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Role of phospholipase A_2 pathway in regulating activation of *Bufo arenarum* oocytes

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Summary

Transient increases in the concentration of cytosolic Ca^{2+} are essential for triggering egg activation events. Increased Ca^{2+} results from its rapid release from intracellular stores, mainly mediated by one or both intracellular calcium channels: the inositol trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR). Several regulatory pathways that tailor the response of these channels to the specific cell type have been proposed. Among its many modulatory actions, calcium can serve as an activator of a cytosolic phospholipase A_2 ($cPLA_2$), which releases arachidonic acid from phospholipids of the endoplasmic reticulum as well as from the nuclear envelope. Previous studies have suggested that arachidonic acid and/or its metabolites were able to modulate the activity of several ion channels. Based on these findings, we have studied the participation of the phospholipase A_2 (PLA_2) pathway in the process of *Bufo arenarum* oocyte activation and the interrelation between any of its metabolites and the ion channels involved in the calcium release from the intracellular reservoirs at fertilization. We found that addition of both melittin, a potent PLA_2 activator, and arachidonic acid, the main PLA_2 reaction metabolite, was able to induce activation events in a bell-shaped manner. Differential regulation of IP_3Rs and RyRs by arachidonic acid and its products could explain melittin and arachidonic acid behaviour in *Bufo arenarum* egg activation. The concerted action of arachidonic acid and/or its metabolites could provide controlled mobilization of calcium from intracellular reservoirs and useful tools for understanding calcium homeostasis in eggs that express both types of receptors.

Keywords: *Bufo arenarum*, Oocytes activation, Phospholipase A_2

Introduction

Fertilization stimulates the eggs of all studied species arrested at different stages of meiosis to resume and complete meiotic cell cycle via intracellular calcium signals (Runft *et al.*, 2002).

Transient increases in the concentration of egg cytosolic Ca^{2+} are essential for cortical granule release, completion of meiosis and pronuclear formation. (Nuccitelli, 1991; Whitaker & Swann, 1993; Schultz & Kopf, 1995; Stricker, 1999; Abbott & Ducibella, 2001; Oterino *et al.*, 2001; Horner *et al.*, 2008).

Two types of intracellular calcium channels, located in specialized regions of the endoplasmic reticulum (ER), have been identified to control calcium signalling in eggs: the universal inositol trisphosphate receptor (IP_3R), and the species-specific ryanodine receptor (RyR), which can be found individually or together depending on the particular egg species (Galione *et al.*, 1991, 1993a,b; McPherson *et al.*, 1992; Swann, 1992; Nuccitelli *et al.*, 1993; Fissore & Robl, 1993; Lee *et al.*, 1993; Kline & Kline, 1994; Ayabe *et al.*, 1995; Yue *et al.*, 1995, 1998; Sousa *et al.*, 1996; Albrieux *et al.*, 1997; Herbert *et al.*, 1997; Macháty *et al.*, 1997; Balakier *et al.*, 2002; Petr *et al.*, 2002; Tesarik, 2002; Wang *et al.*, 2005). Our laboratory has previously reported evidence about the existence and functionality of IP_3Rs and RyRs of *in vitro* matured *Bufo arenarum* oocytes. We observed that IP_3Rs were able to trigger oocyte activation by exerting their effect

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in an independent manner; by contrast, RyRs-induced oocyte activation showed a relationship between both pathways, probably through a calcium-induced calcium-release (CICR) mechanism (Ajmat *et al.*, 2010).

Several regulatory pathways that tailor the response of these channels to the specific cell type have been proposed. Among its many modulatory actions, calcium can serve as an activator of an 85 kDa cytosolic phospholipase A_2 (cPLA$_2$) (Clark *et al.*, 1991). It was demonstrated that sea urchin eggs inseminated in the presence of 4-bromophenacyl bromide (BPB), a specific inhibitor of phospholipase A_2, failed to undergo fertilization envelope formation in a concentration-dependent manner. In contrast, melittin, known to be an activator of PLA$_2$, strongly induced the envelope formation in sea urchin eggs. These observations led to the assumption that the PLA$_2$ reaction participates in the Ca^{2+}-triggered reaction system to cortical vesicles in sea urchin eggs (Ferguson & Shen, 1984; Kamata *et al.*, 1997).

