The integrin-binding motif RGDS induces protein tyrosine phosphorylation without activation in *Bufo arenarum* (Amphibia) oocytes

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Abstract

Integrins are cell adhesion molecules that are thought to be involved in sperm–oocyte interaction. Nevertheless, their function in mammalian fertilization is still controversial, as different species behave differently. In amphibians, their role is mainly supported by *Xenopus laevis* studies, where RGDS peptide induces oocyte activation. We recently provided evidence suggesting the presence and involvement of integrins in the interaction of the oocyte plasma membrane (PM) with sperm in the amphibian *Bufo arenarum*. In order to understand the role of integrin homologs in oocytes and their possible contribution to egg activation mechanisms, we examined the presence of integrin subunits and the effect of RGDS peptide on oocytes and during fertilization. Western blot studies detected integrin subunits $\alpha 5$, αV and $\beta 1$ in oocytes. In sperm, we could detect only the αV integrin subunit. We found that RGDS peptide was unable to elicit egg activation or MAPK dephosphorylation, but can induce reversible inhibition of fertilization. A similar partial inhibition was produced by an anti- $\beta 1$ integrin antibody. Using an anti-phosphotyrosine antibody we found major changes in phosphotyrosine-containing proteins in egg extracts minutes after fertilization. Cytosol and PMs isolated from oocytes and fertilized eggs showed additional fertilization-induced phosphorylated proteins. Some of these were also present in cytosol and PMs from RGDS-treated oocytes (partially mimicking fertilization). These findings suggest that *B. arenarum* fertilization involves integrins (e.g. $\beta 1$ subunit) as adhesion proteins. Our data support the view that RGDS-binding receptors may function as signaling receptors in *B. arenarum* oocytes, but integrin engagement by RGDS is not sufficient for oocyte activation.

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Introduction

Sperm–egg interaction is a complex molecular process leading to gamete fusion mediated by a series of molecular interactions. It is well established that binding of sperm ligands to specific oolemma receptors is a necessary step in sperm–oocyte interaction, leading to fertilization (Kaji & Kudo 2004). Blobel *et al.* (1992) suggested a model for sperm–egg plasma membrane (PM) interaction in the guinea pig, where the binding results from an integrin on the egg adhering to an integrin ligand (disintegrin) on the sperm. In mammalian sperm, the binding to the egg membrane integrins is believed to be mediated by A disintegrin and A metalloprotease domain (ADAM) proteins (also known as metalloprotease/disintegrin/cysteine-rich (MDC)), although other candidates also have been proposed (Boissonnas *et al.* 2010, Yang *et al.* 2010). In different mammals, the role of integrins in fertilization has been the subject of

numerous, and some of them quite contradictory, reports (Almeida *et al.* 1995, Linfor & Berger 2000, He *et al.* 2003, Sessions *et al.* 2006, Tatone & Carbone 2006). Almeida *et al.* (1995) concluded that the integrin $\alpha 6 \beta 1$ serves as a murine sperm receptor. Later, several works argued against a role for integrins in mouse fertilization (Miller *et al.* 2000, He *et al.* 2003). However, in a recent work in mouse, it was suggested that the presence of $\beta 1$ integrin enhances the initial adhesion of sperm to egg PM and that subsequent attachment and fusion are mediated by additional proteins (Baessler *et al.* 2009). In another mammal, bovine, sperm–oocyte interactions and egg activation have been shown to involve integrins (Campbell *et al.* 2000, Sessions *et al.* 2006). Among amphibians, data are mainly restricted to *Xenopus laevis*. In this amphibian model, oocyte integrins have been consistently involved in egg activation during fertilization. Iwao & Fujimura (1996) reported that dejellied oocytes treated with RGDS (an integrin

interacting peptide) at concentrations higher than 250 μ M produced a positive-going activation potential concomitant with intracellular Ca^{2+} release. Shilling *et al.* (1997, 1998) identified and cloned members of the ADAM family from *X. laevis* testis, and peptides derived from the disintegrin domains of three of these were found to inhibit fertilization or activate eggs. Sato *et al.* (1999) showed that parthenogenetic activation by a synthetic RGDS peptide, but not by electrical shock or the calcium ionophore A23187, causes Xyk kinase (Src-related) activation.

