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Activation of maturation promoting factor in *Bufo arenarum* oocytes: injection of mature cytoplasm and germinal vesicle contents

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Summary

Although progesterone is the established maturation inducer in amphibians, *Bufo arenarum* oocytes obtained during the reproductive period (spring–summer) resume meiosis with no need of an exogenous hormonal stimulus if deprived of their enveloping follicle cells, a phenomenon called spontaneous maturation. In this species it is possible to obtain oocytes competent and incompetent to undergo spontaneous maturation according to the seasonal period in which animals are captured. Reinitiation of meiosis is regulated by maturation promoting factor (MPF), a complex of the cyclin-dependent kinase $p34^{cdc2}$ and cyclin B. Although the function and molecule of MPF are common among species, the formation and activation mechanisms of MPF differ according to species. This study was undertaken to evaluate the presence of pre-MPF in *Bufo arenarum* oocytes incompetent to mature spontaneously and the effect of the injection of mature cytoplasm or germinal vesicle contents on the resumption of meiosis. The results of our treatment of *Bufo arenarum* immature oocytes incompetent to mature spontaneously with sodium metavanadate ($NaVO_3$) and dexamethasone (DEX) indicates that these oocytes have a pre-MPF, which activates and induces germinal vesicle breakdown (GVBD) by dephosphorylation on Thr-14/Tyr-15 by cdc25 phosphatase and without cyclin B synthesis. The injection of cytoplasm containing active MPF is sufficient to activate an amplification loop that requires the activation of cdc25 and protein kinase C, the decrease in cAMP levels, and is independent of protein synthesis. However, the injection of germinal vesicle content also induces GVBD in the immature receptor oocyte, a process dependent on protein synthesis but not on cdc25 phosphatase or PKC activity.

Keywords: *Bufo arenarum*, Germinal vesicle breakdown, Maturation promoting factor

Introduction

Although progesterone is the established maturation inducer in amphibians, *Bufo arenarum* oocytes obtained during the reproductive period (spring–summer) resume meiosis with no need of an exogenous hormonal stimulus if deprived of their enveloping follicle cells, a phenomenon called spontaneous maturation. In this species it is possible to obtain oocytes competent and incompetent to undergo spontaneous maturation according to the seasonal period in which animals are captured (Zelarayán *et al*., 1995).

Reinitiation of meiosis represents the transition from the G2 to the M phase of the cell cycle, and is regulated by maturation promoting factor (MPF), a complex of the cyclin-dependent kinase $p34^{cdc2}$ and cyclin B (Masui, 1982; Lohka *et al*., 1987).

Although the function of MPF is common among species, the formation and activation mechanisms of MPF differ according to species (Kishimoto, 1998; Yamashita, 1998; Palmer & Nebreda, 2000; Yamashita *et al*., 2000).

In immature oocytes of *Xenopus* and starfish there is an inactive complex (pre-MPF) that consists of cyclin B-bound cdc2 phosphorylated on both Thr-161 and Thr-14/Tyr-15. In these species, therefore, cdc2 Thr-14/Tyr-15 dephosphorylation is primarily responsible for MPF activation during oocyte maturation (Gautier & Maller, 1991; Kobayashi *et al*., 1991; Minshul *et al*., 1991; Ookata *et al*., 1992; Galas *et al*., 1993).

In many vertebrate species, cyclin B comprises several subtypes. In *Xenopus*, cyclin $B1/p34^{cdc2}$ and cyclin $B2/p34^{cdc2}$ show very similar substrate

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specificities *in vitro* (Nigg, 1993) and the stores of both cyclins are sufficient to permit the activation of the $p34^{cdc2}$ kinase (Minschull *et al*., 1991).

Throughout interphase, MPF accumulates in the cytoplasm, in association with microtubules and centrosomes (Pines & Hunter, 1994).

On the other hand, in immature oocytes of goldfish, cyclin B is absent and all cdc2 is monomeric (Kajiura *et al*., 1993). Immediately before the onset of the germinal vesicle breakdown (GVBD), cyclin B is synthesized *de novo* and binds to a monomeric cdc2, forming the active MPF after phosphorylation on Thr-161 of cyclin-B-bound cdc2 (Katsu *et al*., 1993; Yamashita *et al*., 1995). In goldfish, therefore, the *de novo* synthesis of cyclin B and its binding to cdc2 as well as the subsequent Thr-161 phosphorylation are critical steps for MPF activation, in striking contrast to the mechanism of MPF activation in *Xenopus* and starfish.

The presence of different mechanisms of MPF activation has been reported between *Xenopus* and *Rana*, i.e. even among members of the same phylogenetic group (Schuetz & Samson, 1979; Tanaka & Yamashita, 1995).

Myt1, a protein kinase that is possibly responsible for the phosphorylation of both Thr-14 and Tyr-15, has recently been determined. This kinase is known to phosphorylate at least the Tyr-15 residue (Mueller *et al*., 1995), while cdc25 phosphatase dephosphorylates both residues (Gautier & Maller, 1991; Kumagai & Dunphy, 1991).

In *Xenopus* oocytes, the conversion of pre-MPF into active MPF occurs several hours after progesterone stimulation; this activation precedes GVBD. The downstream signalling pathway activated by progesterone has not yet been elucidated; however, it has been proved to require new protein synthesis and to depend on a phosphorylation/dephosphorylation reaction (Jessus & Ozon, 1993) that converges towards the activation of protein phosphatase cdc25, which catalyses the dephosphorylation of Thr-14 and Tyr-15 of p34 (Jessus & Ozon, 1995). Similarly, during the G2/M transition, the MPF in fully grown *Bufo arenarum* oocytes is activated in a protein-synthesis-dependent manner, followed by GVBD (Zelarayán *et al*., 1996).

In *Xenopus* a property of MPF is its ability to undergo autocatalytic activation in the absence of protein synthesis (Wasserman & Masui, 1975). A small amount of cytoplasm containing active MPF introduced by microinjection triggers the activation of inactive pre-MPF when transferred to a recipient oocyte. Consequently, the microinjection of active cytoplasm in *Xenopus* oocytes is sufficient to activate an amplification loop that promotes the conversion of an inactive cyclin $B/p34^{cdc2}$ complex (pre-MPF) into its active form (MPF).

