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The role of calcium in the nuclear maturation of *Bufo arenarum* oocytes

Liliana I. Zelarayán, Graciela Sánchez Toranzo, Julia M. Oterino and Marta I. Bühler
INSIBIO-UNT, Tucumán, Argentina

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Summary

In *Bufo arenarum*, progesterone is the physiological maturation inducer. However, in this species, oocytes reinitiate meiosis with no need of an exogenous hormonal stimulus when deprived of their enveloping cell, a phenomenon called spontaneous maturation. We demonstrated that in *Bufo arenarum* spontaneous maturation occurs only in oocytes obtained during the reproductive period, which can be considered competent to mature spontaneously, in contrast to those in the non-reproductive period, which are incompetent. Interestingly, full-grown *Bufo arenarum* oocytes always respond to progesterone regardless of the season in which they are obtained. There is a general consensus that both a transient increase in intracellular calcium and a decrease in cAMP-dependent protein kinase activity are the first steps in the mechanisms by which progesterone induces maturation in amphibians. In the present work we analysed the role of calcium in the spontaneous and progesterone-induced maturation of *Bufo arenarum* oocytes. Results demonstrated that the absence of calcium in the incubation medium or the prevention of Ca^{2+} influx by channel blockers such as $CdCl_2$ or $NiCl_2$ did not prevent meiosis reinitiation in either type of maturation. The inhibition of the Ca^{2+}–calmodulin complex in no case affected the maturation of the treated oocytes. However, when the oocytes were deprived of calcium by incubation in Ca^{2+}-free AR + A23187, meiosis resumption was inhibited. In brief, we demonstrated that in *Bufo arenarum* the reinitiation of meiosis is a process independent of extracellular calcium at any period of the year and that oocytes require adequate levels of intracellular calcium for germinal vesicle breakdown to occur.

Keywords: *Bufo arenarum*, Calcium, Nuclear maturation

Introduction

In the ovary of amphibians follicular cells are the source of progesterone, the physiological maturation inducer. Progesterone interacts with the oocyte surface (Godeau *et al.*, 1978) and starts a cascade of events leading to the activation of a cytoplasmic maturation promoting factor (MPF) that induces germinal vesicle breakdown (GVBD).

The role of calcium in progesterone-induced meiotic maturation in amphibian oocytes remains unclear. Tracer flux studies have shown that the rate of $^{45}Ca^{2+}$ efflux from preloaded oocytes increases within minutes after exposure to progesterone in a significant percentage of oocytes injected with the calcium photoprotein aequorin (review by Cork *et al.*, 1987; Cicirelli & Smith, 1987; Smith, 1989). However, other investigators such as Robinson (1985) failed to detect changes in the intracellular concentration of Ca^{2+} when using microelectrodes sensitive to it. In the same way, during the experiments of Cork *et al.* (1987) only a few oocytes from *Xenopus laevis* exhibited a transient increase in $[Ca^{2+}]$ in response to progesterone. The rest of the oocytes, which showed no alteration in Ca^{2+} levels, also achieved maturation in the presence of progesterone.

Ca^{2+} has been proposed as a second messenger for a wide range of physiological and biochemical functions (Masui & Clarke, 1979). Specifically, Ca^{2+} could act by way of calmodulin, a well-characterized protein found in many cells, which changes its conformation over a physiological range of Ca^{2+} concentrations (Waismanne *et al.*, 1978*a*). Calmodulin

All correspondence to: Dra Marta I. Bühler, Departamento de Biología del Desarrollo, INSIBIO-UNT, Chacabuco 461, 4000 Tucumán, Argentina. e-mail: mbuhler@unt.edu.ar

could activate various enzymes such as cyclic nucleotide phosphodiesterase, adenylate cyclase and protein kinases (Chueng *et al.*, 1975; Waismanne *et al.*, 1978*b*).

