Zygote **16** (1), pp. 65–72. © 2008 Cambridge University Press
doi:10.1017/S0967199407004510 Printed in the United Kingdom

Modulators of *Bufo arenarum* ovulation

Inés Ramos[1,2], *Susana B. Cisint*[2], *Claudia A. Crespo*[2], *Marcela F. Medina*[2] *and Silvia N. Fernández*[2]
Instituto Superior de Investigaciones Biológicas (INSIBIO), Departamento de Biología del Desarrollo, Universidad Nacional de Tucumán, Argentina, Chacabuco 461, Tucumán, Argentina

Date submitted: 25.05.07. Date accepted: 16.07.07

Summary

In this study we investigated ovulation *in vitro* using ovary samples from *Bufo arenarum* with respect to their response to stimulation with homologous pituitary homogenate (HPH) or with progesterone and prostaglandins ($PGF_{2\alpha}$ and PGE_1) as intermediates of pituitary action. Ovary samples were obtained from animals captured during the breeding period. Our results demonstrate that the ovulatory response to all different inducers was dose dependent, the highest percentage of ovulated oocytes being obtained with HPH treatment. An important increase in the ovulatory response was obtained by the association of $PGF_{2\alpha}$ with either HPH or progesterone at suboptimal doses, indicating that this prostaglandin induced a synergistic potentiating effect. Incubation with cyclooxygenase inhibitors (indomethacin or diclofenac sodium) produced a significant decrease in the ovulation induced by HPH, demonstrating that prostaglandins are involved in the action of the pituitary gland in this process. According to our results, PGE_1 not only had no participation in the ovulatory process, but also produced an inhibitory effect on ovulation induced by HPH treatment.

Keywords: Amphibian, Ovulation, Progesterone, Prostaglandin

Introduction

Bufo arenarum females exhibit a seasonal sexual cycle characterized by a breeding or reproductive period and a postovulatory one. The former comprises a preovulatory period, before oviposition (end of July–August), in which the gonad presents a great population of follicles with a maximum degree of development (Valdez Toledo & Pisano, 1980). At this time fully grown oocytes, arrested at the first meiotic division (Masui, 1985), are ready to mature (de Romero *et al*., 1998) and almost prepared for ovulation, one of the most relevant reproductive events.

The ovulatory or spawning period, which completes the breeding period, takes place during the spring (September–November), at which time environmental conditions such as temperature, humidity and food supply are appropriate to insure normal embryonic development after the eggs have been fertilized.

The postovulatory period, characterized by the recovery of the reproductive system, comprises the early postovulatory phase, during the summer months (December–March), marked by follicular growth and development (folliculogenesis), and the late postovulatory period or hibernation. This period coincides with the fall and winter months (April–June) and is characterized by the completion of oogenesis and the acquisition of the maturation capacity of the oocyte. Toward the end of the winter, the reinitiation of the breeding period completes the cycle.

Under physiological conditions, ovulation is restricted to those oocytes that have completed both cytoplasmic and nuclear maturation. In this way, it is evident that maturation is a process temporally associated with ovulation. In our laboratory, cytoplasmic maturation is expressed through biochemical changes in the intermediary metabolism of carbohydrates that the oocytes exhibit, while nuclear maturation is represented by nuclear membrane dissolution and meiosis reinitiation (Fernández & Ramos, 2003).

[1] All correspondence to: Inés Ramos. Instituto Superior de Investigaciones Biológicas (INSIBIO), Departamento de Biología del Desarrollo, Universidad Nacional de Tucumán, Argentina, Chacabuco 461, 4000 – S. M. de Tucumán, Argentina. Fax: +54 381 4247752 560. e-mail: inramos@fbqf.unt.edu.ar
[2] Instituto Superior de Investigaciones Biológicas (INSIBIO), Departamento de Biología del Desarrollo, Universidad Nacional de Tucumán, Argentina, Chacabuco 461, 4.000 – Tucumán, Argentina.

It is known that, in amphibians, the coordination of the different events in the reproductive activity is under the control of the hypothalamic–pituitary axis (Whittier & Crews, 1987). During the hibernation period, this axis presents a low degree of activity, probably due to a dopaminergic inhibition, as reported for *Rana temporaria* (Sotowska-Brochocka *et al*., 1994) or to the inhibitory action of the pineal gland (de Atenor *et al*., 1994). At the end of hibernation the central nervous system inhibition decreases, causing the release of GnRH. Concurrently, pituitary contents and circulating gonadotropin levels begin to rise gradually (Itoh & Ishii, 1990; Sotowska-Brochocka *et al*., 1992).

The gonadotropin surge is accompanied by an increase in gonadal steroid production (Polzonetti–Magni *et al*., 1998). The fully grown follicles mainly secrete progesterone and androgens (dihydrotestosterone and testosterone) (Fortune, 1983), whose high circulating levels characterize the preovulatory period (Medina *et al*., 2004).