Integrins are transmembrane glycoproteins with heterodimeric structure (α chain– β chain) that act as co-receptors in many cell–cell interactions (Hynes 1992). Individual integrins can bind to more than one ligand and about half of them recognize the tripeptide sequence Arg-Gly-Asp (RGD) present in extracellular matrix proteins such as fibronectin and vitronectin (Ruoslahti & Pierschbacher 1986). Although the cytoplasmic domains of most integrins are short and do not have intrinsic catalytic properties, they can bind to proteins of the transduction machinery (Harburger & Calderwood 2009). Multiple intracellular signaling molecules are stimulated following integrin-dependent adhesion. These include members of MAPK or ERK signaling pathways, Rho family GTPases, non-receptor tyrosine kinases, such as focal adhesion kinase, integrin-linked kinase and Src, and members of the lipid signaling pathways, such as phosphatidylinositol 3-kinase, and protein kinase C (Rucci *et al.* 2005, Harburger & Calderwood 2009). Some of these signaling proteins are directly associated to the integrin cytoplasmic domain (Harburger & Calderwood 2009).

Earlier work in this laboratory has been focused on the detection of molecules involved in sperm–oocyte PM interaction in the amphibian *Bufo arenarum*. In particular, we recently provided evidence suggesting the presence and involvement of integrins in the interaction of the oocyte PM with sperm (Coux & Cabada 2006). More specifically, chromatographic studies showed oocyte molecules of 100, 70 and 30 kDa with affinity for sperm. Competition studies with an integrin-interacting peptide (RGDS) suggested that the 100 kDa band detected could be a member of the integrin family. This was in agreement with different works that either probed the presence in eggs (Gawantka *et al.* 1992, 1994) or give a role to integrins in *Xenopus* fertilization (Iwao & Fujimura 1996, Shilling *et al.* 1997, 1998). Other amphibians, however, have not received much attention regarding the role of integrins in this process.

In order to understand the role of integrin homologs in *Bufo* oocytes and their possible contribution to the egg activation mechanisms, we examined the presence of integrin subunits and the effect of RGDS peptide over oocytes and during fertilization. In the present study, we have found that although the RGDS peptide was unable to elicit egg activation, it produced a partial inhibition

of fertilization. We also show that the RGDS peptide induced protein tyrosine phosphorylation (partially mimicking fertilization) but not MAPK dephosphorylation or an activated phenotype.

Results

Detection of integrin subunits in oocyte membranes

As β 1 integrin subunit has been involved in fertilization (Almeida *et al.* 1995, Linfor & Berger 2000, Baessler *et al.* 2009), we decided to check its presence in *B. arenarum* oocytes. Total membranes (TMs) were isolated from oocytes and western blots were performed using the 8C8 MAB against *X. laevis* β 1 integrin subunit developed by Gawantka *et al.* (1992). Figure 1 shows a typical Coomassie Blue-stained gel of the TMs and cytosolic fractions obtained. The next panel (panel β 1, 8C8) shows that 8C8 antibody recognizes two bands close to the 100 kDa marker in *B. arenarum* TMs samples but not in cytosol samples. This doublet has been explained due to the ability of the antibody to detect precursors or immature forms of the protein (Gawantka *et al.* 1992). We also analyzed the presence of β 1 integrin with a polyclonal antibody against human β 1 integrin (M-106, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Figure 1 (panel β 1, M-106) shows that this antibody also recognizes a doublet of similar mobility. Then we analyzed the presence of the β 3 integrin subunit in oocytes using a polyclonal antibody against the human homolog (H-96, Santa Cruz Biotechnology), but we could not detect it (Fig. 1, panel β 3). Human platelets lysate showed a band at the expected molecular weight (MW, $\sim$ 135 kDa). Integrins are transmembrane glycoproteins with heterodimeric structure (α chain– β chain), so we decided to analyze the presence of α subunits in *B. arenarum* oocytes. The β 1 subunit can associate with 12 different α subunits, forming the largest subfamily of integrins (Liddington & Ginsberg 2002) but only three of them (α 5 β 1, α V β 1, α 8 β 1) belong to the RGD family (Hynes 1992). We analyzed the presence of α 5 and α V subunits in the oocyte PM. The α 5 panel in Fig. 1 shows the results obtained with the MAB D71E2 against chicken α 5-integrin (Muschler & Horwitz 1991). The first two lanes are positive chicken muscle controls (with and without dithiothreitol (DTT)). In the presence of DTT, the chicken sample shows a band slightly below the 150 kDa marker. In the absence of reducer, there are two bands: one of 150 kDa and the other above 250 kDa (less stained). The cytosol from *B. arenarum* eggs does not show any signal. The lane corresponding to TMs without DTT shows a diffuse signal around 250 kDa, and under reducing conditions, there is a more defined band that migrates slightly above the 150 kDa. We also checked the presence of α V integrin subunit using a polyclonal antibody (H75, Santa Cruz Biotechnology)

