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Studies on the Effects of Dibutyryl Cyclic AMP and Theophylline on RNA Synthesis in Mouse Follicular Oocytes *in Vitro*

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Dibutyryl Cyclic AMP와 Theophylline이 培養중인 생쥐濾胞卵子의 RNA合成에 미치는 영향에 관한 연구

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적 요

自記放射法을 이용하여 dbcAMP와 theophylline이 未成熟卵子의 RNA 合成에 미치는 영향을 관찰하였다. 培養中인 未成熟卵子의 RNA 合成은 dbcAMP 와 theophylline에 의하여 抑制를 받았다. dbcAMP나 theophylline은 培養液(modified Krebs-Ringer bicarbonate solution)內에 100 μg/ml 정도 들어 있으면 핵막(germinal vesicle)이 붕괴되지 못하고 그대로 存在하며 그동안의 RNA 合成은 극히 억제된 채로 남아 있다. 그러나 培養을 시작하여 2~3時間後, 즉 핵막붕괴가 끝난 다음에 이들 억제물질을 배양액에 添加하면 正常卵子와 같이 성숙분열이나 RNA 合成이 억제 됨이 없이 진행된다. 24時間 동안 dbcAMP나 theophylline으로 성숙이 억제 되었던 卵子도 이들 억제물질을 제거하면 즉시 成熟分裂이 진행되며 RNA 合成도 正常的으로 일어난다.

이런 結果로 미루어 dbcAMP등의 RNA 合成 抑制機作에 한 가지 가능성을 추측할 수 있다. 즉 dbcAMP나 theophylline의 처리에 의해 細胞質內 cAMP의 농도가 높아지고 이 cAMP는 핵막붕괴나 染色質의 응집에 관여하는 단백질合成을 誘導할 mRNA合成을 억제하며 이 때문에 卵子는 핵을 보유한채 그대로 남아 있는 것이다.

INTRODUCTION

It has been demonstrated that the mouse oocyte synthesizes RNA within 2~6 hours of incubation (Mintz, 1964; Oakberg, 1967; Bloom and Mukherjee, 1972). Donahue (1968) found that the breakdown of germinal vesicle in mouse oocytes usually occurred in 2~3 hours *in vitro*. It is, therefore, assumed that there might be a particular relation between the RNA synthesis in the early stage of meiosis

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and the breakdown of germinal vesicle, suggesting that the breakdown of germinal vesicle might be induced by specific proteins which are formed by newly synthesized RNA.

Cho *et al.* (1974) found that dibutyryl cyclic AMP (dbcAMP) or theophylline suppressed the breakdown of germinal vesicle in mouse oocytes, and removal of the agents brought an immediate resumption of the delayed maturation. Particularly, such inhibitors were inefficient for the suppression of the meiotic resumption if applied beyond the germinal vesicle stage. This implies that dbcAMP or theophylline would only affect the germinal vesicle, condensation of the chromatin and/or spindle formation.

The present studies were conducted to investigate the effects of dbcAMP or theophylline on RNA synthesis in mouse oocytes in order to clarify the relation between RNA synthesis and germinal vesicle breakdown.

MATERIALS AND METHODS

Mouse oocytes were obtained from the ovaries of 3~5 week old A-strain mice bred randomly in our laboratory. Ovaries were dissected out, placed on the culture dish containing the modified Krebs-Ringer bicarbonate solution (Biggers *et al.*, 1971) and matured ovarian follicles were punctuated with a fine needle under a dissecting microscope, thereby forcing the oocytes out into the medium.

The oocytes denuded from cumulus cells but with a clearly visible germinal vesicle were transferred to the medium for culture. The Brinster's method(1963) was mainly adopted in the present studies for oocyte culture. The oocytes placed in a drop of medium (50 μl) covered by 15 ml of paraffin oil in a culture dish (Falcon #3002) were then incubated at 37°C in an incubator supplied by 5% CO_2 in humidified air. DbcAMP (Sigma) or theophylline (Sigma) was dissolved in Dulbecco's Phosphate Buffered Saline (PBS) at a concentration of 5 mg/ml and then added to each culture medium for a final concentration of 100 μg/ml. ^{3}H-uridine (Dickinson and Co., spec. act., 42 μCi/mM) at a final concentration of 10 μCi/ml was added to the medium before the oocytes were set and cultured. At the end of the culture period, the oocytes were washed with several changes of fresh medium and fixed for 30 minutes in 3 : 1 mixture of alcohol and acetic acid and placed on an albumin coated slide. A coverslip was gently placed over the oocytes on a slide after staining with 0.5% aceto-lacmoid. The slides were then frozen, coverslips were flicked off and placed in a coplin jar containing the fixative for 30 minute. The slides were then washed in 5% trichloroacetic acid at 4°C for 10 minutes, rinsed in cold water, coated with AR-10 film and then stored at 4°C in a light-proof box for 4 weeks. After development, the oocytes were observed under a phase contrast microscope, and grains were counted

and photographed. All glasswares and instruments for the culture were sterilized in a hot air sterilizer and media were filtered with Millipore before use.

