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ROLES OF CYCLIC AMP AND CALCIUM IN MATURATION OF *XENOPUS LAEVIS* OOCYTES

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SUMMARY

Progesterone induces *in vitro* meiotic maturation in *Xenopus* oocytes.

1. Early steps of maturation are sensitive to inhibition by phosphodiesterase inhibitors (methyl xanthines) or by cholera toxin, suggesting that elevated levels of intracellular cyclic-AMP block progesterone action.

2. Progesterone causes a significant decrease in the amount of newly synthesized cyclic AMP from microinjected α-^{32}P-ATP.

3. Ca^{2+} ions are involved both in the initiation of progesterone action and in the whole process of the first meiotic division.

INTRODUCTION

When amphibian oocytes are treated with progesterone they undergo meiotic maturation. The nucleus, blocked in meiotic prophase, migrates to the periphery of the oocyte and the nuclear membrane breaks down, (germinal vesicle breakdown or GVBD). The nuclear membrane breakdown is under the control of a cytoplasmic factor(s) designated maturation promoting factor (MPF). As meiotic maturation proceeds towards metaphase II, the oocyte develops a cytostatic factor (CSF) in its cytoplasm. This factor has the ability to arrest mitosis at metaphase [1, 2]. MPF activity, as well as CSF activity, disappear after fertilization or activation. The whole maturation period can thus be divided into three phases (Fig. 1): (a) The lag period, following hormonal stimulation and the appearance of MPF activity. (b) The GVBD period; cytoplasm taken from hormonally stimulated oocytes at this period can induce nuclear dissolution when microinjected into oocytes that have not been exposed to hormone. This period begins when MPF activity can first be detected. (c) After the occurrence of GVBD, meiotic maturation is blocked in metaphase II; this period lasts until fertilization or activation takes place. What are the molecular connections between the initial progesterone oocyte interaction, MPF appearance and the following steps of meiotic maturation? Different lines of evidence show that Ca^{2+} and cyclic AMP are involved in the initiation of progesterone-induced maturation and that they both play a major role during the whole process of meiotic maturation.

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ROLE OF CYCLIC AMP

Lag period

Intracellular cyclic AMP fluctuations. The endogenous content of cyclic AMP has been investigated in *Rana pipiens* [3, 4] and *Xenopus laevis* [5–7] oocytes and found to be around 1 μM. The time course for fluctuations in cyclic AMP levels during progesterone-induced maturation shows that a significant decrease of cyclic AMP occurs within

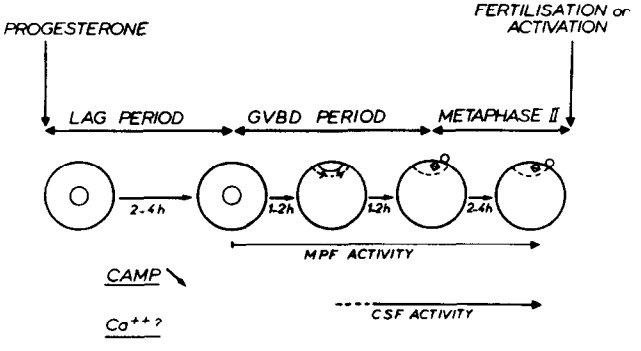

Fig. 1. Meiotic maturation in *Xenopus* oocytes. GVBD, germinal vesicle breakdown; MPF, maturation promoting factor; CSF, cytostatic factor.