Figure 1 $\beta 1$, $\beta 3$, $\alpha 5$ and αV integrin subunits in *Bufo arenarum* oocytes. The expression of $\beta 1$, $\beta 3$, $\alpha 5$ and αV integrins was assayed by immunoblot analysis of oocyte samples. Proteins from total membranes (TM) and cytosolic (Cyt) fractions from oocytes were separated by SDS-PAGE, stained (Coomassie panel) or immunoblotted with an antibody to *Xenopus laevis* $\beta 1$ (8C8) or to *Homo sapiens* $\beta 1$ integrin subunit (M-106), both shown in the figure under the $\beta 1$ label. The brackets indicate the doublet corresponding to $\beta 1$ detected forms (mature and immature). $\beta 3$ integrin subunit was analyzed using an antibody against *H. sapiens* $\beta 3$ integrin subunit (H-96). Positive control is human platelets lysate. The presence of $\alpha 5$ subunit was investigated using a *Gallus gallus* antibody (D71E2). As positive controls, chicken muscle extracts (indicated as C+) were included. The presence (+) or absence (–) of reducer (Red) is indicated below the panel. αV integrin subunit was detected using an antibody to *H. sapiens* αV integrin subunit (H75) and the positive control is HeLa cells whole extract. Ten micrograms protein of each sample were loaded onto each lane. The prestained molecular markers are shown to the right of each panel. The immunoactive bands are indicated with arrows.

against the human homolog. As shown on Fig. 1 panel αV , a specific band at ~ 120 kDa was detected on oocyte TMs as in the HeLa whole cell lysate positive control. The cytosolic fraction did not show any band.

The presence of these integrin subunits in sperm was analyzed (Fig. 2). As the figure shows, $\beta 1$, $\beta 3$ and $\alpha 5$ integrin subunits could not be detected. These experiments were performed with sperm samples from different males, and although the cell number loaded onto the gels was increased, no signal was detected. On the other hand, integrin αV was clearly detected on sperm samples (Fig. 2 panel αV). We observed two bands at a slightly higher MW than the band in oocyte and HeLa extracts (see Discussion).

Effect of RGDS peptide on *B. arenarum* oocyte activation and on MAPK (ERK1/2) phosphorylation

RGDS peptide was examined for its ability to induce parthenogenetic development in *B. arenarum* oocytes but no activation was detected at concentrations up to 1 mM of RGDS (data not shown). This concentration was shown to activate *X. laevis* (Iwao & Fujimura 1996) and bovine eggs (Sessions *et al.* 2006). The oocytes that were unable to undergo activation under RGDS treatment responded to calcium ionophore with all the signs of activation (data not shown).

High levels of MAPK activity are incompatible with pronuclear formation in vertebrate activated/fertilized

eggs (Maller *et al.* 2001, Inoue *et al.* 2007). To test whether inhibition of MAPK activity occurs in RGDS-treated *B. arenarum* eggs, we measured the level of dephosphorylation (inactivation) of MAPK using a specific anti p-ERK 1/2 antibody. First, we studied the physiological dephosphorylation due to fertilization in *B. arenarum* eggs. Figure 3A shows that MAPK is highly phosphorylated in oocytes and short times after fertilization (5 min). Ten minutes post-fertilization, MAPK is almost completely dephosphorylated. Figure 3B shows another batch of oocytes, fertilized and under the effect of 10 min of RGDS treatment. As shown in the figure, RGDS did not induce MAPK dephosphorylation. Even longer times were assayed, and MAPK remained phosphorylated at the initial (oocyte) level. In all the assays, the addition of sperm induced MAPK dephosphorylation, indicating that the oocytes were able to undergo activation (data not shown).

Effect of RGDS peptide and anti- $\beta 1$ integrin antibodies on *B. arenarum* fertilization

As RGDS could not elicit activation, we assessed whether the peptide could impair the ability of sperm to fertilize oocytes; so we performed fertilization inhibition experiments. Figure 4A and B shows the effect of 1 mM RGDS on *B. arenarum* fertilization. Figure 4A shows the effect of the presence of 1 mM RGDS at different sperm concentrations. At all sperm

Figure 2 $\beta 1$, $\beta 3$, $\alpha 5$ and αV integrin subunits in *Bufo arenarum* sperm. Integrin subunits $\beta 1$, $\beta 3$, $\alpha 5$ and αV were assayed by immunoblot analysis of sperm proteins (Sp) extracted with detergent (SDS 0.5% w/v). Proteins were separated by SDS-PAGE, stained (Coomassie panel) or immunoblotted with 8C8, M-106, H-96, D71E2 or H-75 antibodies. Each lane was loaded with the proteins extracted from 4 or 8 millions of sperm, respectively. Positive control is *B. arenarum* oocyte TM for all the immunoblots. The $\beta 3$ immunoblot includes human platelets whole lysate as control. The $\alpha 5$ immunoblot includes a chicken muscle sample (indicated as C+) as another control, and the αV immunoblot includes a HeLa cells whole lysate control. α -Tubulin was used to show sperm sample loading. Positive control protein loadings were lowered to allow long expositions for integrin subunits detection in sperm samples. The prestained molecular markers are shown to the right of each panel. The immunoactive bands are indicated with arrows or a bracket ($\beta 1$ integrin doublet).

