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Involvement of G protein and purines in *Rhinella arenarum* oocyte maturation

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Summary

We investigated the participation of $G_{\alpha i}$ protein and of intracellular cAMP levels on spontaneous and progesterone-mediated maturation in *Rhinella arenarum* fully grown follicles and denuded oocytes.

Although progesterone is the established maturation inducer in amphibians, *Rhinella arenarum* oocytes obtained during the reproductive period (competent oocytes) resume meiosis with no need for an exogenous hormonal stimulus if deprived of their enveloping follicular cells, a phenomenon called spontaneous maturation. In amphibian oocytes, numerous signalling mechanisms have been involved in the rapid, non-genomic, membrane effects of progesterone, but most of these are not fully understood.

The data presented here demonstrate that activation of the $G_{\alpha i}$ protein by Mas-7 induced maturation in non-competent oocytes and also an increase in GVBD (germinal vesicle breakdown) in competent oocytes. Similar results were obtained with intact follicles independent of the season. The activation of adenylyl cyclase (AC) by forskolin seems to inhibit both spontaneous and progesterone-induced GVBD. In addition, the high intracellular levels of cAMP caused by activation of AC by forskolin treatment or addition of db-cAMP inhibited maturation that had been induced by Mas-7 and in a dose-dependent manner. Treatment with H-89, a protein kinase A (PKA) inhibitor, was able to trigger GVBD in a dose-dependent manner in non-competent oocytes and increased the percentages of GVBD in oocytes competent to mature spontaneously. The results obtained with whole follicles and denuded oocytes were similar, which suggested that effects on AC and PKA were not mediated by follicle cells. The fact that Mas-7 was able to induce maturation in non-competent oocytes in a similar manner to progesterone and to increase spontaneous maturation suggests that $G_{\alpha i}$ activation could be an important step in meiosis resumption. Thus, the decrease in cAMP as a result of the regulation of the G proteins on AC and the inactivation of PKA by H-89 could contribute to the activation of MPF (maturation promoting factor) and induce maturation of the oocytes of *Rhinella arenarum.*

Keywords: Adenylyl cyclase, Amphibian, G protein, Oocytes maturation, Protein kinase A

Introduction

In amphibians, during maturation, the fully grown oocyte reinitiates meiosis and acquires the competence needed for its later fertilization. Knowledge of the molecular mechanisms that regulate this process is essential to understand the regulation of the cell cycle and the transduction of signals in the cell (Voronina & Wessell, 2004).

Although maturation takes place in the oocytes of all species, the stimulus varies, and the signal required in each case is specific. The resumption of meiosis in fully grown amphibian oocytes is induced by progesterone produced by the follicular cells. This hormone acts non-transcriptionally to cause germinal vesicle breakdown (GVBD) by interaction with a receptor in the oocytes surface (Maller, 2001). In addition, progesterone has no effect on oocyte maturation when it is injected into the oocyte (Masui & Shibuya, 1987). Progesterone starts a cascade of transmembrane signalling events leading to the activation of a key regulator of G_2 to M phase

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transition, the cytoplasm maturation promoting factor (MPF), a complex of the cyclin-dependent kinase $p34^{cdc2}$ and cyclin B that induces GVBD (Sánchez Toranzo *et al.*, 2006).

The nature of the signal that induces oocytes maturation could be a positive stimulus received by the oocytes or the removal of an inhibitor present in the oocytes or produced by the ovary that maintains prophase arrest. In amphibians, progesterone-induced maturation is evidence of the former hypothesis (Voronina & Wessel, 2003). The oocytes of some species can reinitiate meiosis spontaneously when the surrounding ovarian tissues are removed, which supports the hypothesis of the existence of a maturation inhibitory substance. *Rhinella arenarum (*ex *Bufo arenarum*) oocytes reinitiate meiosis with no need of an exogenous hormonal stimulus when deprived of the 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 that were obtained during the reproductive period. Oocytes collected during this period can be considered to be competent to mature spontaneously, in contrast to those in the non-reproductive period, which are non-competent. Interestingly, fully grown Rhinella *arenarum* oocytes always respond to progesterone regardless of the season in which they are obtained (Zelarayán *et al.*, 1995). Up to now, the signalling pathways involved in spontaneous and progesterone-induced maturation have not been completely understood.