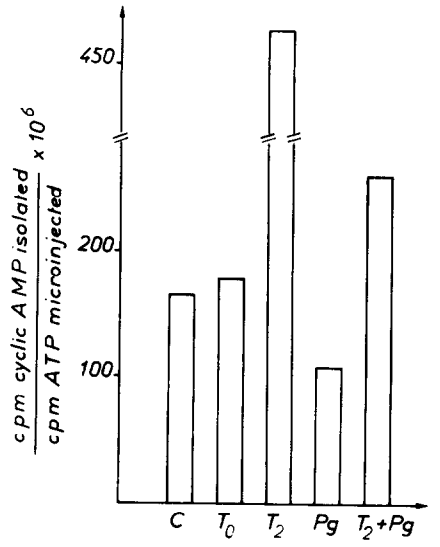

Fig. 2. Action of cholera toxin on cyclic AMP formation in *Xenopus* oocytes. Oocytes were microinjected with α-^{32}P-ATP and the cyclic AMP isolated after 60 min incubation. All experiments were performed in the presence of 3-isobutyl-1-methylxanthine (1 mM). C, Control oocytes; T_0, Cholera toxin (50 ng/ml) was added at the time of microinjection; T_2, Oocytes were preincubated for 2 h in cholera toxin before microinjection; Pg, Progesterone (1 μM) was added at the time of microinjection; Pg + T_2, Oocytes were preincubated for 2 h in cholera toxin then progesterone was added at the time of microinjection.

30–60 minutes after progesterone exposure [3, 4, 7]. The drop in cyclic AMP levels is the first biochemical event which has so far been characterized during the lag period.

Inhibition of progesterone-induced maturation by cAMP, cholera toxin and methylxanthines. Cholera toxin (around 1 ng/ml) irreversibly inhibits progesterone induced maturation [8, 9]. Furthermore, inhibition of maturation occurs only when the toxin is added during the lag period and it appears that an increase in total cyclic AMP content of the oocyte is involved [Schorderet–Slatkine and Baulieu, personal communication]. Parallel to these studies, we injected α-^{32}P-ATP into *Xenopus* oocytes and looked for newly synthesized cyclic AMP. Figure 2 shows that, after 2 h of preincubation, cholera toxin increases the amount of newly synthesized cyclic AMP at least 3-fold (60 min incubation) as compared with controls. The experiments show that increasing the cyclic AMP content of the oocyte during the lag period inhibits maturation.

It is generally assumed that methylxanthines (theophylline or 3-isobutyl-1-methylxanthine) inhibit cyclic AMP phosphodiesterase in isolated cells, and therefore increase the cyclic AMP levels. It was reported that methylxanthines are potent inhibitors of oocyte maturation [4, 6, 7, 10]. The action of the drugs is efficient only when they are added in the external medium during the lag period (microinjected theophylline does not block maturation) [7, 11]. The mechanism of action of methylxanthines on oocytes is not clear. *In vitro*, 3-isobutyl-1-methylxanthine totally blocks cyclic AMP phosphodiesterases activity (unpublished experiments). *In vivo*, the degradation of microinjected ^{3}H-cyclic AMP is only inhibited by 50% when oocytes are incubated in the presence of 3-isobutyl-1-methylxanthine (1 mM). Surprisingly, analysis of total cyclic AMP content of oocytes treated with methylxanthine failed to show any significant increment [3, 6, 7]. A possible explanation could be that methylxanthines *in vivo* inhibit membrane bound cyclic AMP phosphodiesterase without modifying the total cyclic AMP content. An alternative role for methylxanthines could involve inhibition of protein synthesis [7 and unpublished experiments] or a direct action on membrane permeability.

The direct effect of cyclic AMP was also tested. Dibutyryl-cyclic AMP (10^{-3} M) inconsistently inhibits progesterone-induced maturation; however, cyclic AMP entrapped into liposomes was found to be a potent inhibitor of maturation.

Action of progesterone on cyclic AMP synthesis. α-^{32}P-ATP was microinjected into *Xenopus* oocytes and newly synthesized cyclic AMP was recovered after incubation for 60 min (Fig. 2) [9]. The action of progesterone was investigated and we found that this steroid hormone always caused a significant decrease (between 30 and 60%) in the amount of cyclic AMP newly synthesized (Fig. 2). Furthermore, it was shown that progesterone is capable of decreasing the formation of cyclic AMP even in the presence of cholera toxin. It was shown that the decrease in the synthesis of cyclic AMP is specific to maturing steroids. In contrast, non-maturing steroids, such as oestradiol-17β, do not modify cyclic AMP synthesis. There is a good correlation between the percentage of maturation, the concentration of progesterone and the amount of radioactive cyclic AMP formed during the first hour following addition of progesterone (Fig. 3). There are two possible explanations for these results, either (a) progesterone acts on adenylate cyclase (direct inhibition or change in the specific activity of the precursor ATP pool) or (b) progesterone changes the amount of free cyclic AMP accessible to degradation through cyclic AMP phosphodiesterases.