There is a general consensus that both a transient increase in intracellular calcium and a decrease in cAMP-dependent protein kinase activity are the first steps in the mechanisms by which progesterone induces maturation (Smith, 1989; Matten *et al.*, 1994). In this regard, we have demonstrated that high levels of purines such as cAMP or guanosine can inhibit both spontaneous and progesterone-induced maturation in full-grown denuded *Bufo arenarum* oocytes. Moreover, mycophenolic acid, a specific inhibitor of inosine monophosphate dehydrogenase, was able to induce maturation, in a way similar to that of progesterone, in denuded oocytes obtained during the non-reproductive period and also to increase the percentages of spontaneous maturation (Zelarayán *et al.*, 2000).

Many steroids other than progesterone as well as a wide variety of non-hormonal compounds, although active at the surface of the cells, also trigger meiosis either by acting at the cell surface or by displacing calcium from the membrane (Schorderet-Slatkine *et al.*, 1977; Baulieu *et al.*, 1978). The mechanisms by which membrane perturbations may lead to MPF production are poorly understood, although Ca^{2+} has been suggested as a possible second messenger. It has been suggested that the increase in intracellular calcium might be a necessary and sufficient condition to induce maturation in oocytes (Cicirelli & Smith, 1987; Cork *et al.*, 1987). In this regard, Moreau *et al.* (1980) demonstrated that in the oocytes of certain species of anurans and urodeles a marked increase in cytosolic calcium concentration occurs after progesterone stimulation.

Bufo arenarum oocytes reinitiate meiosis with no need of an exogenous hormonal stimulus when deprived of their enveloping cells (Zelarayán *et al.*, 1995). This phenomenon, called spontaneous maturation, is quite rare in amphibians (Vilain *et al.*, 1980; Lin & Schuetz, 1985; Kwon *et al.*, 1989). We demonstrated that in *Bufo arenarum* spontaneous maturation occurs only in oocytes obtained during the reproductive period (September–December) (Zelarayán *et al.*, 1995). Oocytes collected during this period can be considered competent to mature spontaneously, in contrast to those in the non-reproductive period (May–August), which are incompetent. Interestingly, full-grown *Bufo arenarum* oocytes always respond to progesterone regardless of the season in which they are obtained (Zelarayán *et al.*, 1995).

Taking into account the fact that the role of Ca^{2+} in meiosis reinitiation in amphibian oocytes is controversial, in this work we wished to analyse its participation in the spontaneous and *in vitro* progesterone-induced maturation of *Bufo arenarum* full-grown oocytes.

Materials and methods

Animals

Adult specimens of *Bufo arenarum* were collected in northwestern Argentina from May to August (non-reproductive period) and from September to December (reproductive period) and kept at 15 °C until use, generally 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. Full-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 theca layer using fine forceps under 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). Denuded oocytes were kept in AR until use.

Routine *in vitro* cultures were carried out using plastic multiwell culture dishes (Costar 3524, Cambridge, MA). Randomized samples of 20 oocytes were distributed into separate wells containing 2 ml of AR or Ca^{2+}-free AR. The absence of calcium in the Ca^{2+}-free AR was ensured by the addition of EGTA (0.01 mM). Reagents were added (5 µl) directly to the culture medium. Two well duplicates were routinely run in each experimental group.

Oocyte maturation was assessed by detecting germinal vesicle breakdown (GVBD) 10 h after hormone or reagent addition. Meiosis reinitiation was scored both by the presence of a transient white spot in the animal pole and by the absence of a nuclear envelope after subsequent dissection of the oocytes fixed in trichloroacetic acid (TCA). The removal of the follicle cells from the oocytes in the reproductive period and the addition of progesterone (1 µg/ml) to those in the non-reproductive period were considered as time 0 of the treatment. Only viable oocytes were taken into consideration at the end of the incubation period.

Denuded oocytes obtained during the reproductive period (September–December) were considered competent to mature spontaneously, in contrast to those obtained during the non-reproductive period

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(May–August), which were considered incompetent (Zelarayán *et al.*, 1995).