A large number of signalling mechanisms and second messengers have been implicated in the progesterone-induced maturation of oocytes. There is a general consensus that a transient decrease in cAMP intracellular levels, resulting at least in part from the inhibition of membrane adenylyl cyclase (AC) activity, is an obligatory step in the mechanism by which progesterone induces oocytes maturation (Kwon *et al.*, 1989; Zelarayán *et al.*, 2000). In frogs, steroids such as progesterone cause a decrease in AC activity, resulting in a decrease in cAMP levels within minutes of steroid addition (Cicirelli & Smith, 1985). It has been hypothesized that a decrease in cAMP concentrations in the oocytes is sufficient to promote maturation in *Rhinella arenaru*m oocytes (Zelarayán *et al.*, 1995), presumably through inhibition of cAMP-dependent kinase (PKA) activity leading to MPF activation and to GVBD. In *Rhinella arenarum* oocytes, we demonstrated that the increase in intracellular levels of purines such as cAMP or guanosine can inhibit progesterone-induced maturation reversibly. Alternatively, the pharmacological blockade of phosphodiesterase with theophyline prevented meiotic resumption (Zelarayán *et al.*, 2000; Sánchez Toranzo *et al.*, 2006). Experimental results in mouse oocytes have shown that high intra-oocytes cAMP levels prevent the spontaneous resumption of meiosis (Cho *et al.*, 1974). These results suggest that oocyte maturation is governed by a fall in intracellular cAMP levels and subsequent inactivation of PKA, which exerts an important control on oocyte maturation. In fact, in amphibians, it has been initially demonstrated that an active PKA is responsible for the maintenance of oocytes in prophase (Maller & Krebs, 1977).

One important signalling molecule involved in cAMP synthesis is the heterotrimeric G protein that may mediate downregulation of AC. This situation leads to a transient reduction in cAMP levels. In contrast, the inhibitory action of progesterone on GTP-dependent AC associated to the oocytes plasma membrane suggests that the hormone receptor could be linked to a G protein.

There are two alternative models to explain the participation of the G proteins in oocyte maturation. In the first, these proteins are supposed to be actively involved in the arrest of the prophase (mouse and *Xenopus* models) and this arrest is supposedly caused because the increase in cAMP is mediated by the activation of a Gαs whose target is adenylyl cyclase (AC). This increase would be mediated by a Gα or by the dimer βγ (Lutz *et al.*, 2000; Romo *et al.*, 2002). In sea urchin, in contrast, maturation occurs spontaneously when the oocyte is separated from the tissues that surround it in the ovary. In this case, meiosis resumption seems to depend on the activation of a Gαi and on the release of the dimer βγ (Voronina & Wessel, 2004). In this sense, these authors demonstrated that the different types of Gα subunits are present in the oocyte during all growth stages, are associated with the cortical granules and are translocated to the cell surface when the oocyte matures. In brief, the association of G proteins to the plasma membrana could mediate the signalling that leads to maturation.

The aim of this work was to study the participation of $G_{\alpha i}$ proteins in the regulation of cAMP intracellular levels in competent and non-competent oocytes of *Rhinella arenarum* during the maturation process.

Materials and methods

Animals

Adult specimens of *Rhinella 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.

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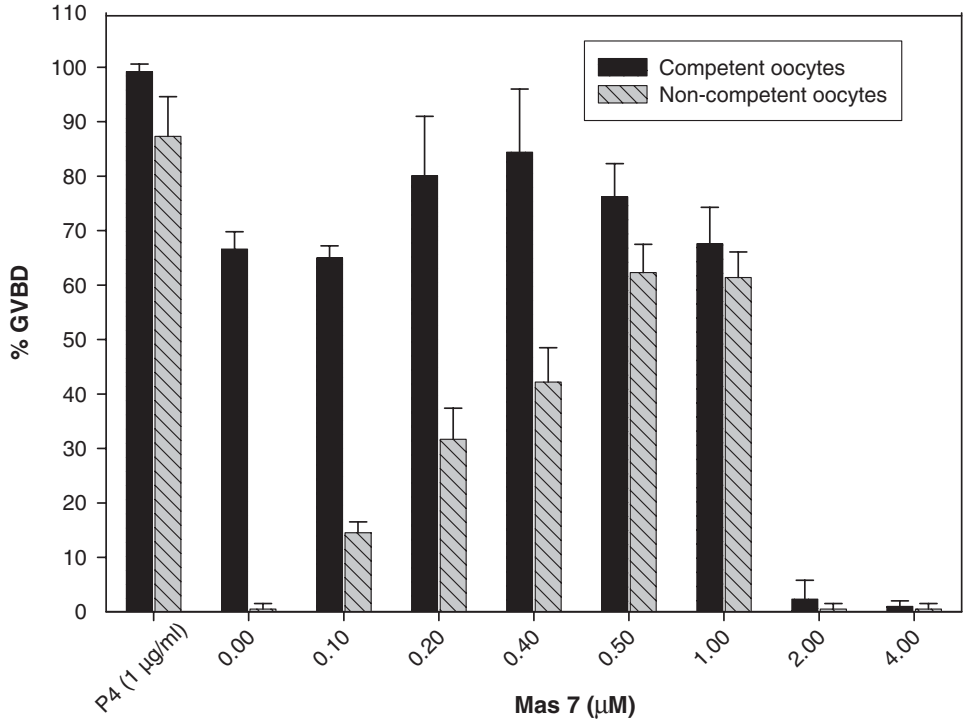

