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Spontaneous and LH-induced maturation in *Bufo arenarum* oocytes: importance of gap junctions

G. Sánchez Toranzo, J. Oterino, L. Zelarayán, F. Bonilla and M.I. Bühler
Departamento de Biología del Desarrollo, San Miguel de Tucumán, Argentina

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Summary

It has been demonstrated in *Bufo arenarum* that fully grown oocytes are capable of meiotic resumption in the absence of a hormonal stimulus if they are deprived of their follicular envelopes. This event, called spontaneous maturation, only takes place in oocytes collected during the reproductive period, which have a metabolically mature cytoplasm.

In *Bufo arenarum*, progesterone acts on the oocyte surface and causes modifications in the activities of important enzymes, such as a decrease in the activity of adenylate cyclase (AC) and the activation of phospholipase C (PLC). PLC activation leads to the formation of diacylglycerol (DAG) and inositol triphosphate ($IP_{3)}$, second messengers that activate protein kinase C (PKC) and cause an increase in intracellular Ca^{2+}. Recent data obtained from *Bufo arenarum* show that progesterone-induced maturation causes significant modifications in the level and composition of neutral lipids and phospholipids of whole fully grown ovarian oocytes and of enriched fractions in the plasma membrane. In amphibians, the luteinizing hormone (LH) is responsible for meiosis resumption through the induction of progesterone production by follicular cells.

The aim of this work was to study the importance of gap junctions in the spontaneous and LH-induced maturation in *Bufo arenarum* oocytes. During the reproductive period, *Bufo arenarum* oocytes are capable of undergoing spontaneous maturation in a similar way to mammalian oocytes while, during the non-reproductive period, they exhibit the behaviour that is characteristic of amphibian oocytes, requiring progesterone stimulation for meiotic resumption (incapable oocytes).

This different ability to mature spontaneously is coincident with differences in the amount and composition of the phospholipids in the oocyte membranes. Capable oocytes exhibit in their membranes higher quantities of phospholipids than incapable oocytes, especially of PC and PI, which are precursors of second messengers such as DAG and IP_3.

The uncoupling of the gap junctions with 1-octanol or halothane fails to induce maturation in follicles from the non-reproductive period, whose oocytes are incapable of maturing spontaneously. However, if the treatment is performed during the reproductive period, with oocytes capable of undergoing spontaneous maturation, meiosis resumption occurs in high percentages, similar to those obtained by manual defolliculation.

Interestingly, results show that LH is capable of inducing GVBD in both incapable oocytes and in oocytes capable of maturing spontaneously as long as follicle cells are present, which would imply the need for a communication pathway between the oocyte and the follicle cells. This possibility was analysed by combining LH treatment with uncoupling agents such as 1-octanol or halothane. Results show that maturation induction with LH requires a cell–cell coupling, as the uncoupling of the gap junctions decreases GVBD percentages. Experiments with LH in the presence of heparin, BAPTA/AM and theophylline suggest that the hormone could induce GVBD by means of the passage of IP_3 or Ca^{2+} through the gap junctions, which would increase the Ca^{2+} level in the oocyte cytoplasm and activate phosphodiesterase (PDE), thus contributing to the decrease in cAMP levels and allowing meiosis resumption.

All correspondence to: Marta I. Bühler, Departamento de Biología del Desarrollo, Chacabuco 461, 4000 – San Miguel de Tucumán, Argentina. Fax: +54 381 424 8025. e-mail: mbuhler@fbqf.unt.edu.ar

Departamento de Biología del Desarrollo, Chacabuco 461, 4000 – San Miguel de Tucumán, Argentina.

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Introduction

In amphibians, meiosis is resumed by the action of gonadotrophins both *in vivo* and *in vitro*, which act indirectly through the stimulation of the intrafollicular production of steroid hormones that act directly on the oocyte (Masui, 1967; Redshaw, 1972; Schuez, 1974; Kim *et al*., 1998; Patiño *et al*., 2001).

In *Bufo arenarum*, progesterone acts on the oocyte surface (Zelarayán *et al*., 1995, 2000) and induces modifications in the activities of important enzymes such as the decrease in the activity of adenylyl cyclase (AC) and the activation of phospholipase C (PLC). PLC activation lead to the formation of diacylglycerol (DAG) and inositol triphosphate (IP_3), second messengers that activate the protein kinase C (PKC) and cause an increase in intracellular Ca^{2+}. Numerous reports suggest that PKC, which is dependent on diacylglycerol, phosphatidylserine and Ca^{2+}, has a positive inducer effect on maturation. In *Bufo arenarum*, it was demonstrated that PKC activation is involved in spontaneous and hormone-induced maturation (Zelarayán *et al*., 1996). In progesterone-treated *Rana* oocytes, Morril & Kostellow (1999) demonstrated that the release of DAGs from membrane phospholipids increase during nuclear maturation.

In this sense, the results of Smith (1989) demonstrated that in progesterone-treated *Xenopus* oocytes DAG levels increase at the time of germinal vesicle breakdown (GVBD). Kobrinsky *et al*., (1999) reported the participation of oesphingomieline-derived compounds in progesterone-induced maturation.

Besides the lipids from inositol, other lipids are involved in maturation, as progesterone also induces the formation of phosphatidylcholine from phosphatidylethanolamine, the subsequent hydrolysis of phosphatidylcholine and the production of arachidonic acid (Godeau *et al*., 1985; Chien *et al*., 1991). In sea urchin oocytes, arachidonic acid has the capacity to induce maturation, imitating the effect of the physiological inducer (Epel, 1982).

In agreement with the above, recent data from *Bufo arenarum* show that progesterone-induced maturation causes important modifications in the level and composition of neutral lipids and phospholipids from whole fully grown ovarian oocytes and enriched fractions in the plasma membrane (Alonso, 2000). These changes might be due to the activation of enzymes, especially phospholipase A_2, which would release unsaturated fatty acids, especially arachidonic acid. Interesting variations were also found in sphingomyelin, a phospholipid that is apparently the generator of second messengers involved in many cellular processes such as growth and apoptosis (Spiegel *et al*., 1996; Kronke, 1999).

Although progesterone is the physiological inducer of maturation in amphibians, in *Bufo arenarum* it has been demonstrated that fully grown oocytes are capable of meiotic resumption in the absence of a hormonal stimulus if they are deprived of their follicular envelopes (Zelarayán *et al*., 1995). This phenomenon, called spontaneous maturation, takes place only in oocytes with a metabolically mature cytoplasm, that is, in those that have modified the biochemical behaviour of their cytoplasm (Petrino *et al*., 1983; Zelarayán *et al*., 1995, 1996, 2000). However, the nature of the relationship between the stage of cytoplasmic maturation, the ability to mature spontaneously and the molecular mechanisms involved in the process are still unknown.

In *Bufo arenarum*, cytoplasmic maturation occurs during the reproductive period. Interestingly, fully grown *Bufo arenarum* oocytes always respond to progesterone, independently of the time of the year in which they are obtained or of their stage of cytoplasmic maturation (Zelarayán *et al*., 1996).

Spontaneous maturation has been well studied in mammals and, although the molecular mechanism that regulates the process is not completely understood yet, it has been suggested that the presence of the follicular envelopes is important in maintaining the meiotic blockade (Dekel & Beers, 1980; Vilaint *et al*., 1980; Eppig & Ward Bailley, 1982; Lin & Schuetz, 1985; Eppig, 1991; Atef *et al*., 2005).

