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Involvement of $GABA_A$ receptor in *Bufo arenarum* oocyte maturation

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Summary

Amphibian oocytes meiotic arrest is released under the stimulus of progesterone; this hormone interacts with the oocyte surface and starts a cascade of events leading to the activation of a cytoplasmic maturation promoting factor (MPF) that induces germinal vesicle breakdown (GVBD), chromosome condensation and extrusion of the first polar body.

The aim of this work was to determine whether the activation of a $GABA_A$ receptor is able to induce GVBD in fully grown denuded oocytes of *Bufo arenarum* and to analyse its possible participation in progesterone-induced maturation. We also evaluated the role of purines and phospholipids in the maturation process induced by a $GABA_A$ receptor agonist such as muscimol.

Our results indicated that the activation of the $GABA_A$ receptor by muscimol induces maturation in a dose- and time-dependent manner and that this activation is a genuine maturation that enables oocytes to form pronuclei. Assays with a receptor antagonist, picrotoxine, showed that the maturation induced by muscimol was inhibited. Treatment with picrotoxine, however, shows that the participation of $GABA_A$ receptor in progesterone-induced maturation is not significant.

In addition, our results indicate that high intracellular levels of purines obtained by the use of db-AMPc and theophylline or the inhibition of the phosphatidylinositol 4,5-bisphosphate (PIP_2 hydrolysis by neomycin and PIP_2 turn over by LiCl, respectively, inhibited the maturation induced by muscimol. Treatment with H-7 indicated, however, that PKC activation is not necessary for GVBD induced by the $GABA_A$ receptor agonist. Results suggest that the transduction pathway used by the $GABA_A$ receptor to induce maturation is different from those used by progesterone.

Keywords: *Bufo arenarum*, $GABA_A$ receptor, Oocyte maturation

Introduction

In amphibians, fully grown oocytes are arrested at G_2, at the beginning of meiosis I. This meiotic arrest is released under the stimulus of progesterone, a process termed oocyte maturation (Fortune *et al.*, 1975; Schuetz, 1985). Progesterone interacts with the oocyte surface to trigger a complex chain of reactions that induce germinal vesicle breakdown (GVBD), chromosome condensation and extrusion of the first polar body to produce a fertilizable egg.

A maturation-inducing substance (MIS) binds to the receptor located on the oocyte plasma membrane to produce rapid changes in intracellular signalling pathways, ultimately leading to the formation of a maturation-promoting factor (MPF), a complex formed by cdc2 kinase and cyclin B protein. While MPF activation is universal to oocyte maturation, the signalling events that lead to MPF activation are quite variable among species (Yamashita *et al.*, 2002; Klima *et al.*, 2004; Voronina *et al.*, 2004).

Although progesterone is the established maturation inducer in *Bufo arenarum*, meiosis resumption also occurs spontaneously when follicular cells are removed and oocytes are cultured *in vitro* under suitable conditions. In this species it is possible to obtain oocytes competent or non-competent to undergo spontaneous maturation according to the seasonal period in which animals were captured (Zelarayán *et al.*, 1995). Interestingly, fully grown *Bufo arenarum* oocytes always respond

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to progesterone, independently of the time of the year in which they are obtained (Zelarayán *et al.*, 1996).

Studies concerning oocyte maturation in species as diverse as amphibians, starfish and mammals report the identical conclusion that levels of the second messenger cAMP in the oocytes play a critical role in maintaining meiotic arrest (Ferrell, 1999). In this sense, it has been proposed that cAMP diffuses from follicle cells to the oocytes through heterologous gap junction coupling (Eppig, 1991; Webb *et al.*, 2002).

One common signalling event initiated upon hormonal induction of oocyte maturation in amphibians, starfish and lower vertebrates is a decrease in oocyte cAMP levels caused, at least partly, by the activity of the G protein in the plasma membrane (Sadler & Maller, 1980, 1981) during the period in which oocytes resume meiosis. cAMP levels would be enhanced by a concomitant stimulation with either membrane-permeable cAMP analogues or phosphodiesterase (PDE) inhibitors that prevent maturation *in vitro* (Sánchez Toranzo *et al.*, 2004). High cAMP levels in the oocyte maintain the oocyte in meiotic arrest, probably via the protein kinase A (PKA) pathway. PKA in an active state causes phosphorylation of unknown protein substrates.

The earliest known biochemical events in oocytes treated with progesterone are a rapid decrease in cAMP caused by the inhibition of adenylate cyclase activity related, at least partly, to the activity of the G protein in the plasma membrane (Sadler & Maller, 1980, 1981) and complex changes in phospholipid metabolism (Morrill & Kostellow, 1999).

