The Role of Cyclic AMP in Oocyte Maturation of Goldfish (*Carassius auratus*)

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Abstract

The role of cyclic AMP in oocyte maturation was investigated using denuded goldfish oocytes cultured in vitro. The oocytes were stimulated with a maturation-inducing steroid (MIS), 17α, 20ß dihydroxy-4-pregnen-3-one (17, 20 P) with or without forskolin or forskolin only. Changes in cAMP concentrations and percent maturation of the oocytes were determined. Results showed that elevated levels of cAMP maintain the oocytes in meiotic arrest while a decrease would trigger the resumption of meiotic maturation.

Introduction

Vertebrate oocytes are arrested in the first meiotic prophase until the preovulatory surge of gonadotropins in vivo. Cyclic AMP (cAMP) has been viewed to mediate in the maintenance of meiotic arrest when present at high concentrations in the oocyte while a decrease in intraoocyte cAMP results in resumption of meiotic maturation (reviewed by Dekel 1988, Schultz et al. 1983). It has been suggested that the meiotic arrest in vivo may be due to gap junction-mediated transmission of follicle cell cAMP to the oocyte (Racowsky and Baldwin 1989, Eppig et al. 1983, Dekel and Beers 1978). The preovulatory surge of gonadotropins which releases the oocyte from meiotic arrest causes a down-regulation of follicular gap junctions disrupting the transfer of cAMP from the follicle cells into the oocyte (Racowsky et al. 1989).

The role of cAMP in oocyte maturation was investigated in this study using denuded goldfish oocytes cultured in vitro. We wanted to test whether elevated levels of cAMP are responsible for the maintenance of oocyte meiotic arrest and

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whether a decrease in the cAMP concentrations of the oocyte is a sufficient signal for the resumption of meiotic maturation in goldfish oocytes.

Materials And Methods

Chemicals

Medium 199, fungizone, penicillin-streptomycin, and L-glutamine were obtained from Gibco Laboratories, Grand Island, N.Y. The labeled antigen, succinyl cAMP-[^{125}I]-tyrosine methyl ester was from Amersham International, Amersham, U.K. while the dye reagent for Bradford protein assay was obtained from Bio-Rad Lab, Richmond, California. Maturation-inducing steroids (MIS), 17α, 20ß dihydroxy-4-pregnen-3-one (17, 20 P) and all other chemicals except forskolin (Calbiochem) were purchased from Sigma.

Oocyte Collection and Culture

Gravid goldfish purchased from fish farms were maintained at 12-16 °C in a 18/6 h light/dark cycle. Fish were chosen for experiments by sucking out some ovarian oocytes with a polyethylene cannula (inner diameter, 0.86 mm; outer diameter, 1.27 mm) inserted through the ovipore. These oocytes were treated with a clearing reagent (glacial acetic: absolute ethanol, 1:1) to reveal the position of the germinal vesicle. Only fish containing oocytes at the tertiary yolk stage (0.8 mm diameter) with central germinal vesicles were decapitated. The ovaries were removed and placed in ice-cold isolation medium, pH 7.2, consisting of Medium 199, 20 mM Hepes, 8mM $NaHCO_3$, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1.25 ug/ml fungizone. Small pieces of the ovary were preincubated for 30-45 min in Ca/Mg-free medium prepared as described by Greeley et al. (1987), except that gentamycin was replaced with 100 IU/ml penicillin, 100 µg/ml streptomycin, 1.25 ug/ml fungizone, and 2 mM L-glutamine, to loosen the follicular envelope surrounding the oocyte. Then, the ovarian fragments were transferred back to ice-cold isolation medium and the follicular wall was removed manually with a fine tipped forcep.

Groups of 10 oocytes were cultured in a multiwell tissue culture dish (NUNC Inter Med, Denmark) containing 1 ml of incubation medium with the same formulation as the isolation medium except that the Hepes was replaced with 30 mM NaHCO3. All cultures were placed in an incubator with a humidified atmosphere of 5% CO_2, 95% air at 25 °C. After culture, the oocytes were scored for GVBD (germinal vesicle breakdown), an indicator that the oocytes had undergone maturation. GVBD was considered to have occurred when the germinal vesicle was no longer visible after treating the oocytes with the clearing reagent. Viability of the

oocytes was ascertained before the GVBD scoring with the trypan blue exclusion test (Patterson 1979).

The osmotic pressure of the media used was determined with a WESCOR 5500 vapor pressure osmometer and was adjusted with NaCl to that of the ovarian fluid when necessary.