Although nuclear maturation is a well known process, the complex mechanism involved in ovulation control has not been completely elucidated. Up to now, it is not clear how gonadotropins and sexual hormones regulate this ovarian function. Prostaglandins (PGs) have been demonstrated to play an important role in regulating gonadotropin induced ovulation in several vertebrates (Goetz *et al*., 1989; Jones *et al*., 1990). In addition, Espey (1980) reported that the rupture of the ovarian wall required for ovulation could be compared to the inflammatory response in which PGs are involved. The ovary of anuran amphibians such as *Rana esculenta* releases PGs ($PGF_{2\alpha}$ and PGE_2), which exhibit seasonal changes at the basal levels (Gobbetti & Zerani, 1993) and exert various effects on reproductive functions at the ovarian and oviductal level.

Taking the above into account, the aim of this work was to investigate *in vitro* the *Bufo arenarum* ovulation in response to endocrine stimulation and to study the role of PGs in this process.

Materials and methods

Animals

Sexually mature *Bufo arenarum* females were collected in the neighbourhood of San Miguel de Tucumán, Argentina. Animals were used immediately after capture or housed for brief periods in boxes with appropriate humidity at room temperature until use.

Ovarian samples

Ovaries, free of extraneous tissue, were rapidly removed from females under ether anaesthesia and placed in amphibian Ringer's solution, pH 7.4. The gonad was cut off in pieces of about 1 ± 0.1 g and washed in the same solution in order to eliminate blood and tissue remains. Each ovary piece was maintained in Ringer's solution containing streptomycin sulphate 0.50 g/l, penicillin G sodium 0.30 g/l and L-ascorbic acid 0.01 mM (hereafter referred to as incubation medium) until use.

Pituitary homogenate

Bufo arenarum pituitary glands were removed from females captured during the breeding period and a pool of them was homogenized in Ringer's solution, using a glass homogenizer, at a ratio of 1 gland/ml. Then, the homogenate was aliquoted and kept at –24 °C. Hereafter the homologous pituitary homogenate is referred to as HPH.

Ovulation induction

Ovulation was induced by incubating ovary pieces in the incubation medium with or without different doses of HPH and/or the hormones or drugs under study. The final incubation volume was 15 ml. After 12 or 24 h of treatment, the percentage of ovulation was scored by determining the number of ovulated oocytes/total number of oocytes present in the tissue sample × 100.

All incubations were performed in duplicate at 25 ± 1 °C in a shaking incubator agitated at 80 oscillations per min. As controls, ovarian pieces were incubated in medium alone under the same experimental conditions.

Hormones and chemicals

Progesterone, obtained from Sigma Chemical Co. was dissolved in ethanol at a ratio of 1 mg/ml and taken to the appropriate volume with Ringer's solution. $PGF_{2\alpha}$, PGE_1, indomethacin and diclofenac sodium, supplied by Sigma Chemical Co., were dissolved in Ringer's solution before addition to the incubation medium.

Data analysis

Results, expressed as percentage of ovulated oocytes, are presented as mean ± SEM of the number of experiments performed with individual toads. The number of animals used and the duration of each treatment are indicated in the legend to each figure or table.

Statistical analysis of the values was carried out by Student's *t*-test and results were considered significantly different at $p < 0.05$.

Results

Preliminary results obtained in our laboratory indicated that the *in vitro* ovulatory response to HPH

https://doi.org/10.1017/S0967199407004510 Published online by Cambridge University Press

was dependent on seasonal variations (Ramos *et al.*, 2005). These data led us to evaluate the optimal HPH dose in the ovulatory response during the breeding period and the influence of progesterone and PGs as intermediates of the pituitary action in this process.

Figure 1 shows the data obtained when ovary pieces were incubated with HPH at doses of 0.01–0.1 gland/ml of incubation medium. Maximum percentage of ovulated oocytes was obtained with 0.03 gland/ml ($p < 0.001$), which is highly significant with respect to the lowest dose assayed. Higher HPH doses did not lead to an increase in the percentage of ovulated oocytes. The results obtained also show that during the time analysed there was a strong response within the first 12 h at all doses assayed. No ovulation was obtained without HPH in the incubation medium (control conditions).

Taking into account that the processes of maturation and ovulation are related temporally, in a second set of experiments we analysed the effect of progesterone, the steroid physiologically responsible for inducing meiosis reinitiation or nuclear maturation, on *Bufo arenarum* ovulation. Figure 2 shows the data obtained when ovary pieces were treated with progesterone at doses of 1–8 μg/ml. Progesterone was able to induce ovulation in a dose-dependent manner at the times analysed. However, the highest percentage of ovulated oocytes obtained with 6 μg/ml of progesterone is low compared with the data obtained after treatment with HPH at 0.03 gland/ml dose ($p < 0.001$). It is important to note that incubation with 1 μg/ml of progesterone, which proved highly effective in inducing nuclear maturation, had little effect on the ovulatory response.

In order to determine the relationship between maturation and ovulation, we analysed the presence of the germinal vesicle in ovulated oocytes after 12 h of treatment with HPH and progesterone. Data (not shown) demonstrated that all gametes released were mature oocytes.

For determining the effect of prostaglandins on the ovulatory process, $PGF_{2\alpha}$ and PGE_1 were tested.

The results obtained indicated that $PGF_{2\alpha}$ per se was able to induce ovulation in a dose-dependent manner during the breeding period. The highest percentage of ovulated oocytes was observed with the 1–5 μg/ml dose (Fig. 3).

