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Activation of spleen tyrosine kinase (Syk) at fertilization in *Rhinella arenarum* eggs

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ABSTRACT Recently, we have provided evidence for the involvement of a cytosolic tyrosine–phosphorylatable 70 kDa oocyte protein in *Rhinella arenarum* (Anura: Bufonidae) fertilization. The aim of the present work was to characterize its phosphorylation, determine the identity of this protein and establish its biological role during the fertilization process. Tyrosine phosphorylation of the 70 kDa protein was not observed in eggs activated with the calcium ionophore A23187. Pretreatment of oocytes with the tyrosine kinase inhibitor genistein effectively blocked the fertilization-dependent phosphorylation of the 70 kDa protein. In order to identify this protein, we examined the presence in amphibian oocytes of non-receptor 70 kDa tyrosine kinase members of the Syk/Zap70 and Tec families by RT-PCR using degenerate primers. We found that *R. arenarum* oocytes contain the transcripts coding for Syk and Tec kinases. Western blot analysis confirmed the presence of Syk protein in unfertilized oocytes and eggs. Studies using phospho-Syk specific antibodies showed that fertilization rapidly (less than 10 minutes) induces phosphorylation on Syk tyrosine residues (352 and 525/526) that are necessary for the activation of the enzyme. Finally, specific inhibition of Syk with the R406 compound provoked a diminished fertilization score, thereby confirming a functional role of the Syk protein during *R. arenarum* fertilization. To our knowledge this is the first time that Syk is described as a player in the signaling cascade activated after fertilization.

KEY WORDS: *tyrosine phosphorylation, amphibian, signaling*

Introduction

Sperm-egg interaction implies a series of physiological and biochemical events, involving species recognition, cell adhesion and fusion between gametes. The ultimate step is egg activation, the starting point of a developmental program leading to the formation of a new individual. Activation of animal eggs has long been recognized as a temporally and spatially regulated sequence of steps (Vacquier, 1998; Singson *et al.*, 2001; Li *et al.*, 2013). In species that fertilize externally like sea invertebrates and frogs, activation includes membrane depolarization which may account for the "fast" block to polyspermy, cortical granule exocytosis which promotes fertilization envelope formation that provides the "slow" and permanent block to polyspermy, an increase in intracellular pH (alkalinization), elevated protein synthesis, pronuclear formation and DNA synthesis (Sato *et al.*, 2000; Santella *et al.*, 2012). Each of these physiological, biochemical and morphological events are strictly regulated by signaling cascades that involve the activation or inactivation of diverse kinases and phosphatases.

The oocyte accomplishes this diverse series of tasks by establishing an array of signal transduction pathway components that include, among others, a select collection of non-receptor protein tyrosine kinases (NRPTKs). This array of NRPTKs includes kinases such as Src-family PTKs and FAK kinases, whose presence in oocytes have been described in several species (Sato *et al.*, 1996; Giusti *et al.*, 2000; Runft and Jaffe, 2000; Sharma and Kinsey, 2006; Townley *et al.*, 2009; McGinnis *et al.*, 2009; Levi *et al.*, 2010; Levi and Shalgi, 2010; McGinnis *et al.*, 2011; Kinsey, 2013). Usually,

Abbreviations used in this paper: mpf, minutes post-fertilization ;Syk, spleen tyrosine kinase.

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these kinases regulate their activity by phosphorylation of tyrosine residues in their own activation loops (Calalb *et al.*, 1995; Xu *et al.*, 1999; Roskoski, 2004; Grigera *et al.*, 2005).

Protein tyrosine phosphorylation plays an important role in the regulation of many biological processes such as cell growth, division, differentiation, cell-cell communication, and fertilization. In fact, it has been described that tyrosine phosphorylation increases in the first minutes following fertilization of *Xenopus* oocytes (Sato *et al.*, 1998), *Rhinella arenarum* oocytes (Mouguelar *et al.*, 2011), rat eggs (Ben-Yosef *et al.*, 1998) and ascidian eggs (Ueki and Yokosawa, 1997), and as early as 1 min after fertilization in sea urchin eggs (Peaucellier *et al.*, 1988). In all these species, this increase can be detected during the latent period, i.e., before the fertilization calcium signal (Ciapa and Epel, 1991). Nevertheless, the identity of most of the key members involved in these signaling pathways has not yet been elucidated.

Recently, we have described the phosphorylation at tyrosine residues of at least two proteins of 60 kDa and 70 kDa in *R. arenarum* egg cytosolic fractions ten minutes post-fertilization (mpf) (Mouguelar *et al.*, 2011).

Due to the growing evidence indicating that members of the Src family protein tyrosine kinases are involved in oocyte signal transduction during fertilization, not only in all the species with external fertilization analyzed until now (Sato *et al.*, 1996; Giusti *et al.*, 2000; Runft and Jaffe, 2000; Sharma and Kinsey, 2006; Townley *et al.*, 2009), but also in mammalian species, (McGinnis *et al.*, 2009; Levi *et al.*, 2010; Levi and Shalgi, 2010; McGinnis *et al.*, 2011), we hypothesize that the *Rhinella arenarum* 60 kDa protein early tyrosine-phosphorylated after oocyte-sperm meeting belongs to the Src family of PTKs. However there is scarce information concerning a role for a 70 kDa tyrosine phosphorylated protein in the fertilization process of any species.