Cyclic AMP Extraction and Radioimmunoassay

At the end of the culture experiment, the medium and the oocytes were separately transferred into 12 x 75 mm glass tubes. The glass tubes with the oocytes contained 0.5 ml of 1 mM theophylline while 100 µl of 10 mM theophylline was added into the glass tubes containing culture medium samples. All the samples were boiled for 5 min and stored at -60 °C until used. The medium was assayed directly for cAMP without further extraction. The oocytes were thawed and twice-distilled absolute ethanol was added to a final concentration of 80%. They were homogenized with a motor-driven Teflon pestle for 2 min. The homogenates were left to settle overnight at 4 °C to precipitate the proteins and then, centrifuged at 3000 rpm for 15 min. The supernatant was decanted and evaporated to dryness in a vacuum oven at 55 °C. The extract was redissolved in 0.05 M sodium acetate, pH 6.2. The pellets were stored for protein assay. In experiments where follicular layers were co-cultured with denuded oocytes and assayed for cAMP, the procedure used for oocytes in processing and extraction was followed.

Cyclic AMP was measured by radioimmunoassay. The labeled antigen was succinyl tyrosine-[^{125}I]-methyl ester derivative of cAMP. The standard curve ranged from 7.8 to 500 femtomole (fm). The sensitivity of the standard curve defined as the dose corresponding to the mean counts per minute (cpm) bound at zero dose minus 2 standard deviations of the mean cpm bound at zero dose (Abraham 1977) was calculated to be 2.14 fm. The intra- and inter-assay coefficients of variation were 8.3% and 9.1%, respectively. RIA data were analyzed using the W.H.O. Immunoassay Data Processing Program developed by P.R. Edwards (1984). Test of parallelism using increasing aliquot sizes or increasing dilutions of extracted samples was done to validate the assay and it was found to be parallel to the linear portion of the standard curve. Recovery of the tracer added to the samples was 92%. The concentrations of cAMP were expressed in terms of pmole cAMP per mg protein in oocytes and folicullar layers while in the medium, it was in pmole cAMP per ml.

Protein Assay

The pellets remaining after centrifugation of homogenized samples were dissolved in 0.01% sodium dodecyl sulfate in 1 N NaOH. They were boiled and assayed for protein following the Bio-Rad microassay procedure for protein assay (Bulletin 1069, Bio-Rad Lab 1987).

Statistical Analyses

The data were analyzed using the software computer program SPSS/PC+. One-way analysis of variance test (ANOVA) followed by Duncan's Multiple Range Test and two-way ANOVA were used to test differences between treatments. Differences of $P<0.05$ were considered to be statistically significant.

Results

Cyclic AMP Levels

The cAMP levels in goldfish oocytes and follicular layers cultured in vitro are shown in Fig. 1. In denuded oocytes cultured with or without free follicular layers, the cAMP content decreases significantly ($P<0.05$) within 30 min and remains low until the 20-h observation period. In contrast, intact oocytes show a fluctuating pattern of cAMP levels (Fig. la). A significant decrease ($P<0.05$) is noted at 4 h, after which, cAMP increases to basal level (0-h level) at 8 h, and decreases again significantly ($P<0.05$) at 20 h. The total cAMP concentrations in the intact oocytes are significantly higher ($P<0.05$) than those of the denuded oocytes whether these are cultured with or without free follicular layers (Fig. la). The follicular cAMP, also shown in Fig. 1a, is about 5 to 10 fold higher than those of the oocytes. However, the presence of follicular layers does not increase the cAMP content of co-cultured denuded oocytes.

To ensure that differences in the intraoocyte cAMP levels were not due to extrusion of cAMP into the medium, cAMP content of the culture media was assayed at different times. The assay showed that the cAMP concentrations in the medium was very minimal, except at 0 h (Fig. lb). Cyclic AMP progressively decreased until the 4 h and then remained low until the 20-h observation period. There were no apparent differences in the overall levels of cAMP in the media of the three different kinds of oocyte preparations.