concentrations, the presence of the peptide induced a lower fertilization rate reaching statistical significance at a sperm concentration of 3.3×10^5 cells/ml, where RGDS induced a decrease in fertilization of nearly 50%. It is remarkable that although the peptide was added 20 min earlier than sperm, at high sperm concentrations the effect of the peptide seemed to be abolished, suggesting a reversible action. Further tests were performed at sperm concentration of 3.3×10^5 cells/ml: i) a peptide with only alanine in its sequence and the RGES peptide did not induce statistically significant inhibition and ii) the pre-incubation of sperm with the RGDS peptide followed by washout showed no inhibition when used in the fertilization assay (Fig. 4B). These results suggest a role for an oocyte RGDS-binding protein (e.g. an integrin) in gamete interaction during fertilization in *B. arenarum*. To further test the role of integrin, we performed IVF assays in the presence of anti- $\beta 1$ integrin polyclonal antibody M-106, which was developed against amino acids 375–480 mapping on the extracellular domain of $\beta 1$ integrin subunit. We first studied $\beta 1$ integrin expression by immunofluorescence and confocal microscopy using

M-106 antibody. Figure 5A–H shows that $\beta 1$ integrin is localized to the PM in both oocyte vegetal and animal poles. In some cases, the animal PM was more heavily stained than the vegetal membrane; in others, the $\beta 1$ integrin subunit signal was evenly distributed (data not shown). Figure 5I shows that the antibody provoked statistically significant inhibition of fertilization when compared with control fertilization or with fertilization in the presence of an equal amount of normal rabbit IgG or an antibody to the Na^+/K^+ -ATPase.

Analysis of the tyrosine phosphorylation pattern during *B. arenarum* fertilization

Fertilization in other species is accompanied by phosphorylation signals (Peaucellier *et al.* 1988, Kinsey 1997, Sato *et al.* 2002, McGinnis *et al.* 2007) and tyrosine phosphorylated proteins have been detected after engagement of RGDS with its receptor (Sato *et al.* 1999, Ortega-Velazquez *et al.* 2003, Plows *et al.* 2006). We decided to check if *B. arenarum* fertilization showed changes in protein tyrosine phosphorylation and if any of these changes could be induced by RGDS treatment.

Figure 3 Effect of sperm and RGDS peptide on p42 MAPK phosphorylation state. (A) Time course of sperm-induced p42 MAPK dephosphorylation. Total extracts were prepared from *Bufo arenarum* oocytes and eggs at different times after fertilization (across the top: oocytes and minutes post-fertilization (mpf)). Protein samples (10 μ g of protein) were separated by SDS-PAGE on 12% polyacrylamide gels, blotted and analyzed using the anti p-ERK 1/2 Thr202/Tyr204 antibody. (B) RGDS effect on p42 MAPK phosphorylation. Total extracts (10 μ g of protein) were prepared from *B. arenarum* oocytes that were incubated 10 min with the RGDS peptide (1 mM). The phosphorylation state of p42 MAPK protein in this sample is shown accompanied by samples of oocytes and eggs at 1 or 10 min after fertilization from the same batch. The position of phospho-p42 MAPK is indicated by closed arrows in both immunoblottings. The prestained molecular markers are shown to the left of each panel. α -Tubulin was used as loading control.

We first analyzed protein tyrosine phosphorylation in egg total extracts by immunoblot before and at short times (1, 5, 10 and 30 min) after fertilization and also in oocytes treated with the RGDS peptide for 10 min. Figure 6 shows a representative Coomassie-stained gel of the total extracts used for blotting and a typical experiment with antibody PY350 alone and in the presence of 100 $\times$ excess of its inhibitory peptide. It also shows tubulin detected on the same blot to ensure equal loading. Several immunoreactive bands were detected. They were phosphotyrosine specific, because addition of its inhibitory peptide in the immunoblotting reaction resulted in a competitive disappearance of all the signals observed. In addition, fertilization caused no dramatic alterations of protein distribution or content (Fig. 6, Coomassie panel). Antibody PY350 recognized phosphotyrosine residues in proteins of apparent MWs of 42, 33, 31 and 25 kDa (arrows in Fig. 6). A 42-kDa protein (p42) was found to be the major phosphotyrosine-containing protein of unfertilized eggs. It is clearly shown that fertilization triggered a decrease in the intensity of this band. It started to decrease at 10 min after insemination and disappeared almost completely by 30 min. The phosphorylation signals of 33, 31 (sometimes as a doublet) and 25 kDa remain till 5 min and then decrease rapidly. The pattern observed in the lane that corresponds to oocytes treated with the RGDS peptide is the same as that of the oocyte lane. These

results indicate that at an early time period after fertilization, a number of egg proteins are subjected to tyrosine phosphorylation or dephosphorylation. Some of these proteins can be detected in total extracts. However, none of these changes can be visualized in oocytes extracts after RGDS treatment.