Hormones and reagents

All hormones and reagents were purchased from Sigma. Progesterone (5 µl) was dissolved in ethanol and added directly to the culture medium to give a final concentration of 1 µg/ml. Final ethanol concentration in the culture medium was 0.25% (v/v). At no time were the maturation percentages affected by ethanol.

The stock solutions of $CdCl_2$, $NiCl_2$ and verapamil (400 mM, 80 mM and 400 mM respectively) were preprared in distilled water. At the time of use they were appropriately diluted and added to the incubation medium at a volume of 5 µl. The calmodulin inhibitor W-7 and the calcium ionophore A23187 were prepared in stock solutions (80 mM and 2 mg/ml respectively) in dimethylsulphoxide.

Results

Participation of extracellular calcium

Culture in Ca^{2+}-free media

To determine whether the presence of calcium in the incubation medium was necessary for spontaneous or progesterone-induced maturation, denuded oocytes obtained during the reproductive period (competent oocytes) and during the non-reproductive period (incompetent oocytes) were incubated in AR or in Ca^{2+}-free AR. In the latter, maturation was induced by progesterone (1 mg/ml). The results obtained (Fig. 1) indicate that the absence of Ca^{2+} in the culture medium did not significantly affect the percentage of either spontaneous or progesterone-induced GVBD.

Similar results were obtained when 0.01 mM EGTA was added to the Ca^{2+}-free AR for both kinds of maturation. However, in the initial experiments the viability of the oocytes decreased when they were incubated in Ca^{2+}-free media for long periods (20 h or more). Consequently, the incubation time was shortened to 10 h in later experiments. After 6.5 h of incubation, 50% of denuded oocytes underwent GVBD in response to progesterone, and by 10 h the response was almost 100%, while in the case of spontaneous maturation maximum GVBD (50%) occurred after 9 h of culture. In these experimental conditions, the viability of the cultured oocytes in Ca^{2+}-free AR was 85%.

Role of calcium channel blockers

The participation of extracellular Ca^{2+} in oocyte maturation was also studied by preventing Ca^{2+} influx through the use of Ca^{2+} channel blockers such as $CdCl_2$, $NiCl_2$ and verapamil.

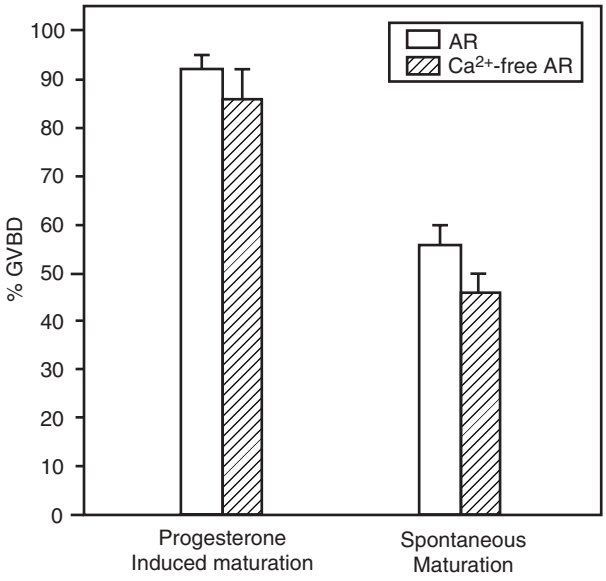

Figure 1 Effect of extracellular calcium on spontaneous and progesterone-induced maturation. Competent denuded oocytes that mature spontaneously and incompetent oocytes treated with progesterone, were cultured in amphibian Ringer solution (AR) or Ca^{2+}-free AR. GVBD occurred after incubation for 10 h. Values represent the mean $\pm$ SEM ($n = 4$) of experiments carried out in different animals.