The culture of MPF-microinjected pig oocytes in the presence of a protein synthesis inhibitor such as cycloheximide does not induce meiosis resumption (Fulka *et al*., 1988; Mattioli *et al*., 1991), probably because pig oocytes require transcription to promote maturation (Fulka *et al*., 1988). This fact suggests that large mammal species may depend on the protein synthesis of cyclin to complete the meiotic process (Tatemoto & Horiuchi, 1995). In contrast to large mammals, mouse oocytes do not require protein synthesis for the initiation of maturation, but, as shown recently, may require cyclin to complete the meiotic process (Hampl & Eppig, 1995).

In fully grown immature starfish oocytes, which have a stock of pre-MPF (Strausfeld *et al*., 1991), the microinjection of cytoplasm containing active MPF is not sufficient to activate the amplification loop. In this regard, it has been suggested that a nuclear factor originating from the germinal vesicle (GV) is required for p34 kinase activation of pre-MPF (Kishimoto *et al*., 1981; Picard & Dorée, 1984; Picard *et al*., 1991). The injection of only GV content from immature oocytes into the cytoplasm is occasionally sufficient to activate cyclin $B/p34^{cdc2}$ and cause GVBD in recipient immature oocytes (Picard & Dorée, 1984; Picard *et al*., 1991).

The effect caused by a factor sequestered in the GV of starfish oocytes could be bypassed by the specific inhibition of type 2A phosphatase (pp2A), suggesting that an unidentified pp2A inhibitor could act synergistically to induce MPF amplification, perhaps by a positive action on cdc25 phosphatase. In immature starfish oocytes (Pondaven & Cohen, 1987; Picard *et al*., 1989) and frog oocytes (Rime *et al*., 1990), it has been demonstrated that okadaic acid (OA), a specific inhibitor of protein phosphatase 1 (pp1) and pp2A, induces both GVBD and MPF activation. In *Xenopus* oocytes the existence of a negative regulator of MPF activity, an OA-sensitive pp2A, has been proposed. When *Xenopus* oocytes are exposed to OA they resume meiosis.

The effects of glucocorticoids on oocyte maturation have been studied in several species of fish, such as the Atlantic croaker (Patiño & Thomas, 1990), and in pig (Yang *et al*., 1999; Wei-Yi *et al*., 2000), with controversial results. In mammals, glucocorticoids have an inhibitory effect on maturation by the decrease in cyclin B synthesis, while in fish they induce GVBD through synergism with progesterone metabolites.

It is well known that the resumption of oocyte meiosis is associated with the decreased concentration of intracellular cAMP and the resulting inactivation of cAMP-dependent protein kinase A (PKA) (Morril *et al*., 1981; Baulieu, 1983; Maller, 1983; Kwon & Schuetz, 1986). However, the specific substrate of PKA during oocyte maturation remains unidentified and the events regulated by the transduction signalling cAMP/PKA are unknown.

Another protein kinase, calcium/phospholipid-dependent protein kinase C (PKC), is also involved in the resumption of meiosis in amphibian oocytes (Kwon & Lee, 1991; Zelarayán *et al*., 1996).

This study was designed to determine the presence or absence of an inactive pre-MPF in the cytoplasm of *Bufo arenarum* oocytes and the molecular mechanisms of MPF activation by the injection of mature cytoplasm or GV content into immature oocytes.

Materials and methods

Sexually mature *Bufo arenarum* females were collected in the northwestern area of Argentina from May to August (winter animals) and from September to December (summer animals) and kept at 15 °C until use, which generally took place 15 days after collection.

In vitro follicle and denuded oocyte culture

Experimental manipulation and culture were performed at room temperature (22–25 °C) in amphibian Ringer solution (AR) (6.6 g NaCl/l, 0.15 g $CaCl_2$/l and 0.15 g KCl/l) containing penicillin G-sodium (30 mg/l) and streptomycin sulphate (50 mg/l), pH 7.4.

Fully grown follicles (1.7–1.8 mm in diameter) were isolated from other ovarian tissues using watchmaker's forceps. Denuded oocytes were obtained by manually pulling off the follicle epithelium and the theca layer using fine forceps with the aid of a dissecting microscope (Lin & Schuetz, 1985). Follicle cells were removed by incubation of defolliculated oocytes in AR for 5 min with gentle shaking (100 oscillations/min) (Zelarayán *et al*., 1995). Denuded oocytes were kept in AR until use.

Freshly denuded oocytes were placed in AR at 22–25 °C. Routine *in vitro* cultures were carried out using plastic multiwell culture dishes (Costar 3524). Randomized samples of 20 oocytes were distributed into separate wells containing 2 ml of AR; the reagents were added (5 µl) directly to the culture medium. Two-well duplicates were routinely run in each experimental group.

Oocyte maturation was assessed 24 h after addition of hormone or reagent. Meiosis reinitiation was scored both by the presence of a transient white spot in the animal pole and by the absence of GVBD after dissection of the oocytes fixed in trichloroacetic acid (TCA).

Hormones and reagents

All hormones and reagents were purchased from Sigma. Progesterone was dissolved in ethanol and added (5 µl) directly to the culture medium to give a final concentration of 2.5 µM, at which concentration GVBD was almost 98%.

Sodium metavanadate ($NaVO_3$) was dissolved in dd H_2O at 75 °C and various doses were added to the culture medium at a constant volume (5 µl).

Cycloheximide, an inhibitor of protein synthesis, was dissolved in AR and various doses were added to the culture medium at a constant volume (5 µl).

Cytoplasm transfers

Cytoplasm from mature oocytes was obtained according to Hedeimanns method, as modified by Bühler & Petrino (1983). Oocytes matured with progesterone (2.5 µM) (mature cytoplasm) or immature oocytes were suspended at the interface of 60% Ficoll (w/v) in Ringer solution and then centrifuged for 30 min at 2500 *g* at 4 °C in a Sorval HB 4 rotor. After this procedure the oocytes were stratified into four layers: yolk platelets, pigment granules, clear cytoplasm and oil cap.