Phosphorylation of proteins in PM and cytosol during fertilization and RGDS treatment

To further explore if RGDS could induce any change in tyrosine protein phosphorylation, we worked with cytosol and purified PM fractions. The aim of these experiments was to improve the detection by enriching and purifying the samples to be analyzed as well as to determine the subcellular localization of phosphorylated bands. The amphibian oocyte is a large cell with abundant intracellular organelles that could compromise the detection of fertilization and RGDS-induced protein tyrosine phosphorylation. Figure 7 shows a typical experiment in which cytosol and PMs samples were isolated from oocytes, eggs 1 and 10 min after fertilization and oocytes treated with RGDS 1 mM for 10 min. Coomassie Blue-stained gels, the PY350 immunoblot (with the loading control) and the inhibitory peptide control are included in the figure. The panel of PM samples shows a strong 42 kDa band that is markedly reduced only in the 10 min post-fertilization lane. In the 1 mpf lane, there are a couple of faint bands

Figure 4 Effect of the presence of RGDS peptide in *Bufo arenarum* fertilization. (A) Sperm concentration dependence of RGDS action. Fertilization percentages were obtained at different sperm concentrations (1×10^4 , 5×10^4 , 1×10^5 , 3.3×10^5 and 1×10^6 cells/ml) in two conditions, presence (triangles) or absence (closed circles) of RGDS peptide 1 mM. $*P < 0.05$ vs 3.3×10^5 cells/ml in the absence of peptide. Oocytes (18–30 each treatment) were obtained from three to four animals. (B) Specificity of RGDS action over oocytes. Fertilization percentages were acquired at a sperm concentration of 3.3×10^5 cells/ml during different treatments. Control: fertilization in the absence of any peptide. AAA: fertilization in the presence of 1 mM of the AAA peptide. RGES: fertilization in the presence of 1 mM of the RGES peptide. Pre-inc: fertilization using sperm that were pre-incubated with the RGDS peptide. RGDS: fertilization in the presence of 1 mM of the RGDS peptide. $**P < 0.01$ versus Control, AAA, RGES and Pre-inc. In all the experiments, fertilization was scored by the occurrence of the first cleavage. Oocytes (24–40 each treatment) were obtained from three animals. In both experiments, different sperm donors were used. Values are expressed as relative percentages to the control of each animal.

Figure 5 $\beta 1$ integrin subunit immunofluorescence localization in oocytes and effect of anti- $\beta 1$ integrin antibody presence in *Bufo arenarum* fertilization. (A–F) Oocyte sections were incubated with anti- $\beta 1$ integrin subunit antibody M-106, followed by staining with Cy3 conjugated secondary antibody (red signal). The arrows show integrin $\beta 1$ subunit expression at the oocyte plasma membrane. (G and H) Controls in the absence of primary antibody. The images were taken with a confocal laser scanner. (A–D–G) DIC/Nomarski images, (B–E–H) Cy3 fluorescence images ($\beta 1$ integrin), (C–F) overlapped images of DIC and $\beta 1$ integrin. v.p., vegetal pole; a.p., animal pole. The arrowhead in D and F shows the vitelline envelope. Scale bars represent 100 μ m in A, B, C, G and H and 25 μ m in D, E and F. Pictures are representative of three independent experiments. (I) Effect of anti- $\beta 1$ integrin polyclonal antibody M-106 in fertilization. Fertilization percentages were acquired at a sperm concentration of 3.3×10^5 cells/ml. Control: fertilization in the absence of any antibody, M-106: fertilization in the presence of 50 μ g/ml of anti-human $\beta 1$ integrin rabbit polyclonal antibody M-106, IgG: fertilization in the presence of 50 μ g/ml of normal rabbit IgG, a-NKA: fertilization in the presence of 50 μ g/ml of anti- Na^+/K^+ -ATPase antibody. $^{##}P < 0.01$ versus Control, IgG or a-NKA. In all the experiments, fertilization was scored by the occurrence of the first cleavage. Oocytes (20–40 each treatment) were obtained from three animals. In both experiments, different sperm donors were used. Values are expressed as relative percentages to the control of each animal.

with apparent MWs of 70 and 58 kDa (arrows). Finally, there is a band with an apparent molecular mass of 30 kDa that appears in the 10 mpf and RGDS lanes. The cytosol samples show a strong band at an apparent MW of 42 that is less intense in the 10 mpf lane. Also, the behavior of the phosphorylation signals of 33 and 31 kDa follows the same pattern. In the 1, 10 mpf and RGDS lanes, there is an additional band with an apparent mass of 58 kDa. Another band of about 70 kDa can be found in the 10 mpf and RGDS lanes. These results suggest that RGDS treatment can induce protein tyrosine phosphorylation, although not completely mimicking the fertilization-induced changes in tyrosine phosphorylation.