Oocytes competent to mature spontaneously were incubated in AR in the presence of $CdCl_2$ (50–400 µM) or $NiCl_2$ (100–400 µM) for 30 min before removing the follicle cells. Incompetent oocytes were denuded and preincubated in the presence of the blockers for 30 min and maturation was then induced by progesterone (1 µg/ml). Incubation continued for 10 h in both types of oocytes. Results (Fig. 2) agree with those obtained in Ca^{2+}-free media since both spontaneous and progesterone-induced maturation were resumed in the presence of $CdCl_2$ or $NiCl_2$. The percentages of GVBD observed were similar to those of the controls.

Another blocker of the Ca^{2+} channels assayed was verapamil, a tertiary amine that inhibits calcium flow through the plasma membrane and alters the interaction between its phospholipids and proteins (Baulieu *et al.*, 1978).

The effect of verapamil on nuclear maturation was studied by incubating competent and incompetent denuded oocytes in AR in the presence of different doses of the blocker (0.01–1.00 mM). Another batch of incompetent oocytes was incubated in the same way, but in order to obtain low GVBD percentages (about 30%) (Zelarayán *et al.*, 1996) maturation was induced with low progesterone doses (0.1 µg/ml). Results (Fig. 3*A*) indicate that verapamil is capable of inducing maturation in oocytes incompetent to mature spontaneously in the absence of progesterone. Verapamil can also increase the percentage of GVBD in

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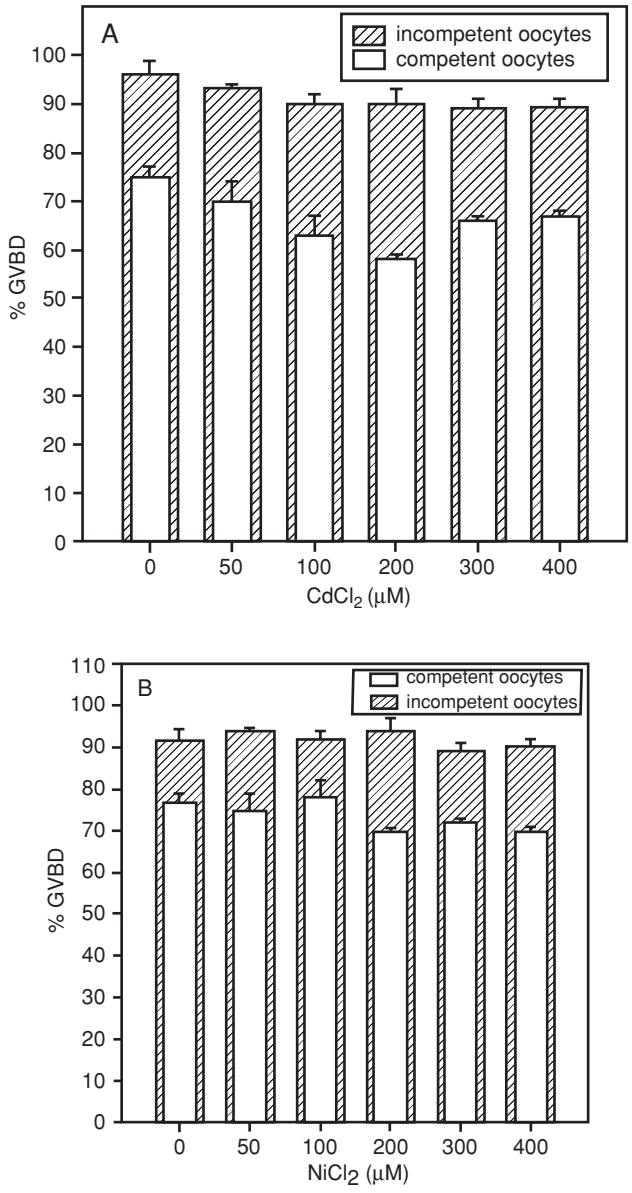

Figure 2 Effect of calcium channel blockers on meiosis reinitiation. Oocytes competent and incompetent to undergo spontaneous maturation were incubated for 30 min before follicle cell extraction or addition of 1 μg/ml progesterone in AR, in the presence of different doses of: (*A*) $CdCl_2$ (50–400 μM) or (*B*) $NiCl_2$ (50–400 μM). GVBD was scored after 10 h of culture. Values represent the mean SEM ($n = 3$) of experiments carried out in different animals.

oocytes that mature either spontaneously or through the action of the hormone (Fig. 3*B*, *C*).