Once activated, cPLA$_2$ releases arachidonic acid, a poly-*c*is unsaturated fatty acid (20:4), from phospholipids of the ER as well as from the nuclear envelope. Arachidonic acid acts as a lipophilic messenger and it is the precursor of the synthesis of the eicosanoids.

Previous studies have suggested that arachidonic acid is able to modulate the activity of several ion channels and furthermore, that arachidonic acid and/or its metabolites might be involved in the regulation of the intracellular calcium homeostasis. (Maruyama, 1993; Rowles & Gallacher, 1996). Striggow & Erlich (1997) have investigated the influence of arachidonic acid and some of the eicosanoids on the single channel properties of the IP_3R and the RyR on microsomes of canine cerebellum. They showed that IP_3Rs and the RyRs are regulated differently by arachidonic acid and its product leukotriene B4; IP_3Rs are inhibited by arachidonic acid, whereas RyRs are activated by arachidonic acid through its metabolite leukotriene B_4.

It has been proposed that cyclic ADP ribose could be the endogenous activator of some types of RyRs, but this view remains controversial. Recent observations have suggested that arachidonic acid acts as a physiological activator of ryanodine receptors in pancreatic beta-cells (Woolcott *et al.*, 2006).

However, there is no evidence of the effect of arachidonic acid nor of its metabolites on the calcium signalling pathways that led to the intracellular calcium increase during egg activation in frogs. Therefore, we have studied the participation of the PLA$_2$ pathway in the process of *Bufo arenarum* oocyte activation and the probable interrelation between any of its metabolites and the ion channels involved in the calcium release from the intracellular reservoirs at fertilization.

Materials and methods

Animals

Adult specimens of *Bufo arenarum* were collected in the northwestern area of Argentina and kept at 15 °C until use, which generally took place 7 days after collection. Experimental manipulation and oocyte culture were conducted at room temperature (22–25 °C) using amphibian Ringer solution (AR): NaCl 6.6 g /l, KCl 0.15 g /l, $CaCl_2$ 0.15 g /l, that contains penicillin G-sodium (30 mg/l), streptomycin sulphate (50 mg/l) and 0.005 M Tris–HCl buffer (pH 7.4).

Hormones and reagents

Progesterone (Sigma) was dissolved in ethanol and added directly to the culture medium to give a final concentration of 1 μg/ml. Melittin (Sigma) was dissolved in dimethylsulphoxide (DMSO) and stored in a stock solution of 250 μg/ml and quinacrine (Sigma) was dissolved in DMSO (stock solution 4 mM). Arachidonic acid (Sigma) was dissolved in DMSO in order to obtain a stock solution of 20 mM. Aliquots of 100 μl were purged with gaseous nitrogen and stored at –20 °C until use to avoid oxidation by oxygen, light or heat. All experiments were performed under dim light. Thimerosal (Lilly) was added directly to the culture medium. Ruthenium red (Ted Pella) was dissolved in ddH_2O in order to obtain a suspension; then it was heated at 60 °C for 5 min while shaking and centrifuged at 1600 rpm for 10 min. The supernatant resulted in a stock solution < 1%.

All the compounds injected were diluted into calcium-free Tris-buffered saline solution containing NaCl 7.59 g /l, Tris–HCl 2.40 g /l pH 7.4.

Gametes collection

Fully grown ovarian oocytes were obtained from adult female specimens of *Bufo arenarum.* Oocytes were denuded by manually pulling off the follicle epithelium and theca layer using fine forceps under a stereoscopic microscope. Follicle cells were removed by shaking in AR for 5 min with gentle shaking (100 oscillations/min). Oocytes remained with only the vitelline envelope.

In vitro maturation of oocytes

Hormonal maturation was induced by treatment of denuded oocytes with progesterone 2.5 μM. Oocyte maturation was assessed 18 h after follicle cells removal or after hormone addition. Meiosis reinitiation was scored both by the presence of a transient white spot at the animal pole and by cytological examination. In our working conditions, oocytes reached metaphase

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(M)II in 16 h after defolliculating or progesterone treatment.