Figure 1 Effect of $G_{\alpha i}$ activation by mastoparan on nuclear maturation. Fully grown denuded oocytes competent and incompetent to mature spontaneously were incubated in amphibian Ringer solution (AR) with different doses of Mas-7 (0.10–4.00 μM) for 20–40 min. After careful washing with AR, the oocytes were incubated in AR for 18–20 h. Control oocytes were exposed to progesterone (1 μg/ml) all the time. Germinal vesicle breakdown (GVBD) was scored after 18–20 h of culture at 25 °C. Values represent the mean ± standard error of the mean (SEM) ($n = 4$) of experiments carried out in different animals.

In vitro follicle and denuded oocytes culture

Experimental manipulation and culture were performed at room temperature (22–25 °C) in amphibian Ringer solution (AR) (6.6 g/l NaCl, 0.15 g/l $CaCl_2$ and 0.15 g/l KCl) 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 under a dissecting microscope (Zelarayán et at., 1995). 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, USA). Randomized samples of 20 oocytes were distributed into separate wells that contained 2 ml of AR. 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 detection of germinal vesicle breakdown (GVBD) 18–20 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 in 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 with those obtained during the non-reproductive period (May–August), which was considered non-competent (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.

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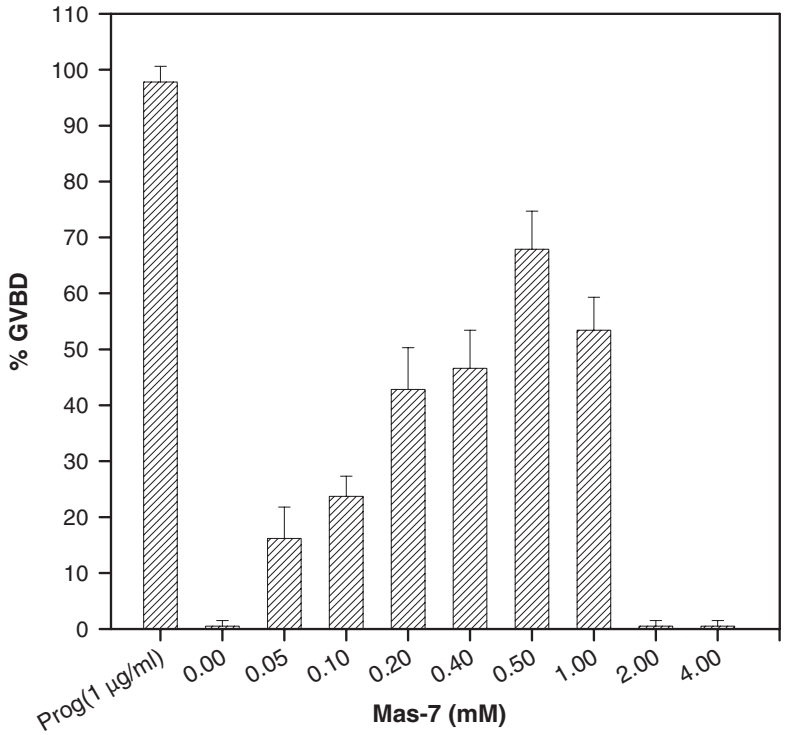

Figure 2 Effect of Mas-7 on *Rhinella arenarum* follicles. The fully grown follicles were exposed to different doses of Mas-7 (0.10–4.00 μM) for 20–40 min. After careful washing with amphibian Ringer solution (AR), the follicles were incubated in AR for 18–20 h. Control follicles were exposed to progesterone (1 μg/ml) all the time. Germinal vesicle breakdown (GVBD) was scored after 18–20 h of culture at 25 °C. Values represent the mean ± standard error of the mean (SEM) ($n = 5$) of experiments carried out in different animals.

The stock solution of H-89 (1 mg/ml), a PKA inhibitor, was prepared in AR:ethanol (4:6). This solution was maintained at –80 °C.

Mas-7, a Gi activator, was dissolved in AR distilled water in order to obtain a stock solution of 60 mM. This solution was kept at –20 °C.

Forskolin, an AC activator (Seamond *et al.*, 1981) was prepared in ethanol: propylene glycol (1:1) and kept at –20 °C (stock solution 4 mM).

Dibutyryl cAMP (db-cAMP) was dissolved in AR and various doses were added to the culture medium at a constant volume (5 μl) 60 min before the extraction of follicular cells or supplemented with progesterone or Mas-7.