Participation of intracellular calcium

Changes in the intracellular levels of free calcium are known to be involved in the triggering of meiosis in numerous amphibian and mammalian species (Smith, 1989; Homa, 1995). However, little is known concerning the effect of the movement of calcium on the maturation of *Bufo arenarum* oocytes.

Effect of the ionophore A23187

A comparative study was carried out concerning the effect of calcium flow on the resumption of spontaneous and progesterone-induced maturation using the ionophore A23187. Ionophores are molecules that increase lipid bilayer permeability to specific inorganics ions. Since ionophores are not coupled to energy sources, they permit net movement of ions only down their electrochemical gradient. The ionophore A23187 is a mobile carrier. It transports divalent cations such as Ca^{2+} or Mg^{2+}. It normally acts as an ion-exchange shuttle, carryng two H^+ out of the cell for every divalent cation it carries in.

Competent and incompetent oocytes were incubated in AR or in Ca^{2+}-free AR in the presence of the ionophore A23187 (1 μg/ml) after removing the follicle cells. The absence of extracellular calcium was ensured by adding EGTA (0.01 mM) to the culture medium.

Results are summarized in Fig. 4. As shown there, when oocytes were incubated in AR in the presence of the ionophore (calcium influx) neither spontaneous nor progesterone-induced maturation was affected. Also, Ca^{2+} influx is not capable by itself of inducing GVBD in oocytes incompetent to mature spontaneously. On the other hand, when oocytes were incubated in Ca^{2+}-free AR in the presence of A23187 (calcium efflux), both spontaneous and progesterone-induced maturation decreased.

These results indicate that, although an increase in intracellular calcium cannot induce maturation by itself, adequate intracellular calcium levels are required for both spontaneous and progesterone-induced maturation.

Participation of calmodulin

Another calcium target in many cellular processes is calmodulin and the calmodulin-dependent protein kinase (Dorée *et al.*, 1982; Lorca *et al.*, 1993; Xu *et al.*, 1996). Calmodulin, a highly conserved protein, has been found in *Xenopus* oocytes (Wasserman & Smith, 1981), in which a microinjection of the calcium–calmodulin complex can trigger meiosis reinitiation.

For the purpose of evaluating the participation of the above complex in both types of maturation, we studied the effect of one of its antagonists: W-7 (*N*(6-aminohexyl) 5-chloro-1-naphthalenesulphonamide).

Competent denuded oocytes were incubated in AR with different doses of W-7 (50–100 μM) for 30 min before removing the follicle cells. Incompetent oocytes were denuded and preincubated in the presence of diferent doses of W-7 for 30 min and maturation