Microinjection procedures

Oocytes were microinjected using a Leitz Wetzlar Micromanipulator. Glass micropipettes HUMAGEN™ FERTILITY DIAGNOSTICS were filled by suction of a microdrop (30 nl) that contained either 2.5–40 μg/ml melittin, 25–200 μM arachidonic acid or 20–200 μM ruthenium red. All injections were carried out at room temperature while oocytes were held in calcium-free Tris-buffered saline solution. Microinjections were performed at approximately 18 to 20 h after progesterone addition.

Cytological preparations

Oocytes were fixed in Ancel and Vintemberger's solution (10% formaldehyde, 0.5% acetic acid and 0.5% NaCl), embedded in paraffin and sliced into 5 μm thick sections. Slides were bleached in 30% hydrogen peroxide solution for 3 days to prevent pigment from interfering with microscopic observations. Then they were stained with Alcian blue at pH 2.5 and counterstained with eosin. This method allowed us to observe cortical granules.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Comparisons among different treatments were carried out using Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Participation of the PLA_2 pathway in oocyte activation

In order to determine the participation of the PLA_2 pathway in the activation process we tested the effect of different doses of melittin, a 26 amino acid peptide that represents 50% of the total protein of the bee venom and it is known to able to activate the PLA_2.

Denuded ovarian oocytes matured *in vitro* with progesterone (2.5 μM) were microinjected with different doses of melittin (2.5–40 μg/ml). Egg activation was scored after 20 min of culture.

Results show that melittin is able to induce activation in a dose-dependent manner up to a concentration of 10 μg/ml. We found a decrease in egg activation levels at higher concentrations of melittin (Fig. 1).

Subsequently, in order to test whether melittin exerted its activator effect specifically through the stimulation of the PLA_2 pathway, oocytes were exposed to quinacrine, an inhibitor of PLA_2, before treatment with melittin.

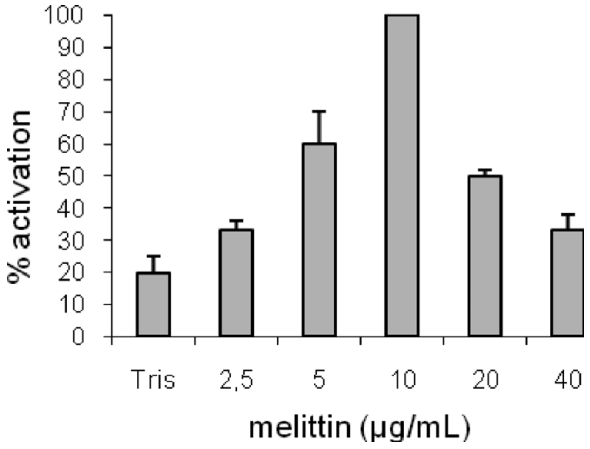

Figure 1 Effect of melitin on the activation of in vitro matured oocytes. Dose–response curve for melittin-induced egg activation. Groups of 20 denuded fully grown ovarian oocytes matured *in vitro* with progesterone were microinjected in amphibian Ringer solution (AR) with different doses of mellitin (2.5–40 μg/ml) and parameters were evaluated after 20 min activation. Values are the mean ± standard error of the mean (SEM) of six experiments. Each experiment was performed on a different animal.

Results that showed the specificity of the melittin activation effect are summarized in Fig. 2. Data show that pre-treatment with quinacrine was able to inhibit significantly melittin-induced egg activation, so we could infer that melittin exerts its effect through phospholipase A_2 pathway.

The influence of PLA_2 pathway on the oocyte activation process was also studied by direct stimulation with one of the products of the enzyme reaction: arachidonic acid.

Results exhibited a bell-shaped curve activation dependence with arachidonic acid levels (Fig. 3), similar to that of melittin. At doses less than 100 μM, we observed a stimulatory effect of arachidonic acid in a dose-dependent manner, whereas at higher concentrations, we noted a decrease in activation levels. Maximum effective concentration of arachidonic acid was 100 μM and at this concentration the activation response was not higher than 50%.