There are currently at least 32 known vertebrate genes that encode NRPTKs. These can be divided into ten families: Abl, Fes/Fer, Frk, Syk/Zap70, Jak, Tec, Fak, Ack, Src, and Csk (Neet and Hunter, 1996; Robinson *et al.*, 2000). An examination of their molecular weights allowed us to discard most of them as possible candidates. Only members of two of these families have the expected molecular mass: the 70 kDa Syk/ Zap70 and Tec families.

The Tec family has five kinases (Bmx, Btk, Itk, Tec, Txk) while the spleen tyrosine kinase (Syk) family has only two members (Syk and Zap-70) (Chu *et al.*, 1998; Smith 2001). Members of the Syk and Tec family of kinases operates downstream of many different receptors (Mócsai 2010; Lowell 2011). Syk was first described playing a key role in signaling downstream of the B cell receptor, but subsequently it has been found to play a broad role in hematopoietic cell signaling including downstream of the FCεRI receptor in mast cells, FCγRIIA receptor in macrophages, monocytes, and platelets, and sometimes the T cell receptor in T cells (Chan *et al.*, 1994; Turner *et al.*, 1997, 2000; Oliver *et al.*, 2000; Sada *et al.*, 2001; Sedlik *et al.*, 2003). Zap-70 (Zeta-chain associated kinase of 70 kDa) was first identified as a protein associated with the ζ chain of the T cell receptor. Unlike Syk, Zap-70 showed a narrow distribution pattern with expression restricted to T cells and natural killer cells (Chan *et al.*, 1992; Béné, 2006; Wang *et al.*, 2010). On the other hand, Tec family was found to be expressed primarily in a variety of hematopoietic cells including B cells, T cells, mast cell, and macrophages (Mano, 1999; Yang *et al.*, 2000; Ellmeier *et al.*, 2011). The finding of expression of any of these NRPTKs in reproductive tissue of any vertebrate would be the first report regarding the issue. So, our hypothesis was that the phosphorylatable 70 kDa protein (p70) we detected in *R. arenarum* could be a lead pointing towards the presence in oocytes of a yet not described NRPTK family member (e.g. Syk/Zap70) participating in fertilization signalling.

The high degree of conservation of most essential cellular and molecular mechanisms compared with other vertebrate like mammals, its ease of manipulation and the large amounts of material that can be readily obtained for a variety of experimental procedures make *R. arenarum* the model of choice for our experiments. Moreover, as *R. arenarum* fertilization is external, the setup of fertilization assays is simple, allowing the testing of the effect of inhibitors and other compounds in a relatively straight-forward manner.

In this work, our aim was to characterize the phosphorylation of the 70 kDa protein, determine the identity of this protein and establish a biological role during the *Rhinella arenarum* fertilization process.

Results

Fertilization but not calcium ionophore induces p70 phosphorylation

Our first experiments were directed to determine if the fertilization-induced p70 phosphorylation could be mimicked by artificial intracellular calcium rise. This parthenogenetic activation can be induced by egg incubation with calcium ionophore A23187 (see Methods). Cytosolic protein samples of oocytes, eggs ten minutes after fertilization and A23187-treated eggs were subjected

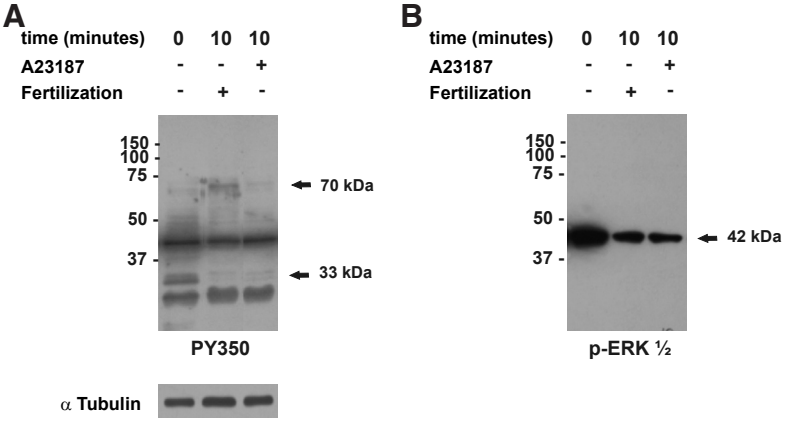

Fig. 1. Effect of calcium ionophore A23187 treatment on cytosol protein tyrosine phosphorylation and on p42 MAPK phosphorylation state in R. arenarum oocytes. *Cytosolic fraction was prepared from* Rhinella arenarum *oocytes, eggs at 10 minutes post-fertilization and artificially activated eggs by treatment with A23187 (2,5 μM). Protein samples (20 μg of protein) were separated by SDS-PAGE on 12% polyacrylamide gels, blotted and analyzed using the anti phosphotyrosine antibody (PY-350)* **(A)** *and the anti p-ERK1/2Thr202/Tyr204 antibody* **(B)**. *Closed arrows indicate proteins that change their phosphorylation state due to one or both treatment (fertilization/artificial activation). α-Tubulin was used as loading control. Molecular standards are indicated to the left of each panel.*