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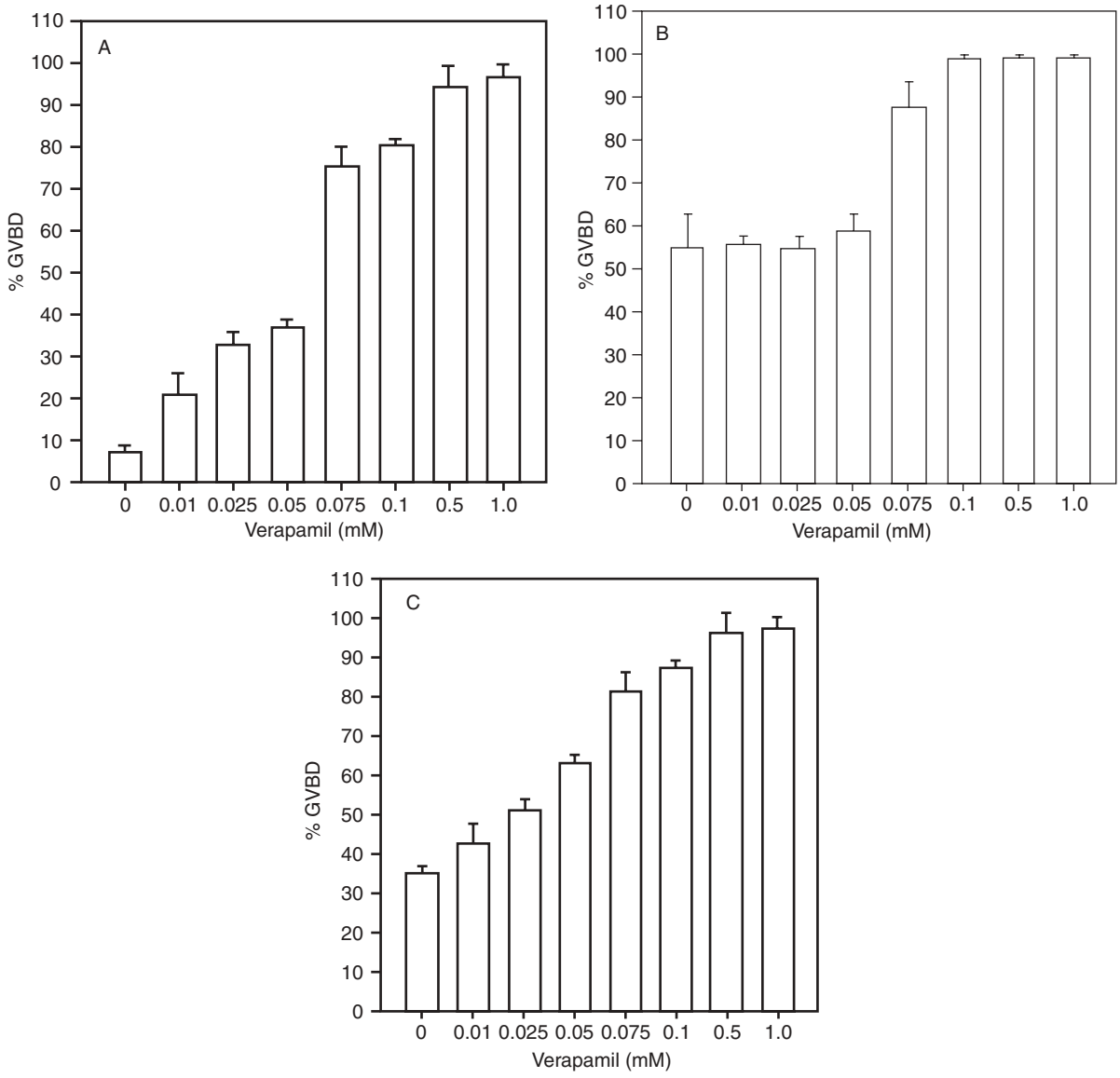

Figure 3 Effect of verapamil on meiosis resumption in oocytes competent and incompetent to mature spontaneously. (*A*) Denuded incompetent oocytes (May–August) and (*B*) denuded competent oocytes (September–December) were incubated in AR with different doses of verapamil (0.01–1.0 mM). GVBD was scored after incubation for 10 h. Values represent the mean SEM ($n = 3$) of experiments carried out in different animals. (*C*) Denuded incompetent oocytes (May–August) were incubated in AR with different doses of verapamil (0.01–1.0 mM). GVBD was induced with 0.1 μg/ml progesterone. GVBD was scored after incubation for 10 h. Values represent the mean SEM ($n = 4$) of experiments carried out in different animals.