Gap junctions provide a structural mechanism for the transference of small molecules from one cell to another (Warner, 1988). The cell–cell communication through homologous and heterologous gap junctions has been described in the ovary of mammals (Albertini & Anderson, 1974; Anderson & Albertini, 1976; Gilula *et al*., 1978) and amphibians (Browne *et al*., 1979; Browne & Werner, 1984; Van den Hoef *et al*., 1984; Villeco *et al*., 2000). Gap junctions participate in the passage of substances that allow oocyte growth; besides, there is a co-operative coupling between the somatic cells and the oocyte that contributes to maintaining the meiotic blockade. However, the role played by gap junctions in oocyte maturation is still controversial.

Racowsky (1984); Racowsky & Satterlie (1985); Dekel *et al*., (1988); Thomas *et al*., (2004), demonstrated that the loss of the coupling between the oocyte and the cumulus cells or the decrease in the number of the gap junctions between these cells coincides with GVBD and proposed that the physiological separation of the

oocyte from its somatic compartment might be the cause of the removal of the meiotic block. These authors propose two basic models to explain spontaneous maturation in mammls. One of them propounds that gap junctions mediate the transference from the follicle cells towards the oocyte of an inhibitory molecule, cAMP, which arrests meiosis. This model is supported by experiments in which the levels of cAMP in the cumulus cells were kept high through stimulation by FSH, cholera toxin, forskolin or db-AMP and where maturation failed to occur (Racowsky, 1984; Dekel *et al*., 1988; Webb *et al*., 2002).

A first interpretation of this phenomenon is that the cAMP produced in the somatic cells diffuses towards the oocyte through the gap junctions, thus blocking meiosis. The closure or uncoupling of gap junctions, which would prevent the passage of the inhibitory signal, would trigger maturation, as is the case in numerous mammals. In some species, however, meiosis resumption has been observed before the detection of the metabolic uncoupling between the oocyte and the follicle cells (Moor *et al*., 1980; Eppig, 1982; Racowsky & Satterlie, 1985; Larsen *et al*., 1986). More detailed investigations have revealed a more complex mechanism in which the granulosa cells stimulated by the luteinizing hormone (LH) produce a soluble factor that would act directly on the oocyte membrane, activating adenylyl cyclase (AC) (Mattioli, 1994; Grondahl *et al*., 2003).

Another model involves the production by follicle cells of a positive signal that, reaching the oocyte through the gap junctions, is capable of inducing GVBD even in the presence of the inhibitory influence of the follicle (Eppig, 1982; Eppig & Downs, 1987; Downs *et al*., 1987). This hypothesis is supported by numerous experiments such as meiotic resumption induced by growth factors even when the oocytes are inside their cumuli (Eppig & Downs, 1987; Down *et al*., 2002; O'Donnell *et al*., 2004; Park *et al*., 2004).

Gap junctions have also been studied in fish follicles, where the works of Iwamatsu & Ohta (1981) in *Oryzias lapites* and of Weber & Sullivan (2005) in *Morone chrysopt* demonstrated the necessity of the association between the granulosa cells and the oocyte for the normal development of the latter. *Fundulus heteroclitus* oocytes reinitiate meiosis *in vitro* in the absence of a hormonal stimulus when the somatic cell layer in contact with the oocyte is removed manually (Greeley *et al*., 1987). Cerdá *et al*., (1993), using Lucifer Yellow stain. This fact demonstrated, in *Fundulus heteroclitus* ovaries, that gap junctions are normally open and that when this condition is modified by the addition of chemicals, such as phorbol esters (PMA) or long-chain alcohols, meiosis is resumed.

The n-alcohols, 1-heptanol and 1-octanol, are commonly used to handle cell–cell coupling (Ramón *et al*., 1985; Meda, 1989; Stagg & Fletcher, 1990; Rozental *et al*., 2001). These alcohols were described specifically by Sandberg *et al*. (1990) as being responsible for the inhibition of molecules transference between the oocyte and the follicle cells in *Xenopus laevis.* In mice (Fagbohun & Downs, 1991) and pigs (Coskun & Lin, 1994), the use of heptanol reduces the coupling between the cumulus cells and the oocyte in the cumulus–oocyte complex (COC), stimulating meiosis resumption.

If we postulate that gap junctions mediate the transference of maturation inhibiting substances from the cumulus to the oocyte (Dekel *et al*, 1978), it seems logical to conclude that LH stimulates meiotic resumption by removing the cell–cell coupling and, consequently, the flow of maturation inhibitors (Dekel *et al*., 1978; Tsafriri *et al*., 1982; Sela *et al*., 2005). However, the cumulus cells and the oocyte remain coupled until GVBD takes place (Eppig, 1982; Larsen *et al*., 1986). This fact could suggest, in mice at least, that a factor other than uncoupling between cells would be required for oocyte maturation (Downs & Eppig, 1988; Fagbohun & Downs, 1990).

In vertebrates, meiosis is arrested in prophase I while the ovarian follicle is being formed and growing. In all mammalian species, the signal responsible for meiotic resumption is a sudden increase in LH, which would be responsible for the progress to metaphase II.

As in mammals (Tsafriri *et al*., 2005), in amphibians and fish LH is the hormone responsible for meiosis resumption. The first *in vitro* studies in amphibians were performed by Masui & Clarke (1979), Nagahama (1987) and Dettlaff (1988), who demonstrated that LH induced the production of a steroid similar to progestin, which they called maturation inducing hormone (MIH) and which is responsible for meiosis resumption. The experiments *in vivo* performed in fishes and amphibians (Kahn & Thomas, 1999; Kagawa *et al*., 2003) showed that the preovulatory increase in LH is related to an increase in MIH production and to meiosis resumption by the oocyte. Consequently, in fishes and amphibians, the need for the follicular production of MIH for meiosis resumption is a well-established paradigm (Bolamba *et al*., 2003; Patiño *et al*., 2003).

The *in vivo* administration of chorionic gonadotrophin (hCG) or pituitary homogenate in *Xenopus laevis* (Reynhout *et al*., 1975) and carp, *Cyprinus carpio* (Jalabert *et al*., 1977) increases the maturational sensitivity of the ovarian follicle by stimulating MIH production. In amphibians and fishes, Patiño *et al*., (2001) proposed a model of response to LH in two stages. During the first, the ovarian follicle acquires the ability to produce MIH and the oocyte the capacity to respond to it. During the second, the follicle produces MIH and the oocyte responds by reinitiating meiosis.

There is no doubt that, in mammals, meiosis is resumed in preovulatory follicles by a peak of LH. However, no receptor for LH was found in the oocyte plasma membrane (Mattioli, 1994). The LH receptor is present in the theca and granulosa cells (Zelesnik *et al*., 1981). The hormone acts on the follicular wall by inducing the theca and/or granulosa cells to secrete the second messenger into the follicular fluid in which the oocyte is immersed (Mattioli, 1996). Moreover, LH can act directly on the cumulus cells that, though few, are able to communicate directly with the oocyte via the gap junctions (Peng *et al*., 1991).