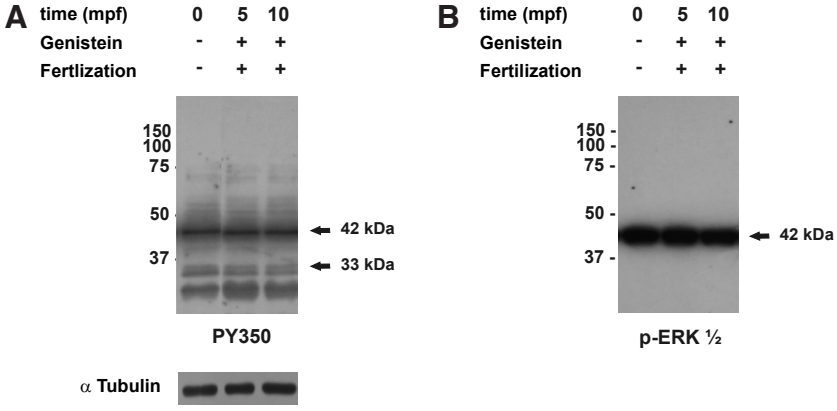

Fig. 2. Effect of genistein treatment on cytosol protein tyrosine phosphorylation and on p42 MAPK phosphorylation state in R. arenarum oocytes. *Cytosolic fractions were prepared from* Rhinella arenarum *oocytes and fertilizated eggs. Oocytes were pre-incubated with the tyrosine kinases inhibitor, genistein (100 μM) for 1 hour, washed and finally fertilized. Fertilization was stopped 5 and 10 minutes post-fertilization (mpf). Protein samples (20 μg of protein) were separated by SDS-PAGE on 12% polyacrylamide gels, blotted and analyzed using the anti phosphotyrosine antibody (PY-350, cat. sc-18182)* **(A)** *and the anti p-ERK1/2 Thr202/Tyr204 antibody* **(B)**. *Closed arrows indicate proteins that usually are dephosphorylated due to fertilization. α-Tubulin was used as loading control. Molecular standards are indicated to the left of each panel.*

to electrophoresis, transferred to nitrocellulose and proved with anti-phosphotyrosine antibodies (Fig. 1A) and anti-phospho-MAPK antibodies (Fig. 1B). High levels of MAPK activity are incompatible with pronuclear formation in vertebrate activated/fertilized eggs (Tunquist and Maller, 2003; Inoue *et al*., 2007). To test whether inhibition of MAPK activity occurs in A23187 treated *R. arenarum* eggs, we measured the level of dephosphorylation (inactivation) of MAPK using a specific anti p-ERK 1/2 antibody. Calcium ionophore treatment did activate the eggs, as shown by MAPK expected typical dephosphorylation pattern (Fig. 1 B) and the disappearance of the signal corresponding to the 33 kDa phosphorylated doublet (Fig. 1A). However, tyrosine phosphorylation of the 70 kDa protein was not observed in samples from A23187 treated eggs while it can be clearly seen in fertilized eggs (Fig. 1A). Possible explanations for these results are that p70 may act upstream of fertilization-calcium transient or that its phosphorylation is independent of cytosolic calcium rise.

Genistein blocks increase in p70 tyrosine phosphorylation associated with Rhinella arenarum fertilization

Next, we examined the effect of genistein on fertilization-induced p70 phosphorylation at tyrosine residues. Unfertilized eggs were pre-incubated in 50% SB solution supplemented with 1% DMSO (as control) and genistein 100 μM for 1 h. Treated eggs were washed and then inseminated. Subsequently, tyrosine phosphorylation pattern was analyzed in cytosolic protein samples obtained from oocytes and embryos at 1, 5 and 10 mpf. Genistein was found to abolish some of the typical phosphorylation changes that were previously associated with fertilization in *Rhinella arenarum* (Mouguelar *et al*., 2011), like dephosphorylation of proteins of 33 and 42 kDa, and phosphorylation of 70 kDa proteins (Fig. 1A).We also evaluated the effect of genistein on the physiological dephosphorylation due to fertilization of the p42-MAPK protein using a specific anti p-ERK 1/2 antibody (see above). Treatment with genistein impaired this normal pattern of dephosphorylation (Fig.2B).

Oocyte contains maternal mRNAs that code for Syk and Tec proteins

In order to determine if Syk, ZAP70 or Tec mRNA are expressed in *Rhinella arenarum* oocyte, we designed degenerate primers (see Methods for details) to amplify cDNAs encoding fragments of these NRPTK sequences. RNA was isolated from fresh oocytes and retro-transcribed. The obtained cDNA was used in PCR reactions with the designed degenerate primers to amplify Syk, ZAP70 and members of the Tec family. As these tyrosine kinases were described mainly in hematopoietic tissues, spleen was used as positive control of the reaction. In this tissue an amplicon of the expected size was detected for the three genes evaluated (Lanes: Spleen, Fig. 3). Meanwhile oocytes presented positive signals only for the Syk and Tec genes, but not for the ZAP-70 gene. The excised bands were extracted, cloned and sequenced. The analysis of the obtained sequences (see Suppl. Fig. 1) showed strong homology to Syk and Tec, respectively. So, this methodology allowed us to determine that *Rhinella* oocytes contain maternal mRNAs that code for Syk and also for Tec kinases.