Results

Effect of $G_{\alpha i}$ activation on nuclear maturation

The effect of $G_{\alpha i}$ activation on oocytes maturation was assayed using mastoparan (Mas-7), a bee venom toxin that, *in vitro*, activates selectively $G_{\alpha i}$ and $G_{\alpha o}$ but not $G_{\alpha s}$ (Higashijima *et al.*, 1990).

Denuded oocytes that were competent and incompetent to mature spontaneously were incubated in AR at different doses of Mas-7 (0.10–4.00 μM) for 20–40 min. Then the oocytes were carefully washed in AR and the incubation time was completed at 18–20 h only in AR. Continuous exposure to Mas-7 at any of the assayed doses caused lysis. The control denuded oocytes treated only with progesterone were incubated permanently with the hormone. GVBD was scored after 18–20 h of culture at 25 °C.

Incubation in Mas-7 (0.10–1.00 μM) stimulated GVBD in a dose-dependent manner (Fig. 1) in competent and non-competent oocytes. However, in both cases concentrations above 2 μM were toxic to the oocytes, as shown by gradual cellular lyses or excessive increase in oocytes volume during the first incubation hours. On the basis of the above results, the dose chosen for Mas-7 for the subsequent experiments was 0.50 μM. Figure 1 shows that with this dose about 61 ± 4% of the incompetent oocytes matured without impairment of oocyte viability. In the case of competent oocytes, a dose of 0.40 μM induced the greatest increase in GVBD, about 20% with respect to the controls, which suggested that these oocytes were more sensitive to treatment with Mas-7.

Similar results were obtained when intact follicles, obtained during the non-reproductive period, were incubated with different doses of Mas-7 in the absence of progesterone (Fig. 2). A significant increase in the

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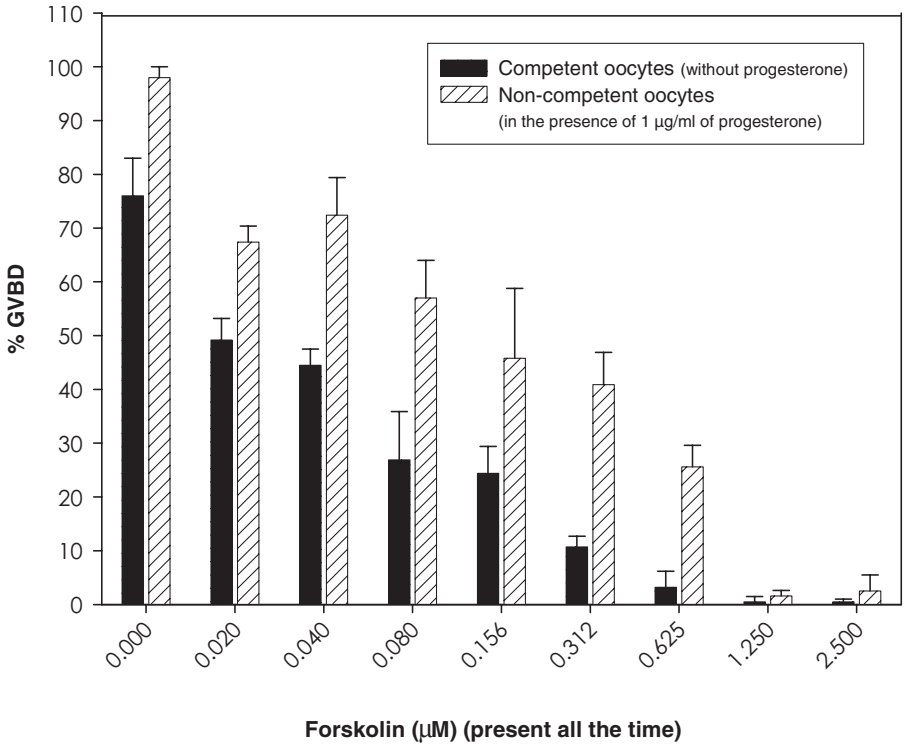

Figure 3 Effects of adenylyl cyclase (AC) activation on oocytes nuclear maturation. Dose–response studies of forskolin, on spontaneous and progesterone-induced oocytes maturation *in vitro.* Fully grown denuded oocytes incompetent to mature spontaneously were incubated in progesterone for 20 h with different doses of forskolin (0.020–2.500 µM). Fully grown denuded oocytes competent to mature spontaneously were cultured in forskolin (0.020–2.500 µM) for 30 min before removal of follicle cells. Germinal vesicle breakdown (GVBD) was checked after 20 h in both cases. Values represent the mean ± standard error of the mean (SEM) ($n = 4$) of experiments carried out in different animals.

percentage of GVBD was obtained with Mas-7 doses higher than 0.20 µM.