GVBD period

Since agents which increase (or block the decrease of) intracellular cyclic AMP concentrations during the lag period inhibit hormone-induced maturation, it was important to know whether these agents block the appearance or affect the mechanism of action of MPF. To find a solution to this, MPF was microinjected into oocytes treated with either 3-isobutyl-1-methylxanthine or with cholera toxin; neither of these agents inhibited the ability of MPF to initiate GVBD. Cytological analysis has shown that although GVBD was obtained under all the experimental conditions after MPF microinjection, numerous anomalies were observed in the formation of the maturation

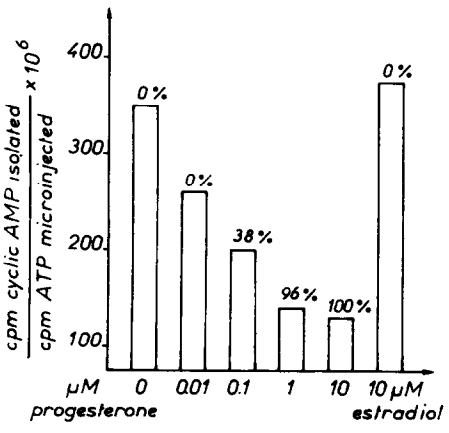

Fig. 3. Cyclic AMP formation as a function of the progesterone concentration in the incubation medium. Duplicate groups of 5 oocytes each were microinjected with α-^{32}P-ATP and incubated for 60 min in medium A containing 3-isobutyl-1-methylxanthine (1 mM) and various concentrations of progesterone (0.01–10 μM) or oestradiol-17β (10 μM). At the end of the incubation period, oocytes were homogenized and cyclic AMP isolated. The results are expressed as the amount of cyclic AMP recovered versus the amount of α-^{32}P-ATP microinjected. Parallel experiments were conducted with 50 non-microinjected oocytes incubated until maturation in the presence of the same amount of progesterone or oestradiol, but without 3-isobutyl-1-methylxanthine. The number above each bar represents the percentage of GVBD.

spindles [12]. These observations demonstrate that the mechanism of action of MPF (as far as GVBD is concerned) does not involve cyclic AMP fluctuations.

ROLE OF Ca^{2+}

Lag period

Action of Ca^{2+} mobilizing agents. The chelation of extracellular Ca^{2+} by EGTA does not inhibit progesterone-induced maturation, although the presence of divalent cations (Mg^{2+}, Co^{2+} or Mn^{2+}) is absolutely required [2, 13, 14]. This demonstrates that uptake of Ca^{2+} is not a necessary step for the induction of maturation. In contrast, chelation of intracellular Ca^{2+} by EGTA microinjection inhibits GVBD [2]. The reaction initiating maturation appears, therefore, to involve intracellular Ca^{2+} mobilization. Numerous Ca^{2+} mobilizing agents were tested: La^{3+} [15], propranolol [16] and ionophore A 23187 in the presence of a high concentration (10–20 mM) of divalent cations [2, 17]. They all induced maturation when applied to the surface of the oocyte (external medium) but not when introduced directly by microinjection into the endoplasm of the oocyte. It appears then that sequestered Ca^{2+} could be released from intracellular store(s) during the maturation process.

Intracellular free Ca^{2+} concentration. Free intracellular Ca^{2+} was monitored in isolated *Xenopus* oocytes using Ca^{2+}-sensitive luminescent protein aequorin and was found to be in the micromolar range [18]. No changes in the internal free Ca^{2+} concentration could be detected during the lag period following progesterone stimulation. The absence of light response might be interpreted differently: (a) Ca^{2+} fluctuations are too small to be detected by the aequorin method. (b) Ca^{2+} change occurs in a compartment other than the aequorin diffusion compartment. (c) Free Ca^{2+} concentration remains constant during the lag period. The last possibility seems unlikely since indirect evidence for a change in Ca^{2+} concentration was recently suggested [19]. The kinetics of Ca^{2+} exchange by *Xenopus* oocytes were monitored during progesterone action; an increase in the rate of Ca^{2+} efflux begins within 40 min of progesterone exposure. The increased release of Ca^{2+} during the lag period could therefore reflect an increased Ca^{2+} concentration in the cytoplasm.