Syk protein is expressed in oocytes and early embryos

Once the presence of mRNA coding for Syk was established,

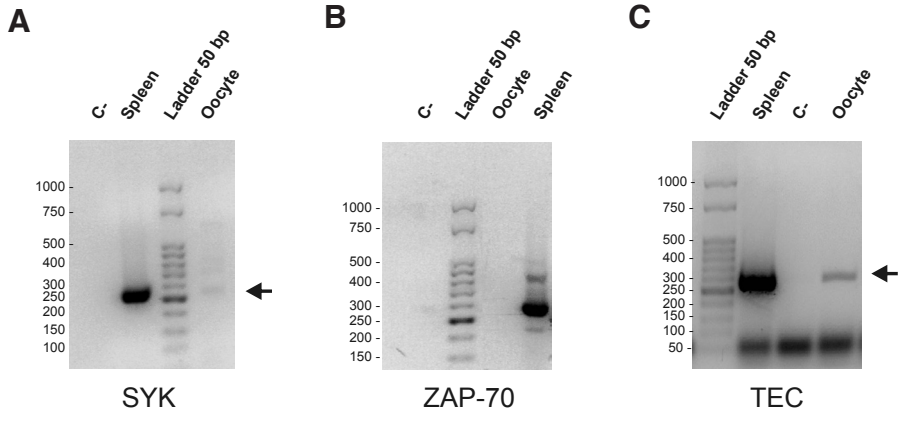

Fig. 3. *Syk* (A)*, ZAP-70* (B) and *Tec* (C) mRNA expression analyzed by RT-PCR. *RNA was isolated and retro-transcribed from oocytes and spleen. The obtained cDNA were used in PCR experiments as described in Methods. Oocyte: PCR performed using* R. arenaum *oocytes cDNA as template; Spleen (positive control): PCR performed using spleen cDNA as template; C- (negative control): PCR in the presence of not retro-transcribed oocyte RNA. Ladder 50 bp: The size is indicated on the left, the most intense band corresponds to 250 bp (base pairs). In all the positive cases the band obtained had the expected molecular size: 266 bp for Syk, 295 bp for ZAP-70 and 283 bp for Tec.*

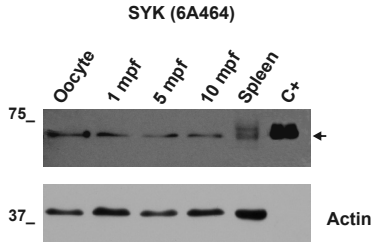

Fig. 4. **Syk protein expression analyzed by western blot.** *Cytosolic fractions were prepared from* Rhinella arenarum *oocytes and eggs at different times after fertilization (minutes post-fertilization (mpf)). Protein samples (20 μg of protein) were separated by SDS-PAGE on 12% polyacrylamide gels, blotted and analyzed using the anti Syk antibody (6A464). Positive controls:* Rhinella arenarum *spleen tissue (Spleen) and recombinant GST-human Syk (6-370) (C+). Actin was used as loading control. Molecular standards are indicated to the left of each panel.*

we analyzed if the enzyme could also be detected in oocytes and early embryos (< 10 mpf). To accomplish this, western blot analysis was performed using a mouse monoclonal antibody raised against amino acids 5-360 of Syk of human origin. The high degree of sequence homology that this protein has with several species (including *H. sapiens*, see Suppl. Fig. 1) encouraged us to test this antibody to detect the *R. arenarum* orthologous protein. Fig.4 shows cytosolic fractions of oocytes and early embryos that were subjected to electrophoresis, transferred to nitrocellulose and proved with the anti Syk antibody. As positive controls a *Rhinella arenarum* spleen homogenate (lane 5) and recombinant GST-human Syk fragment (amino acids 6-370) (lane 6) were also analyzed to discard possible false negatives. Syk protein expression was in this way corroborated in all the stages analyzed.

Syk is activated by tyrosine phosphorylation after fertilization and R406 (Syk inhibitor) impairs fertilization

To evaluate the phosphorylation state of the Syk proteins before and after fertilization, we used two different antibodies, one that is able to recognize Tyr352 phospho-Syk (65E4) and the other that detect Tyr525/526 phospho-Syk (C87C1). Fig.5 shows cytosolic fractions prepared from *Rhinella arenarum* oocytes and eggs at different times after fertilization (1, 5 and 10 mpf). Protein samples were subjected to electrophoresis, blotted and analyzed using the antibodies previously mentioned. As the western blot analysis shows, Tyr352 Syk is phosphorylated as a consequence of the fertilization process, and this modification occurs gradually since the first minute after gamete interaction (Fig. 5A). Fertilization also induces phosphorylation of Syk on tyrosine residues 525/526 (Fig. 5B). This phosphorylation seems to be slower than Tyr352, as it takes 10 min to be evident. This two changes result in a full activated state of the enzyme (see Conclusions).

As Syk becomes phosphorylated at activating tyrosine residues during fertilization, we assessed whether a specific inhibitor of Syk (R406) had any effect on fertilization. We performed fertilization inhibition experiments. Fig. 5C shows that the inhibitor provoked a diminished fertilization score at the two concentrations tested, reaching statistical significance at the concentration of 200 μM when compared with fertilization in the presence of vehicle (control). These experiments suggest a role for Syk at fertilization in *R. arenarum*.