Discussion

In mammals, the role of integrins in fertilization has been controversial, as different results were observed in different species (Sessions *et al.* 2006, Baessler *et al.* 2009) or different laboratories reported different results in the same animal model (He *et al.* 2003, Tatone & Carbone 2006, Baessler *et al.* 2009). Amphibians have been accepted models for the study of early development at the molecular and cell biological levels. This is mainly because these animals can provide a large

amount of gametes (i.e. egg and sperm), zygotes (i.e. embryos) and they are easy to manipulate. Use of the frog system has become fruitful in the study of fertilization. In amphibians, a role for integrins has been studied almost exclusively in *X. laevis* eggs. Information from other amphibian species may help understanding the whole process and distinguish general features from species-specific steps.

A previous work from our laboratory pointed to integrins as candidates for sperm receptors in the oolemma of *B. arenarum* (Coux & Cabada 2006). In this work, we show that *B. arenarum* oocytes express the $\beta 1$ integrin subunit by western blot analysis using two different antibodies. The 8C8 antibody is a MAB developed against the *X. laevis* protein, while the M-106 is a polyclonal antibody commercially available against the human $\beta 1$ integrin. Both antibodies recognized a doublet at about the same apparent MWs. The doublet has been attributed to the ability to detect immature forms of the protein (Gawantka *et al.* 1992). Moreover, $\beta 1$ integrin has been detected at the protein level in the oocyte of several mammals (Campbell *et al.* 2000, Linfor & Berger 2000, He *et al.* 2003) and, at high levels, in *X. laevis* eggs (Gawantka *et al.* 1992). All these facts strengthen the idea that $\beta 1$ integrin subunit is present in *B. arenarum* oocytes. We used a MAB against

Figure 6 Effect of fertilization and RGDS treatment on protein tyrosine phosphorylation in *B. arenarum* oocytes. Total extracts (20 μ g of protein) of *B. arenarum* oocytes were prepared before and at different times after fertilization (across the top: oocytes and minutes post-fertilization (mpf)) and also from *Bufo* oocytes that were incubated 10 min with the RGDS peptide at a concentration of 1 mM (RGDS lane). Protein samples were separated by SDS-PAGE on 12% polyacrylamide gels and stained (Coomassie panel) or blotted and analyzed using the anti-phosphotyrosine antibody (PY350 panel) or the antibody plus 100 $\times$ excess of antibody inhibitory peptide (PY350+INH panel). Arrows indicate proteins that change their phosphorylation state due to fertilization. The prestained molecular markers are shown to the left of each panel. α -Tubulin was used as loading control. Results are representative of three independent experiments.

chicken $\alpha 5$ integrin subunit to detect the presence of this subunit in *B. arenarum* oocytes. In the positive chicken control, the antibody detects a band migrating somewhat below the 150 kDa marker in the presence of a reducing agent or two bands with apparent MWs of 250 and 150 kDa in its absence. In *B. arenarum*, there is a blurry signal at 250 kDa without reducer and a definite band of about 165 kDa in its presence. We suggest that the 250 kDa signal may represent integrin dimers (e.g. $\alpha + \beta$) that in the absence of reducer preserve their interaction. The difference in molecular mass of the reduced forms between the chicken and *B. arenarum* samples

(150 vs 165 kDa) may reflect species-specific characteristics (e.g. different glycosylation patterns). The $\alpha 5$ subunit has been detected in the oocyte PM of different mammals such as mice, cows and humans (Evans *et al.* 1995, Sengoku *et al.* 2004, Pate *et al.* 2007), and in *X. laevis* oocytes (Joos *et al.* 1995). We also found that the αV integrin subunit is expressed by *B. arenarum* oocytes. αV integrin subunit expression has been reported in mammalian oocytes from mice, cows, humans and pigs (Almeida *et al.* 1995, Linfor & Berger 2000, Sengoku *et al.* 2004, Pate *et al.* 2007) and in amphibian oocytes from *X. laevis* and *Pleurodeles* (Alfandari *et al.* 1995,