It has been hypothesized that a decrease in cAMP concentrations in the oocytes is sufficient to promote maturation in *Bufo arenarum* (Zelarayán *et al.*, 1996) and mouse oocytes, presumably through inhibition of cAMP-dependent protein kinase activity leading to MPF activation.

Another event during oocyte maturation in many species is the generation of second messengers, such as the ones generated by the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2). The hormonal stimulus involves a receptor-dependent hydrolysis of inositol phospholipids by phospholipase C (PLC) to generate two second messengers, inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). In this sense, DAG is involved in the regulation of protein kinase C (PKC) activity (Nishizuka, 1984). The activation of PKC is thought to be involved in the initial response of amphibian oocytes to agonists that induce GVBD (Smith, 1989; Zelarayán *et al.*, 1996). In addition, IP3 is released into the cytosol and acts to mobilize intracellular Ca^{2+} (Berridge & Irvine, 1989; Downes, 1989; Fissore *et al.*, 1999).

Recent studies have shown that, in *Bufo arenarum*, the inhibition of PIP_2 hydrolysis by treatment of denuded oocytes with neomycin totally blocks progesterone-induced maturation, suggesting that the products of this hydrolysis (DAG and IP3) are involved in the pathway normally used by progesterone in this species (Zelarayán *et al.*, 2000).

Lithium, which blocks phosphoinositide metabolism (Hanson, 1991), has been reported to exert an inhibitory effect on the maturation of mouse (Pesty *et al.*, 1994), starfish (Picard & Dorée, 1983) and *Bufo arenarum* oocytes (Sánchez Toranzo *et al.*, 2004).

$GABA_A$ receptor, which is coupled to chloride ion channels, is an oligomeric complex with binding sites for direct $GABA_A$ receptor agonists such as muscimol and antagonists such as picrotoxine. $GABA_A$ receptor has been found both in neuronal and in several non-neuronal mammalian cells including pancreatic α_2-cells (Rorsman *et al.*, 1989), pars intermedia melanotrophs (Taraskevich *et al.*, 1985) and in mammalian oviducal tissue (Laszlo *et al.*, 1989). It has been suggested that this receptor is also involved in the progesterone induction of the acrosome reaction of human sperm *in vitro* (Wistrom & Meizel, 1993). This finding agrees with the results obtained in mouse sperm that underwent acrosomal exocytose when stimulated with progesterone. This effect was inhibited by picrotoxin, the $GABA_A$ receptor antagonist (Sih & Roldan, 1995). This finding indicates that the action of progesterone in the sperm is mediated by a $GABA_A$ receptor and apparently linked to Cl^- channels (Sih & Roldan, 1995). Sperm have $GABA_A$ receptors, but the signal transduction that mediates the effect of progesterone on the acrosome reaction is not clear (Meizel, 1997).

Although the non-genomic actions of progesterone in oocyte maturation are clearly mediated by a receptor located at the oocytes plasma membrane, this receptor has not yet been characterized.

Membrane-bound $GABA_A$ receptors have not been described in oocytes. These cells, however, were used to study the $GABA_A$ receptor. In *Xenopus* oocytes the experimental procedures were carried out by injecting cells with cRNAs composed of the α_1 and β_1 subunits of the bovine $GABA_A$ receptor to express the receptor.

There is no evidence that progesterone action on oocyte meiosis resumption is mediated by a $GABA_A$-receptor-like receptor. Nevertheless, this fact was demonstrated in the acrosome reaction (Sih & Roldan, 1995). In this respect, studies performed by Covey (2001) suggest that progesterone action in oocytes involves a coupling place different from that of the $GABA_A$ receptor.

The aim of this work was to determine whether the activation of a $GABA_A$ receptor is able to induce GVBD in denuded oocytes and to analyse its possible participation in progesterone-induced

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maturation, as well as to evaluate the role of purines and phospholipids in the maturation process induced by a $GABA_A$-receptor agonist such as muscimol in fully grown denuded oocytes of *Bufo arenarum*.

Materials and methods

Animals

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

In vitro follicle and denuded oocyte culture

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

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

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

Oocyte maturation was assessed 24 h after 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 subsequent dissection of the oocytes fixed in trichloroacetic acid (TCA).

Hormones and reagents

All hormones and reagents were purchased from Sigma. Progesterone was dissolved in ethanol and added (5 μl) directly to the culture medium to give a final concentration of 2.5 μM, concentration at which the GVBD reach almost 98%. Muscimol was dissolved in NaCl until used and was used at a final concentration of 1 μM.

Defolliculated oocytes were incubated with various concentrations (0.0025–2.5 μM) of muscimol.

Dibutyryl cAMP (dbcAMP) was dissolved in AR and various doses were added to the culture medium at a constant volume (5 μl). Picrotoxin was dissolved in ethanol and different doses (10–50 μM) were added to the culture medium.