Conclusions

The oocyte is a highly specialized cell poised to respond to fertilization with a unique set of actions needed to recognize and incorporate a single sperm, complete meiosis, reprogram maternal and paternal genomes and assemble them into a unique zygotic genome, and finally initiate the mitotic cell cycle. Oocytes accomplish this diverse series of events through an array of signal transduction pathway components that include a characteristic collection of protein tyrosine kinases.

This study had as its primary goal to try to determine the identity of the 70 kDa protein that showed tyrosine phosphorylation as a consequence of fertilization in *Rhinella arenarum* (Mouguelar *et al.*, 2011). To narrow the search we decided to put some restriction characteristics to the possible candidates. The protein must: 1) have a molecular weight of 70 kDa, 2) be phosphorylatable in tyrosine residues, 3) participate in signaling cascades, 4) be able to interact with oolema receptors (previously we observed that the tyrosine phosphorylated 70 kDa protein appeared first in the plasma membrane fraction and was re-localized to the cytosol minutes later). Candidates turned out to be members of the Syk/ZAP70 family and Tec family of tyrosine kinases.

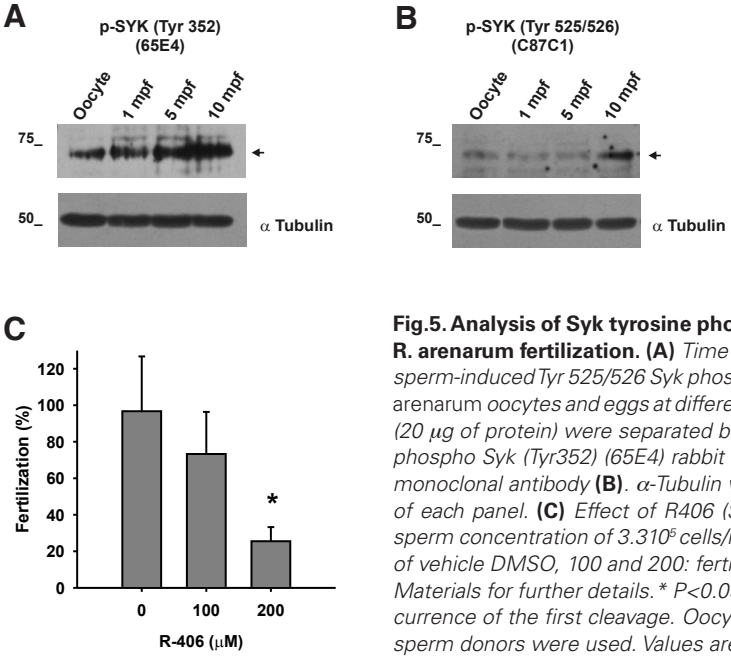

Fig.5. Analysis of Syk tyrosine phosphorylation state and effect of a specific Syk inhibitor (R406) during R. arenarum fertilization. (A) *Time course of sperm-induced Tyr 352 Syk phosphorylation.* **(B)** *Time course of sperm-induced Tyr 525/526 Syk phosphorylation. In both cases, cytosolic fractions were prepared from* Rhinella arenarum *oocytes and eggs at different times after fertilization (minutes post-fertilization (mpf)). Protein samples (20 μg of protein) were separated by SDS-PAGE on 12% polyacrylamide gels, blotted and analyzed using the phospho Syk (Tyr352) (65E4) rabbit monoclonal antibody* **(A)** *or the phospho Syk (Tyr525/526) (C87C1) rabbit monoclonal antibody* **(B)**. *α-Tubulin was used as loading control. Molecular standards are indicated to the left of each panel.* **(C)** *Effect of R406 (Syk inhibitor) in fertilization. Fertilization percentages were acquired at a sperm concentration of 3.310[5] cells/ml. 0: fertilization in the absence of inhibitor but with an equivalent volume of vehicle DMSO, 100 and 200: fertilization in the presence of 100 μM and 200 μM of R406 respectively. See Materials for further details.* P<0.05 versus 0 R406. In all the experiments, fertilization was scored by the occurrence of the first cleavage. Oocytes (80–150 each treatment) were obtained from three animals. Different sperm donors were used. Values are expressed as relative percentages to the control of each animal.*

In the present article we detected the presence of maternal mRNAs that code for the Syk and Tec kinases in *R. arenarum* oocytes. Until now, presence of Syk and Tec mRNAs in oocytes has only been described in two invertebrate species: *Anthocidaris crassispina* and *Schistosoma mansoni* (Sakuma *et al.*, 1997; Knobloch *et al.*, 2002). So, to our knowledge this is the first time that Syk and Tec expression in oocytes are informed in any vertebrate. Meanwhile ZAP-70 expression is relatively restricted to T cells and NK cells (Chan *et al.*, 1992; Béné, 2006; Wang *et al.*, 2010) so it was not surprising that we could not detect mRNA for ZAP-70 in *R. arenarum* oocytes.

Syk is widely expressed in hematopoietic cells, including mast cells and basophils (Oliver *et al.*, 2000), neutrophils, T-cells, B-cells, NK cells, platelets (Turner *et al.*, 1997, 2000; Sada *et al.*, 2001), monocytes, macrophages, dendritic cells (Sedlik *et al.*, 2003) and even red blood cells (Harrison *et al.*, 1994). More recently, the distribution of Syk kinase was found not only restricted to hematopoietic cells, and Syk expression has been identified in several cell types of non-hematopoietic origin: breast (Coopman *et al.*, 2000), lung (Ulanova *et al.*, 2005), epithelial cells, fibroblasts (Yamada *et al.*, 2001), hepatocytes (Tsuchida *et al.*, 2000), endothelial cells (Yanagi *et al.*, 2001), neuronal cells (Tsujimura *et al.*, 2001), osteoclasts (Faccio *et al.*, 2005) and kidney glomerular mesangial cells (Tsuge *et al.*, 2003).