The effect of $PGF_{2\alpha}$ on ovulation was also tested in ovaries from animals captured during all periods of the sexual cycle. The highest effect of $PGF_{2\alpha}$ was visible in gonads obtained from animals captured during the breeding period (Fig. 4), while a marked decrease was observed during both the early and late post ovulatory periods.

In contrast, the results found using PGE_1 demonstrated that, in our experimental conditions, it did not affect the ovulation process at the doses assayed (0.25–5.0 μg/ml).

Experiments were carried out to determine whether $PGF_{2\alpha}$ affected the response of HPH- or progesterone-induced ovulation. Ovary pieces were incubated in a

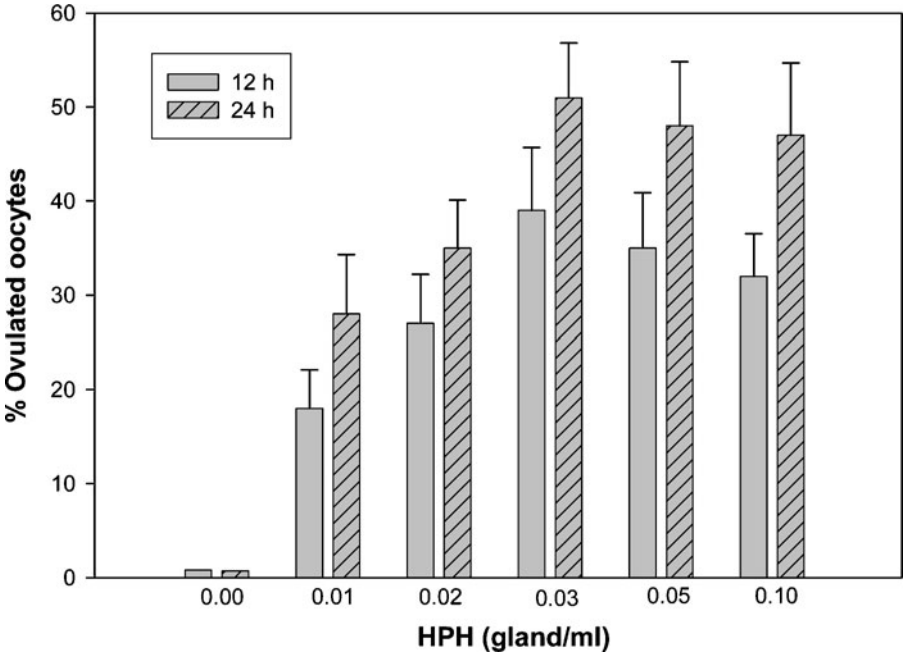

Figure 1 Effect of homologous pituitary homogenate (HPH) on *in vitro* ovulation in *Bufo arenarum*. Ovary pieces obtained from animals captured during the breeding period were incubated with different doses of HPH. Data obtained from experiments using different animals ($n = 7$) represent the percentage mean ± SEM of ovulated oocytes after 12 and 24 h of treatment.

https://doi.org/10.1017/S0967199407004510 Published online by Cambridge University Press

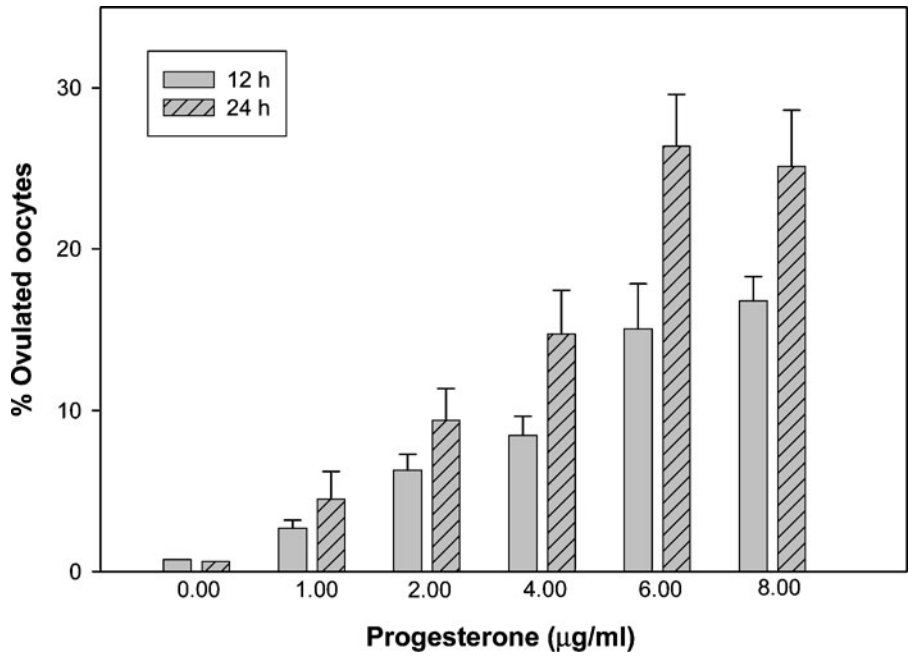

Figure 2 Ovulatory response in *Bufo arenarum* under progesterone treatment. Ovary pieces obtained during the breeding period were incubated with progesterone at different doses for 12 and 24 h. Data obtained by experiments performed with different animals ($n = 6$) represent the percentage mean ± SEM of ovulated oocytes.