Denuded non-competent oocytes and intact follicles were cultured in AR with Mas-7 (0.50 µM) for 24 h at 25 °C and examined for GVBD at different times during culture. After 8 h of incubation, oocytes and follicles underwent GVBD (with a white spot in the animal pole) but by 16–18 h the maximal response was achieved. In both cases they exhibited metaphase II 18–20 h after Mas-7 had induced maturation.

In brief, stimulation of $G_{\alpha i}$ by Mas-7 induced meiosis resumption in denuded competent and incompetent oocytes as well as in the follicles at times similar to those obtained with progesterone treatment.

Effects of AC activation on nuclear maturation

In order to evaluate the effects of AC activation on spontaneous and progesterone-induced oocytes maturation, different doses (0.020–1.250 µM) of forskolin, an adenylyl cyclase activator, were added to the culture medium 30 min before hormone addition (1 µg/ml) or before removal of follicular cells.

As shown in Fig. 3, forskolin inhibited maturation in a dose-dependent manner in competent oocytes and progesterone-induced maturation in non-competent ones, although the former were more sensitive to this treatment than the latter. With this treatment, at doses of 0.625 µM of forskolin, nuclear oocytes maturation was abolished.

Activation of AC by forskolin appears to inhibit both spontaneous and progesterone-induced GVBD.

Next, we assayed the ability of Mas-7 to induce oocytes maturation in the presence of forskolin.

Denuded oocytes non-competent to mature spontaneously were cultured in AR with different doses of forskolin (0.312–5.000 µM) 30 min before the addition of Mas-7 (0.5 µM). Oocytes were left in the presence of forskolin plus Mas-7 for an additional 20–40 min, then washed to remove this medium, and placed back in the presence of forskolin alone for the rest of the culture time (18–20 h). Results (Fig. 4) indicated that activation of AC with forskolin inhibited the maturation induced by Mas-7 in a dose-dependent manner. The GVBD induced by Mas-7 was significantly inhibited by doses of 1.250 µM of forskolin.

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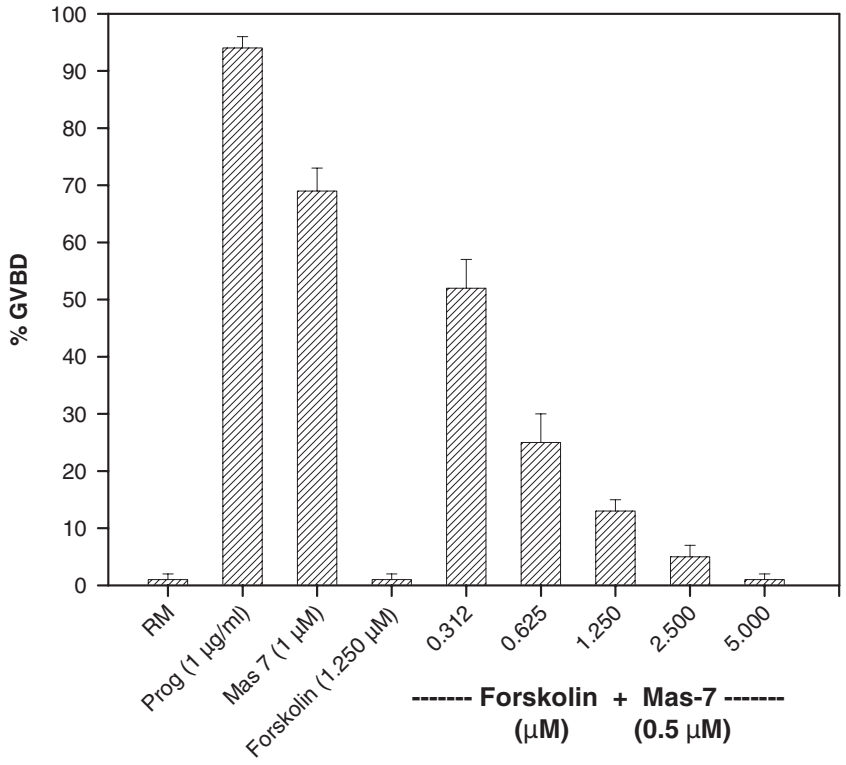

Figure 4 Effects of adenylyl cyclase (AC) activation on Mas- 7 induced Germinal vesicle breakdown (GVBD). Dose-response studies of forskolin on Mas-7 (0.5 μM) induced oocytes maturation. Fully grown non-competent oocytes were precultured for 30 min in different doses of forskolin (0.312–5.000 μM) and then Mas-7 was added. The oocytes were incubated for an additional 20 h and the GVBD was examined after culture. Values represent the mean ± standard error of the mean (SEM) ($n = 5$) of experiments carried out in different animals.

Effects of db-cAMP on Mas-7 induced maturation

There is a general consensus that a transient decrease in cAMP levels is an obligatory step in the mechanism by which progesterone induces oocytes maturation (Morrill *et al.*, 1977).