Electrophysiological experiments. The exact role of the oocyte membrane during progesterone-induced maturation is far from clear. Electrophysiological studies indicate that steroid responsive oocytes have a negative membrane potential (Em from −80 to −30 mV) [20]. Recordings of the membrane potential during the lag period after progesterone exposure do not change until GVBD occurs; at this time a pronounced depolarization (Em ≃ 10 mV) takes place [20, 18].

We have studied the electrical properties of fully grown *Xenopus* oocytes (R. Kado, K. Marcher and R. Ozon, unpublished results) and we discovered that defollicularized oocytes, when depolarized by a current pulse (1 s), can be blocked at a membrane potential of between +80 mV and +60mV for 1–5 min (Fig. 4). This large regenerative depolarization indicates an opening of channels to either Na^+ or Ca^{2+} or both.

GVBD period

The Ca^{2+} requirement changes at the time when the oocyte begins to produce MPF activity in the cytoplasm [2]. If ionophore A 23187 and Ca^{2+} (14 mM) are applied to the oocyte at the time of appearance of MPF (after progesterone stimulation), maturation is blocked. This shows that raised intracellular Ca^{2+} at the beginning of the GVBD period is an inhibitor of MPF activity. It was also demonstrated *in vitro* that MPF extracts are inhibited by Ca^{2+} [21]. Both *in vivo* and *in vitro* experiments indicate that Ca^{2+} at high concentration causes the inhibition of maturation during the GVBD period.

ROLE OF PHOSPHORYLATION

Maturation can be induced, in the oocyte in the absence of progesterone, after injection of the purified regulatory subunit of cyclic AMP-dependent protein kinase, or by injection of the heat stable inhibitor of the catalytic subunit of cyclic AMP-dependent protein kinase [22]. This result, together with the known functions of Ca^{2+} and cyclic AMP in *Xenopus*

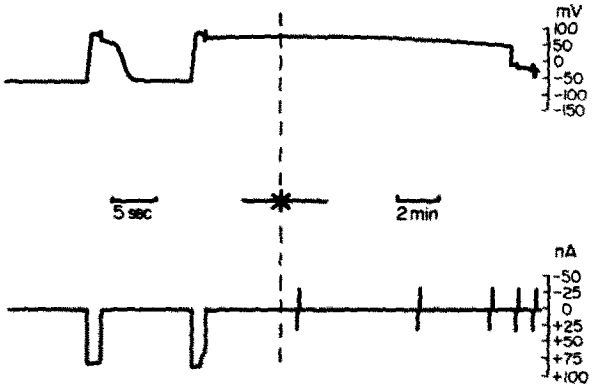

Fig. 4. Effect of current pulses (lower traces) on membrane potential (top traces) of a defolliculanized *Xenopus* oocyte. When current pulses (1 s) reach threshold values (from 50 to 80 nA) a large regenerative depolarisation occurs.

oocytes (first and second sections), suggests the possibility that a phosphorylation—dephosphorylation system plays a major role during the process of progesterone-induced maturation. Endogenous protein phosphorylation following progesterone exposure was analyzed in *Xenopus* oocytes [23, 11, 24]. During the lag period no quantitative changes in ^{32}P-labelled phosphoproteins could be observed. As maturation proceeded towards the GVBD period a burst in protein phosphorylation occurred. Qualitative analysis by SDS polyacrylamide electrophoresis and autoradiography of ^{32}P-labeled phosphoproteins indicated that in the cytosoluble fraction the relative amount of a specific phosphoprotein (45,000 molecular weight region) decreased [24]. Under our experimental conditions no qualitative change during the lag period has so far been detected.

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