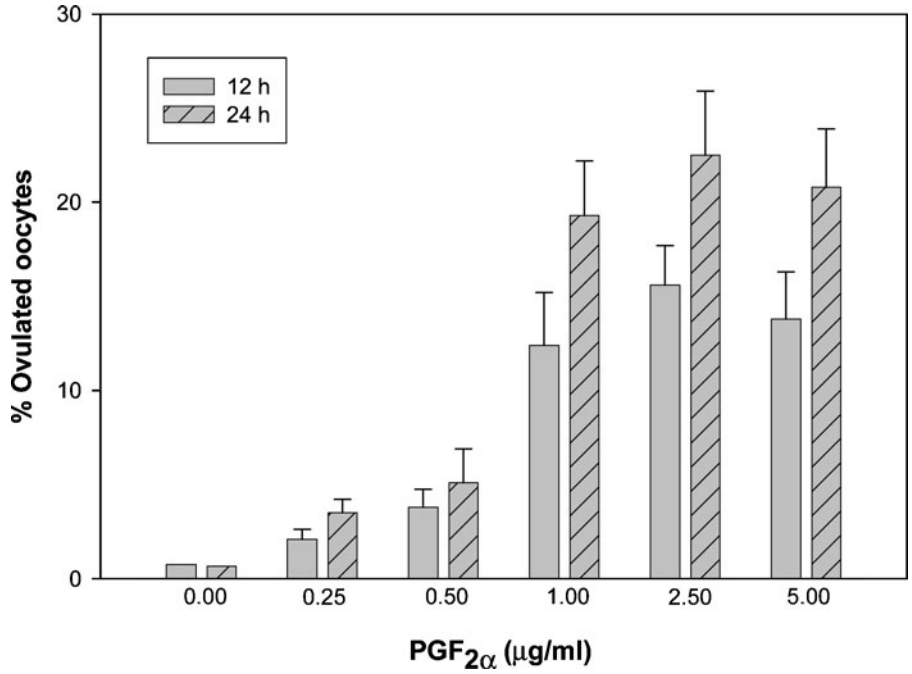

Figure 3 Effect of $PGF_{2\alpha}$ on *in vitro* ovulation in *Bufo arenarum*. Ovary pieces were incubated with different doses of $PGF_{2\alpha}$. Results obtained from experiments using different animals ($n = 6$) are presented as the percentage mean ± SEM of ovulated oocytes after 12 and 24 h of treatment.

medium containing different doses of $PGF_{2\alpha}$ associated with HPH or progesterone, both at suboptimal doses. Under these conditions, a progressive increase in the percentage of ovulated oocytes was observed, a maximum being reached at the dose of 1.0 μg/ml of $PGF_{2\alpha}$ (Fig. 5).

The association of $PGF_{2\alpha}$ with HPH had a stronger effect on ovulation (90% ± 12.4) than with progesterone (30% ± 11.2).

When we assayed the association of PGE_1 with HPH an inhibition of 70% in the ovulatory response was observed (Fig. 6).

https://doi.org/10.1017/S0967199407004510 Published online by Cambridge University Press

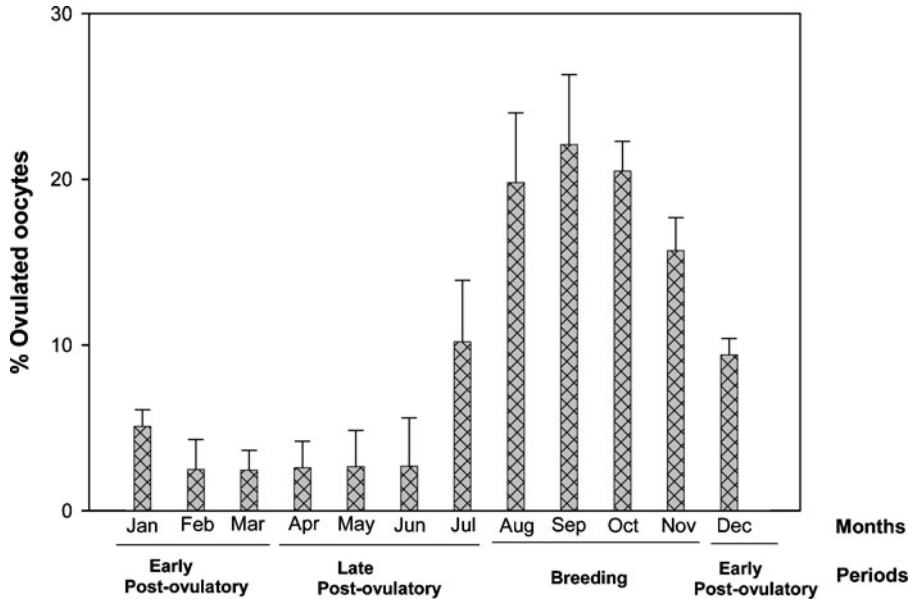

Figure 4 Effect of $PGF_{2\alpha}$ on *in vitro* ovulation in *Bufo arenarum* throughout the sexual cycle. Ovary pieces obtained from animals captured monthly during 2 consecutive years were induced to ovulate with $PGF_{2\alpha}$ 1.5 µg/ml. Each bar represents the percentage mean ± SEM of ovulated oocytes after 12 h of treatment ($n = 4$–7).

