in Table 1. The eight samples (*i.e.* 2 h post stimulation, 1 h with cAMP and 1 h without), after the preincubation were compared with that just prior to stimulation. It can be seen that at the end of 1 h with cAMP there is a significant rise in androgen and progestin secretion ($P < 0{\cdot}001$) which declined 1 h after removal of cAMP ($P < 0{\cdot}001$). The rapid fall in steroid secretion is also seen in Fig. 5 where high levels of both androgen and progestin were found as long as cAMP was present in the medium.

As shown in Fig. 6, theophylline and cAMP did

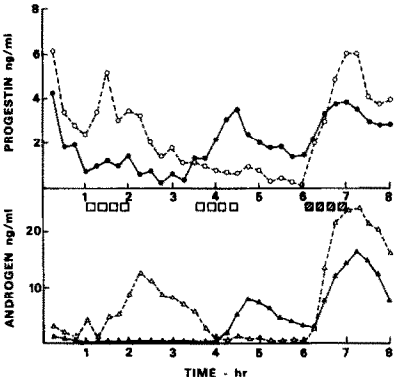

Fig. 7. Effects of cAMP and theophylline on steroidogenesis. □ cAMP or theophylline. ■ cAMP + theophylline. △—△, ○—○ cAMP was added at 1–2 h; ▲—▲, ●—● cAMP was added at 3·5–4·5 h. Two follicles were used per incubation from 1 rabbit with uterine weight 18·7 g.

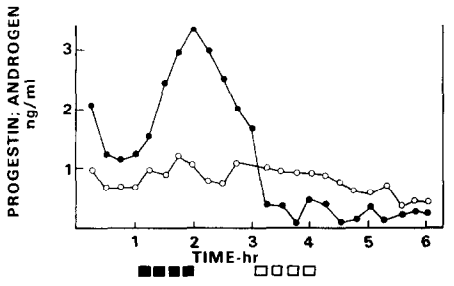

Fig. 8. Effects of 5'-AMP and cIMP on steroidogenesis. Solid bars refer to 5'-AMP and open bars to cIMP. Symbols are the same as for Fig. 2. Eighteen follicles from two rabbits with uterine weights 1·4 and 0·8 g were used.

not appear to have a synergistic affect on androgen production when compared to either test substance alone. However, the effects on progestin secretion do seem to be additive. When the order of adding the stimulants was changed (Fig. 7) the results were similar to those of Fig. 6 with some synergism observed.

The addition of 5' AMP to the medum led to an increase in androgen secretion whereas cIMP had no significant effect (Fig. 8). Again, the effect on androgen production was very marked.

Sodium fluoride which stimulates adenylate cyclase activity in a variety of tissues was ineffective in the rabbit ovarian follicle system. Figure 9 shows the results obtained when sodium fluoride (10 mM) was added to the medium. Similar results are obtained when media and follicles were incubated without any additions [8]. Concentrations of 20 mM and 20 μM sodium fluoride gave essentially the same results.

DISCUSSION

LH can stimulate the synthesis of cAMP in rabbit ovarian follicles [4], isolated rat follicles [12] and corpora lutea tissue of cows [13, 14] and man [15]. LH, FSH and prostaglandin E_2 stimulate synthesis of cAMP in sliced ovarian tissue of rats [16]. Rabbit interstitial tissue responded to cAMP in the same way as to LH [17]. Dorrington and Kilpatrick [18] found

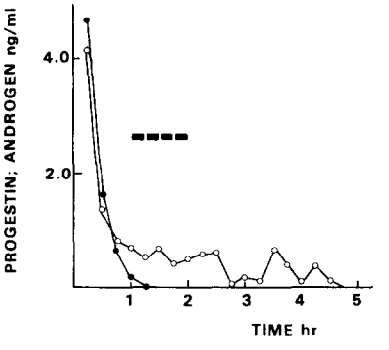

Fig. 9. Effect of sodium fluoride on steroidogenesis by the isolated rabbit follicle. Solid bars refer to times when 10 mM NaF was added. Symbols are the same as for Fig. 2. Sixteen follicles were obtained from a rabbit with uterine weight 10·4 g.

that chopped ovarian tissue of rabbits responded to as little as 8 μM cAMP with a 4-fold increase in progestin formation.