Interrelation between arachidonic acid and ryanodine receptors

Several effects of arachidonic acid on intracellular ion channels have been described. Striggow & Erlich (1997) showed that inositol 1,4,5-trisphosphate receptors and ryanodine receptors are modulated in an opposing manner by arachidonic acid and its

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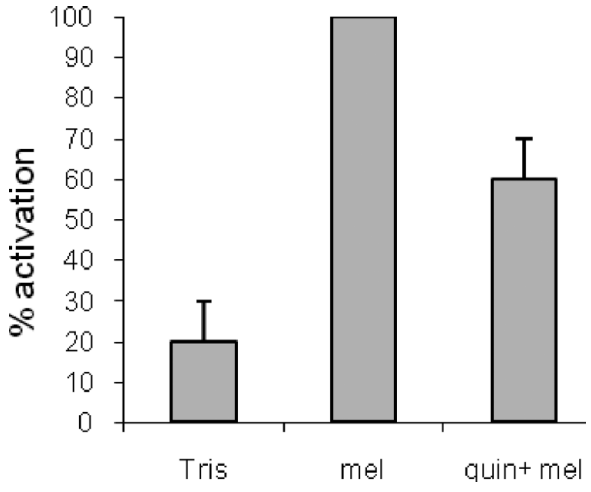

Figure 2 Effect of quinacrine on the mellitin-induced egg activation. Groups of 20 denuded fully grown ovarian oocytes matured *in vitro* with progesterone 2.5 μM were exposed to quinacrine 10 μM before microinjection of 10 μg/ml melitin. Activation was scored after 20 min of insemination. Values are the mean ± standard error of the mean (SEM) of three experiments. Each experiment was performed on a different animal.

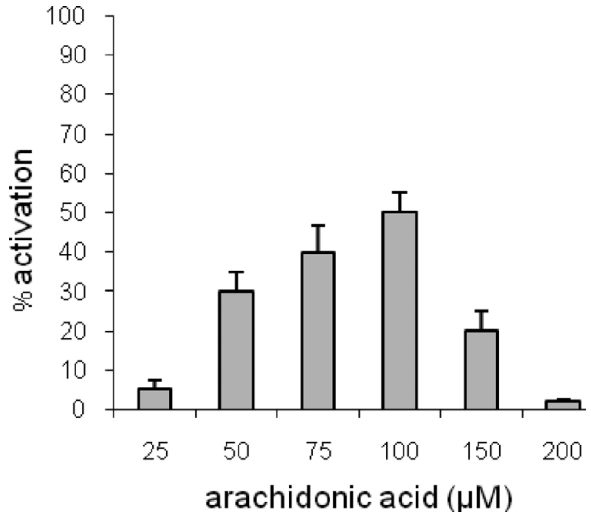

Figure 3 Effect of arachidonic acid on the activation of in vitro matured oocytes. Dose–response curve for arachidonic acid-induced egg activation. Groups of 20 denuded fully grown ovarian oocytes matured *in vitro* with progesterone 2.5μM were microinjected with different doses of arachidonic acid (25–200 μM) and activation parameters were evaluated after 20 min. Values are the mean ± standard error of the mean (SEM) of five experiments. Each experiment was performed on a different animal.

product leukotriene B_4. The IP_3Rs were inhibited by arachidonic acid, whereas the RyRs were unaffected by this compound. In contrast, LTB_4 was able to fully activate the RyRs but did not influence the IP_3Rs.

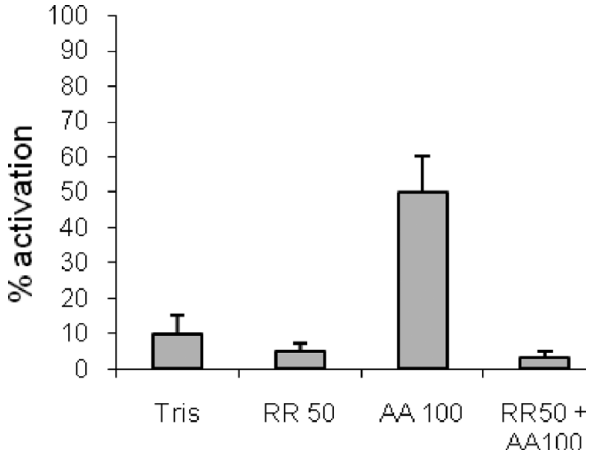

Figure 4 Effect of ruthenium red (RR) on arachidonic acid (AA)-induced oocyte activation. Groups of 20 denuded fully grown ovarian oocytes matured *in vitro* with progesterone were microinjected with RR 50 μM before treatment with AA 100 μM and activation parameters were evaluated after 20 min. Values are the mean ± standard error of the mean (SEM) of three experiments. Each experiment was performed on a different animal.