In order to determine whether Mas-7-induced maturation was dependent on intracellular levels of cAMP, denuded non-competent oocytes were cultured in AR or AR plus Mas-7 (0.5 μM) with different doses of db-cAMP.

Non-competent denuded oocytes were preincubated for 30 min with different doses of db-cAMP (0.025–1.0 mM). GVBD was induced with Mas-7 (0.5 μM) and was controlled after 18–20 h of culture.

The results shown in Fig. 5 indicate that db-cAMP induced a decrease in the percentage of GVBD in Mas-7-induced maturation. These experiments indicated that the increase in cAMP intracellular levels inhibited mastoparan-induced maturation in a dose-dependent manner. The GVBD induced by Mas-7 was inhibited significantly by doses of 0.25 mM of db-cAMP.

We demonstrated that high intracellular levels of purines can inhibit both spontaneous and progesterone-induced maturation in fully grown denuded Rhinella *arenarum* oocytes (Zelarayán *et al.*, 2000). However, when maturation was induced by Mas-7, lower doses were required to inhibit maturation, as 0.25 mM of db-cAMP reduced the percentages of GVBD to 18 ± 4%.

In the case of competent oocytes treated with Mas-7, meiosis resumption was also inhibited by db-cAMP (results not shown).

Effect of PKA inhibition on oocytes maturation

In amphibians, cAMP levels regulate the activity of PKA, whose inhibition leads to MPF activation and to GVBD.

In order to evaluate the role of PKA during the process of oocyte maturation, we investigated the effect of several doses of H-89, a synthetic inhibitor of this enzyme, on non-competent denuded oocytes.

Denuded oocytes competent and non-competent to mature spontaneously were incubated in different doses of H-89 (2.5–50.0 μM) at 25 °C. In all cases, GVBD was scored after 18 h of culture in AR.

The competence of the oocytes to undergo GVBD was tested using exogenous added progesterone

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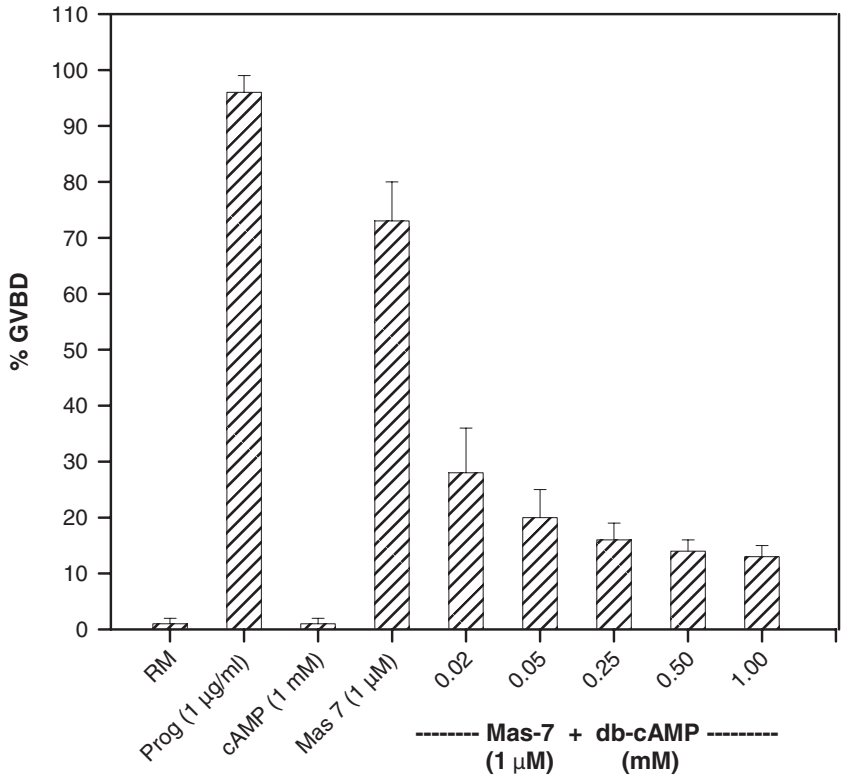

Figure 5 Effect of db-AMP on oocyte maturation induced by Mas-7. Fully grown denuded oocytes non-competent to mature spontaneously were preincubated for 30 min in AR with in different doses of db-cAMP (0.025–1.0 mM) before the addition of Mas-7 (0.5 µM). Full-grown denuded oocytes competent to mature spontaneously were preincubated in the same manner before the extraction of follicular layers. Germinal vesicle breakdown (GVBD) was controlled after 18–20 h of culture at 25 °C. Values represent the mean ± SEM ($n = 4$) of experiments carried out in different animals.

(1 µg/ml), the physiological oocyte maturation inducer in *Rhinella arenarum.*

Results (Fig. 6) showed that PKA inhibition with H-89 was able to trigger GVBD in a dose-dependent manner in competent and non-competent oocytes.