Syk contains two tandem SH2 and C-terminal kinase domains, interrupted by two interdomains: A and B (Sada *et al.*, 2001; Bradshaw, 2010; Mócsai *et al.*, 2010). A major mechanism for regulating Syk family kinases is via changes in phosphorylation. Several phosphorylation sites in Syk kinases have been identified (Furlong *et al.*, 1997; Geahlen, 2009). These include two Tyr residues within the activation loop (Y525, Y526), two Tyr residues within the SH2-kinase linker (Y348, Y352), and Tyr residues near the C-terminus of Syk (Y630). Three of these sites (Y352, Y525, Y526), have been frequently associated with the enzymatic activation of the kinase (Zhang *et al.*, 2000; Speich *et al.*, 2008; Geahlen, 2009; Bradshaw, 2010). Syk is autoinhibited in its resting state owing to the binding of interdomains A and B to the kinase domain. Phosphorylation of the Tyr 352 residue, present in the interdomain B, causes a conformational change that abolishes the autoinhibition, partially activating the enzyme. Nevertheless, for a full activation of the enzyme residues Y525 and Y526 have to be also phosphorylated.

In this work, we not only described the expression of the Syk protein in *Rhinella arenarum* oocytes, but also its full activation through the phosphorylation of tyrosines residues 352, 525/526 in the early fertilized embryo. Moreover, the presence of a specific inhibitor of Syk (R406) in fertilization media partially impaired it, suggesting that Syk activation is important for fertilization success.

It is well known that once activated, Syk catalyzes the phosphorylation of multiple protein substrates that are important for transducing the ligand-receptor interaction into the appropriate physiological response. It remains to be determined which are the downstream effectors that are consequently phosphorylated after Syk activation in the *R. arenarum* oocyte. Nevertheless, we propose that during fertilization Syk may be involved: 1) in the signal transduction pathway that mediates the actin cytoskeletal re-organization that finally leads to sperm engulfment. Evidences that support this hypothesis are those papers that propose Syk as a phagocytosis mediator (Greenberg *et al.*, 1996; Crowley *et al.*, 1997; Raeder *et al.*, 1999; Berton *et al.*, 2005; Tohyama and Yamamura, 2009) and also those reports that postulate the oocyte as a non-professional phagocyte (Bronson, 1998; Bronson 2009); and/or 2) in the signaling pathway that cause the transient increase in intracellular calcium that ultimately provoke the cortical granule exocytosis, like in mast cell and basophils, where Syk mediates the signaling that provoke degranulation (Siraganian *et al.*, 2010; Ninomiya *et al.*, 2010; Lu *et al.*, 2012, 2014; Matsuo *et al.*, 2013).

The Syk tyrosine kinase was originally thought to only contribute to signaling responses of immunoreceptors of the adaptive immune response. However, more recent studies have identified a large number of new functions of Syk both in the immune system and beyond. In this work we present Syk as a participant in the fertilization process, a role not described until now in any animal species.

Methods and Methods

Materials, antibodies and recombinant proteins

The following antibodies were used: rabbit polyclonal antibodies anti-Phosphotyrosine (PY350, sc-18182), rabbit polyclonal antibodies anti-Phospho-Extracellular Regulated Kinase (p-ERK 1/2 Thr202/Tyr204, R-sc-16982), mouse monoclonal antibodies anti-Syk (6A464, sc-73089) and rabbit antibodies anti-actin (R-sc-1616) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Mouse monoclonal antibodies anti-α-tubulin (T5168) was from Sigma–Aldrich (St. Louis, MO, USA). Rabbit monoclonal antibodies anti- Phospho-Syk (Tyr525/526) (C87C1) and anti-Phospho-Syk (Tyr352) (65E4) were from Cell Signaling Technology (Beverly, MA, USA).

All these antibodies (except PY350) were produced by immunizing animals with recombinant fragments of the corresponding human proteins. The immunogen used to produce the PY350 antibody was O-phospho-L-tyrosine.

Syk inhibitor R406 (6-(5-fluoro-2-(3,4,5-trimethoxyphenylamino) pyrimidin-4-ylamino)-2,2-dimethyl-2H-pyrido[3,2-b][1,4]oxazin-3(4H)-one benzenesulfonate) was from Selleckchem (Houston, TX, USA). R406 stock solution was prepared in DMSO at a concentration of 10 mM.

Goat anti-mouse IgG (HCL)–HRP Conjugate and Precision Plus Protein Standards Dual Color were from Bio-Rad (Hercules, CA, USA) and donkey anti-rabbit HRP-conjugated F(ab)2 was from Amersham (Piscataway, NJ, USA). Supersignal West Pico Chemiluminescent Substrate was from Pierce (Rockford, IL, USA). Amersham Hybond ECL Nitrocellulose was from GE Healthcare (Buckinghamshire, UK). X-ray films were Amersham Hyperfilm ECL High Performance chemiluminescence film or AGFA ortho CP-G PLUS (Medical X-Ray Film Agfa – Gevaert Argentina S.A.). All other reagents were purchased from Sigma-Aldrich.