Theophylline was used at a final concentration of 0.1 μM. Stock solution of neomycin sulfate (10 mm) and LiCl (1 M) was prepared in ddH_2O. 1-(5-Isoquinolinyl sulphonyl)-2-methyl-piperazine (H-7), a stock solution of PKC antagonist was dissolved in DMSO. Various doses were added directly to the culture medium in a constant volume of vehicle (5 μl).

Insemination of ovarian oocytes

Sperm suspensions were obtained by gently disrupting the testes in 4 ml AR and centrifuging at 1085 *g* for 10 min; then, the pellet was resuspended in AR. Denuded ovarian oocytes were treated with trypsin according to Elinson (1973).

Trypsin-treated denuded oocytes (Elinson, 1973) were inseminated in 10% AR containing the diffusible factor (1 : 1) obtained according to Barbieri & Oterino (1972). A 20 μl sperm suspension (final concentration 2×10^6 sperm/ml) was added; 10 min later oocytes were placed in 10% AR for different periods and fixed for cytological examination (Bühler *et al.*, 1987).

Cytological preparations

The oocytes were fixed in Ancel & Vintemberger's solution (10% formol; 0.5% acetic acid and 0.5% NaCl), embedded in paraffin and sliced into 7 μm thick sections, which were then stained with Ehrlich hematoxylin and eosin. This method allowed us to easily observe the pronucleus (Bühler *et al.*, 1987).

Results

Effect of muscimol on denuded oocytes incompetent to mature spontaneously

Fully grown *Bufo arenarum* oocytes obtained during the winter period did not mature spontaneously, but they were able to respond to progesterone. We examined whether the treatment of an agonist of the $GABA_A$ receptor such as muscimol is able to induced GVBD.

Denuded oocytes not competent to mature spontaneously were cultured in AR with different doses of muscimol (0.0025–10 μM) for 24 h at 25 °C and examined for GVBD. Results were compared with the response of these oocytes to the same doses of progesterone. The results obtained (Fig. 1) show that muscimol induced GVBD in a dose-dependent manner. The maximum response obtained with muscimol,

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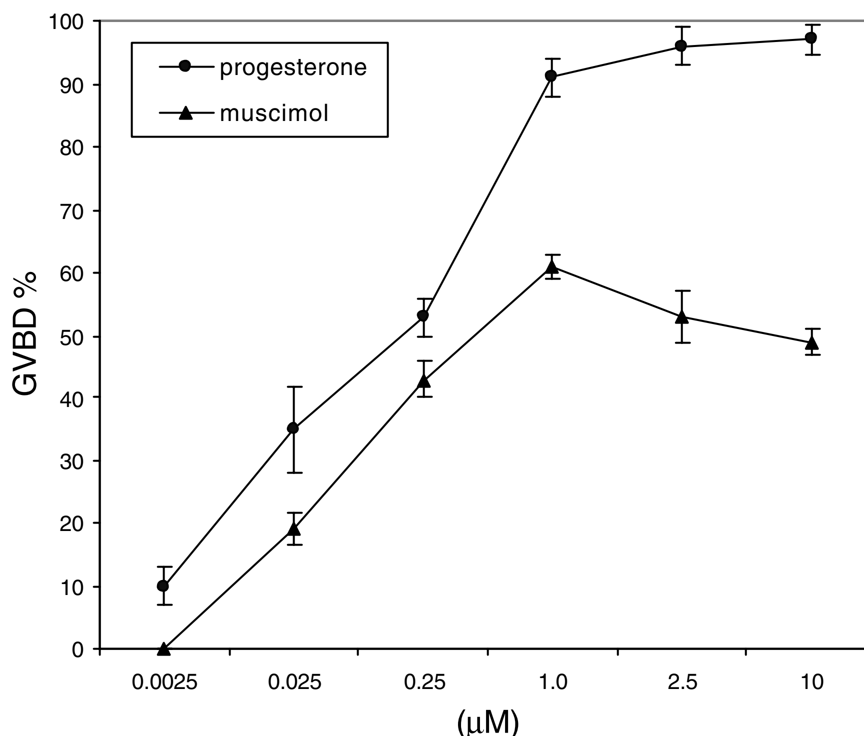

Figure 1 Dose–response curves for muscimol-induced and progesterone-induced GVBD in denuded oocytes. Oocytes not competent to mature spontaneously were cultured in AR with different doses of muscimol or progesterone (0.0025–2.5 μM). GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different animal.

however, was 60%, which is quite lower than the response obtained with progesterone.