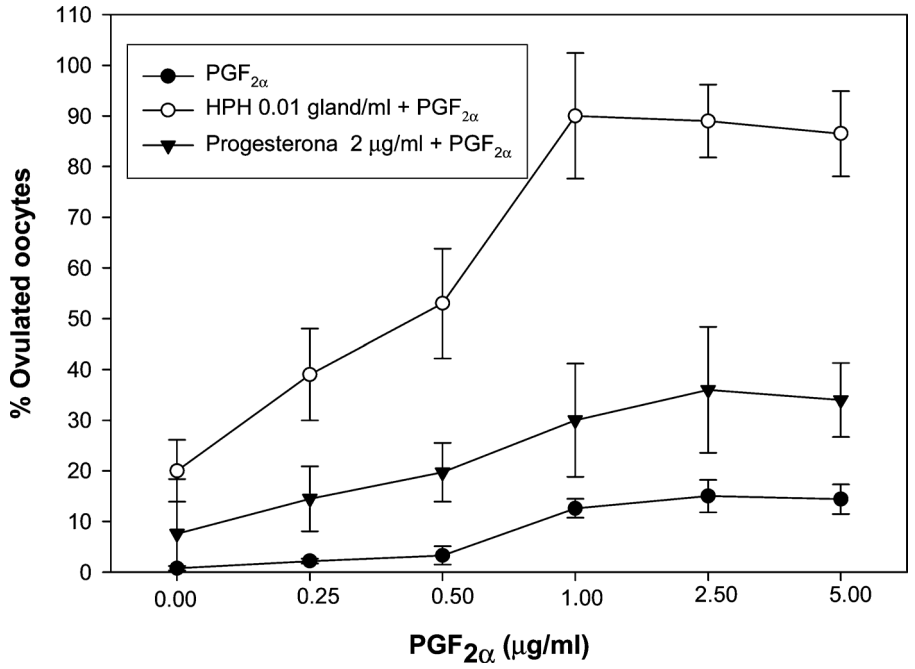

Figure 5 Effect of $PGF_{2\alpha}$ on *in vitro* ovulation induced by HPH or progesterone in *Bufo arenarum*. Ovary pieces were incubated in a medium containing different doses of $PGF_{2\alpha}$ associated with subliminal doses of HPH (0.01 gland/ml) or progesterone (2 µg/ml). Each point in the figure represents the percentage mean ± SEM of ovulated oocytes after 12 h of treatment. ($n = 6$).

In order to test if the effect of HPH was mediated by PG synthesis, ovary pieces were preincubated for 1 h in the presence of inhibitors of cyclooxygenase such as indomethacin or diclofenac sodium at different doses. After that time HPH (0.03 gland/ml) was added to the incubation medium. Table 1 shows that both inhibitors induced a significant decrease in the percentage of ovulated oocytes in a dose-dependent manner. The highest doses of inhibitors assayed blocked ovulation completely.

Discussion

Our results demonstrate that not only HPH, but also progesterone and $PGF_{2\alpha}$, acting at the ovarian level, were able to induce *in vitro* ovulation in *Bufo arenarum*.

https://doi.org/10.1017/S0967199407004510 Published online by Cambridge University Press

Table 1 Effect of cyclooxygenase inhibitors on HPH-induced ovulation

	Indomethacin (μg/ml)				Diclofenac sodium (μg/ml)			
	0	30	60	100	0	30	60	100
Ovulation (%)	39 ± 5.6	23 ± 3.9	15 ± 2.7	0	39 ± 5.6	18 ± 4.5	5 ± 1.4	0

Ovary pieces were preincubated (1 h) with indomethacin or diclofenac sodium and then HPH (0.03 gland/ml) was added to the incubation medium. Results obtained from experiments performed with different animals ($n = 5$) are expressed as mean ± SEM of ovulated oocytes after 12 h of treatment.