Microinjection was performed with a sharpened glass micropipette (outer diameter 40–50 µm) attached to a micromanipulator (Leitz). Fully grown denuded immature oocytes were microinjected with 50 nl of cytoplasm obtained from the clear cytoplasm layer of oocytes matured with progesterone (2.5 µM) (mature cytoplasm) or with 50 nl of immature cytoplasm (control) obtained using the same procedure. Following injection, the oocytes were placed in AR or in AR with an inhibitor (cycloheximide or $NaVO_3$). The recipient oocytes were scored for GVBD after incubation for 24 h.

Microinjection of germinal vesicle contents

In order to obtain the contents of GVs, fully grown oocytes were isolated manually and pricked at the animal pole with a fine glass pipette. The GVs were squeezed out into AR Tris-HCl (pH 7.4). Forty GVs were packed into a capillary tube with AR to a final volume of 18 µl and centrifuged at 10 000 *g* for 10 min at 15 °C. The supernatant is referred to as 'GV content'.

Microinjection was performed with a sharpened glass micropipette (outer diameter 40–50 µm) attached to a micromanipulator (Leitz). Fifty nanolitres of the GV content was injected into immature fully grown oocytes. Following injection, the oocytes were placed in AR or in AR with an inhibitor (cycloheximide or $NaVO_3$), and cultured for 24 h.

Immunofluorescence

The oocytes were fixed in Ancel & Vintemberger's solution (10% formol, 0.5% acetic acid and 0.5% NaCl) at room temperature, followed by postfixation overnight in absolute methanol at −20 °C, embedded in paraffin and sliced into 8 mm thick sections. Oocytes were then rehydrated in phosphate-buffered saline

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(PBS) (128 mM NaCl, 2 mM KCl, 8 mM NaH_2PO_4, 2 mM KH_2PO_4, pH 7.2), treated with 0.9% Triton X-100 in PBS for 30 min at room temperature, washed in PBS, incubated in 1% bovine serum albumin (BSA) for 10 min and washed in PBS. Then they were incubated overnight in rabbit monoclonal anti-cyclin B1(1:25) at 4 °C, washed repeatedly in PBS and incubated in fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:50) for 2 h at room temperature in the dark. After extensive washing in PBS oocytes were dehydrated in several changes of absolute methanol and cleared in benzyl benzoate–benzyl alcohol (1:2) (Dent & Klymkowsky, 1989). Observations were made with an epifluorescence microscope. In each experiment, control sections were incubated only with the second antibody. Under these conditions no fluorescence was observed.

Results

Effect of cdc25 phosphatase inhibition on the GVBD of denuded oocytes incompetent to mature spontaneously

In order to determine the presence or not of a pre-MPF, *Bufo arenarum* denuded oocytes incompetent to mature spontaneously were treated with $NaVO_3$, which is known to selectively inhibit the activity of the cdc25 phosphatase. Denuded oocytes incompetent to mature spontaneously were pre-incubated for 30 min with different doses of $NaVO_3$ before the addition of progesterone (2.5 μM). GVBD was scored after incubation for 24 h. The results (Fig. 1) indicate that the inhibition of the cdc25 phosphatase by $NaVO_3$ induced a decrease in the percentage of GVBD in a dose-dependent manner. A significant inhibition in the percentage of GVBD was obtained with doses of $NaVO_3$ lower than 0.5 mM. The inhibitory effect of $NaVO_3$ was reversible and 80% of the oocytes exhibited GVBD 24 h after the removal of this agent from the culture medium.

Effect of dexamethasone on GVBD of denuded oocytes incompetent to mature spontaneously

The effect of glucocorticoids was assayed using dexamethasone (DEX), a glucocorticoid that has an inhibitory effect on GVBD in several species. Denuded oocytes incompetent to mature spontaneously were preincubated for 30 min with different doses of DEX (10–40 μg/ml) before the addition of progesterone (2.5 μM). GVBD was scored after incubation for 24 h at 25 °C. Results showed that DEX at the doses assayed has no effect on GVBD. In our experimental conditions, oocytes incompetent to mature spontaneously treated with progesterone and those incubated with DEX and progesterone behaved similarly, suggesting that the synthesis of cyclin B is not required in the activation of MPF.

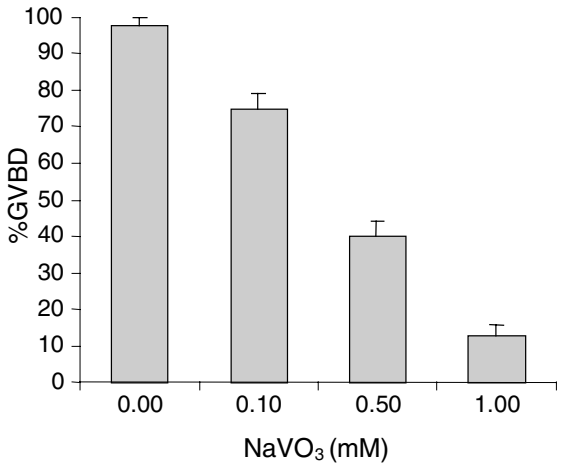

Figure 1 Effect of cdc25 phosphatase inhibition on the GVBD of denuded oocytes incompetent to mature spontaneously. Denuded oocytes incompetent to mature spontaneously were preincubated for 60 min in AR in the presence of different doses of $NaVO_3$ (0.1–1.0 mM) before the addition of progesterone (2.5 μM). Oocytes were then incubated in AR with the inhibitor. GVBD was scored after incubation for 24 h. Values are the mean ± SEM ($n = 5$). Each experiment was performed on a different animal.

Presence and location of cyclin B1 in immature oocytes incompetent to mature spontaneously

Denuded oocytes incompetent to mature spontaneously treated with anti-cyclin B1 show a perinuclear fluorescence, especially noticeable in the basal part of the GV. In this case no fluorescence could be observed inside the GV (Fig. 2). This result indicates that cyclin B1 is present in the immature oocytes of this species.