Recombinant human SYK fragment containing an N-terminal Glutathione-S-Transferase tag (pGEX-2T plasmid generously provided by Dr. Woodside, D., La Jolla, California, USA) was produced in a prokaryotic system (*E. coli* BL21 [DE3] pLysS) and purified using Glutathione Sepharose 4 Fast Flow matrix (GE Healthcare; Uppsala, Sweden) according to the manufacturer's protocol. The protein was dialyzed and concentrated using filter devices (Amicon; Millipore). Afterwards the recombinant protein was used as a control in immunoblot assays.

Animals, gametes and eggs

Sexually mature *R. arenarum* (Anura: Bufonidae) (Hensel, 1867) specimens (15) were collected in the neighborhoods of Rosario City and kept in a moist chamber at 12°C until used. All experiments were performed in conformity with the guide for the care and use of laboratory animals promulgated by the National Institute of Health (National Center for Research Resources), Bethesda, MD, USA.

Testes were dissected from male toads, and spermatozoa were obtained by mincing the organs in Ringer–Tris solution (0.11 M NaCl, 2 mM KCl, 1.4 mM $CaCl_2$, 10 mM Tris, pH 7.2) at 4°C. The homogenate was filtered through gauze and the suspension was centrifuged for 10 min at 130 *g* at 4°C to remove blood cells and tissue debris. The sperm suspension was centrifuged for 10 min at 650 *g* at 4°C. Pelleted spermatozoa were

resuspended in Ringer–Tris solution and the concentration of cells was estimated measuring optical density at 410 nm.

Female specimens were kept in a moist chamber at 20–22°C for 1 day before stimulation with one homologous hypophysis homogenate injected intracoelomically. After 10–12 h, deposed oocyte strings were collected. Fertilized eggs were obtained by adding sperm suspension to a monolayer of oocytes in plastic dishes followed by the addition of excess 0.1X Ringer solution. Sperm at a final concentration of at least $5x10^5$ cells/ml were used to fertilize 600–800 oocytes/dish. Fertilization was stopped at the desired times with ice-cold 1X Ringer and dejelling was performed by a short exposure to 1% v/v thioglycolic acid pH 8.0. Oocytes were thoroughly washed with Ringer solution and kept frozen until used.

Egg water

Egg water (EW) was obtained as described previously (O'Brien *et al.*, 2011). Briefly, strings of *R. arenarum* oocytes were removed from the ovisacs and extracted with distilled water with occasional stirring at 20°C–22°C. After 20 min the diffusible fraction (EW) was decanted. Oocytes not visibly perfect were not used.

Oocyte artificial activation

Parthenogenetic activation of *Rhinella arenarum* oocytes was accomplished by oocyte incubation in 50% Steinberg's solution (SB) containing A23187 (5-(Methylamino)-2-[[2*R*,3*R*,6*S*,8*S*,9*R*,11*R*)-3,9,11-trimethyl-8-[(1*S*)-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid) at a final concentration of 2,5 μM. To stop the activation procedure, after 10 min of incubation eggs were washed with 50% SB. After washing and removal of excess buffer, eggs were immediately frozen by liquid nitrogen and kept at -80°C until use. Steinberg's solution contained: 58 mM NaCl, 0.67 mM KCl, 0.34 mM $CaCl_2$, 0.85 mM $MgSO_4$, and 4.6 mM Tris-HCl (pH 7.4).

Pretreatment of oocytes with the tyrosine kinase inhibitor genistein

Unfertilized eggs were preincubated in 50% SB containing 100 μM genistein for 1 h, washed with 50% SB and inseminated in an inhibitor free medium. A concentrated stock solution of the inhibitor was prepared and diluted 100-fold with SB so that the final DMSO concentration was 1% in SB. Thus, control eggs were pretreated with 1% DMSO and inseminated.

Fertilization inhibition studies

These assays were performed essentially as described in Mouguelar *et al.,* (2011). Briefly, groups of 6–8 jelly-intact oocytes were placed in wells filled with 100 μl of R406 (100 μM, 200 μM or vehicle control) solution in 50% SB. After 20 min, the lack of activation was visualized under the stereoscopic magnifier and oocytes were washed thoroughly with SB. Then, 40 μl of egg water were added. Egg water was supplemented to eliminate any dilution of the factors that are necessary for successful fertilization (Arranz and Cabada, 2000). Fertilization was carried out adding 5 μl of sperm stock solution to reach the desired final sperm concentration (3.3 10^5 cells/ml, see Mouguelar *et al.,* (2011) for details on the sperm concentration). After 25 min, the eggs were washed three times with 100 μl of 50% SB each. Finally, fertilization was scored as the occurrence of the first cleavage, since this was the most accurately measurable indicator of fertilization.

Statistical analysis

Results are expressed as mean ± SEM. Data were analyzed using Student t-test or the one-way analysis of variance followed by the Holm-Sidak method. The 0.05 level of probability was used as the criterion of significance.