RESULTS

After 6 hours of culture, oocytes cultured in the plain medium showed breakdown of nuclear membrane and condensation of chromatin, while those cultured in the medium containing dbcAMP or theophylline kept their germinal vesicles unchanged. ^{3}H-uridine was added to this culture period to see its incorporation into the oocyte RNA. The grains counted were classified according to the following density system (Table 1).

Table 1. Criteria of grain counts per ovum.

Density	0	1	2	3	4	5
Grain count	0—5	5—10	10—20	20—30	30—40	40<

Grain density of control group was 3~4, while that of experimental group of dbcAMP or theophylline was as low as 1 (Table 2, Figs. 1, 2, 3).

Table 2. Grain densities in the oocytes exposed to ^{3}H-uridine for 6 hours in the medium containing dbcAMP or theophylline.

Treatment	No. of oocytes	Density						
		0	1	2	3	4	5	mean
None[1)]	15	—	2	3	6	3	1	2.9
dbcAMP[2)]	15	2	9	4	—	—	—	1.1
theophylline[3)]	15	3	8	4	—	—	—	1.1

1) basic medium+^{3}H-uridine.
2) basic medium+^{3}H-uridine+dbcAMP (100μg/ml).
3) basic medium+^{3}H-uridine+theophylline (100μg/ml).

In order to see the incorporation of ^{3}H-uridine into the RNA of oocytes whose germinal vesicle had already been broken down, oocytes were cultured initially for 4 hours in the plain medium and then transferred to the medium containing ^{3}H-uridine together with dbcAMP or theophylline and kept for another 3 hours. The oocytes cultured in the plain medium for 4 hours normally showed complete breakdown of germinal vesicle, and those transferred to the medium containing inhibitors continued their further meiotic division without showing any abnormality. The results are presented in Table 3.

As shown in the table, grain densities were 0~1 in all three groups (Figs. 4, 5, 6). This result indicates that RNA synthesis of the oocytes is almost completed during the first 4 hours of culture.

The oocytes whose meiotic resumption had been inhibited for 24 hours by dbcAMP or theophylline restored the maturation processes immediately after they

Table 3. Grain densities in the oocytes exposed to ^{3}H-uridine in the medium containing dbcAMP or theophylline for 3 hours after incubation in the plain medium for 4 hours.

Post-treatment	No. of oocytes	Density						
		0	1	2	3	4	5	mean
None[1)]	15	4	7	2	2	—	—	1.1
dbcAMP[2)] (100 μg/ml)	15	6	7	1	1	—	—	0.7
theophylline[3)] (100 μg/ml)	15	6	3	3	3	—	—	1.2

1) basic medium (4 hrs), then+^{3}H-uridine (3 hrs).
2) basic medium (4 hrs), then+^{3}H-uridine+dbcAMP (3 hrs).
3) basic medium (4 hrs), then+^{3}H-uridine+theophylline (3 hrs).

were transferred into the plain medium. To see whether these inhibited oocytes for 24 hours possess the ability to synthesize RNA as seen in normal one or not, the oocytes whose germinal vesicles remained unchanged under the presence of the inhibitors were transferred to the plain medium and cultured for another 6 hours. The results are shown in Table 4.

Table 4. Grain densities in the oocytes cultured in the plain medium containing ^{3}H-uridine for 6 hours after exposed to dbcAMP or theophylline for 24 hours.

Treatment	No. of oocytes	Density						
		0	1	2	3	4	5	mean
None[1)]	15	—	2	6	4	3	—	2.5
dbcAMP[2)] (100 μg/ml)	15	—	3	6	5	1	—	2.3
theophylline[3)] (100 μg/ml)	15	1	1	4	6	3	—	2.6

1) basic medium+^{3}H-uridine (6 hrs).
2) basic medium+dbcAMP (24 hrs), then basic medium+^{3}H-uridine (6 hrs).
3) basic medium+theophylline (24 hrs), then basic medium+^{3}H-uridine (6 hrs).

Even though their meiosis had been inhibited for a period as long as 24 hours, the oocytes were found to have an ability for the meiotic resumption upon removal of the inhibitors. Their germinal vesicles were brocken down within 2~3 hours and the condensed chromatins appeared in the plain medium normally. RNA synthesis by such oocytes, of course, occurred within 6 hours. Grain densities of these oocytes were 2~3 (Figs. 7, 8, 9).