MPF amplification in oocytes incompetent to mature spontaneously

The MPF amplification in *Bufo arenarum* oocytes was tested by microinjection of cytoplasm from mature oocytes, obtained according to Hedeimann's method modified as by Bühler & Petrino (1983), and GV content of immature oocytes.

Fully grown denuded oocytes incompetent to mature spontaneously were microinjected with cytoplasm from oocytes matured with progesterone (2.5 μM) (mature cytoplasm). The injection of 50 nl of mature cytoplasm is sufficient to promote 90% of GVBD in recipient oocytes. As a control, fully grown denuded oocytes were injected with the same amount (50 nl) of the cytoplasm obtained from immature oocytes (immature

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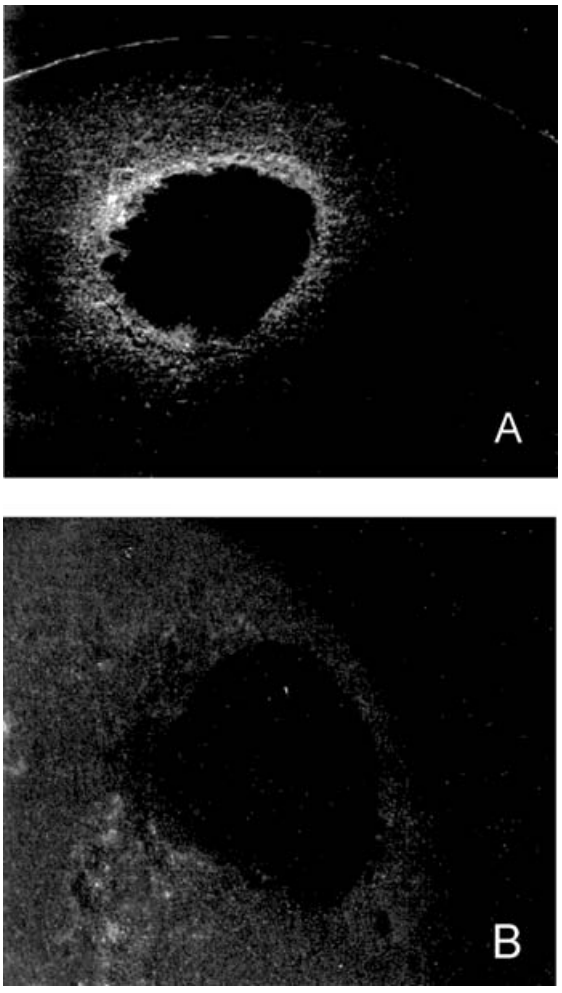

Figure 2 Immature oocyte. (*A*) Immunofluorescence staining (anti-cyclin B1) of the perinuclear cytoplasm. The upper part of the GV appears brightly stained; ×10. (*B*) Control oocyte was stained only with the second antibody; ×10.

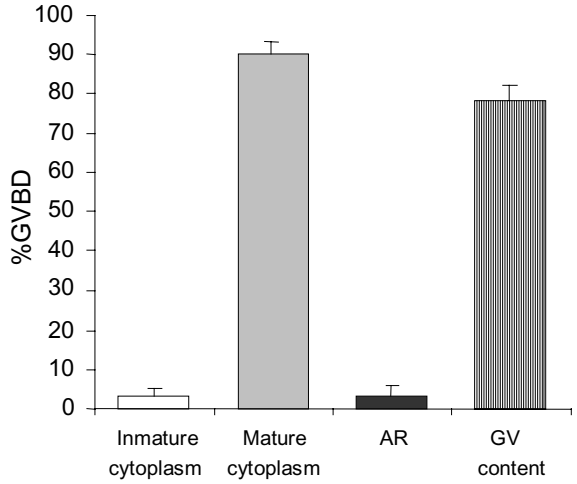

Figure 3 MPF amplification in oocytes incompetent to mature spontaneously. Denuded oocytes incompetent to mature spontaneously were injected with 50 nl of: (*A*) mature cytoplasm or 50 nl of immature cytoplasm; (*B*) 50 nl of GV content or 50 nl of AR, and cultured in AR. GVBD was scored after incubation for 24 h. Values are the mean ± SEM ($n = 5$). Each experiment was performed on a different animal.

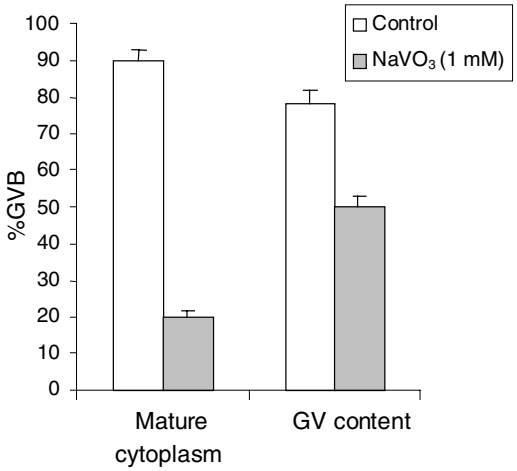

Figure 4 Effect of the inhibition of cdc25 phosphatase on MPF amplification. Denuded oocytes incompetent to mature spontaneously were preincubated for 60 min in AR in the presence of $NaVO_3$ (1 mM) before the injection of 50 nl of mature cytoplasm or 50 nl of GV content. The oocytes were incubated in the same medium and GVBD was scored after incubation for 24 h. Controls were injected and incubated in AR. Values are the mean ± SEM ($n = 4$). Each experiment was performed on a different animal.

cytoplasm). In the controls GVBD was not obtained in any case (Fig. 3).

In another series of experiments, the effect of the microinjection of the GV content was tested in fully grown denuded oocytes incompetent to mature spontaneously. The GV content of immature oocytes, obtained according to Materials and methods, was injected into these oocytes, which were incubated in AR for 16 h before scoring GVBD. In these experiments 78% GVBD was obtained (Fig. 3), indicating that the GV content is able to activate the MPF in the recipient oocytes.