Table 1 Effect of W-7 on spontaneous and progesterone-induced maturation

Type of oocyte	Treatment	% GVBD
Competent (September–December)	Control	42 ± 6
	W-7 (100 μM)	37 ± 4
Incompetent (May–August)	Control	4 ± 2
	W-7 (100 μM)	5 ± 2
	Progesterone (1 μg/ml)	87 ± 5
	Progesterone (1 μg/ml) + W-7 (100 μM)	87 ± 2

was then induced by progesterone (1 μg/ml). In our experimental conditions, none of the doses assayed affected the percentage of GVBD in either type of oocyte (Table 1). Table 1 shows the results obtained with the highest W-7 doses assayed.

Discussion

It has been proposed that the release of calcium in amphibian oocytes is a consequence of progesterone action and that the increase in free calcium is a

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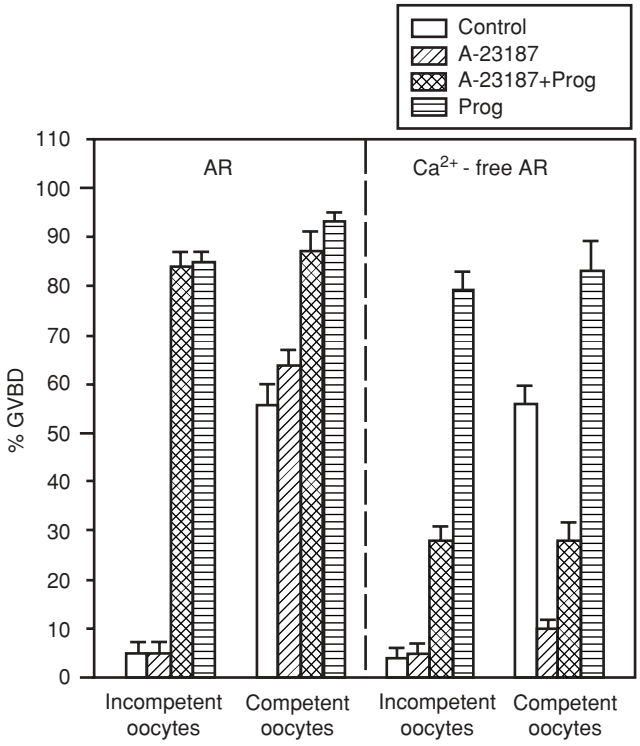

Figure 4 Effect of $Ca^{2\pm}$ flow on spontaneous and progesterone-induced maturation. Competent and incompetent oocytes obtained during the September–December or May–August period were incubated in AR or in Ca^{2+}-free AR in the presence of A23187 (1 μg/ml), of A23187 (1 μg/ml) + progesterone (1 μg/ml), or of progesterone (1 μg/ml). Control oocytes were incubated in AR or in Ca^{2+}-free AR without A23187 or progesterone. GVBD occurred after incubation for 10 h. Values represent the mean SEM ($n = 4$) of experiments carried out in different animals.

necesary condition for meiosis reinitiation. The increase in Ca^{2+} levels precedes MPF formation (Cicirelli & Smith, 1987; Cork *et al.*, 1987). This increase, necessary for maturation, might result from ionic flow through the oocyte membrane or might be released from the intracellular reservoirs as demonstrated in the case of starfish (Moreau *et al.*, 1978 *a*, *b*). On the other hand, the oocytes of *Xenopus laevis* and of pleurodeles are able to resume meiosis in Ca^{2+}- and Mg^{2+}-free media supplemented with 10 mM of EDTA (Moreau *et al.*, 1980).