Time course of muscimol-induced maturation in denuded oocytes

Denuded oocytes that did not show spontaneous maturation were cultured in AR with muscimol (1 μM) or progesterone (2.5 μM) at 25 °C and examined for GVBD at different times during culture (Fig. 2). After 16 h of incubation, 50% of the oocytes underwent GVBD in response to muscimol and after 22 h the response was almost 60%. The maturation of muscimol-treated oocytes was slow when compared with the response of those treated with progesterone, in which 50% of GVBD was observed after 6 h of culture.

Effect of muscimol on progesterone-induced maturation

The effect of combination of low doses of muscimol (0.025 μM) and progesterone (0.025 μM) was assayed in denuded oocytes not competent to mature spontaneously.

In progesterone-induced maturation we used a low dose of progesterone (0.025 μM), with which GVBD reached almost 30%. Figure 3 shows that the percentage of GVBD increased to 75% when low doses of muscimol (0.025 μM) were added. This fact suggests a synergistic effect between muscimol and progesterone-induced maturation.

Effect of $GABA_A$ antagonist

The effect of picrotoxin (antagonist of $GABA_A$ receptor) on nuclear maturation was studied by incubating non-competent denuded oocytes in AR in the presence of different doses of the antagonist (10–50 μM) for 60 min before maturation was induced with progesterone (2.5 μM) or muscimol (1 μM). The GVBD was scored after 24 h of culture at 25 °C.

Results presented in Fig. 4 indicated that picrotoxin blocked the ability of muscimol to stimulate maturation. Nevertheless, the GVBD induced by progesterone was slightly inhibited by same doses of picrotoxin.

Pronuclear formation in oocytes matured with muscimol

To determine whether the muscimol-induced maturation was genuine, oocytes was inseminated with homologous sperm (see Material and methods). Pronuclear formation was followed through cytological examination of oocytes fixed at various time after insemination. Two hours after insemination, oocytes

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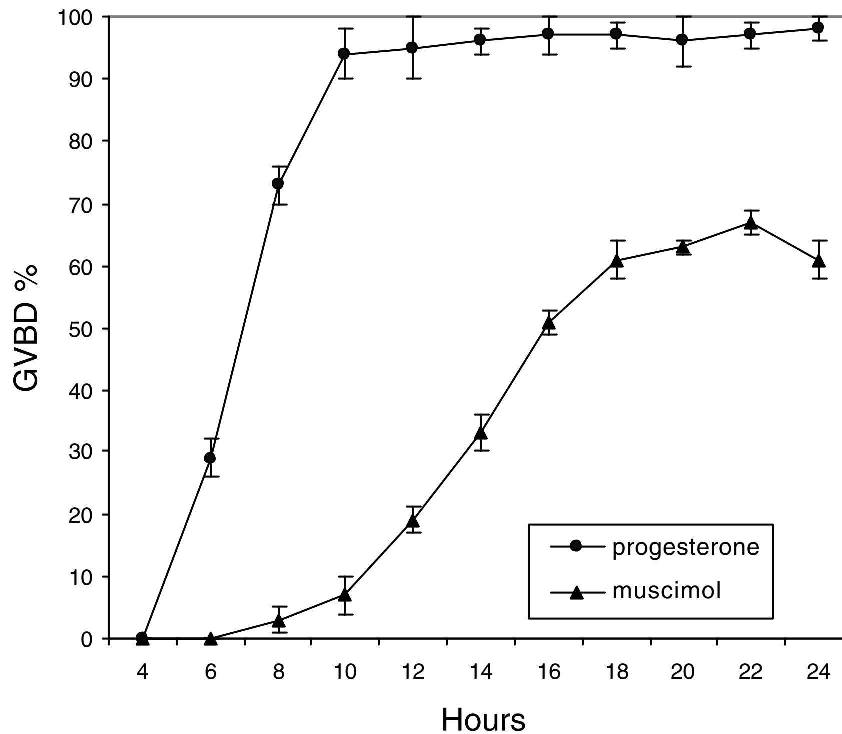

Figure 2 Time course of muscimol-induced GVBD in denuded oocytes. Oocytes not competent to mature spontaneously were cultured with muscimol (1 μM) or progesterone (2.5 μM) at 25 °C and examined for GVBD at different times during culture. Values are the mean ± SEM of three experiments. Each experiment was performed on a different animal.