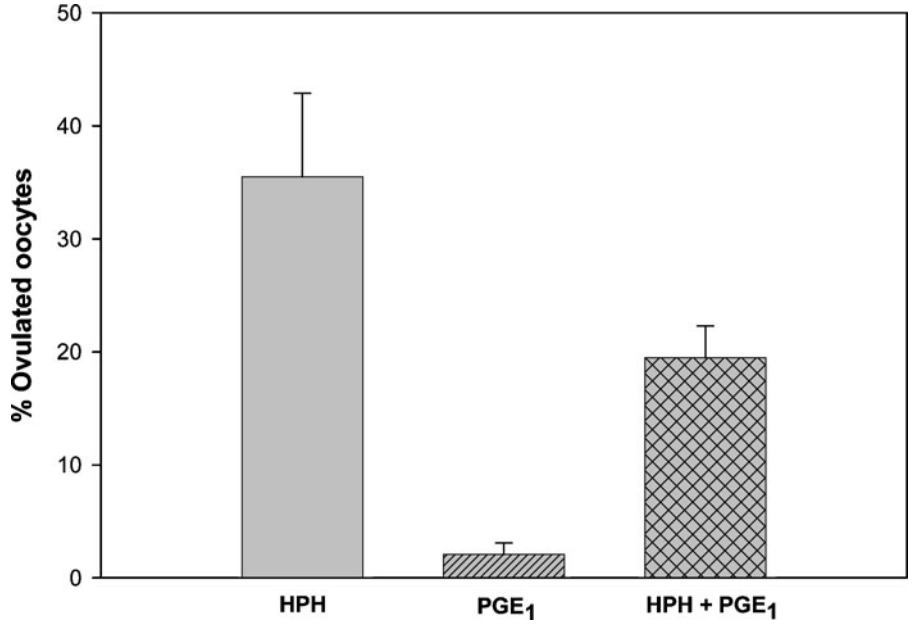

Figure 6 Inhibitory effect of PGE_1 on *in vitro* HPH-induced ovulation in *Bufo arenarum*. Ovary pieces from individual animals ($n = 4$) were incubated in the presence of HPH (0.03 gland/ml), PGE_1 (1.5 μg/ml) or HPH + PGE_1. Data represent the percentage mean ± SEM of ovulated oocytes after 12 h of treatment.

The ovulatory response obtained by treatment with HPH from the pituitaries of females captured during the breeding period could be caused by the effect of gonadotropins, especially LH, which is considered the physiological inducer of ovulation in amphibians (Itoh & Ishii, 1990; Kim *et al*., 1998). In agreement with the above, a preovulatory increase in gonadotropin circulating levels, as well as in pituitary content, has been reported for *Rana temporaria* (Sotowska-Brochocka *et al*., 1992).

At the beginning of the reproductive period, under the influence of gonadotropins, progesterone is secreted by follicle cells that surrounded the fully grown oocytes (Fortune, 1983; Medina *et al*., 2004; Morril *et al*., 2006). It is known that progesterone induces oocyte nuclear maturation (Maller & Krebs, 1980; de Romero *et al*., 1998). Furthermore, our results show that progesterone also induces ovulation per se. Similar results were reported for *Rana pipiens* (Schuetz, 1986) and *Rana temporaria* (Skoblina *et al*., 1997).

In amphibians the exact role of progesterone in this process has not been established yet. It is important to point out that progesterone-induced ovulation could be associated with the interruption of the microvilli and gap junction connections between the follicle cells and the oocytes that takes place previously during nuclear maturation (Schuetz, 1986; Lessman & Kessel, 1992; Ramos *et al*., 1999).

Additional studies performed in mammals have also shown that progesterone is involved in the ovulatory process. Thus, as reported for rat ovary, the use of inhibitors of progesterone synthesis decreases the ovulation rate (Hellberg *et al*., 1996). Likewise, progesterone receptor (PR)-knockout mice exhibited no ovulation in response to hCG due to an abnormal expression of the proteases involved in follicular rupture (Robker *et al*., 2000).

In relation to the quality of the ovulated oocytes obtained after 12 h treatment with HPH or progesterone it is important to note that 100% of them are mature oocytes. These results are in agreement with those obtained for *Rana* (Chang *et al*., 1997). These oocytes, fertilizable in a high percentage, exhibited normal embryonic development.

https://doi.org/10.1017/S0967199407004510 Published online by Cambridge University Press

The percentage of ovulatory response observed under progesterone treatment was lower than the ones obtained after incubation with HPH, suggesting that, for the ovulatory process, pituitary action requires not only the action of LH and progesterone secretion but also of other compounds. It is known that the high levels of gonadotropins before ovulation cause an increase in PGs production through the induction of cyclooxygenase, as reported for mammals (Sirois, 1994). This enzyme transforms arachidonic acid into cycle endoperoxide intermediators that are precursors of prostanoids (Murdoch *et al*., 1993).

Experiments performed with $PGF_{2\alpha}$ and PGE_1 showed that only the former was able to induce ovulation in a dose dependent manner.

The analysis of the completely sexual cycle demonstrated that the action of $PGF_{2\alpha}$ was significantly higher during the reproductive period than during the postovulatory period ($p < 0.001$). This positive effect of $PGF_{2\alpha}$ agrees with data for *Rana esculenta* that report a marked increase in ovary content and plasma circulating level during the ovulatory phase (Gobetti & Zerani, 1993).

As with progesterone, the percentage of ovulation obtained with $PGF_{2\alpha}$ alone was lower than the ones observed after treatment with HPH. This finding suggests that PGs alone are not enough for follicular rupture, which agrees with the results reported for mammals (Espey *et al*., 1992). However, when ovary pieces were treated with $PGF_{2\alpha}$ associated with HPH or progesterone, both at suboptimal doses, a significant increase ($p < 0.001$) in the percentage of ovulated oocytes was obtained with respect to progesterone, $PGF_{2\alpha}$ or HPH alone. Our data suggest a synergistic potentiation effect of $PGF_{2\alpha}$. These results are in agreement with those reported for *Rana temporaria*, in which $PGF_{2\alpha}$ stimulated *in vitro* ovulation and potentiated the effect of progesterone (Skoblina *et al*., 1997).