Doses of 30 µM of H-89 were able to obtain GVBD percentages (94 ± 8) similar to the ones obtained with progesterone (98 ± 2) in both types of oocytes. The time required for GVBD was similar in both cases (results not shown).

As in all cases our experiments were performed in denuded oocytes, we consider that the effect of H-89 on meiosis resumption is mediated by the direct action of the PKA activator on the oocytes and not by its effect on the follicle cells.

Discussion

The results obtained in this work further ones in our previous published reports with respect to signalling mechanisms in the spontaneous and progesterone-induced maturation in oocytes of *Rhinella arenarum.*

Our results indicate that the activation of the $G_{\alpha i}$ protein with Mas-7 was sufficient to induce maturation in non-competent oocytes. The maximal response was obtained with doses of 0.5–1.0 µM. Similar results were obtained when the follicles were treated with the $G_{\alpha i}$ activator during reproductive and non-reproductive periods.

Both the chronology and the morphological signs of Mas-7-induced maturation in denuded oocytes and follicles were similar to the ones obtained by progesterone treatment in which 60% GVBD was observed after 6–7 h of culture (Zelarayán *et al.*, 1995).

The activation of Gi-mediated signalling by Mas-7 enhanced spontaneous maturation in *Rhinella arenarum* competent oocytes that had been deprived of follicular cells in a dose-dependent manner.

Our results agree with those reported for sea urchin (Voronina & Wesell, 2000), in which meiosis resumption seems to depend on the activation of a $G_{\alpha i}$. It has been reported that $G_{\alpha i}$, $G_{\alpha s}$ and $G_{\alpha q}$ are present on the plasma membrane of sea urchin oocytes during maturation and that these proteins remain in eggs and embryonic tissues. They concluded that $G_{\alpha i}$ is involved in the maturation of sea urchin oocytes and probably Gαi protein localization would change to the cell surface when GVBD occurred. In this way, when

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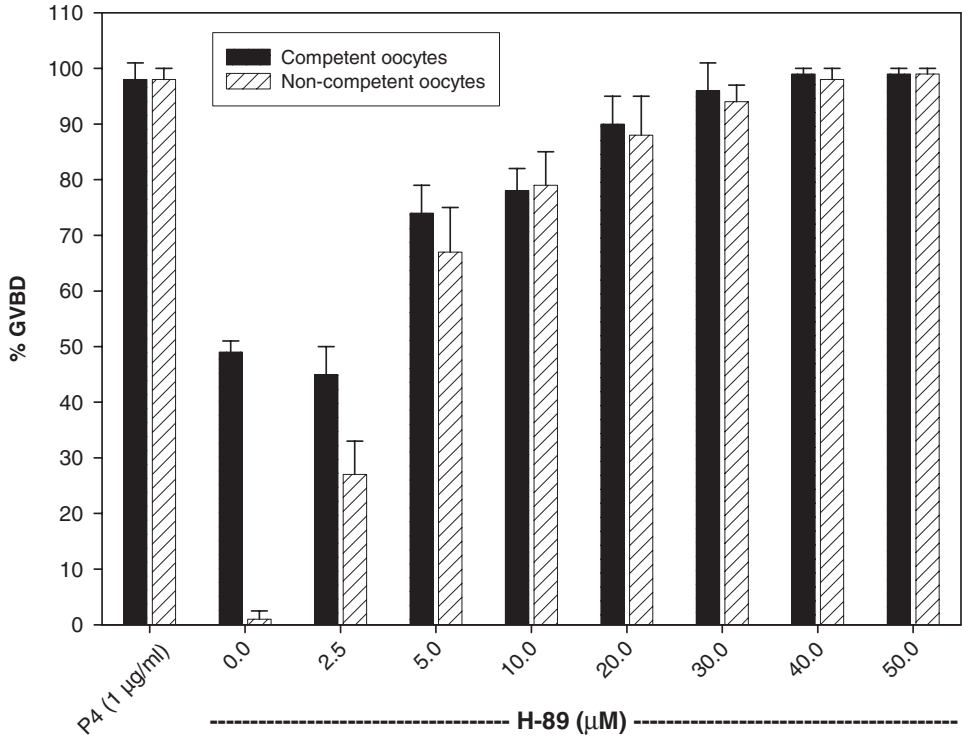

Figure 6 Effect of H-89 on oocytes maturation. Fully grown denuded oocytes competent and non-competent to mature spontaneously were incubated in different doses of H-89 (2.5–50.0 µM) at 25 °C. In all cases the germinal vesicle breakdown (GVBD) was scored after 18 h of culture in AR. Values represent the mean ± standard error of the mean (SEM) ($n = 5$) of experiments carried out in different animals.