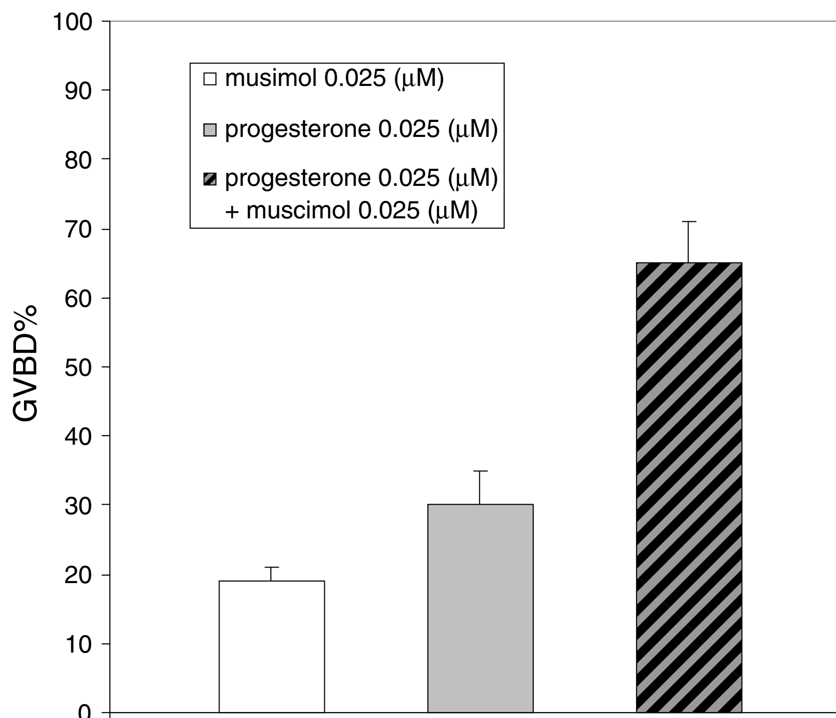

Figure 3 Effect of muscimol on progesterone-induced maturation. Denuded oocytes not competent to mature spontaneously were treated with a low dose of muscimol (0.025 μM) and progesterone (0.025 μM). The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of three experiments. Each experiment was performed on a different animal.

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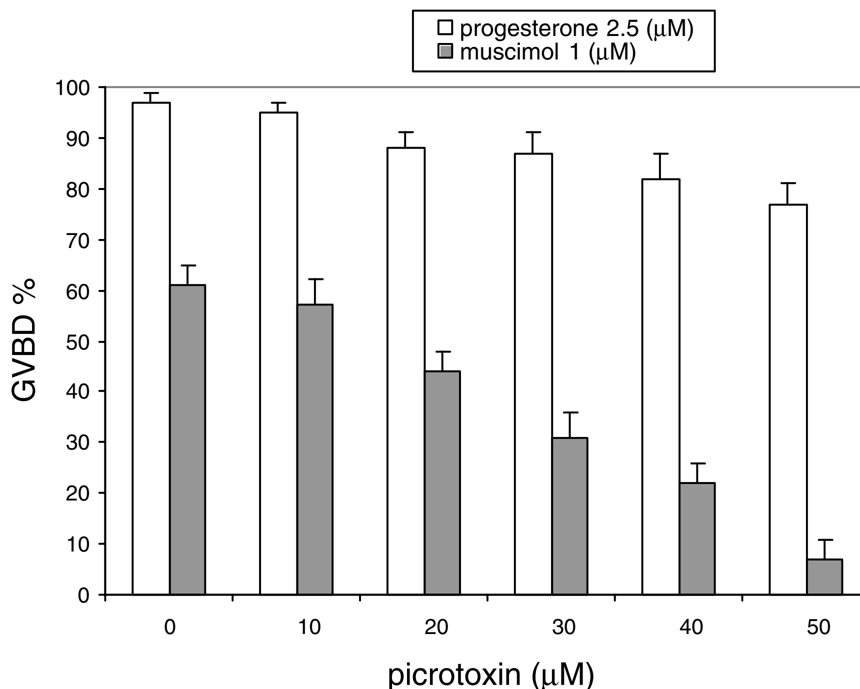

Figure 4 Effect of picrotoxin on muscimol and progesterone-induced maturation. Denuded oocytes not competent to undergo spontaneous maturation were incubated for 60 min in AR with different doses of picrotoxin (10–50 μM) before muscimol (1 μM) or progesterone addition (2.5 μM). The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different animal.