Effect of the inhibition of protein synthesis on MPF amplification

Denuded oocytes were preincubated for 1 h in cycloheximide (CHX) (10 µg/ml) before the injection of 50 nl of mature cytoplasm or GV content. Then the injected oocytes were cultured in the same medium for 16 h and scored for GVBD. Results (Fig. 4) showed 80% GVBD when oocytes are injected with mature cytoplasm, indicating that the amplification of MPF can occur in the absence of protein synthesis.

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These results suggest that cyclin B is already present in immature oocytes and may indicate the presence of a pre-MPF.

The injection of the GV content into oocytes pretreated with CHX (10 μg/ml) decreased the percentages of GVBD breakdown by up to 40% (Fig. 4) This suggests that the GV content requires the synthesis of proteins to produce its effect on maturation.

Effect of cdc25 activity inhibition on MPF amplification

It has been recognized that the phosphatase from gene cdc25 is responsible for p34 activation through tyrosine and threonine dephosphorylation. To verify whether this regulatory pathway is operative when immature oocytes are microinjected with mature cytoplasm or GV content, we incubated the oocytes for 1 h in the presence of different doses of the specific inhibitor $NaVO_3$ before injection of 50 nl of mature cytoplasm or GV content. Oocytes were then cultured in AR with the inhibitor for 16 h before scoring GVBD.

Results (Fig. 5) indicated a decrease of about 25% in percentage GVBD when oocytes are injected with GV content, and a decrease of about 70% in percentage GVBD in the oocytes injected with mature cytoplasm.

The inactivation of cdc25 by incubation in $NaVO_3$ (1 mM) interferes with the amplification process when oocytes are microinjected with mature cytoplasm. This could suggest that the amplification loop has need of cdc25 activity. However, the injection of GV content was less sensitive to $NaVO_3$ treatment, suggesting the participation of more than one route in the process.

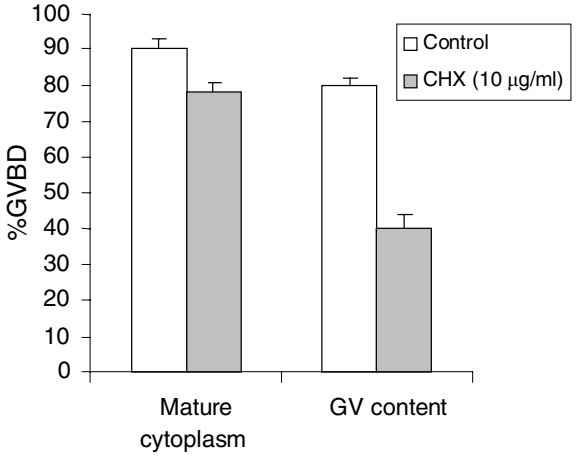

Figure 5 Effect of the inhibition of protein synthesis on MPF amplification. Denuded oocytes incompetent to mature spontaneously were preincubated for 60 min in AR in the presence of cycloheximide (CHX; 10 μg/ml) before the injection of 50 nl of mature cytoplasm or 50 nl of GV content. Then the injected oocytes were cultured in AR for 24 h and scored for GVBD. Controls were injected and incubated in AR. Values are the mean ± SEM ($n = 5$). Each experiment was performed on a different animal.

Effect of cAMP levels on MPF activation by injection of mature cytoplasm and GV content

An obligatory step in the mechanisms by which the progesterone induces oocyte maturation is a transient decrease in cAMP levels, produced by inhibition of adenylate cyclase (AC) and activation of phosphodiesterase (PDE), which is thought to result in decreased PKA activity.

In order to analyse whether the GVBD induced by injection of mature cytoplasm or GV content is dependent on intracellular cAMP levels, denuded oocytes incompetent to mature spontaneously were preincubated for 60 min in forskoline (1.25 μM), an activator of AC, or theophylline (1 μM), an inhibitor of PDE activity, before the injection of 50 nl of mature cytoplasm or GV contents. The injected oocytes were cultured in the presence of forskoline or theophylline for 20 h and the GVBD was scored.

The results (Fig. 6) show that the increase in the intracellular levels of cAMP has no effect on the percentages of GVBD induced by injection of GV content, but significantly diminished GVBD in oocytes injected with mature cytoplasm. This may suggest the participation of PKA in the amplification of MPF but not in the mechanisms of GV-content-induced maturation.

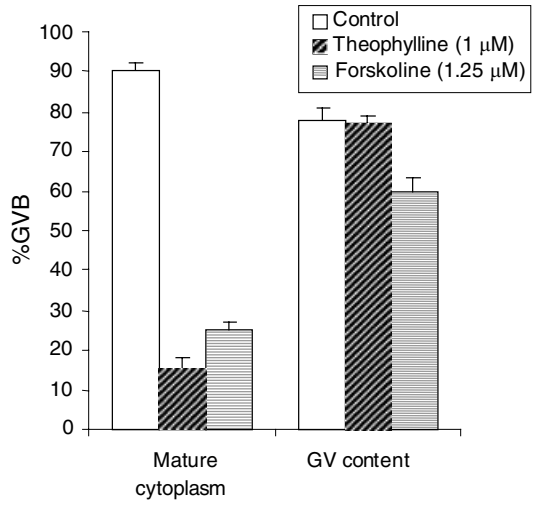

Figure 6 Effect of cAMP levels on MPF amplification and on GV-content-induced maturation. Denuded oocytes incompetent to mature spontaneously were preincubated for 60 min in AR in the presence of theophylline (1 μM) or forskoline (1.25 μM) before: (*A*) injection of 50 nl of GV content, (*B*) injection of 50 nl of mature cytoplasm. Oocytes were then incubated in AR with the corresponding inhibitors. Controls were injected and incubated in AR . GVBD was scored after incubation for 24 h. Values are the mean ± SEM ($n = 3$). Each experiment was performed on a different animal.