In the experiments described here steroidogenesis was not affected to the same extent by the cyclic nucleotides as with LH. Within an hour of the removal of the nucleotide steroid secretion had declined (Table 1, Figs. 2–5). In the case of LH stimulation, steroidogenesis usually continued for about 4 h [8] suggesting that LH initiates events which are more lasting than those initiated by cAMP. Similar results were obtained with LH and cAMP in Leydig tumor cells [7]. In the rat adrenal gland cyclic nucleotides and adrenocorticotrophic hormone had effects on corticosteroidogenesis similar to the follicles and Leydig tumor [19]. This is in contrast to the situation in the whole rabbit ovary where both LH and cAMP (5 μM) stimulate the synthesis of progestins [20]. In our follicular system 60 μM cAMP or less had no effect. On the other hand, Keyes *et al.*[21] demonstrated that LH but not cAMP could cause autotransplanted rabbit follicles to luteinize, indicating that LH influences more than steroidogenesis in the follicle. The results with cAMP also suggest that the continuous presence of the nucleotide is necessary for the production of an essential labile factor involved in promoting steroidogenesis.

From the uterine weights it was also possible to get an idea of the stage of maturity of the rabbits from which ovarian follicles were obtained. Thus in Figs. 2 and 3, which represent responses of follicles from immature and mature rabbits respectively, it is evident that a greater increase in the secretion of androgen and progestin is elicited by follicles from mature rabbits.

In the presence of theophylline, androgen synthesis and secretion were identical to the effects shown in the presence of cAMP, dbcAMP and cCMP. This implies that cAMP has accumulated in sufficient amounts to stimulate steroidogenesis. The rapid return of androgen production to pre-stimulation levels after the removal of theophylline suggests that the cAMP which was produced was rapidly metabolized by the phosphodiesterase.

The response of the follicles to cCMP which is mimicked by cAMP is similar to that found for adrenal cells in culture [22, 23]. It has been suggested that this action of cCMP may be due to stimulation of adenylate cyclase enzyme, inhibition of phosphodiesterase, or specific binding proteins or protein kinases in the cells which are sensitive to cCMP [23]. In the same communication cyclic CMP was shown to displace cAMP from cAMP binding proteins.

The lack of response of the follicular cells to the other nucleotides may be due to the differences in permeability of the compounds, the lower concentrations used or to the fact that there are no specific binding proteins for these nucleotides which could stimulate the protein kinases needed for steroidogenesis. The response to 5'-AMP is of interest since

this has also been found to stimulate steroidogenesis in the adrenal cortex [23]. The high concentration of 5'-AMP may inhibit the phosphodiesterase causing cAMP to accumulate. This observation also points to the danger of using non-cyclic nucleotides as controls. However, Dorrington and Kilpartick[18] were unable to show any effects of 5'-AMP, adenosine diphosphate or adenosine triphosphate on progesterone synthesis under the same conditions where cAMP had a significant effect.

It was also of interest that no response could be elicited with NaF which is known to stimulate adenylate cyclase enzyme systems [2, 18]. Even a 1000-fold decrease in NaF which has been shown to stimulate progestin synthesis in the bovine corpus luteum [17] was found to be ineffective in the rabbit follicular system. Thus the high NaF concentration could not be an inhibitory influence. The lack of response to NaF in our experiments is probably due to the fact that intact cells were used [24]. Robison *et al.*[25] have pointed out that NaF does not cause accumulation of cAMP in whole-cell preparations and have concluded that NaF does not activate adenylate cyclase in intact cells. Since it is known that fluoride ions can enter freely into intact cells it has been suggested that the process of membrane fragmentation alters the enzyme system rendering it susceptible to activation by NaF [24].

The additive effects of cAMP and theophylline on steroidogenesis is noteworthy. Similar results on progestin synthesis by rabbit ovarian homogenates have been reported [18].

The increased production of androgen in these incubations is similar to that seen in previous studies from this laboratory [8, 26] when LH was used as the stimulant. Since incubation of media alone gave no responses it was suggested that alternate pathways for the synthesis of androgen were operative in the rabbit follicle. Some evidence for such an alternative pathway has been presented by de la Torre and Diczfalusy [27]. Other workers have also shown that testosterone is a dominant product of the rabbit follicle [28].

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