DISCUSSION

It has been shown that mouse oocytes synthesize RNA within 2~6 hour culture period (Bloom and Mukherjee, 1972), lose their germinal vesicles within 2~4 hours and reach metaphase II stage in 18 hours of culture (Donahue, 1968). In other

words, the period of RNA synthesis in the oocytes seemed to be related to the time of germinal vesicle breakdown.

As shown in the present results, dbcAMP or theophylline apparently affects the RNA synthesis and suppresses the further maturation *in vitro*. Cho *et al.* (1974) and Stern and Wassarman (1974) showed that the oocytes in the presence of dbcAMP undertook protein synthesis as untreated oocytes. Fertilized mouse eggs continued their cleavages even under the influence of actinomycin D which is an inhibitor of RNA synthesis (Mintz, 1964). These findings suggest that the ordinary synthesis of proteins by the oocytes or by the embryonal cells even under the influence of the inhibitors of RNA synthesis would occur rather by the long-lived RNA. We assume, therefore, that only some specific RNA synthesized at the early stage of meiotic division would be responsible for the initiation of meiosis, producing some specific proteins which act on the breakdown of germinal vesicle, chromatin condensation and spindle formation. Thus, dbcAMP or theophylline would affect such RNA synthesis, and eventually suppresses the oocyte maturation.

It has been known that dbcAMP is more resistant to hydrolysis by the phosphodiesterase which converts cAMP to 5'-AMP and is more permeable than cAMP (Butcher *et al.*, 1970; Kaukel *et al.*, 1972). DbcAMP penetrated into the cell is supposed to be converted to cAMP, or to bind with specific proteins instead of cAMP, and acts in the same way as cAMP does. Theophylline raises the intracellular concentration of cAMP level. Thus, two agents are closely related to the increase of cAMP concentration in the cell. Actually, Cho and Yoon (1975) found that oocytes lost their glycogen concentrations in the presence of dbcAMP or theophylline in the culture. In general, it has been well known that cAMP induces glycogenolysis in various cells. Consequently, it is reasonable to assess that dbcAMP or theophylline acts likely as cAMP does.

There are several investigations on the RNA synthesis with cAMP. Nussdorfer and Mazzochi (1972) in rat liver cells found that the increase of cAMP in cytoplasm enhanced RNA synthesis. Chambers and Zubay (1969), working with a cell free system, had obtained an evidence to suggest that the effect of cAMP is to promote the initiation of RNA synthesis. Kuo and Greengart (1969) mentioned that cAMP stimulates the protein kinase capable of phosphorylating histones in *E. coli*. Thus, many of the previous studies strongly suggest a possible initiation of the RNA synthesis by the presence of cAMP.

On the other hand, in the present studies, RNA synthesis which must be occurred before or during germinal vesicle breakdown of the oocytes is apparently inhibited by dbcAMP or theophylline which is responsible for increasing cAMP in the cell.

Based on the present results, we could make assumptions that (1) the increased

cAMP inhibits the transcription of RNA by inactivating a protein kinase for the phosphorylation of histones, and (2) it would inactivate the RNA polymerase activity.

Further investigation on the role of cAMP in oocytes with special relation to the oocyte maturation is remained.

REFERENCES

Biggers, J.D., W.K. Whitten and D.G. Whittingham, 1971. The Culture of Mouse Oocytes *in vitro*. *In*: Methods in Mammalian Embryology. (J.C. Daniel, Jr., editor). W. H. Freeman & Co., San San Francisco, Calif. U.S.A., p. 86.

Bloom, A.M. and B.B. Mukherjee, 1972. RNA synthesis in maturing mouse oocytes. *Exptl. Cell Res.* **74** : 577.

Brinster, R.L., 1963. A method for in vitro cultivation of mouse ova from two-cell to blastocyst. *Exptl. Cell Res.* **32** : 205.

Butcher, R.W., G.A. Robison and E.W. Sutherland, 1970. The role of cyclic AMP in certain biological control systems. *In*: A Ciba Foundation Symposium. (G. E. W. Wolskenholm & J. Knight, editors). J. & A. Churchill, London, U.K. pp 64—85.

Chambers, D.A. and G. Zubay, 1969. The stimulatory effect of cyclic adenosine 3′, 5′—monophosphate on DNA-directed synthesis of β-galactosidase in a cell free system. *Proc. Nat. Acad. Sci.* U.S. **66** : 104.

Cho, W.K., S. Stern and J. D. Biggers, 1974. Inhibitory effect of dibutyryl cyclic AMP on mouse oocyte maturation *in viro*. *J. Exp. Zool.* **187** : 383.

Cho, W.K. and Y.D. Yoon, 1975. Studies on the effects of dibutyryl cyclic AMP and theophylline on intracellular contents of glycogen of mouse follicular oocytes *in vitro*. *Korean J. Zool.* **18** : 27—40.

Donahue, R.P., 1968. Maturation of the mouse oocyte *in vitro*. I: Sequence and timing of nuclear progression. *J. Exp. Zool.* **169** : 237.