In order to determine whether a similar regulatory mechanism is underlying the experimental findings about arachidonic acid-induced activation mentioned above (Fig. 3), we tested the effect of arachidonic acid in combination with agonists and antagonists of the two types of ion channels.

To test the hypothesis that arachidonic acid-induced activation is mediated by RyRs, we incubated oocytes with ruthenium red, a specific blocker of RyRs before treatment with arachidonic acid.

We found that the pre-injection of 50 μM ruthenium red strongly inhibited 100 μM arachidonic acid-induced egg activation (Fig. 4). These observations allow us to confirm that arachidonic acid-induced egg activation is carried out through RyRs.

To determine whether arachidonic acid released calcium through IP_3Rs we used heparin (1 μg/ml), a specific blocker of IP_3Rs, before treatment with arachidonic acid 100 μM.

As expected, we found that arachidonic acid-induced activation was not affected by pre-treatment with heparin (Fig. 5). These results indicate that IP_3Rs do not mediate the release of calcium by arachidonic acid.

To examine whether arachidonic acid exerted an inhibitory effect on IP_3Rs in *Bufo arenarum* oocytes, previous injections with arachidonic acid were performed, before exposing oocytes to thimerosal 200 μM, which is a potent activator of IP_3Rs.

A significant decrease on the activation induced by thimerosal was observed when mature oocytes were microinjected with arachidonic acid 100 μM, before

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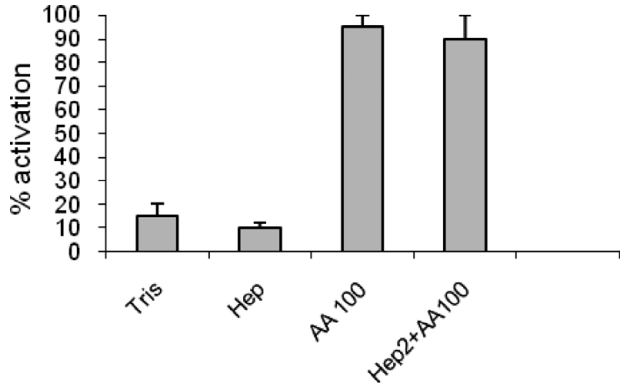

Figure 5 Effect of heparin on arachidonic acid (AA)-induced oocyte activation. Groups of 20 denuded fully grown ovarian oocytes matured *in vitro* with progesterone were microinjected with heparin 1 μg/ml before treatment with AA 100 μM and activation parameters were evaluated after 20 min. Values are the mean ± SEM of three experiments. Each experiment was performed on a different animal.

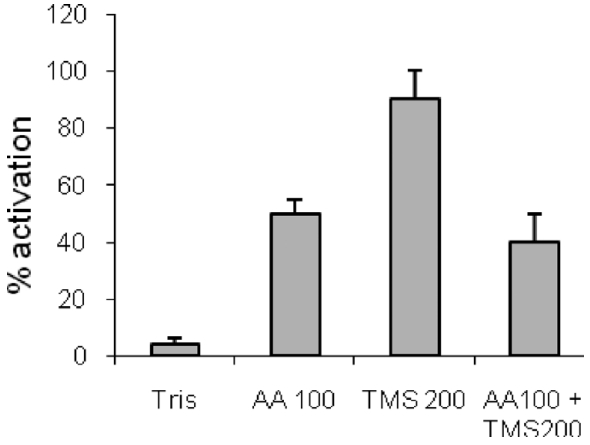

Figure 6 Effect of arachidonic acid (AA)-induced on thimerosal-induced oocyte activation. Groups of 20 denuded fully grown ovarian oocytes matured *in vitro* with 2.5 μM progesterone were microinjected with 100 μM AA (AA 100) before treatment with 200 μM thimerosal (TMS 200) and after 20 min activation parameters were evaluated. Values are the mean ± standard error of the mean (SEM) of three experiments. Each experiment was performed on a different animal.

exposing to high doses of thimerosal (200 μM). This response indicates that arachidonic acid is able to inhibit egg activation through IP_3Rs (Fig. 6).