The functional roles of PGs in ovulation have not been fully defined yet. In rats, PGs increased the vascular permeability and caused the activation of collagenase enzymes required for digestion of the follicular wall (Reich *et al*., 1991). An additional mechanism was proposed by Gobetti & Zerani (1993), who suggested that, in *Rana esculenta*, $PGF_{2\alpha}$ could favour ovulation through the increase in the release of ovarian corticosteroids, whose highest levels characterize the ovulatory phase.

The negative ovulatory response found when PGE_1 was assayed could be due to an increase in the cAMP levels produced by this prostaglandin. It is known that the second messenger inhibits not only maturation but also strongly suppresses *in vitro* ovulation (Kwon *et al*., 1992).

In the present work the participation of PGs as a component of the ovulatory process triggered by the pituitary gland was confirmed by using cyclooxygenase inhibitors in the incubation medium. In fact, indomethacin and sodium diclofenac significantly reduced the percentage of HPH-induced ovulation in a dose-dependent manner. These results are in agreement with those obtained with indomethacin in *Rana* (Skoblina *et al*., 1997) and mouse (Rose *et al*., 1999).

In *Bufo arenarum* the inhibition of ovulation induced by HPH by the action of melatonin (de Atenor *et al*., 1994) supports the above results. In this connection, it is known that the pineal hormone is a potent inhibitor of cyclooxygenase (Takamura & Kogo, 1989; Hardeland *et al*., 2006).

The present results suggest that several ovarian factors such as progesterone and $PGF_{2\alpha}$ could be involved in the control of ovulation in *Bufo arenarum*. It should be noted that, although our results were obtained under *in vitro* conditions, the experimental model used could have a physiological relevance for the study and understanding of the ovulatory process and the factors involved in it.

Acknowledgements

The present work was supported by Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT).

References

Chang, K.J., Kim, J.W., Im, W.B., Kang, H.M. & Kwon, H.B. (1997). Differential effects of gonadotropin and orthovanadate on oocyte maturation, ovulation and prostaglandin synthesis by *Rana* ovarian follicles *in vitro*. *J. Exp. Zool.* **277**, 155–65.

de Atenor, M.B., de Romero, I.R., Brauckmann, E., Pisanó, A. & Legname, A.H. (1994). Effect of the pineal gland and melatonin on the metabolism of oocytes *in vitro* and on ovulation in *Bufo arenarum*. *J. Exp. Zool.* **268**, 436–41.

de Romero, I.R., de Atenor, M.B. & Legname, A.H. (1998). Nuclear maturation inhibitors in *Bufo arenarum* oocytes. *Biocell* **22**, 27–34.

Espey, L.L. (1980). Ovulation as an inflammatory reaction – a hypothesis. *Biol. Reprod.* **22**, 73–106.

Espey, L.L., Tanaka, S., Stacy, S. & Okamura, H. (1992). Inhibition of ovulation in the gonadotropin-primed immature rat by exogenous prostaglandin E2. *Prostaglandins* **43**, 67–74.

Fernández, S.N. & Ramos, I. (2003). *Endocrinology* of Reproduction. In *Reproductive Biology and Phylogeny of Anura*, vol. **4** (ed. B.G.M. JAMIESON), pp. 73–117. Enfield, New Hampshire, USA: Science Publisher, Inc.

Fortune, J. E. (1983). Steroid production by *Xenopus* ovarian follicles at different developmental stages. *Develop. Biol.* **99**, 502–9.

Gobbetti, A. & Zerani, M. (1993). Possible roles for prostaglandins E2 and F2 alpha in seasonal changes in ovarian steroidogenesis in the frog (*Rana esculenta*). *J. Reprod. Fertil.* **98**, 27–32.

Goez, F.W., Duman, P., Ranjan, M. & Herman, C.A. (1989). Prostaglandin F and E synthesis by specific tissue components of the brook trout (*Salvelinus fontinalis*). *J. Exp. Zool.* **250**, 196–205.

Hardeland, R., Pandi-Perumai, S.R. & Cardinali, D.P. (2006). Melatonin. *Int. J. Biochem. Cell Biol.* **38**, 313–6.

Hellberg, P., Larson, L., Olofsson, J., Brännström, M. & Hedin, L. (1996). Regulation of the inducible form of prostaglandin endoperoxide synthase in the perfused rat ovary. *Mol. Hum. Reprod.* **2**, 111–6.

Itoh, M. & Ishii, S. (1990). Changes in plasma levels of gonadotropins and sex steroids in the toad, *Bufo japonicus*, in association with behavior during the breeding season. *Gen. Comp. Endocrinol.* **80**, 451–64.

Jones, R.E., Orlicky, D.J., Austin, H.E., Rand, M.S. & Lopez, K.J. (1990). Indomethacin inhibits ovarian PGE secretion and gonadotropin-induced ovulation in a reptile (*Anolis carolinensis*). *J. Exp. Zool.* **255**, 57–62.

Kim, J.W., Im, W.B., Choi, H.H., Ishii, S. & Kwon, H.B. (1998). Seasonal fluctuations in pituitary gland and plasma levels of gonadotropic hormones in *Rana*. *Gen. Comp. Endocrinol.* **109**, 13–23.