Kaukel, E., U. Fuhrmann and H. Hilz, 1972. Divergent action of cAMP and dbcAMP on macromolecular synthesis in HeLa S3 cultures. *Biochem. Biophys. Res. Comm.* **48** : 6.

Kuo, J.F. and P. Greengart, 1969. Cyclic nucleotide-dependent protein kinases. Ⅳ. Wide spread occurrence of adenosine 3′, 5′-monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom. *Proc. Nat. Acad. Sci.* U.S. **64** : 1349.

Mintz, B., 1964. Synthetic processes and early development in the mammalian egg. *J. Exp. Zool.* **157** : 85.

Nussdorfer G.G. and G. Mazzocchi, 1972. Effect of 3′, 5′-cyclic AMP on nuclear and mitochondrial RNA synthesis of rat adrenocortical cells. *Acta Endocrinol.* **70** : 81.

Oakberg, E.K., 1967. ^{3}H-uridine labeling of mouse oocytes. *Arch Anat. microscop.* **56**, Suppl. 3-4 pp. 171—184.

Stern, S. and P.M. Wassarman, 1974. Meiotic maturation of the mammalian oocyte *in vitro*: Effect of dibutyryl cyclic AMP on protein synthesis. *J. Exp. Zool.* **189** : 275.

EXPLANATION OF FIGURES

(Figs. 1 and 7.) Oocyte cultured in control medium containing ^{3}H-uridine for 6 hours. A number of grains are seen through the egg cytoplasm (600×).

Fig. 2. Oocyte cultured in dbcAMP medium containing ^{3}H-uridine for 6 hours (600×).

Fig. 3. Oocyte cultured in theophylline medium containing ^{3}H-uridine for 6 hours (600×).

Fig. 4. Oocyte cultured in control medium containing ^{3}H-uridine for 3 hours after incubation in the plain medium for 4 hours (600×).

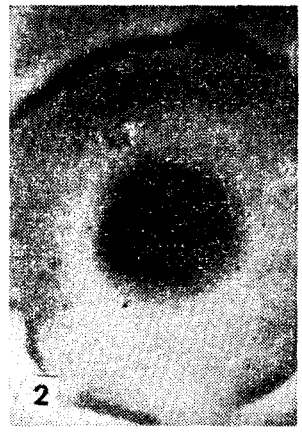
Fig. 5. Oocyte cultured in dbcAMP medium containing ^{3}H-uridine for 3 hours after incubation in the plain medium for 4 hours (600×).

Fig. 6. Oocyte cultured in theophylline medium containing ^{3}H-uridine for 3 hours after incubation in the plain medium for 4 hours (600×).

Fig. 8. Oocyte cultured in plain medium containing ^{3}H-uridine for 6 hours after exposed to dbcAMP for 24 hours (600×).

Fig. 9. Oocyte cultured in plain medium containing ^{3}H-uridine for 6 hours after exposed to theophylline for 24 hours (600×).


1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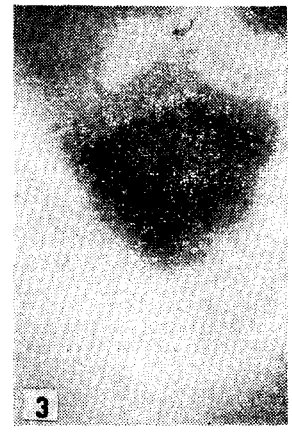
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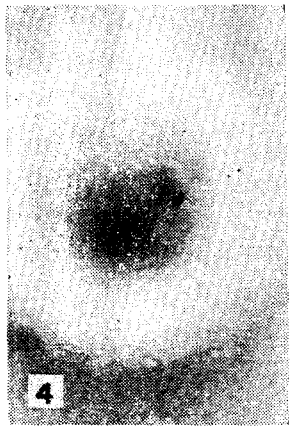
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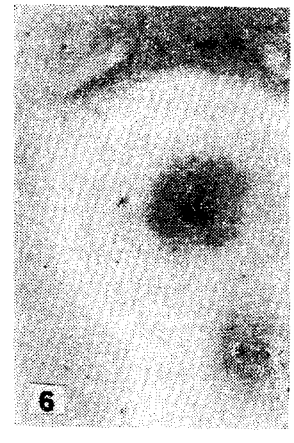
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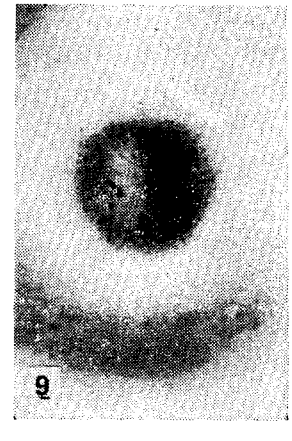
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