Discussion

Participation of the PLA_2 pathway in egg activation

Probable participation of PLA_2 pathway in the process of fertilization has not been elucidated yet. Many investigations have focused on the involvement of this intracellular mechanism in the sperm acrosomal reaction but only a few have reported its participation in triggering egg activation.

As mentioned earlier, PLA_2 inhibition was able to block some activation events such as cortical vesicle exocytosis and subsequent fertilization envelope formation probably through calcium-triggered sequential reactions (Kamata *et al*., 1997).

Evidence has suggested the implication of the PLA_2 pathway in sperm-zona pellucida interaction. Hydrolysis of phospholipids by PLA_2 generates free fatty acids and lysophospholipids that are important either as substrates for the generation of other metabolites (e.g., eicosanoids) or as they have a direct, essential action in the final stages of membrane fusion. (Roldan & Shi, 2007)

It was suggested that sperm PLA_2 and one of its modulators, lysophosphatidylcholine (LPC), may contribute to membrane-fusion events in mammalian fertilization (Riffo & Párraga, 1997). Studies in guinea pig spermatozoa suggested that PLA_2 plays a fundamental role in agonist-stimulated acrosomal exocytosis (Chen *et al*., 2005).

Activation of PLA_2 at fertilization in *Xenopus* is a matter of discussion. There were no detectable lipid changes that reflect significant activation of PLA_2 during fertilization in *Xenopus laevis* eggs (Petcoff *et al*., 2008).

We found that addition of both melittin, a potent PLA_2 activator, and arachidonic acid, the main PLA_2 reaction metabolite, was able to induce activation events such as cortical granule exocytosis. Moreover, quinacrine treatment before melittin stimulation was proved to be inhibitory. These findings suggest that phospholipase A_2 pathway is involved in the calcium-release mechanism that led to *Bufo arenarum* egg activation.

Results from melittin–induced egg activation as well as arachidonic acid-induced egg activation showed a Gauss curve (Figs. 1 and 3), suggesting the involvement of an underlying regulation mechanism.

Interrelation between arachidonic acid and ryanodine receptors

Arachidonic acid has been shown to interact with intracellular ion channels in many cell types. For instance, it was proved that arachidonic acid inhibits L-type calcium channel currents in cardiac myocytes (Petit-Jacques & Hartzell, 1996) and in CA1 pyramidal cells of hippocampus (Keyser & Alger, 1990).

Mobilization of stored calcium from the ER through IP_3Rs and RyRs is a crucial step of intracellular calcium signalling at egg activation.

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In contrast, it has been proposed that arachidonic acid could be the endogenous activator of ryanodine receptors in pancreatic beta-cells (Woolcott *et al.*, 2006).

It is based on these findings that we have approached the study of the effect of arachidonic acid in combination with agonists and antagonists of the two main families of ion channels in mature *Bufo arenarum* oocytes. The findings presented here at a cellular level are in line with previous studies undertaken on microsomal fractions in other cell models (Striggow & Erlich, 1997). Differential regulation of IP_3Rs and RyRs by arachidonic acid and its products could explain melittin and arachidonic acid behaviour in *Bufo arenarum* egg activation shown in Figs. 1 and 3. The concerted action of arachidonic acid and/or its metabolites could provide controlled mobilization of calcium from intracellular reservoirs by terminating IP_3-induced calcium release and activating the RyR calcium-release channel.

Which of the products of the arachidonic acid cascade is involved in this complex intracellular calcium signalling? Preliminary reports have shown that LTB_4 is the only eicosanoid able to induce intracellular calcium release in oocytes (Silver *et al.*, 1994). Additional experiments would be necessary to identify the specific arachidonic acid-derived compounds that exert its effects on activation events of *Bufo arenarum* oocytes.

Knowledge of these recently identified agonists of the calcium channels will provide useful tools to understand the calcium homeostasis in eggs expressing both types of receptors and to improve in vitro protocols of egg activation in frogs.

Acknowledgements

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