Kwon, H.B., Chang, K.J., Yoo, Y.R., Lee, Ch. C. & Schuetz, A.W. (1992). Induction of ovulation and oocyte maturation of amphibian (*Rana dybowski*) ovarian follicles by protein kinase C activation *in vitro*. *Biol. Reprod.* **47**, 169–76.

Lessman, C.A. & Kessel, R.G. (1992). Effect of acrylamide on germinal vesicle migration and dissolution in oocytes of the frog, *Rana pipiens*. *Exp. Cell Res.* **202**, 151–60.

Maller, J.L. & Krebs, E.G. (1980). Regulation of oocyte maturation. *Curr. Topics Cell Regul.* **16**, 271–311.

Masui, Y. (1985). Meiotic arrest in animal oocytes. In *Biology of Fertilization*, vol. **1** (ed. C.B. Metz & A. Monroy), pp. 189–219. Orlando: Academic Press.

Medina, M.F., Ramos, I., Crespo, C.A., González, S. & Fernández, S.N. (2004). Changes in serum sex steroid levels throughout the reproductive cycle of *Bufo arenarum* females. *Gen. Comp. Endocrinol.* **136**, 143–51.

Morril, G.A., Schatz, F., Kostellow, A. & Bloch, E. (2006). Gonadotropin stimulation of steroid síntesis and metabolism in the *Rana pipiens* ovarian follicle: sequential changes in endogenous steroids during ovulation, fertilization and cleavage stages. *J. Steroid Biochem. Mol. Biol.* **99**, 129–38.

Murdoch, W.J., Hansen, T.R. & McPherson, L.A. (1993). Role of eicosanoids in vertebrales ovulation. *Prostaglandin* **46**, 85–115.

Polzonetti-Magni, A.M., Mosconi, G., Carnevali, O., Yamamoto, K., Hanaoka, Y. & Kikuyama, S. (1998). Gonadotropins and reproductive function in the anuran amphibian *Rana esculenta*. *Biol. Reprod.* **58**, 88–93.

Ramos, I., Winik, B. C., Cisint, S., Crespo, C., Medina, M. & Fernández, S. N. (1999). Ultrastructural changes during nuclear maturation in *Bufo arenarum* oocytes. *Zygote* **7**, 261–9.

Ramos, I., Cisint, S., Medina, M.F., Crespo, C. & Fernández, S.N. (2005). Role of prolactin in nuclear maturation and ovulation in amphibian oocytes. *Zygote* **134**, 265–68.

Reich, R., Daphna-Iken, D., Chun, S.Y., Popliker, M., Slager, R., Adelmann-Grill, B.C., Tsafriri, A. (1991). Preovulatory changes in ovarian expression of collagenases and tissue metalloproteinase inhibitor messenger ribonucleic acid: role of eicosanoids. *Endocrinology* **129**, 1869–75.

Robker, R.L., Russell, D.L., Espey, L.L., Lydon, J.P., O'Malley, B.W. & Richards, J.S. (2000). Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proc. Natl. Acad. Sci. USA* **97**, 4689–94.

Rose, U.M., Hanssen, E.G.J.M. & Kloosterboer, H.J. (1999). Development and characterization of an *in vitro* ovulation model using mouse ovarian follicles. *Biol. Reprod.* **61**, 503–11.

Schuetz, A.W. (1986). Hormonal dissociation of ovulation and maturation of oocytes. Ovulation of immature amphibian oocytes by prostaglandin. *Gamete Res.* **15**, 99–113.

Sirois, J. (1994). Induction of prostaglandin endoperoxide synthase-2 by human chorionic gonadotropin in bovine preovulatory follicles *in vivo*. *Endocrinology* **135**, 841–8.

Skoblina, M.N., Kondrat'eva, O.T., Nikiforova, G.P. & Huhtaniemi, I. (1997). The role of eicosanoids and progesterone in the ovulation of the oocytes in the common frog. *Ontogenez* **28**, 211–6.

Sotowska-Brochocka, J., Martynska, L. & Licht, P. (1992). Changes of LH level in the pituitary gland and plasma in hibernating frogs, *Rana temporaria*. *Gen. Comp. Endocrinol.* **87**, 286–91.

Sotowska-Brochocka, J., Martynska, L. & Licht, P. (1994). Dopaminergic inhibition of gonadotropic release in hibernating frogs, *Rana temporaria*. *Gen. Comp. Endocrinol.* **93**, 192–6.

Takamura, K. & Kogo, H. (1989). The mode of action of indomethacin, aspirin and melatonin on the blockage of the first ovulation in immature rat pretreated with PMSG. *Jpn J. Pharmacol.* **50**, 491–4.

Valdez Toledo, C.L. & Pisanó, A. (1980). Fases ovogenéticas en *Bufo arenarum*. *Reproduccion* **4**, 315–30.

Whittier, J. M. & Crews, D. (1987). Seasonal reproduction: patterns and control. In *Hormone and Reproduction in Fishes, Amphibians and Reptiles*, (ed. D.O. Norris & R.E. Jones), pp. 385–409. New York: Plenum Press.