The existence of a receptor for LH in the cumulus cells described by Peng *et al*. (1991) suggests a direct effect of LH on the cumulus. This hypothesis was confirmed by the observation that the *de novo* synthesis of mRNA in the cumulus cells is necessary for gonadotrophin to induce maturation (Meinecke & Meinecke Tillmann, 1993). However, the way in which this signal is transferred towards the oocyte and stimulates meiotic maturation is still unknown.

In order to overcome the meiotic blockade, LH must suppress the inhibitory signal from the follicle or, alternatively, must provide a positive stimulus to overcome follicular inhibition. This action is apparently mediated by the cumulus cells and transmitted via the gap junctions (Fagbohun & Downs, 1991; Downs, 2001). A common event in the response to LH is that follicles increase the number of gap junctions. In this sense, Browne *et al*., (1979) described that in *Xenopus* an increase in heterologous contacts (oocyte–follicle cells) can be observed after hCG injection. In fish as well, after stimulation with hCG, an increase is observed in homologous and heterologous contacts (York *et al*., 1993), which disappear during the second maturation stage (Patiño & Kagawa, 1999; Patiño *et al*., 2000; Bolamba *et al*., 2003).

In mammals, it has been reported that LH induces maturation through a dual effect on whole follicles, stimulating AC, which causes a rapid increase in cAMP concentration (Sánchez Yague *et al*., 1993; Davis, 1994) and activating PLC, which hydrolyzes PIP_2 and produces IP_3, which in turn releases intracellular calcium (Davis *et al*., 1986, 1994; Dimino *et al*., 1987; Goren *et al*., 1990; Gudermann *et al*., 1992; Flores *et al*., 1998). These second messengers, IP_3, Ca^{2+} and cAMP, are also important regulators of the gap junction's function in numerous tissues, including the ovary and are also responsible for the stimulation of oocyte maturation.

It is possible that these molecules generated in the granulosa cells are transferred towards the oocyte via gap junctions in order to induce maturation. In this sense, numerous authors have reported modifications in Ca^{2+} concentration after the exposure of the follicles to LH. The passage of this signal from the somatic compartment towards the oocyte depends on the integrity of the heterologous gap junctions and their mechanical rupture prevents the increase of intracellular Ca^{2+} in the oocyte.

On the basis of the above, the aim of this work was to study the importance of gap junctions in the spontaneous and LH-induced maturation of *Bufo arenarum* oocytes.

Materials and methods

Sexually mature *Bufo arenarun* 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 culture

Experimental manipulation and culture were performed at room temperature (22–25 °C) in amphibian Ringer solution (AR) (6.6 g NaCl/1, 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. Oocytes surrounded by follicular cells 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) and kept in AR at 22–25 °C until use.

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 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 GVBD after dissection of the oocytes fixed in trichloroacetic acid (TCA).

Fluorescent stain

The experiments were performed with isolated ovarian follicles containing fully grown oocytes. These were microinjected with 50 nl of an aqueous solution at 2% of Lucifer Yellow CH (PM: 457.2) under a stereoscopic microscope at room temperature.

To evaluate stain transference to the follicle cells, the follicular envelopes were manually dissected 2 h after injection. All experiments were carried out in duplicate. Photographs of the envelopes, corresponding to the

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distribution of the fluorescent stain, were obtained with a Kodak film (ASA 400). The samples were observed with a fluorescent microscope (OLYMPUS BX 40).

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%.

Dibutyryl cAMP (dbcAMP) was dissolved in AR and various doses were added to the culture medium at a constant volume (5 μl). Theophylline was used at a final concentration of 1×10^{-5} M. Heparin: the low molecular weight sodium salt was used to prepare a 40 mg/ml stock solution. Dose–response assays were performed with dilutions of the solution, adding 5 μl to the incubation medium. Theophylline: 200 mg aminophilin (Laboratorio Phoenix) was used. Different doses were added to the incubation medium to a constant volume of 5 μl; 1-octanol dilutions:one part of the drug was diluted in nine parts of absolute ethanol. This was added directly to the incubation medium to obtain the appropriate final concentration. Halothane from Laboratorio Ayerst was used. Different doses were added directly to the incubation medium to obtain the appropriate final concentration. LH: 'Luteoliverina', Laboratorio Elea, was used. Different doses were added to the incubation medium to obtain the appropriate final concentration. Dilutions were performed in AR. BAPTA/AM: this was dissolved in dimethylsulphoxide (DMSO) in a 20 mM stock solution. A constant volume of 5 μl of the dilutions made in the same vehicle was added to the incubation medium.

Enriched plasma membrane preparations

We used the methodology originally developed to obtain an enriched preparation of plasma membranes from sea urchin oocytes (Barber & Foy, 1973) adapted by Caldironi *et al*. (1996). Oocytes were transferred to 10 volumes of a solution containing 0.1 M $MgCl_2$, 0.01 M TRIS at pH 7.5 and kept there for 30 min for membrane stabilization and surface washing. The oocytes were centrifuged at 3000 r.p.m. for 5 min and the supernatant was discarded. Oocytes were osmotically lysed by resuspending them in 0.01 M $MgCl_2$, 0.01 M TRIS, pH 8.0 for 30 min and by gently homogenization with a Teflon pestle. The lysed cells were centrifuged and the resulting pellet was resuspended in diluted $MgCl_2$ and then centrifuged again. A 40% sucrose solution was added to the pellet containing the plasma membranes, which was then centrifuged at 3000 r.p.m. for 5 min. The enriched preparations of purified membranes were recovered from the interphase and washed with diluted $MgCl_2$ to remove sucrose.

Lipids extraction

Extraction of lipids was performed by the method of Folch *et al*. (1957). Phosphoglycerides were separated by two-dimensional thin layer chromatography (TLC) in silica gel H according to Rousel *et al*. (1970). The phosphoglycerides were located after exposure of the plates to I_2 vapours and quantified by phosphorus analysis after digestion with perchloric acid (Rouser *et al*., 1970).

Results

Seasonal variation of the response to follicle cells removal

Bearing in mind that in *Bufo arenarum* it is possible to obtain fully grown oocytes all year round, we studied the ability of the oocytes to mature in response to the removal of follicle cells during two seasonal periods: September to December (reproductive period) and March to August (non-reproductive period). The oocytes were deprived of their follicular envelopes (denuded oocytes) and incubated in AR for 20 h at 25 °C.

Results indicate that only the oocytes from the reproductive period respond to the removal of follicle cells by resuming meiosis spontaneously (capable oocytes), reaching a GVBD percentage of about 70% (Fig. 1). The

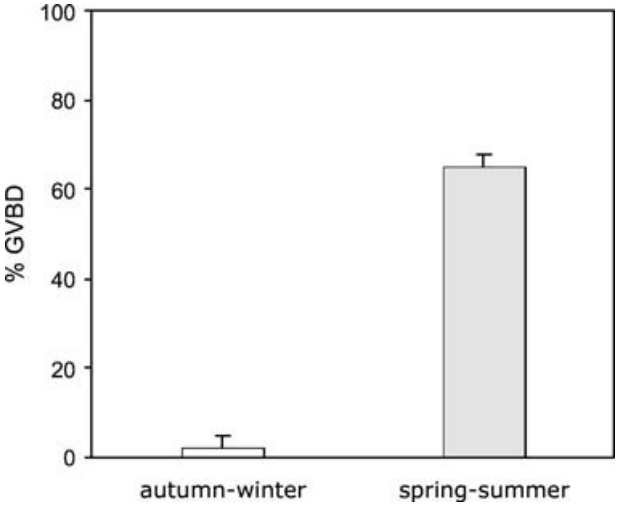

Figure 1 Seasonal variation in the response to the removal of follicle cells. Oocytes were removed from the ovary during the autumn–winter and spring–summer periods, deprived of follicle cells and incubated in AR. GVBD was scored after 20 h of incubation. Values represent the mean ± SEM ($n = 6$) of experiment performed on different animals.