Figure 7 Effect of fertilization and RGDS treatment on protein tyrosine phosphorylation in cytosol and plasma membrane fractions of *Bufo arenarum* oocytes. Cytosol (Cyt, 20 μ g of protein) and plasma membranes (PM, 20 μ g of protein) were prepared from *Bufo* oocytes (oocyte lanes), eggs at 1 and 10 min post-fertilization (1 and 10 mpf lanes) and oocytes that were incubated 10 min with the RGDS peptide at a concentration of 1 mM (RGDS lane). Protein samples were separated by SDS-PAGE on 12% polyacrylamide gels stained (Coomassie panel) or blotted and analyzed using the anti-phosphotyrosine antibody (PY350 panel) or the antibody plus 100 $\times$ excess of antibody inhibitory peptide (PY350+INH panel). Closed arrows indicate proteins that change their phosphorylation state due to fertilization and RGDS treatment. The prestained molecular markers are shown to the left of each panel. α -Tubulin was used as loading control. Results are representative of three independent experiments.

Joos *et al.* 1998). Both $\alpha 5$ and αV integrin subunits have been involved in fertilization (Linfor & Berger 2000, Thys *et al.* 2009), supporting our findings.

It is known that αV and $\alpha 5$ integrin subunits usually pair with $\beta 1$ subunit to form fibronectin receptors. So, the presence of the αV and $\alpha 5$ subunit, combined with a high expression of $\beta 1$ subunit, suggests that both heterodimers are likely to be present in *B. arenarum* oocytes. $\alpha V\beta 1$ and $\alpha 5\beta 1$ integrins are heterodimers that recognize the RGDS motif (Hynes 1992). We also analyzed the expression of the $\beta 3$ integrin subunit in the PM of *B. arenarum* oocytes but we could not detect it. The anti- $\beta 3$ antibody was raised against amino acids 635–730 of integrin $\beta 3$ of human origin. This region displays a high homology to amphibian $\beta 3$ integrin and is recommended for detection of this protein in *X. laevis* samples. These facts suggest that $\beta 3$ integrin is present at low levels or not present at all in *Bufo* oocytes.

The presence of integrin subunits $\alpha 5$, αV , $\beta 1$ and $\beta 3$ was also studied in *B. arenarum* sperm. With the exception of the αV integrin subunit, we did not detect their presence on sperm, suggesting that these cells do not express these integrin subunits. However, $\alpha 5$, $\beta 1$ and $\beta 3$ integrin subunits have been reported as present in mammalian sperm (Thys *et al.* 2009, Boissonnas *et al.* 2010). The possibility of a very low expression of these proteins to explain our results is lowered by the fact that the proteins extracted from 8×10^6 sperm cells were loaded onto each lane for the western blot studies. We did detect αV integrin subunit in sperm. This subunit displayed a slightly higher MW in sperm samples compared with oocyte samples. This heterogeneity is not surprising as differential electrophoretic mobility (resulting from differential glycosylation) of this subunit during early development or in different embryonic tissue has been described in *Pleurodeles* (Alfandari *et al.* 1995). To our knowledge, integrin expression has not been studied in sperm from any amphibian and this report would be the first about the topic. Taking into account the dissimilar environments that mammalian and *B. arenarum* sperm have to face (internal versus external fertilization), it is possible to suggest that the set of proteins displayed by these cells from these different animals may vary broadly.

The action of the well-known integrin-interacting peptide RGDS on the oocytes was studied. Oocyte activation due to RGDS peptide was not detected either in dejellied or in the presence of intact jelly coat. We performed IVF assays and analyzed the effect of the presence of RGDS. In this case, we found an inhibition of ~50% of fertilization. This action seems not to be due to an effect on sperm, as suggested by the lack of detection of $\alpha 5$, $\beta 1$ and $\beta 3$ integrins on these cells. However, αV was found to be present on *B. arenarum* sperm. We do not know the identity of the β partner of αV subunit in sperm, but all the possible combinations of αV subunit described so far belong to the RGD subfamily