$G_{\alpha i}$ subunits are located in the oocytes membrane, they interact with the maturation inducer and mediate the events leading to maturation.

In *Rhinella arenarum* oocytes the activation of $G_{\alpha i}$ signalling in response to Mas-7 led to meiosis resumption within a time period similar to the one required for spontaneous or progesterone-induced maturation. This factor means that, by 6–8 h, 50% of the oocytes stimulated with Mas-7 were able to undergo GVBD.

Different results suggest that in *Xenopus* and in mouse oocytes (Romo *et al.*, 2002; Lutz *et al.*, 2000) the constitutive activation of $G_{\alpha s}$ would be responsible for maintaining meiotic arrest by stimulation of AC activity and maintainance of elevated cAMP levels (Kalinowsky *et al.*, 2004). The inhibition of the activity of this Gαs and the reduction in cAMP levels and PKA activity would enable meiosis resumption (Gallo *et al.*, 1995).

Other evidence suggests that activation of $G_{\alpha i}$ is necessary for maturation (Thomas *et al.*, 2002). In *Xenopus* oocytes, Sheng *et al.* (2001) suggested that the action of progesterone is not mediated by the activation of classical $G_{\alpha i}$ proteins. However, the injection of neutralizing antibodies against $G_{\alpha s}$ causes oocyte maturation, suggesting that *Xenopus* endogenous $G_{\alpha s}$ plays a dominant role in maintainance of prophase arrest (Sheng *et al.*, 2004). Interestingly, Gallo *et al.* (1995) showed that an antibody against $G_{\alpha s}$ inhibited αs activity in frog oocyte membranes and stimulated maturation when injected into frog oocytes. Their results suggest that progesterone acts by inhibiting Gαs at an early point in the pathway and leads to oocytes maturation (Gallo *et al.*, 1995).

In amphibian oocytes, one signalling pathway regulated by progesterone appears to be a decrease in cAMP levels that is necessary for progesterone-induced maturation. In this sense, inhibition of oocytes AC is involved in hormone binding to the plasma membrane receptor (Sadler & Maller, 1982).

Many results suggest that progesterone induces a reduction in cAMP probably by inhibiting membrane-bound AC. Consequently, as the αi subunits of the G proteins regulate AC activity negatively and decrease cAMP intracellular levels, our results might indicate that Mas-7-induced oocyte maturation could be due to a transient decrease in intracellular cAMP.

In agreement with the above, our results (Fig. 3) show that forskolin, an activator of AC, inhibited spontaneous and progesterone-induced maturation in a dose-dependent manner. In general, spontaneous maturation was more sensitive to forskolin inhibition

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than was progesterone-induced maturation on denuded oocytes.

In addition, we demonstrated previously that higher intracellular levels of purines (cAMP or guanosine) can inhibit reversibly the progesterone and insulin-induced maturation process in *Rhinella arenarum* as well as spontaneous maturation (Zelarayan *et al.*, 2000; Sánchez Toranzo *et al.*, 2004).

In *Rana dybowskii* (Kwon *et al.*, 1990), the inhibition of AC by transient exposure of follicles to forskolin induced the increase in intracellular levels of cAMP in follicle cells that had stimulated progesterone production and the hormone acted on the oocytes to trigger maturation when cAMP decreased after removal of forskolin from the medium. In *Rhinella arenarum,* using a similar procedure, we precultured isolated follicles in AR plus forskolin and then the follicles were incubated in AR alone for an additional 20 h. These follicles did not mature, although control follicles from the same animal matured in the presence of progesterone (results not shown).

In contrast, preincubation of competent and non-competent oocytes with forskolin blocked both spontaneous and progesterone-induced maturation. In a similar way, when the oocytes were exposed to forskolin (Fig. 4) the maturation induced by Mas-7 was abolished.

These results suggest that while AC activity is stimulated by forskolin, $G_{\alpha i}$ activation by Mas-7 is not able to induce meiosis resumption in denuded oocytes. In our experimental conditions, probably the intracellular level of cAMP does not decline, which does not allow oocytes to mature in response to $G_{\alpha i}$ activation.

With respect to the participation of PKA in meiosis resumption, our results would indicate that PKA inhibition with H-89 could be related to the induction of GVBD in competent and non-competent oocytes of *Rhinella arenarum,* in a way similar to that found in follicles of *Fundulus heteroclitus* (Cerdá *et al.*, 1997).

In conclusion, our results suggest that $G_{\alpha i}$ activation by Mas-7 could be an important step in the resumption of meiosis of denuded competent and incompetent oocytes and follicles. In addition, the decreases in cAMP as a result of the regulation of the G proteins on AC and PKA inactivation by H-89 could contribute to MPF activation and induce maturation in the oocytes of *Rhinella arenarum.*

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