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oocytes obtained during the non-reproductive period were incapable of maturing when follicular envelopes were removed.

Response of *Bufo arenarum* oocytes that are capable of maturing spontaneously to the uncoupling of the gap junctions

For the purpose of studying whether the oocyte has the ability to mature spontaneously is associated with metabolic modifications in the oocyte itself or in its relation to follicle cells, experiments were carried out using chemicals such as 1-octanol and halothane, commonly used to modify coupling between cells.

Experiments were carried out in animals captured between September and December (capable oocytes). Fully grown *Bufo arenarum* follicles were incubated in the presence of different doses of 1-octanol (1–6 mM) and halothane (1–8 mM). GVBD was determined after incubation for 24 h.

Results shown in Fig. 2a, b indicate that capable oocytes can mature spontaneously, even when surrounded by follicle cells, when they are treated with gap junction uncoupling agents. A dose-dependent increase in GVBD percentages can also be seen with doses lower than 2 mM 1-octanol and 4 mM halothane. However, when the dose of the uncoupling agents was increased, GVBD percentages decreased.

On the basis of the results obtained, the doses of choice for the subsequent experiments were 2 mM for 1-octanol and 4 mM for halothane. In order to determine whether 1-octanol was responsible for the uncoupling of the gap junctions between the follicle cells and the oocyte, fully grown *Bufo arenarum* oocytes were preincubated for 2 h in the presence of the uncoupling agent and then injected with 50 nl of fluorescent Lucifer Yellow, a fluorescent stain used as a marker of the existence of operating gap junctions. Later, the follicles were cultured for 2 h in the same medium.

All the follicles injected with LY and incubated in AR showed fluorescence in their follicle cells (Fig. 3), which indicates the passage of the stain from the oocyte through the gap junctions. However, the follicle cells of the follicles cultured in the presence of the uncoupler did not show fluorescence, which would indicate that the gap junctions are not operating, thus demonstrating the effectiveness of the uncoupling agent used.

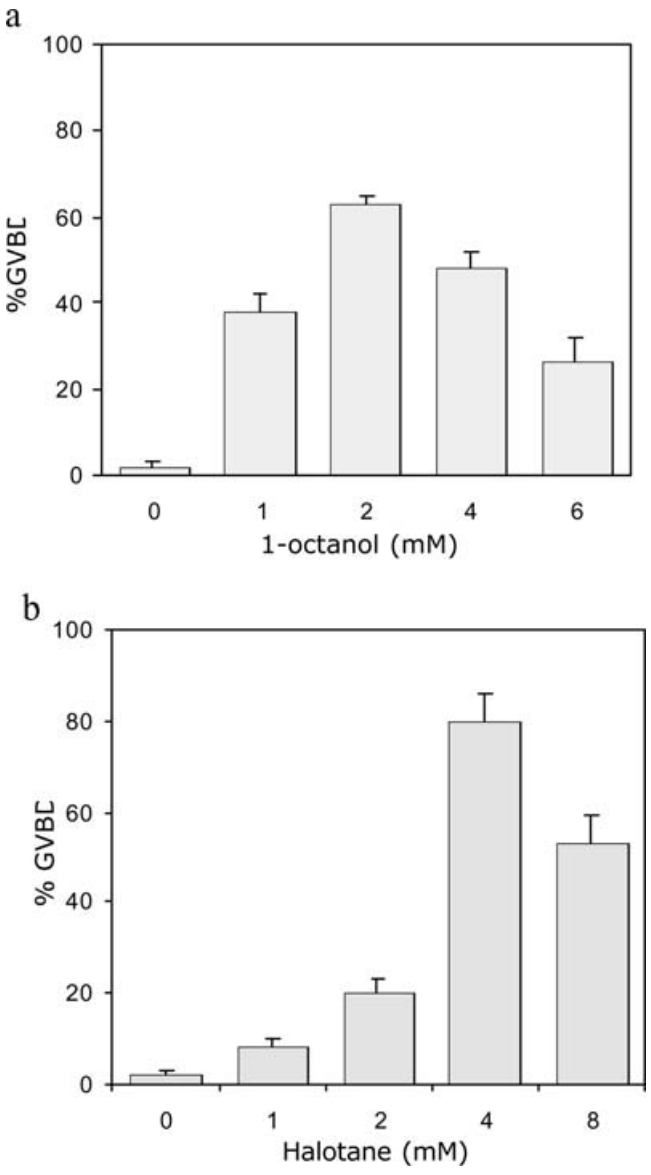

Figure 2 Dose–response of gap junction uncoupling agents on GVBD. Oocytes capable of maturing spontaneously, surrounded by follicle cells, were incubated in (*a*) different doses of 1-octanol (1–6 mM); or (*b*) different doses of halothane (1–8 mM). GVBD was scored after 20 h of incubation at 25 °C. Values represent the mean ± SEM ($n = 4$) of experiments performed on different animals.

Effect of gap junction uncouplers on the induced nuclear maturation of oocytes with follicle cells capable and incapable of maturing spontaneously

Capable and incapable ovarian oocytes surrounded by follicle cells were preincubated in 1-octanol (2 mM) or halothane (4 mM) for 2 h, after which they were transferred to AR with or without progesterone (2.5 μM). GVBD was scored after 24 h of incubation. The results (Fig. 4) show that the uncoupling of the gap junctions with 1-octanol or halothane was sufficient to induce meiotic resumption in capable oocytes, but had no effect on incapable oocytes. However, both types of oocytes responded to the subsequent progesterone treatment, indicating that the uncoupling agents at the doses used had no deleterious effects on the oocytes.

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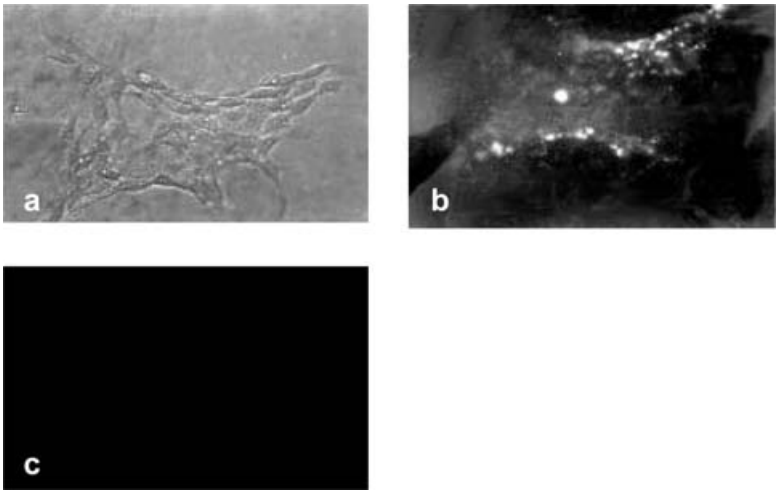

Figure 3 Control of oocyte–follicle cells communication. (*a*) Phase-contrast microscopy of the follicle cells–vitelline envelope. (*b*) Image of a similar preparation of follicle cells–vitelline envelope obtained from an oocyte injected with fluorescent stain (LY), without previous treatment with the uncoupler. (*c*) Image of a preparation obtained from an oocyte treated with the uncoupler prior to the fluorescent stain (LY) injection.