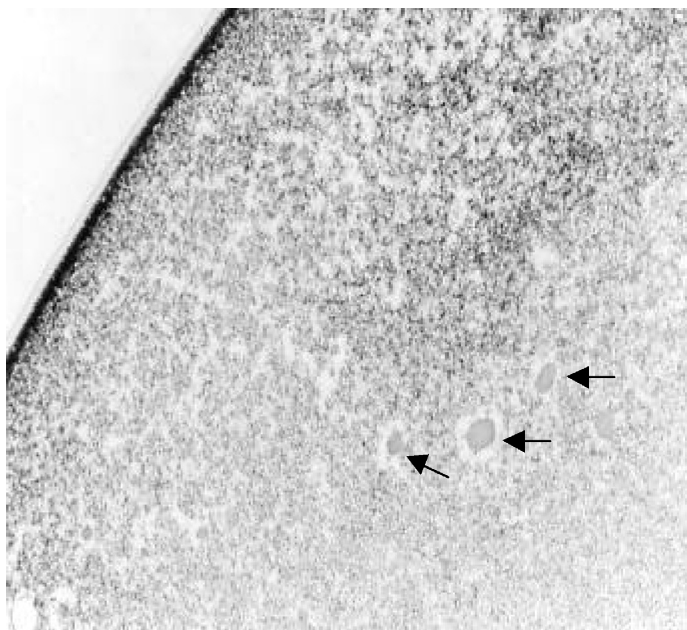

Figure 5 Pronuclear formation in denuded oocytes matured with muscimol. Oocytes matured with muscimol were inseminated with homologous sperm and fixed at various times after insemination. Two hours after insemination, oocytes exhibited well developed pronuclei (arrows).

exhibited well developed pronuclei, similar to that observed in oocytes matured with progesterone (Fig. 5).

Effect of dbcAMP on muscimol-induced maturation

In order to determine whether muscimol-induced maturation was dependent on intracellular levels of

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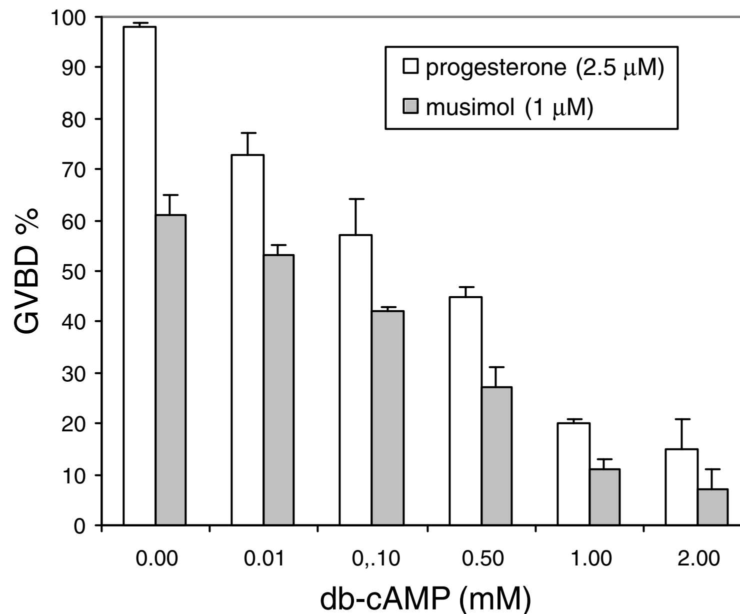

Figure 6 Effect of dibutyryl cAMP (dbcAMP) on oocytes incompetent to undergo spontaneous maturation. Oocytes incompetent to mature spontaneously were cultured in AR with different doses of dbcAMP (0.01–2.0 mM) 60 min before muscimol (1 μM) or progesterone addition (2.5 μM) and examined for GVBD after 24 h of culture. Values are the mean $\pm$ SEM of three experiments. Each experiment was performed on a different animal.

cAMP, denuded oocytes not competent to mature spontaneously were cultured in AR and muscimol (1 μM) or progesterone (2.5 μM) with different doses of dbcAMP (0.1–2.0 mM). The GVBD was scored after 24 h of culture at 25 °C.

Results (Fig. 6) indicated that the increase in intracellular levels of dbcAMP inhibited maturation in both cases in a dose-dependent manner.

Effect of phosphodiesterase inhibition on muscimol-induced and progesterone-induced maturation

The effect of PDE inhibition on GVBD was assayed using theophyllin, an inhibitor of the enzyme. Denuded oocytes not competent to mature spontaneously were cultured in AR with different doses of theophyllin (0.25–1.00 μM) for 60 min before the addition of muscimol (1 μM) or progesterone (2.5 μM). Oocytes were cultured at 25 °C and examined for GVBD after 24 h of culture (Fig. 7). Results indicated that the inactivation of PDE with theophyllin inhibited maturation in a dose-dependent manner in all cases.

Effect of neomycin and LiCl on muscimol-induced and progesterone-induced maturation

In order to analyse the participation of membrane phospholipids hydrolysis during maturation in *Bufo arenarum*, we studied the effect of neomycin and LiCl on denuded oocytes. Neomycin is an antibiotic that binds to PIP and PIP_2, then preventing their hydrolysis in DAG and IP3 and LiCl inhibits the turnover of membrane lipids.

Denuded oocytes not competent to mature spontaneously were preincubated in neomycin (1 mM) or LiCl (20 μM) for 60 min before muscimol (1 μM) or progesterone (2.5 μM) treatment. GVBD was scored after 24 h of culture at 25 °C.