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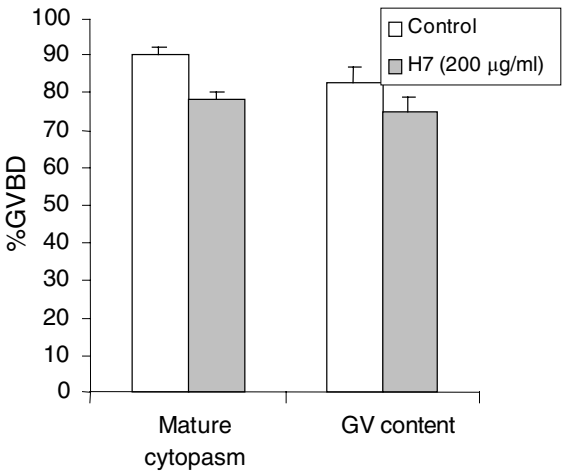

Figure 7 Participation of PKC in the amplification of MPF and in GV-content-induced maturation. Denuded oocytes incompetent to mature spontaneously were preincubated for 60 min in AR in the presence of H7 (200 μg/ml) before the injection of 50 nl of mature cytoplasm or 50 nl of GV content. Controls were injected and incubated in AR. GVBD was scored after incubation for 24 h. Values are the mean ± SEM ($n = 4$). Each experiment was performed on a different animal.

Participation of PKC in the activation of MPF by injection of mature cytoplasm and GV content

The study of PKC involvement in the amplification of MPF was assayed by treatment of oocytes with 1-(5-isoquinolilsulfonil)-2-metil pipirazine (H7), an inhibitor of PKC. Denuded oocytes incompetent to mature spontaneously were preincubated in the presence of H7 (200 μg/ml) for 60 min. Maturation was induced by injection of 50 nl of mature cytoplasm or 50 nl of GV content. GVBD was scored after 20 h of culture.

The results (Fig. 7) indicated that the inhibition of PKC by treatment with H7 did not modify the percentages of GVBD obtained, suggesting that this second messenger is not implicated in the signalling route of the cytoplasm or of the GV content.

Discussion

Our results indicate that the inhibition of cdc25 phosphatase by $NaVO_3$ caused a decrease in the percentages of GVBD in a dose-dependent manner in oocytes incompetent to mature spontaneously that had been treated with progesterone.

Several authors have described cdc25 phosphatase as a positive regulator of GVBD in species whose immature oocytes contain pre-MPF, since this phosphatase dephosphorylates Thr-14 and Tyr-15, which inactivate this complex. cdc25 phosphatase is active only when it is phosphorylated, so that $NaVO_3$, by preventing its phosphorylation, inhibits its activity in a reversible dose-dependent manner.

The use of $NaVO_3$ to inhibit the dephosphorylation of p34 kinase has been described in rodent oocytes by Choi *et al*. (1991, 1992) and Goren & Dekel (1994) and their results agree with those obtained for *Bufo arenarum*. The participation of cdc25 phosphatase in the dephosphorylation of $p34^{cdc2}$ has also been implicated in the resumption of meiosis in *Xenopus* oocytes (Rime *et al*., 1994; Minshull *et al*., 1991; Galas *et al*., 1993).

Our results demonstrate that the activity of cdc25 phosphatase is necessary in the signalling pathway leading to meiosis resumption in progesterone-induced maturation, which suggests the existence of a significant amount of pre-MPF in immature *Bufo arenarum* oocytes incompetent to mature spontaneously.

Treatment of incompetent oocytes for 20 h with DEX, a synthetic glucocorticoid reported as being responsible for the decrease in the levels of cyclin B synthesis in pig oocytes (Wei-Yi *et al*., 2000), did not modify the percentages of GVBD at any of the assayed doses.

Since cyclin B is a component of MPF together with $p34^{cdc2}$, these results would suggest that, in immature *Bufo arenarum* oocytes, there would exist cyclin B levels high enough to allow the formation of pre-MPF, which would be activated in response to maturation inducers. The results of the treatments with DEX indicate that immature *Bufo arenarum* oocytes are able to break down the GV in response to progesterone without new synthesis of cyclin B, suggesting the existence of maternal deposits.

Similar results were described by Gautier & Maller (1991) and Kobayashi *et al*. (1991), who demonstrated that cyclin B is present in immature *Xenopus* oocytes in sufficient amounts to induce meiosis resumption. In the same way, the investigations of Minshull *et al*. (1991) demonstrated that in the immature *Xenopus* oocyte there is a deposit of cyclin B in the form of pre-MPF and that injection of an anti-mRNA antibody for cyclin B does not inhibit hormone-induced maturation.

On the other hand, our findings concerning the fact that DEX does not affect maturation in progesterone-treated incompetent *Bufo arenarum* oocytes also agree with what has been reported for clam and starfish oocytes, which contain a cyclin B deposit and can break down the GV with no need for protein synthesis (Labbé *et al*., 1989; Meijer & Guerrier, 1984; Westerndorf *et al*., 1989).

In contrast, in the immature oocytes of certain mammals, as well as those of goldfish, carp, *Rana* sp. and *Bufo japonicus*, there is no cyclin B deposit (Katsu *et al*., 1993) so that the pre-MPF is absent. In these species, cyclin B is synthesized during oocyte maturation in response to the inducer (Hirai *et al*., 1992), which implies the need for the *de novo* synthesis of cyclin B (Jantzen & Schulze, 1994; Tanaka & Yamashita,1995; Yamashita *et al*., 1995) to form the active MPF. Kalous

et al. (1993) demonstrated that in bovine oocytes cyclin B is synthesized just before GVBD and that this synthesis is coincidental with $p34^{cdc2}$ activation.

Wei-Yi *et al*. (2000) demonstrated in pig oocytes that 1 μg/mg of DEX causes a decrease in the levels of cyclin B synthesis, which reduces the formation of active $p34^{cdc2}$ kinase, thus preventing the disassembly of the nuclear envelope. These authors also suggested that the inhibition of GVBD by DEX is not caused by inadequate levels of p34, since the levels found in the control oocytes are the same as those in treated oocytes.