Intraoocyte cAMP was also assayed in denuded goldfish oocytes stimulated with MIS (1 μg/ml 17, 20 P) only, MIS (1 μg/ml) in the presence of forskolin (0.01 μM), and forskolin (0.01 μM) only (Fig. 1c). There was an immediate significant decrease ($P<0.05$) in intraoocyte cAMP within 15 min. in MIS-stimulated denuded oocytes. It continued to decrease until 4 h by about 73% from the basal level (at 0 h), and began to rise again back to the basal level at 16 h. In MIS and forskolin-stimulated denuded oocytes. It continued to decrease until 4 h by about 73% from the basal level (at 0 h), and began to rise again back to the basal level at 16 h in MIS- and forskolin-stimulated oocytes, intraoocyte cAMP decreased by 25% within 15 min, and became significantly lower by 41% ($P<0.05$) than the basal level at 1 h. Intraoocyte cAMP decreased greatly (65%) at 4 h, then it increased again but way

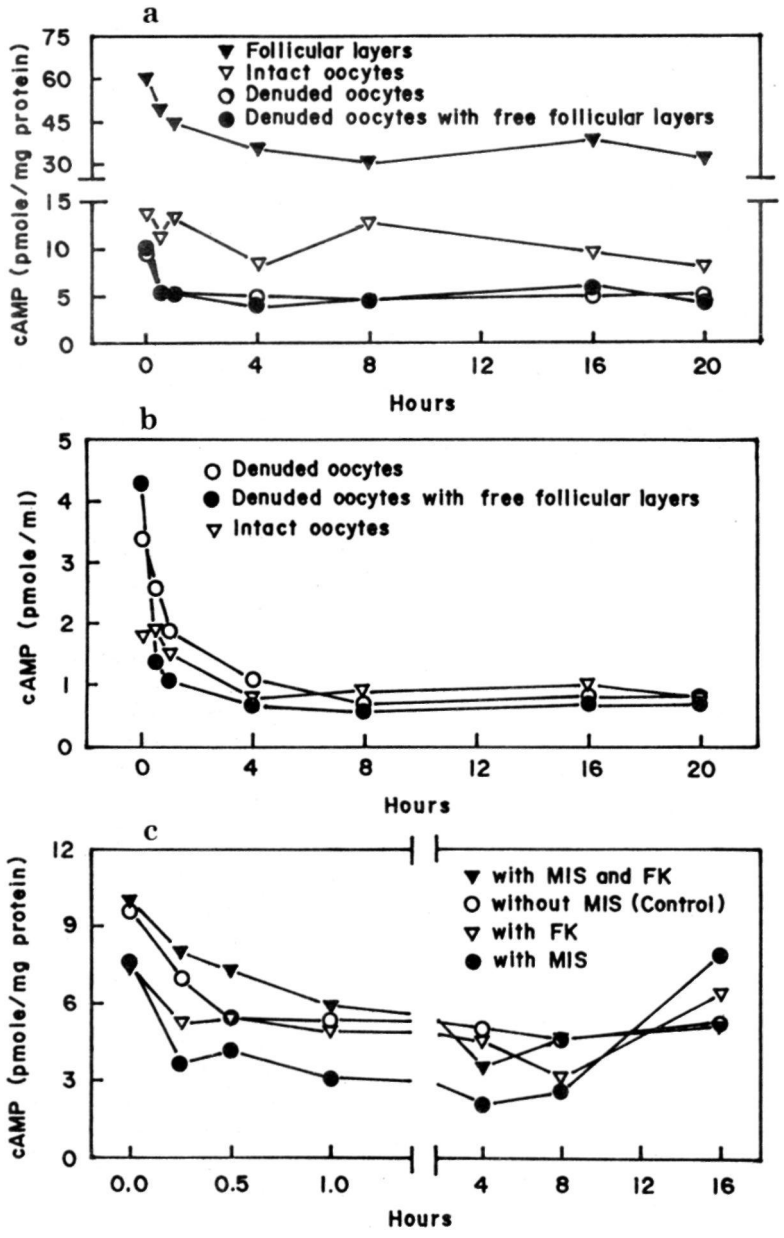

Fig. 1. Cyclic AMP levels assayed in goldfish oocytes. All the experiments were repeated twice with SEM not more than 10 percent. a. Levels (pmole/mg protein) in oocytes and follicular layers cultured at 0.Q5, 1, 4, 8, 16 and 20 h in vitro without MIS (17,20 P) stimulation. b. Levels (pmole/ml) in the media of oocytes cultured in vitro without MIS stimulation. c. Levels (pmole/mg protein) in denuded oocytes stimulated with MIS (1 μ/ml) only, MIS and 0.01 μM forskolin (FK), and μM FK only. Oocytes without MIS stimulation served as control.

below the basal level at 16 h. In forskolin-treated denuded oocytes, changes in intraoocyte cAMP levels are not significant ($P>0.05$). The decrease in intraoocyte cAMP within 15 min was only 30% from the basal level as compared to 52% in MIS-stimulated denuded oocytes. In denuded oocytes that matured spontaneously (control), intraoocyte cAMP decreased only by 27% from the basal level in 15 min but subsequently decreased to a significantly lower level until the end of the experiment (16 h).