Content and distribution of phospholipids in oocytes capable and incapable of maturing spontaneously

It is known that certain phospholipids and fatty acids from the cell membrane, such as PKC, AC and guanylate cyclase (GC), are crucial for the activity of enzymes that participate in the oocyte maturation process. To determine whether there are differences in the content and distribution of phospholipids between oocytes either capable or incapable of maturing spontaneously, we worked with enriched membrane fractions from both types of oocytes.

The results in Table 1 show that the enriched membrane fractions from capable oocytes contain a larger amount of phospholipids than those from oocytes incapable of undergoing spontaneous maturation. The most important difference appears in the PC content, this difference being less marked for levels PE, PI and AP, precursors of second messengers such as DAG and IP_3.

Dose–response curve of the effect of LH on GVBD in oocytes incapable of undergoing spontaneous maturation

Studies in mammals suggest a double action of LH on the ovarian follicle, both activating AC and promoting a rapid increase in cAMP and causing the activation of PLC, with the corresponding production of IP_3 responsible for Ca^{2+} release from intracellular deposits. In addition, an uncoupling effect of LH on the gap junctions between oocytes and follicle cells has been postulated. On this basis, a dose–responsee curve of the effect of LH on GVBD in oocytes incapable of undergoing spontaneous maturation was plotted.

Oocytes incapable of maturing spontaneously surrounded by follicle cells were incubated in AR in the presence of different doses of LH (5–20 μg/ml) and GVBD was scored after 24 h of incubation. The results in Fig. 5 indicate that LH is capable of inducing maturation in a dose-dependent manner in oocytes incapable of maturing spontaneously but when follicle cells are present. On the basis of the above results, the dose chosen for LH was 15 μg/ml, with which maturation percentages of about 90% were obtained without impairing oocyte viability.

Effect of LH on oocytes with follicle cells capable and incapable of maturing spontaneously

In order to determine whether the effect of LH on GVBD is dependent or not on the capability or incapability of oocytes to mature spontaneously, we performed a comparative analysis of GVBD in capable and incapable oocytes plus follicle cells.

Oocytes were incubated in AR plus LH (15 μg/ml) at 25 °C. GVBD was scored after 24 h of incubation. The results in Fig. 6 show that LH can induce GVBD in both capable and incapable oocytes as long as there are follicle cells present.

Effect of the uncoupling of gap junctions on LH-induced maturation in incapable oocytes with follicle cells

Groups of incapable oocytes with follicle cells were cultured in a medium containing 1-octanol (2 mM) or halothane (4 mM). Each group was supplemented with

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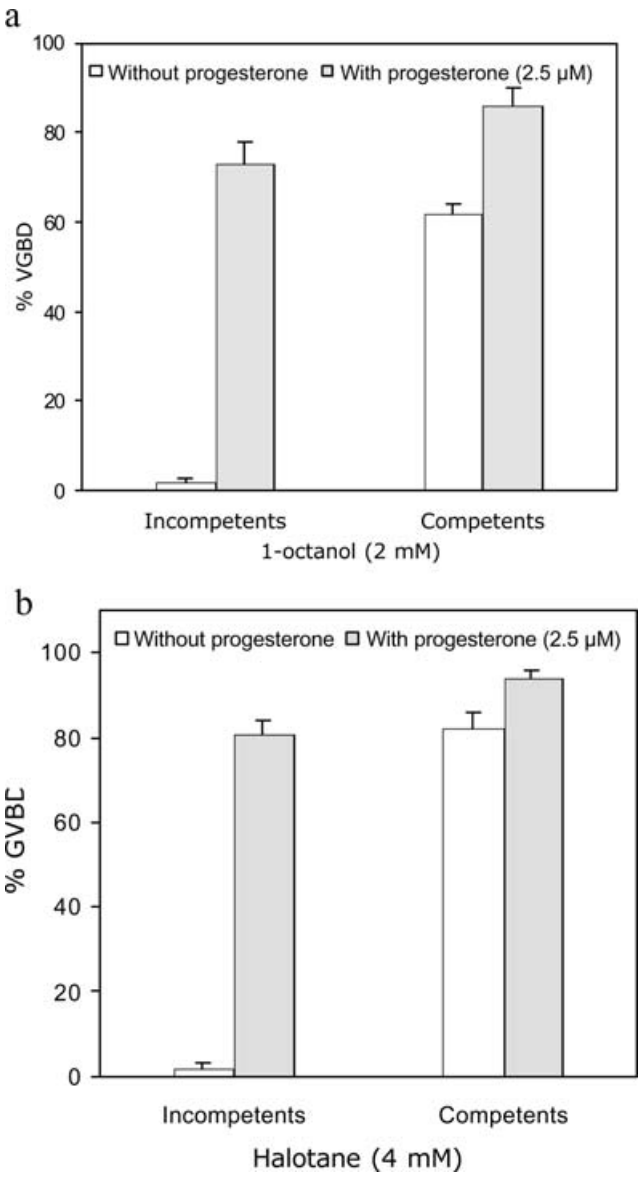

Figure 4 Effect of 1-octanol or halothane on the nuclear maturation in oocytes with follicle cells capable or not capable of maturing spontaneously. Capable (competent) and incapable (incompetent) oocytes surrounded by their follicular cells were preincubated in the presence of (*a*) 1-octanol (2 mM); or (*b*) halothane (4 mM) for 120 min. The oocytes were cultured in AR or in AR with progesterone (2.5 μM). GVBD was scored after 24 h of incubation. Values are the mean ± SEM ($n = 4$) experiments performed on different animals.

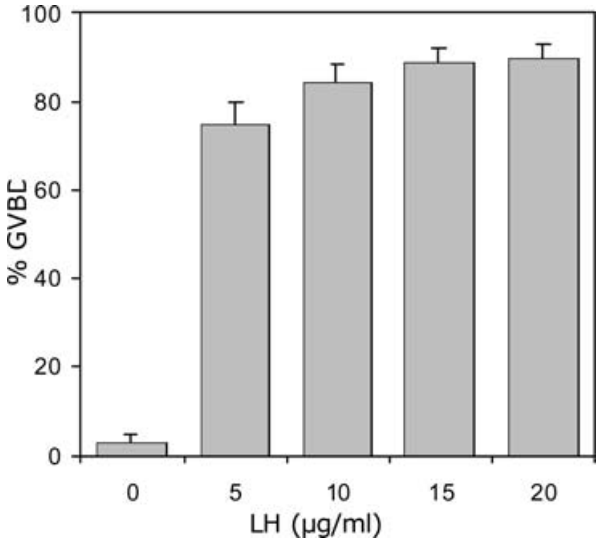

Figure 5 Dose–response curve of LH in oocytes with follicle cells incapable of undergoing spontaneous maturation. Oocytes with follicle cells were incubated in AR by itself or in AR with different doses of LH (5–20 μg/ml). GVBD was scored at 24 h of incubation. Values represent the mean ± SEM ($n = 3$) of experiments performed on different animals.