On the other hand, the observations that DEX is unable to induce maturation in incompetent *Bufo arenarum* oocytes do not agree with the results of Goswani & Sundararaj (1974) for fish such as catfish, *Heteropneustes fossilis*, since these authors propose DEX as responsible for promoting maturation. In other species, such as the Atlantic croaker, however, Patiño *et al*. (1990) suggest that the action of DEX on maturation is not direct but results from its synergistic association with the 20β-hydroxymetabolites of progesterone. The discrepancies in the results among the species studied could be due to differences in the species and/or to the experimental conditions used.

Since cyclin B is a component of MPF together with $p34^{cdc2}$, these results would allow us to infer that immature *Bufo arenarum* oocytes would contain sufficient levels of cyclin B to allow the formation of pre-MPF that would be activated in response to maturation inducers. This idea is supported by indirect immunofluorescence assays that demonstrate the presence of cyclin B1, located mainly around the GV, in immature oocytes. A similar localization of pre-MPF has been described for human and chicken cells (Pines & Hunter, 1994) and in starfish cells (Ookada *et al.*, 1992). In *Bufo arenarum*, the presence of cyclin B, possibly bound to cdc2, coincides with the localization of the microtubules, which, during interphase, are preferentially located in the perinuclear zone (Giunta *et al.*, 1996).

Similar results have been described by Gautier & Maller (1991) and by Kobayashi *et al*. (1991), who demonstrated that cyclin B is present in the immature oocytes of *Xenopus* in sufficient amounts to induce resumption of meiosis. In the same way, the investigations of Minshull *et al*. (1991) demonstrated that in immature *Xenopus* oocytes there is a deposit of cyclin B in the form of pre-MPF and that the injection of an anti-mRNA antibody for cyclin B does not inhibit hormone-induced maturation.

Taken as a whole, the results of our experiments with $NaVO_3$, DEX and anti-cyclin B1 antibodies suggest that fully grown immature *Bufo arenarum* oocytes contain a deposit of pre-MPF, located mainly in the perinuclear cytoplasm, which remains inactive by means of phosphorylations on the sites Thr-14/Tyr-15 of cdc2 and is activated by the action of the cdc25 phosphatase that dephosphorylates these sites. This activation of pre-MPF would be sufficient to induce GVBD in oocytes incompetent to undergo spontaneous maturation.

The idea that *Bufo arenarum* oocytes contain pre-MPF is supported by the results of our microinjection experiments, which showed that the microinjection of cytoplasm from a mature oocyte containing active MPF is sufficient to activate an amplification loop that induces the conversion of the inactive MPF complex or pre-MPF into an active form of MPF that induces meiotic resumption.

These results agree with those of Smith & Ecker (1969), Dabauvalle *et al.* (1988), Dunphy & Kumagai (1991) and Gautier & Maller (1991), who found that injection of mature cytoplasm is sufficient to induce the amplification loop of MPF in the recipient oocyte with no need for the participation of the GV content. In this case, a small amount of active MPF can catalyse the autocatalytic transformation of existing pre-MPF in the oocyte.

The auto-amplification capacity of MPF is not common to all species. In the case of starfish oocytes, Labbé *et al.*, (1989), Picard *et al*. (1989), Gautier *et al.* (1989) and Dunphy & Newport (1989) have demonstrated that active MPF injected into a recipient oocyte undergoes rapid inactivation. No cyclin B degradation was detected in this process, so the authors have suggested that a factor other than cyclin B levels would exert a negative control capable of neutralizing the primary effect of the injection.

The existence of pre-MPF in immature oocytes of *Bufo arenarum* is also supported by our amplification assays, performed in the presence of a specific inhibitor of protein synthesis such as CHX, which indicate that in this species a small amount of active MPF is capable of activating the pre-MPF in the recipient oocyte in the absence of protein synthesis.

When the synthesis of proteins is inhibited by treatment with CHX (10 μg/ml) for 1 h before injection of mature cytoplasm, high percentages of GVBD are obtained in the recipient oocytes. These results support the idea that, in immature *Bufo arenarum* oocytes, cyclin B would already be present to form sufficient levels of the complex.

Although protein synthesis has been implicated in the progesterone induced maturation of *Bufo arenarum* oocytes (Zelarayán *et al.*, 1996), our results suggest that this synthesis would be important at other stages of the maturation process.

A similar phenomenon of amplification with transformation of the pre-MPF into active MPF, even when protein synthesis is inhibited, has been described for *Xenopus* by Wasserman & Masui (1975).

It is interesting to note that, although the immature oocytes of *Xenopus tropicalis* contain a pre-MPF deposit

similar to that of *Xenopus laevis* (Gautier & Maller, 1991), when cytoplasm containing active MPF is injected in the absence of protein synthesis *X. tropicalis* is unable to activate its pre-MPF and induce the recipient oocyte to mature (Bodart *et al*., 2002).

This difference shown by *X. tropicalis* oocytes is not due to the amount of cytoplasm injected, since the same quantity is capable of inducing GVBD in *X. laevis* even in the presence of CHX (Bodart *et al.,* 2002), but rather to the existence of important differences in the mechanisms of activation of MPF, even between closely related species.

When studying the mechanisms through which MPF amplification occurs in *Bufo arenarum*, we evaluated the effect of specific cdc25 phosphatase inhibitors such as $NaVO_3$ in the experiments with mature cytoplasm injection.

A clear correlation between intracellular levels of cAMP and meiosis stage has been demonstrated in both mammalian and non-mammalian species, where cAMP-dependent PKA is known to form part of a negative pathway that participates in the maintenance of the meiotic blockade by inhibition of MPF activity (Eppig *et al*., 1985; Törnell *et al*., 1990; Zelarayán *et al*., 2000; Lu *et al*., 2001). In this sense, our results indicate that MPF amplification by mature cytoplasm injection is also inhibited when the intracellular concentration of cAMP, associated with PKA activation, is increased by treatment with theophylline or forskoline.