Oocyte Maturation

More than 50% of untreated denuded goldfish oocytes (control) underwent spontaneous maturation (Fig. 2). Forskolin (0.01 μM) lowered the percent maturation of oocytes to about 20%. Forskolin also significantly ($P<0.05$) decreased MIS-induced maturation from almost 100% to that of the control level.

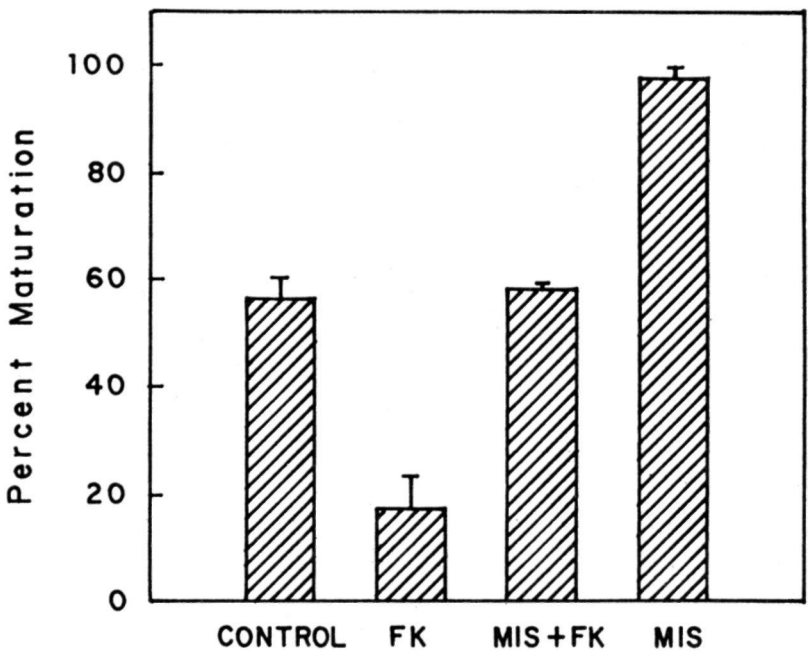

Fig. 2. Percent maturation of denuded goldfish oocytes stimulated with MIS (17, 20 P at 1 μg/ml) only, MIS in the presence of forskolin (FK) at 0.01 μM, and in forskolin (0.01 μM) only. GVBD was scored after 24 h. Oocytes cultured without any stimulation served as control. Forskolin decreased percent maturation of MIS-stimulated oocytes to that of the control level. Results are expressed as mean ± SEM of five experiments.

Forskolin inhibited MIS-induced maturation in a dose-dependent manner (Fig. 3). Complete inhibition of maturation of MIS-stimulated denuded oocytes was observed at 1 and 10 μM forskolin. Even at low concentrations, forskolin significantly ($P<0.05$) decreased maturation of MIS-stimulated denuded goldfish oocytes.