The results obtained in this study agree with those found for *Xenopus* since *Bufo arenarum* oocytes resume meiosis in Ca^{2+}-free media. During the reproductive period, the mere denuding of those oocytes competent to mature spontaneously triggers meiosis reinitiation even when they are incubated in Ca^{2+}-free AR or in Ca^{2+}-free AR plus EGTA. We found that the percentages of GVBD were similar to those in the controls and that the competent oocytes incubated in Ca^{2+}-free AR broke down the germinal vesicle and reached metaphase II as observed in the histological slices of oocytes picked at random. In the same way, the absence of Ca^{2+} in the medium did not alter the GVBD percentages in the case of progesterone-induced maturation in incompetent oocytes.

Results suggest that oocytes possess sufficient intracellular Ca^{2+} for meiosis reinitiation so they do not have to incorporate it from external sources. The experiments carried out with calcium channel blockers such as $CdCl_2$ and $NiCl_2$ would support this hypothesis, since neither affected the maturation rate. These results suggest that both spontaneous and progesterone-induced maturation are independent of extracellular calcium influx.

In contrast with other channel blockers assayed, verapamil produced unexpected results since it increased the percentages of GVBD in incompetent oocytes and induced meiosis reinitiation in them in the absence of progesterone in a dose-dependent manner. As regards competent oocytes, verapamil increased GVBD percentages. It seems probable that verapamil, apart from its effect as a calcium channel blocker, exerts other effects that mimic the action of progesterone on the plasma membrane. In this connection, Baulieu *et al.* (1978) reported that amphibian oocyte maturation can be induced by several substances not chemically related to one another nor to the physiological inducer of the process. Verapamil could exert the above effect on the resumption of meiosis due to its interaction with the phospholipids of the oocyte membrane. This possibility could be supported by previous results obtained in our laboratory in which we demonstrated that neomycin, an inhibitor of phosphoinositide hydrolysis, totally blocks spontaneous and progesterone-induced maturation in *Bufo arenarum* oocytes. Moreover, some of the products of membrane phospholipid hydrolysis, such as DAG, might be involved in the maturation process of this species since the activation of PKC with the phorbol ester induced GVBD in incompetent oocytes (Zelarayán *et al.*, 1996, 2000).

As regards the participation of intracellular calcium, it is agreed that the increase in cytoplasmic Ca^{2+} is a part of the signalling process through which progesterone induces maturation in *Xenopus* oocytes. Studies of calcium efflux from ^{45}Ca-labelled *Xenopus* oocytes demonstrated an increase efflux shortly after progesterone addition (O'Connor *et al.*, 1977). Such an efflux migh be predicted to occur following an increase in cytoplasmic-free calcium. In *Bufo arenarum* oocytes the depletion of calcium caused by its efflux due to the treatment with A23187 would be accompanied by the influx of H^+, which might induce a decrease in intracellular pH. Bearing in mind the fact that in progesterone-induced maturation an alkalinization of the cytoplasm has been observed (Canaux *et al.*, 1995), we may suggest that the decrease in GVBD percentages

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could also be attributable to this effect. Therefore in *Bufo arenarum* oocytes the depletion of calcium caused by its efflux inhibited meiosis reinitiation in both competent and incompetent oocytes, a fact that suggests that oocytes require a certain intracellular level of calcium for GVBD to occur.

Calcium interacts with calmodulin to regulate a series of cellular events, among them meiosis (Wasserman & Masui, 1975; Wasserman & Smith, 1981). It has been reported that this complex is involved in the maturation of *Rana* oocytes induced by phorbol esters and by progesterone (Kwon & Lee, 1991). In *Bufo arenarum*, on the contrary, in our experimental conditions, the inhibition of the Ca^{2+}–calmodulin complex in no case affected the maturation of the treated oocytes.

In conclusion, the results of this work demonstrate that in *Bufo arenarum* the reinitiation of meiosis is a process independent of extracellular calcium at any period of the year and that calcium influx is not sufficient by itself to trigger meiosis in incompetent oocytes. We also demonstrated that GVBD is dependent on the presence of cytosolic calcium, since its depletion inhibited the breakdown of the germinal vesicle in both competent and incompetent oocytes.

Acknowledgements

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