Similar results have been described for *Xenopus* oocytes by Rime *et al*. (1992), who demonstrated the participation of PKA in the autocatalytic activation of MPF and its blockade when the intracellular levels of cAMP are increased. Moreover, Duckworth *et al*. (2002) and Schmit & Nebreda (2002) demonstrated that in amphibian oocytes the levels of cAMP interfere with the activity that controls cdc25 phosphorylation.

On the basis of our results and those of the above authors, we suggest that the substrate of cAMP/PKA could be cdc25 phosphatase. However, the way in which PKA controls cdc25 phosphorylation has not been determined.

Masui & Markert (1971) demonstrated the existence of an active MPF in the cytoplasm of *Rana* oocytes with hormone-induced maturation prior to GVBD. However, in other species, such as starfish, no activity of MPF was detected until GVBD (Kishimoto & Kanatani, 1976), indicating the requirement for the nuclear content for MPF activation to occur.

The requirement for the GV content in the process of activation of MPF is not restricted to starfish, since certain amphibian species are unable to amplify their MPF by hormone treatment if oocytes are enucleated (Gautier, 1987; Skoblina, 1984).

In *Bufo arenarum*, GV content injection was sufficient to induce GVBD in the immature recipient oocyte. These data suggest that a small amount of GV content is able to induce MPF amplification in the recipient oocyte.

Similar results have been described for starfish oocytes, where injection of GV content induced MPF activity in an immature oocyte without hormone treatment (Picard & Dorée, 1984). In this respect, Picard *et al*. (1988), Kishimoto *et al*. (1981) and Picard & Dorée (1984) have suggested that in starfish oocytes a certain factor in the GV content added to the kinase activity of cdc2 is required for MPF activation.

The GV component responsible for this activity has not been identified. However, Picard *et al*. (1991) found that in a starfish oocytes homogenate, pp2A activity was high in comparison with that in an enucleated oocyte. They also reported that the effect of the injection of the GV content in starfish is similar the injection of 1 μM okadaic acid. This suggests that the GV contains an element that inhibits pp2A phosphatase activity in the same way as okadaic acid, which has been reported as being responsible for inducing meiotic resumption in echinoderm (Picard *et al*., 1989), bovine (Lévesque & Sirard, 1996), mouse (Rime & Ozon, 1990) and amphibian (Rime *et al*., 1990) oocytes.

Our results agree with those of Picard *et al*. (1991), so we can suggest that the GV content would contain an inhibitor of type 2A phosphatase capable of inducing MPF amplification through a loop of unidentified regulatory events.

Although the injection of GV content is capable of inducing amplification in the recipient oocyte in the same way as the injection of mature cytoplasm, there are important differences in the mechanisms of action in the two cases. The results of the treatments with cycloheximide show that the effect of the GV content is dependent on protein synthesis. However, when transcription is blocked with actinomycin D, the GV content causes the transformation of the pre-MPF into active MPF. These results suggest that GV content possesses a type 2A phosphatase inhibitor capable of promoting MPF amplification through a loop of unidentified regulatory events that depend on protein synthesis but not on protein transcription.

With respect to the participation of the cdc25 phosphatase in the amplification process, Karaiskou *et al*. (1998, 1999) pointed out that immature *Xenopus* oocytes injected with okadaic acid, similar to the effect of GV content, undergo a sudden auto-amplification that includes hyperphosphorylation of cdc25. Interestingly, our results show that injection of GV content, similar to the effect of okadaic acid, in an immature oocyte treated with $NaVO_3$ induces GVBD and MPF activation, indicating that this amplification is independent of phosphatase cdc25 activity.

The increases in cAMP levels with forskoline or theophylline do not affect the amplification induced

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by injection of the GV content, which suggests that in *Bufo arenarum* the GV material induces MPF activation independently of PKA activity.

Similar results have been described in mouse (Lu *et al*., 2001) and *Xenopus* (Rime *et al.,* 1990), indicating that pp2A, sensitive to okadaic acid, decreases the inhibitory effect produced by cAMP on MPF activation.

It has also been suggested that PKC activity is involved in maturation inhibition in mouse denuded oocytes (Gotoh & Nishida, 1995; Verlhac *et al*., 1993). However, Zelarayán *et al.* (1996) in *Bufo arenarum* and Aberdan & Dekel (1985) in rat oocytes still surrounded by cumulus cells have demonstrated that PKC activators such as phorbol esters (PMA) induce GVBD.

Our results show that PKC inhibition with H7 in assays with mature cytoplasm or GV content injection does not affect MPF amplification, probably because PKC acts upstream in the process.

Conclusions

The results of the experiments with $NaVO_3$ and DEX suggest that fully grown immature *Bufo arenarum* oocytes contain a deposit of pre-MPF that remains inactive by means of phosphorylations on the sites Thr-14/Tyr-15 of the cdc2 and that is activated by dephosphorylation of these sites by the action of the cdc25 phosphatase. This activation of the pre-MPF would be sufficient to induce GVBD.

The presence of pre-MPF in immature oocytes incompetent to mature spontaneously is supported by the detection by immunofluorescence of cyclin B1 in the perinuclear region.

The microinjection of cytoplasm from a mature oocyte containing active MPF is sufficient to activate an amplification loop that promotes the conversion of inactive pre-MPF into its active form, MPF, which induces meiotic resumption.

The mechanism of amplification of MPF by injection of mature cytoplasm requires the presence of an active cdc25 phosphatase and is inhibited when the intracellular concentration of cAMP, associated with PKA activation, is increased by treatment with theophylline or forskoline. On the basis of our results, we suggest that the substrate of cAMP/PKA could be cdc25 phosphatase; however, the way in which PKA controls phosphorylation has not been determined.

In *Bufo arenarum*, the injection of GV content obtained from immature oocytes is sufficient to induce GVBD in the immature recipient oocyte. These results suggest that the GV content possesses a type 2A phosphatase inhibitor capable of promoting MPF amplification through a loop of unidentified regulatory events that depend on protein synthesis but not on protein transcription and are independent of the activity of the cdc25 phosphatase and of PKC.

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