The results, shown in Fig. 8, indicate that the maturation induced by muscimol or progesterone was inhibited when we used neomycin. In the case of LiCl, the GVBD was blockaded. This finding suggests that the participation of membrane phospholipids could be the same in progesterone-induced and muscimol-induced maturation.

Effect of PKC inactivation on muscimol-induced and progesterone-induced resumption of meiosis

The effect of PKC inhibition on GVBD was assayed using H-7, which is known to inhibit purified PKC. Denuded oocytes incompetent to mature spontaneously were cultured with H-7 (200 μg/ml) for 60 min before muscimol (1 μM) or progesterone (2.5 μM) treatment and examined for GVBD after 24 h of culture at 25 °C (Fig. 8).

The inactivation of PKC with H-7 slightly inhibited only the maturation induced by muscimol. H-7, however, caused a decrease in the percentage of GVBD in progesterone-induced maturation.

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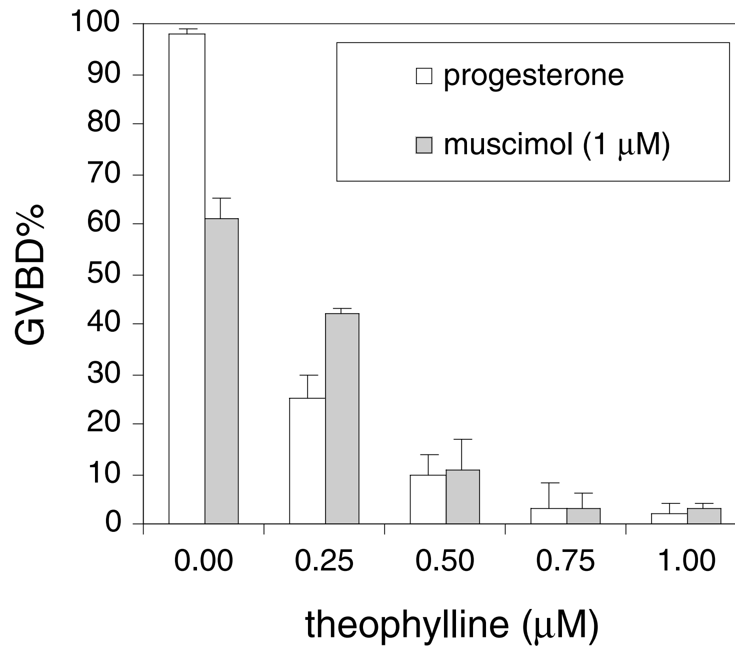

Figure 7 Effect of phosphodiesterase inhibition on muscimol-induced and progesterone-induced maturation. Oocytes incompetent to mature spontaneously were cultured in AR with different doses of theophylline (0.25–1.0 μM) 60 min before muscimol (1 μM) or progesterone addition (2.5 μM). The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different animal.

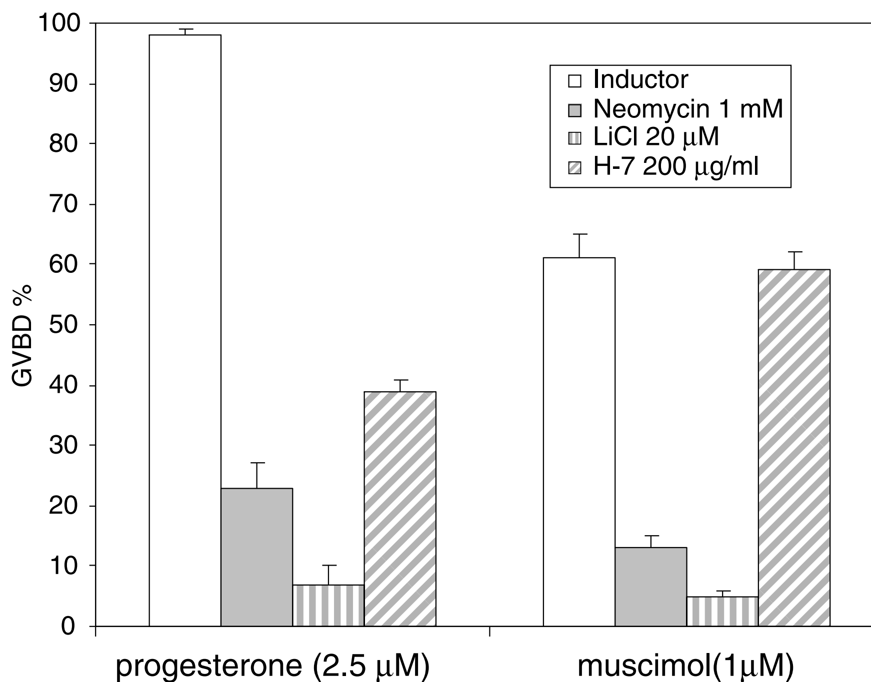

Figure 8 Effect of neomycin, LiCl and H-7 on muscimol-induced and progesterone-induced maturation. Oocytes incompetent to mature spontaneously were preincubated in AR with neomycin (1 mM) or LiCl (20 μM) or H-7 (200 μg/ml) 60 min before muscimol (1 μM) or progesterone addition (2.5 μM). The GVBD was scored after 24 h of incubation. Values are the mean ± SEM of four experiments. Each experiment was performed on a different animal.