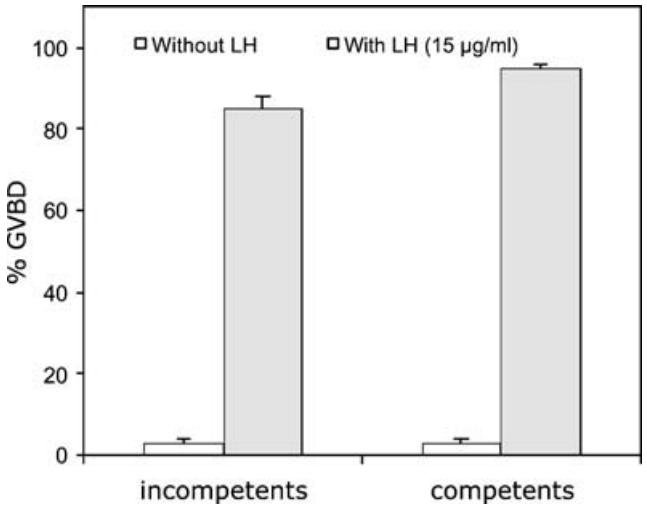

Figure 6 Effect of LH on meiosis resumption in oocytes with follicle cells capable and incapable of maturing spontaneously. Capable (competent) and incapable (incompetent) oocytes with follicle cells were incubated in AR in the presence or absence of LH (15 μg/ml). GVBD was scored at 24 h of culture. Values represent the mean ± SEM ($n = 4$) of experiments performed on different animals.

15 mg/ml of LH at different times after the start of the culture. GVBD was scored 24 h after adding the hormone.

The results in Fig. 7 show that after treatment for 2 h with the uncoupling agents, the percentage of GVBD drops abruptly. This time period coincides with the one required for gap junctions to uncouple due to the effects of 1-octanol or halothane, which suggests that the existence of operating gap junctions is necessary for LH to exert its effect on GVBD. LH probably induces meiotic resumption through the transference of factors from follicle cells to oocytes via gap junctions.

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Table 1 Phospholipids contents in enriched fractions of membranes of oocytes capable and incapable of mature spontaneously

	Capable oocytes nmol/mg of protein	Incapable oocytes nmol/mg of protein
Phosphoglycerides	134.99	186.23
Phosphatidylcholine (PC)	79.10	124.81
Phosphatidylethanolamine (PE)	30.04	41.21
Phosphatidylinositol (PI)	8.53	11.36
Sphingomyelin (SM)	12.16	8.57
Phosphatidylserine (FS)	4.59	4.00
Phosphatidic acid (AP)	0.57	0.78

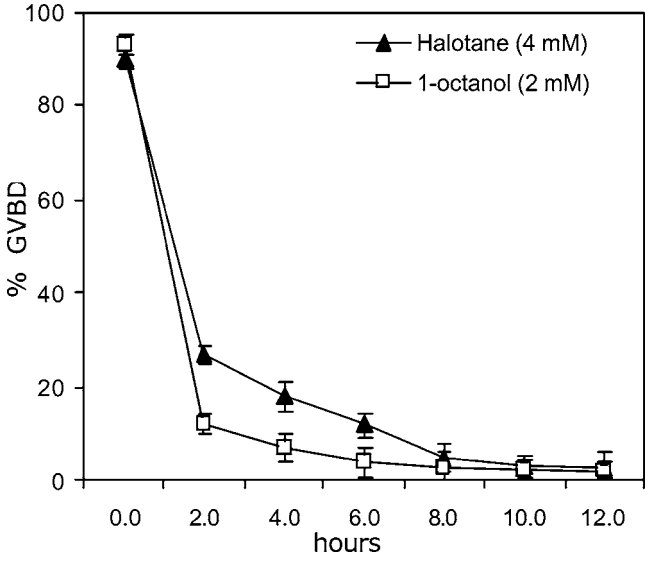

Figure 7 Effects of uncoupling agents on LH-induced meiotic resumption in incapable oocytes plus follicle cells. Oocytes with follicle cells were incubated in the presence of 1-octanol (2 mM) or halothane (4 mM) and maturation was induced by the addition of LH at different intervals. GVBD was scored at 24 h after the addition of the hormone. Values represent the mean ± SEM ($n = 4$) of experiments performed on different animals.

Analysis of Ca^{2+} participation in LH-induced GVBD

LH has been reported to be capable of increasing IP_3 production in somatic cells of mammalian follicles, with the concomitant increase in free intracellular calcium. The increase in this ion in the follicular compartment might diffuse towards the oocyte via gap junctions, causing an increase in Ca^{2+} concentration inside the oocyte. This possibility would be supported by our observation that the combination of LH and uncouplers does not cause GVBD in *Bufo arenarum* oocytes (Fig. 7).

To verify this hypothesis, we designed experiments using different agents that modify the levels of intracellular Ca^{2+}. In order to determine whether free intracellular calcium is necessary for the resumption of LH-induced meiosis, the incapable oocytes plus follicle cells were treated with different doses of a calcium chelating agent such as BAPTA/AM.

Incapable oocytes plus follicle cells were pre-incubated for 30 min in the presence of different concentrations of the permeable chelating agent (50–200 mM) before the addition of 15 ug/ml of LH. As a control, another group of oocytes was incubated directly in AR containing LH without the addition of BAPTA/AM.

The results obtained (Fig. 8) show a significant difference in the percentage of GVBD in oocytes incubated with the chelating agent in comparison with the control group, which indicates that treatment with

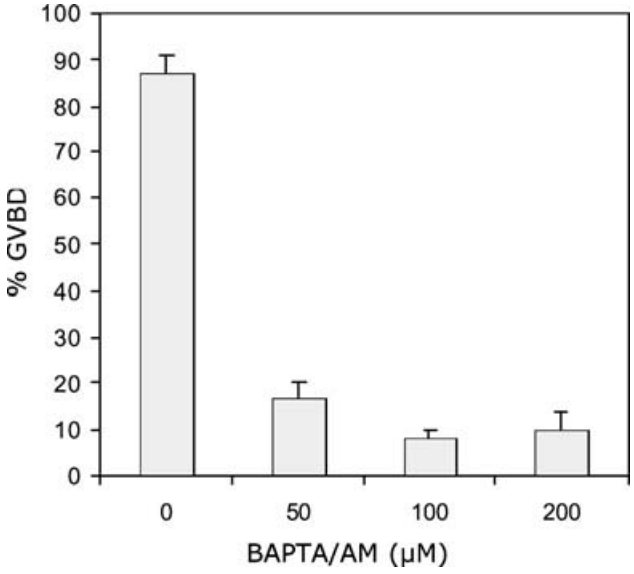

Figure 8 Effect of treatment with BAPTA/AM on LH-induced meiosis resumption in incapable oocytes. Oocytes with follicle cells incapable of maturing spontaneously were preincubated for 30 min with different doses of chelating agent of Ca^{2+} (50–200 μM). Maturation was induced by addition of LH (15 μg/ml) to the medium. Values represent the mean ± SEM ($n = 3$) of experiments performed on different animals.