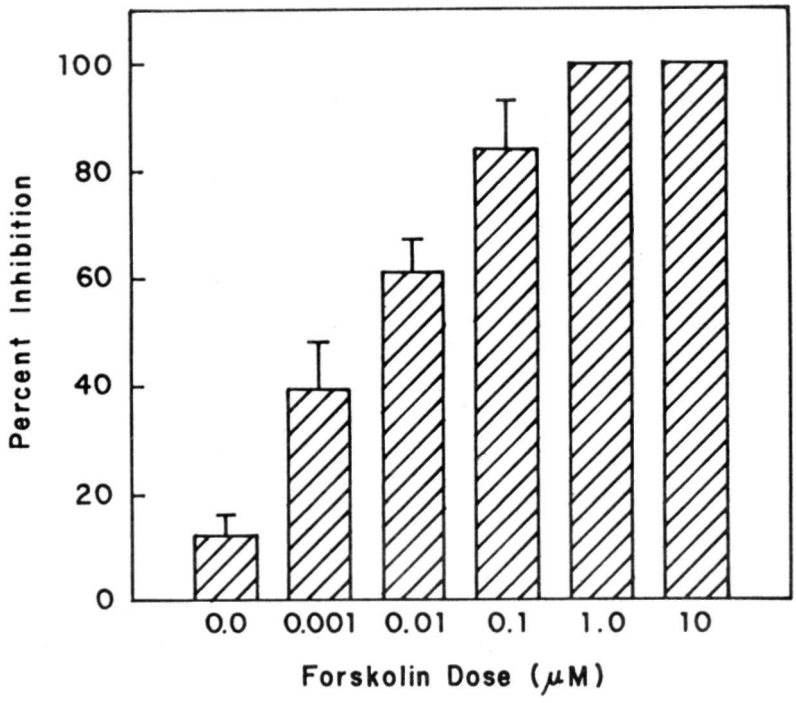

Fig. 3. Effects of different concentrations of forskolin, an activator of adenylate cyclase on MIS (1 μg/ml of 17, 20 P) - induced maturation of denuded goldfish oocytes. Oocytes cultured at zero dose were MIS-stimulated and served as control. Results are expressed as mean ± SEM of three experiments.

Discussion

It has been demonstrated in many studies (reviewed by First et al. 1988, Eckberg 1988, Leibfried-Rutledge et al. 1989, Smith 1989) that the decrease in cAMP levels is associated with reinitiation of oocyte maturation. It has also been shown in amphibians that following hormonal stimulation, the level of intraoocyte cAMP decreased via the inhibition of adenylate cyclase activity (Sadler and Mailer 1983; Jordana et al. 1984). If a similar pathway is involved in goldfish oocyte,

stimulation of denuded oocytes with MIS, would result in a greater decrease in intraoocyte cAMP. Fig. 1c shows that intraoocyte cAMP did decrease significantly by 52% within 15 min after MIS addition while without MIS (control), cAMP decrease of about 43% occurred only within 30 min. Forskolin (0.01 μM), an activator of adenylate cyclase when added to MIS, delays the onset of cAMP decrease to a significant low level by one hour. Forskolin also decreases percent maturation of MIS-stimulated denuded goldfish oocytes to control level (Fig. 2). Thus, it could be inferred that MIS inhibits adenylate cyclase activity, resulting in a more rapid decrease of intraoocyte cAMP compared to cAMP decrease after removal of the follicular wall. Goldfish oocytes devoid of their follicular wall spontaneously matured (control, Fig. 2) as a result of a decrease in intraoocyte cAMP. Removal of the follicular wall disrupts the transfer of follicular cAMP to the oocytes. This decrease in intraoocyte cAMP level subsequently drives the early commitment of MIS-stimulated denuded goldfish oocytes to resume meiosis. The findings presented here confirm that a decrease in cAMP could trigger resumption of meiotic maturation.

Many studies have reported a 10-50 percent drop in cAMP levels during maturation. Cicirelli and Smith (1983) found a 20% decrease in the cAMP content of *Xenopus* during the first 2-50 min following progesterone addition. Mailer (1985) reviewed that within 0.1 GVBD, a rapid drop occurs in the level of cAMP to about 40-60% of basal after progesterone addition. Jalabert and Finet (1986) have also observed a significant decrease in cAMP levels in rainbow trout oocytes incubated with 3 μM 17,20 P. The magnitude of cAMP decrease is still being disputed currently. Some authors contend that the reported drop in cAMP levels is small compared to those associated with the regulation of cAMP by hormones in somatic cells. But, Thibier et al. (1982) have calculated that 10-20% decrease in cAMP levels would be sufficient to trigger oocyte maturation. This could explain the maturation of some denuded goldfish oocytes treated with 0.01 μM forskolin (Fig. 2), which shows a cAMP decrease of 30% at 15 min (Fig. 1c).

Chemical agents that are able to elevate the intraoocyte concentrations of cAMP maintain the oocytes in meiotic arrest. Forskolin, elevated cAMP concentrations thus activating adenylate cyclase activity, blocked MIS-induced maturation of denuded goldfish oocytes. This is consistent with the findings that elevated levels of cAMP are inhibitory to maturation (Urner et al. 1983, Schultz et al. 1983, Racowsky 1985).

The results of the study showed that cAMP is not only involved in the maintenance of meiotic arrest but also acts as a signal to trigger resumption of meiotic maturation. Elevated levels of cAMP maintain meiotic arrest in competent cells while a decrease in the level of cAMP is necessary and sufficient to trigger oocyte maturation.

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