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Discussion

Our results indicate that treatment of fully grown denuded *Bufo arenarum* oocytes not competent to undergo spontaneous maturation with an agonist of the $GABA_A$ receptor, such as muscimol, induces meiosis resumption in a dose-dependent manner. Maximum GVBD values of 60% being reached, these values did not increase with the increase in the dose used. These results suggest the presence of $GABA_A$ receptors in the oocyte plasma membrane of this species and, for the first time, demonstrate their participation in the process of nuclear maturation.

The time required for oocytes to reach 50% GVBD was longer when maturation was induced with muscimol with respect to the time required for progesterone (16 versus 6.5 h, respectively). This difference could indicate that the response to muscimol could involve a signalling pathway different from the one used by progesterone or the need to synthesize some of the enzymes involved.

The enhancement of the effect of the combination of low doses of muscimol and progesterone observed in denuded oocytes not competent to mature spontaneously suggests that these inducers would act at the level of the plasma membrane using different receptors. The possibility of progesterone acting on its own receptor, however, and in turn activating the $GABA_A$ receptor, then increasing the percentage of GVBD, cannot be ruled out (Sih & Roldán, 1995).

The participation of the $GABA_A$ receptor in the resumption of meiosis was confirmed by the use of the specific inhibitor for this receptor, picrotoxine. Treatment of oocytes with picrotoxine inhibited muscimol-induced maturation in a dose-dependent manner, but did not affect the action of progesterone. This result supports the idea that in oocytes progesterone acts through a receptor different from the $GABA_A$ receptor.

Taking into account that there are no reports in the existing literature concerning the participation of the $GABA_A$ receptor in the maturation of amphibian oocytes, the effect of muscimol on meiosis resumption was confirmed, analysing the ability of the mature oocytes obtained to form pronuclei after fertilization. The cytological analysis showed that muscimol-treated oocytes are capable of forming pronuclei, suggesting that the $GABA_A$-receptor agonist induces GVBD and the subsequent progression to metaphase II.

The decrease in cAMP intracellular levels is one of the key events for progesterone to induce meiosis resumption in the oocytes of *Bufo arenarum* (Zelarayán *et al.*, 1996, 2000, Sánchez Toranzo *et al.*, 2004), *Rana pipiens* and *Rana dykoski* (Kwon & Schuetz, 1986; Kwon *et al.*, 1989). In this sense, adenylate cyclase inhibition has been associated with the binding of the hormone to its receptor in the membrane of *Xenopus* oocytes (Sadler & Maller, 1982).

Our results demonstrate that high dbcAMP levels inhibit progesterone or muscimol-induced maturation of incompetent oocytes. Similar results were obtained when PDE was inhibited with theophylline, which suggests the participation of the purine pathway in the maturation induced by agonists of the $GABA_A$ receptor.

In previous works we demonstrated that PIP_2 hydrolysis and PKC activation with phorbol esthers (PMA) induced a genuine maturation in *Bufo arenarum* oocytes (Zelarayán *et al.*, 1996). The inhibition assays of the hydrolysis of PIP_2 with neomycin and of its exchange in the membrane by treatment with LiCl showed a significant inhibitory response when maturation was induced with muscimol, a response similar to the one obtained with progesterone (Sánchez Toranzo *et al.*, 2006). These results suggest that both the exchange of lipids in the membrane and the PIP_2 hydrolysis are involved in the maturation induced by agonists of the $GABA_A$ receptor.

The analysis of the importance of the PKC in the signalling pathways used by muscimol, however, shows that the inhibition of this enzyme by H-7 does not significantly modify the percentages of GVBD, but inhibits GVBD by 60% in progesterone-treated oocytes, which agrees with the reports of Zelarayán *et al.* (2000) in *Bufo arenarum*. These results indicate certain differences in the signalling pathways used by progesterone and muscimol.

In conclusion, the results presented in this work suggest the presence of $GABA_A$ receptors in the plasma membrane of the oocytes of *Bufo arenarum* that would not be used by progesterone to induce GVBD. We also demonstrate that an agonist of the $GABA_A$ receptor such as muscimol is able to induce genuine maturation in denuded oocytes incompetent to mature spontaneously, using signalling pathways similar but not identical to the ones used by progesterone.

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