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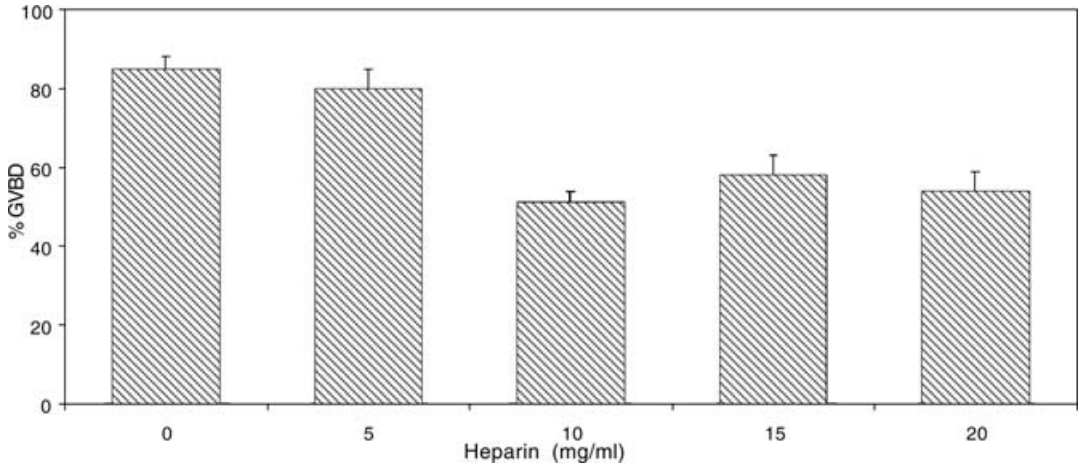

Figure 9 Effect of heparin on meiosis resumption in oocytes with follicle cells. Oocytes with follicle cells incapable of maturing spontaneously were preincubated for 30 min with different doses of low molecular weight sodium heparin (5–20 mg/ml). Maturation was induced by the addition of LH (15 μg/ml) to the culture medium. Values represent the mean ± SEM ($n = 4$) of experiments performed on different animals.

BAPTA/AM causes a dose-dependent inhibition of GVBD. These results suggest that the presence of free intracellular calcium is critical for LH-induced meiosis resumption (Fig. 8).

Effect of an R-IP_3 antagonist on LH-induced maturation in incapable oocytes

Having demonstrated the need of free intracellular calcium for LH to exert its action on GVBD, we tried to determine whether the effect of the hormone was mediated by the activation of the IP_3 receptor. We used heparin, described as a competitive antagonist agent of R-IP_3 capable of blocking Ca^{2+} release by preventing meiosis progress in the oocytes of numerous species.

The oocytes incapable of maturing with follicle cells were preincubated for 60 min in AR in the presence of different doses of low molecular weight sodium heparin (5–20 mg/ml) before the addition of 15 μg/ml of LH. GVBD was scored after 20 h of incubation.

The results in Fig. 9 show that the inhibition of the IP_3 receptor with heparin inhibits by up to 30% the percentage of LH-induced GVBD. The percentage of inhibition did not increase with the increase in heparin dose.

Participation of PDE in LH-induced GVBD in incapable oocytes

PDE has been implicated in the degradation of cyclic nucleotides, such as the enzyme responsible for the hydrolysis of cAMP. In order to study the participation of PDE in LH-induced maturation, oocytes plus follicle cells were preincubated for 60 min in AR in the presence of different amounts of theophylline (0.5–2.0 μM), an inhibitor of PDE. After this time, LH (15 μg/ml) was added to the medium to induce maturation. In all cases, GVBD was scored after 20 h of incubation.

The results in Fig. 10 indicate that treatment with theophylline significantly inhibits LH-induced GVBD, suggesting that PDE activity could be involved in the resumption of LH-induced meiosis.

Discussion

Analysis of the effects of follicle cell extraction on GVBD shows a seasonal variation in the response of *Bufo arenarum* oocytes, as only oocytes from the reproductive period are capable of resuming meiosis with this treatment (spontaneous maturation). These results, which agree with those of Zelarayán *et al*., (1995), indicate that *Bufo arenarum* oocytes present seasonal variations in their capacity for spontaneous meiosis resumption.

During the reproductive period, *Bufo arenarum* oocytes are capable of maturing spontaneously in the same way as mammalian oocytes do, while during the non-reproductive period they exhibit the behavioural characteristics of amphibian oocytes, which require progesterone stimulus for meiotic resumption (incapable oocytes). This different ability to mature spontaneously concurs with differences in the quantity and composition of the phospholipids in the oocytes membranes. Capable oocytes exhibit in their membranes a higher phospholipid content than incapable oocytes, especially PC and PI, which are precursors of second messengers such as DAG and IP_3.

It has been demonstrated that, in *Rana*, progesterone activates *N*-methyltransferase, which converts phosphatidylethanolamine to PC and to DAG (Morril

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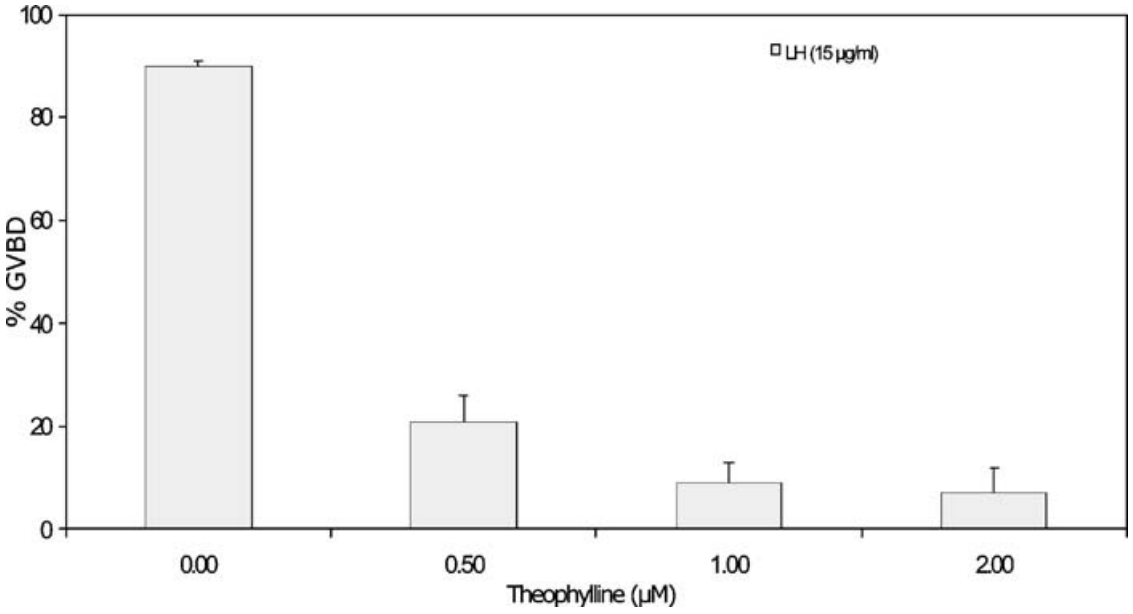

Figure 10 Effect of theophylline on LH-induced meiosis resumption in incapable oocytes plus follicle cells. Oocytes with follicle cells incapable of maturing spontaneously were preincubated for 30 min in different doses of theophylline (0.5–2.0 μg/ml). Maturation was induced by the addition of LH (15 μg/ml) to the culture medium. Values represent the mean ± SEM ($n = 4$) of experiments performed on different animals.