Cytosol isolation of oocytes and eggs

Sub-cellular fractionation was performed according to the procedure described by Sato *et al.,* (1999) with modifications (Mouguelar and Coux, 2012; Fernández-Bussy *et al.*, 2013). All procedures were carried out at 4°C. Approximately 200 dejellied eggs/embryos were mixed with 2 ml of buffer A (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4) supplemented with 1% protein inhibitor cocktail (Sigma-Aldrich) and 2 mM of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF) and homogenized in a Teflon-glass potter. The homogenate was centrifuged 10 min at 1000 *g* (SS34 Rotor, Sorvall Instruments) to remove cellular debris and yolk platelets. The supernatant was collected and centrifuged at 150000 *g* for 1 h (Ti80 rotor, Beckman L-80 ultracentrifuge). After centrifugation, the clear supernatant was set aside as the cytosolic fraction.

Protein assays

Protein concentrations were determined according to Sedmak and Grossberg (1977), using bovine serum albumin as standard.

Electrophoresis, electrotransfer and western blot analysis

Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was performed essentially according to the Laemmli's Method (1970). Samples were diluted with an appropriate volume of 6X sample buffer with 0.1 M dithiothreitol, boiled for 5 min, and loaded onto 12% acrylamide mini-gels with 5% stacking gel, and electrophoresed at 20 mA/gel (MiniProtean III Gel System, *Bio-Rad*, Hercules, CA, USA). The apparent molecular weights were estimated with molecular mass standards (*Amersham Pharmacia Biotech*, Piscataway, NJ, USA). Gels were processed for Coomassie Brilliant Blue staining or electrotransferred to nitrocellulose membranes according to Towbin's method (Towbin *et al.*, 1979).

Membranes were blocked with TBS buffer supplemented with the appropriate blocker (5% w/v nonfat dry milk for α-tubulin, actin and Syk; 1% w/v gelatine for PY-350, p-ERK1/2, p-Syk (Tyr352), and p-Syk (Tyr525/526)). Membranes were washed with TBS–Tween-20 0.1% v/v and incubated with the primary antibody dilution (PY350: 1:500, p-ERK1/2: 1:500, Syk: 1:500, p-Syk (Tyr352): 1:1000, p-Syk (Tyr525/526): 1:1000, Actin: 1:600, α-Tubulin: 1:5000). After washing, membranes were incubated with the appropriate HRP-conjugated antibody (1:8000–1:10000 dilution), washed and developed using chemiluminiscence and X-ray films. Actin and α-Tubulin detection on membranes was performed to ensure equal loading.

mRNA expression analysis

RNA was isolated from *Rhinella arenarum* unfertilized eggs or spleen tissue using the TRIzol Reagent (*Invitrogen*, Carlsbad, CA, USA) following the manufacturer's instructions. Purified RNA was incubated with RQ1 DNAse (*Promega Corporation*, Madison, WI, USA). Total RNA was retro-transcribed with SuperScript II enzyme (Invitrogen, Carlsbad, CA, USA) and 16-18 oligo(dT).

RT-PCR experiments were performed with degenerate primers directed against highly conserved regions of the proteins. PCR amplification was carried out using the following primers:

Syk_{forw}: 5'-CATCAGGTYTCCATGGGMATGAA-3',
Syk_{rev}: 5'-TCCCACATYTRAAYTCCAAAGCTCCA-3';
$ZAP\text{-}70_{forw}$: 5'-TGGARCAGGCMATMATYAGCCAGGC-3',
$ZAP\text{-}70_{rev}$: 5'-ACMAGYTGCCAKAGSGTGTCAAA-3;
Tec_{forw}: 5'-GBATGTGYCWRGATGTRTGTGA-3',
Tec_{rev}: 5'-CCYTCASTRAACACYTCCCACA -3'.

The amplification reaction consisted of 2 μl of the cDNA template, 1 U Taq DNA Polymerase (*Invitrogen*, Carlsbad, CA, USA), 200 μM dNTPs, 20 pmol of each primer, 2.5 mM $MgCl_2$ (total reaction volume = 50 μl). The reaction mix was subjected to 35 cycles of amplification (denaturation: 95°C for 1 min, annealing: 30 sec at 50°C for Syk and ZAP-70 and at 58°C for Tec, extension: 72°C for 30 sec), followed by a final extension step of 10 min at 72°C. Products were resolved in 2% (w/v) agarose gels stained with Gel Green, extracted from them and finally cloned into pGEM-T Easy Vector (*Promega Corporation*, Madison, WI, USA). Sequencing was performed by the University of Maine DNA Sequencing Facility. Sequence identity was confirmed using the basic local alignment search tool (BLAST) supported by the National Center for Biotechnology Information. Multiple sequence alignments were carried out using the CLUSTAL W (1.82) algorithm (Higgins *et al.*, 1996).

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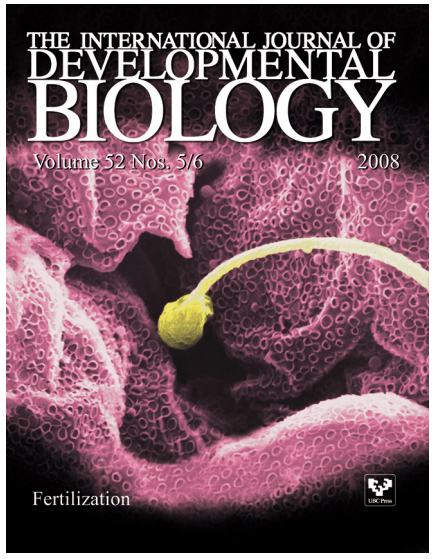

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