& Kostellow, 1994; Bandyopadhayay *et al*., 1998). It has also been suggested that progesterone activates a phospholipase from the plasma membrane specific for PC, which causes a DAG increase that accompanies meiosis resumption (Kostellow *et al*., 1999). These results agree those for *Bufo arenarum* described by Zelarayán *et al*. (1996), which demonstrate meiosis resumption by treatment of oocytes with phorbol esters that mimic the effect of DAG on PKC.

It seems possible that capable oocytes, as they contain a larger amount of precursors of second messengers such as DAG and IP_3, do not require progesterone stimulus for formation, so that the release of the inhibitory influence of the surroundings follicle cells is sufficient to trigger meiosis resumption. The experiments of Homa *et al*. (1991) in mammals demonstrated that spontaneous maturation is related to an increase in IP_3, necessary to increase the levels of free Ca^{2+}, a phenomenon also described for amphibians (Bandyopadhyay *et al*., 1998; Kobrinsky *et al*., 1999).

During the maturation of oocytes from rat, pig and bovines, an increase in PI, PC and PE (Homa *et al*., 1991) and in PA (Davis *et al*., 1983; Dimino *et al*., 1987) has been observed, in which the increase in PC and PE is interpreted as a simulation of the *de novo* synthesis of these phospholipids (Homa, 1991). The fact that the same phospholipids should be increased in capable oocytes suggests that these oocytes do not need to synthesize them, which would contribute to facilitate maturation when the inhibitory influence of the follicle cells is removed.

The experiments carried out with oocytes surrounded by follicle cells point to the presence of communication or gap junctions between these cells and the oocyte, as shown by the passage of LY, and confirm the existence of metabolic coupling in these follicles as found in numerous vertebrates (Gilula *et al*., 1978; Browne *et al*., 1979; Racowski & Satterlie, 1985, Atef *et al*., 2005), and in amphibians (Sandberg *et al*., 1990, Villeco *et al*., 2000, Skoblina, 2004).

The capacity of uncoupling agents (anesthetics and long chain alcohols) to inhibit intracellular communication between the oocyte and the follicle cells is shown by a block in the passage of LY stain in all follicles studied. The n-alcohols inhibit coupling between cells because they interact specifically with connexin proteins that form the gap junctions (Richards *et al*., 1978; Johnson *et al*., 1980; Peracchia, 1991). The block of stain passage in the follicles treated with halothane or 1-octanol indicates that, in this species, the integrity of the gap junctions is sensitive to these agents, which act in a dose-dependent manner.

In *Bufo arenarum*, the uncoupling of the gap junctions with 1-octanol or halothane does not induce maturation in follicles from the non-reproductive period, whose oocytes are incapable of maturing spontaneously. However, if treatment is performed during the reproductive period, with oocytes capable of undergoing spontaneous maturation, meiotic resumption occurs at high levels similar to that obtained by manual defolliculation.

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These observations suggest that block of the passage of inhibitory molecules, such as cAMP, towards the oocyte by uncoupling gap junctions (Eppig, 1991; Zelarayán *et al*., 1995; Webb *et al*., 2002; Atef *et al*., 2005), is a sufficient stimulus to trigger maturation when the oocyte is cytoplasmically mature and capable of undergoing spontaneous maturation. However, when oocytes are cytoplasmically immature and incapable of maturing spontaneously, the interruption of communication via gap junctions is not sufficient to induce GVBD.

Our results support the idea that communication between oocytes and follicle cells during the reproductive period is necessary to maintain meiotic arrest through the passage of inhibitory molecules towards the oocyte. However, these results also show that during the non-reproductive period some other factor is required to induce GVBD, probably an activator. The results also indicate that the chemical agents employed exerted no harmful effects on the follicle cell membrane or on the oocyte plasma membrane that could alter the normal mechanism of interaction between progesterone and its receptor.

The analysis of the effects of LH treatment on incapable oocytes plus follicle cells and incapable denuded oocytes shows that this hormone stimulates meiotic resumption in follicles but not in the denuded oocytes, in a way similar to that described for mammals (Lawrence *et al*., 1980; Buccione *et al*., 1990; Mattioli, 1994). This would indicate that oocytes from this species have no LH receptors, so that maturation induction should be mediated by the somatic components of the follicle, which possess these receptors.

Interestingly, our results show that LH can induce GVBD in both capable and incapable oocytes as long as follicle cells are present, which leads us to assume the need for a communication pathway between oocyte and follicle cells. This possibility was analysed by combining LH treatment with uncoupling agents and the results showed that maturation induction with LH required a cell–cell coupling pathway, as the uncoupling of gap junctions decreases the percentages of GVBD. The necessity for oocyte–follicle cell communication via gap junctions via the LH effect on meiotic resumption suggests that the hormone could also act through the transference of a maturation stimulatory signal.

These results are supported by studies in mouse (Eppig, 1982; Salustri & Siracusa, 1983; Eppig & Downs, 1988) and in pig oocytes (Motlik *et al*., 1989), which show that cumulus–oocyte coupling is maintained during the period of meiotic induction and does not decrease until maturation has started. Current reports indicate that LH-induced maturation involves an increase in Ca^{2+} in granulosa cells and that Ca^{2+} reaches the oocyte via gap junctions (Eppig, 1993; Homa *et al*., 1993; Mattioli *et al*., 1998; Mattioli & Barboni, 2000). The studies of Davis *et al*. (1986) and of Dimino *et al*. (1987) show that LH is responsible for the increase in Ca^{2+} in granulosa cells. Moreover, Allbritton *et al*. (1992) proposed that IP_3 is the messenger that diffuses towards the oocyte, as Ca^{2+} is rapidly sequestered in the cytosol.

The observation that, in *Bufo arenarum*, the inhibition of the IP_3 receptor by heparin is capable of inhibiting LH-induced GVBD by up to 30% supports the idea that the maturation stimulator transferred from the follicle cells to the oocyte could be IP_3. Conversely, the observation that the inhibition of PDE by theophylline almost completely inhibits GVBD in incapable oocytes with follicle cells treated with LH suggests that the activity of this enzyme is important in the process. During the non-reproductive period, stimulation with LH would increase the passage of IP_3 and Ca^{2+} through gap junctions, which would induce an activation of phosphodiesterase, responsible for hydrolyzing cAMP to 5′AMP and thus allowing meiotic resumption.

On the basis of the results obtained, we propose that the higher quantity of phospholipids present in the oocytes during the reproductive period (capable oocytes), especially of PC, PE, AP and PI, precursors of second messengers such as DAG and IP_3, would permit meiotic resumption when passage of inhibitors, such as cAMP from the follicle cells via the gap junctions is blocked. In the case of oocytes from the non-reproductive period (incapable oocytes), the block in oocyte–follicle cell communication would not be sufficient for meiotic resumption. In this case, a positive stimulus would be required to bypass the effect of the inhibitors. The experiments with LH suggest that this hormone might induce GVBD through the passage of IP_3 or Ca^{2+} via gap junctions, which would increase the level of Ca^{2+} in the cytoplasm of the oocyte, activating the PDE and thus contributing to the decrease in the levels of cAMP and permitting meiotic resumption.

Acknowledgements

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