(Hynes 1992). Despite this, RGDS pre-incubation assays did not show statistically significant effects on fertilization scores. Many integrins are not constitutively active; they can be, and often are, expressed on cell surfaces in an inactive or 'OFF' state, in which they do not bind ligands and do not signal (Hynes 2002). Moreover, the fertilization inhibition studies in the presence of antibodies point to a $\beta 1$ -containing integrin, and $\beta 1$ subunit could not be detected in *Bufo* sperm. In our IVF assays, the peptide was present before the addition of sperm. At high sperm concentrations, the inhibition was abolished. If inhibition was due to activation of the egg membrane block to polyspermy, the block would not be expected to be reverted by increasing sperm concentration. This behavior is consistent with a competitive binding mechanism and not with an activation mechanism, supporting the results of the above activation experiments. In *X. laevis*, it was reported that dejellied oocytes treated with RGDS at concentrations higher than 250 μ M underwent cortical granule breakdown, cortical contraction and resumption of meiosis (Iwao & Fujimura 1996). *B. arenarum* showed a different behavior more compatible with recent works in mouse (Baessler *et al.* 2009) which suggest that the inhibition of fertilization by integrin-interacting peptides is a consequence of competition with sperm binding to the cell surface more than generation of a full egg-activation pathway. To further test the inability of RGDS to activate eggs, we studied MAPK phosphorylation levels using an anti p-ERK1/2 antibody. This kind of analysis provides a biochemical confirmation for our morphological observations and has been used previously in the search for activating factors (Sette *et al.* 1997, Mammadova *et al.* 2009). A dephosphorylation-dependent decrease in MAPK activity is required for meiosis II resumption, the ultimate oocyte activation goal (Inoue *et al.* 2007, Wu & Kornbluth 2008). Normal activation by sperm of *B. arenarum* eggs was shown to induce rapid MAPK dephosphorylation within 10 min. RGDS was unable to induce MAPK dephosphorylation at any time of incubation, confirming our previous visual examination.

The RGDS motif is recognized by $\alpha 5\beta 1$ and $\alpha V\beta 1$ integrins, also known as fibronectin receptors (Pytela *et al.* 1985, Vogel *et al.* 1990). Fertilization inhibition assays using antibodies have been widely used (He *et al.* 2003, Sakakibara *et al.* 2005). The immunofluorescent microscopy experiments showed $\beta 1$ integrin expression on the egg surface. So, in order to reinforce $\beta 1$ integrin role in fertilization, we performed IVF in the presence of anti- $\beta 1$ integrin antibodies. M-106 antibody is a polyclonal antibody raised against part of the extracellular domain of the protein where epitopes of integrin inhibitory antibodies usually map (Byron *et al.* 2009). These kinds of experiments suggest a direct functional link between the process of fertilization and the antigen ($\beta 1$ integrin in this case). Our results were consistent with

RGDS results and supported a role in sperm adhesion for $\beta 1$ integrin.

In T cells, integrins act as co-stimulating receptors in synchrony with the T-cell receptor (Hynes 1992). A similar model can be suggested for oocyte integrins, in which co-stimulation of one or several sperm receptor(s) and integrins could then promote adhesion, fusion and stimulation of cellular metabolism. These receptors may have tyrosine kinase activity or may be indirectly linked to kinases (Hynes 1992). Following this hypothesis, integrin stimulation alone may not be enough to induce all the events of activation, but should be close or have a role in part of the signaling. To explore this hypothesis, we studied fertilization-induced egg signaling events, in particular changes in the protein phosphorylation pattern. We analyzed protein phosphorylation/dephosphorylation in tyrosine residues in total oocyte and egg extracts. Although fertilization induced several major changes, our results failed to detect any RGDS-induced tyrosine-phosphorylation difference with respect to the oocyte pattern. Some tyrosine-phosphorylated proteins may not be detected by PY350 antibody for different reasons such as, among others, low or no affinity at all for certain epitopes or low abundance of an epitope. In order to improve the detection level, we analyzed more purified samples. By this approach, we could also gain some information regarding the subcellular localization of the signaling. We isolated PM fractions without detergent treatment, using a discontinuous sucrose density gradient. This method has been developed by Luria *et al.* (2002) for *Xenopus* eggs, and adapted by us (Coux & Cabada 2006) and others (Buschiazzo *et al.* 2008) to *B. arenarum* eggs. The method provides membranes enriched in PM markers, such as Na^+/K^+ -ATPase, 5'-nucleotidase and caveolin, and with some properties similar to rafts (Luria *et al.* 2002, Coux & Cabada 2006, Buschiazzo *et al.* 2008). In this fraction, we detected a band with an apparent weight of 30 kDa that becomes phosphorylated 10 min after fertilization. A similar band appears in RGDS-treated PM samples. We can only speculate regarding the identity of this tyrosine-phosphorylated band. In this connection, recently, Sakakibara *et al.* (2005) reported that 5 min after fertilization they found a raft-associated 30 kDa protein that becomes tyrosine-phosphorylated in *Xenopus* eggs. This protein was identified as *X. laevis* homolog of uroplakin III and antibodies against its extracellular domain blocked sperm-egg interaction suggesting its involvement in this process (Sakakibara *et al.* 2005).

We also analyzed cytosolic fractions obtained by ultracentrifugation. In this fraction, we found at least two bands (with apparent masses of 58 and 70 kDa) that become phosphorylated due to fertilization and also appear in cytosols isolated from RGDS-treated oocytes. Non-receptor protein tyrosine kinases (PTKs) with SH2 domains (Src and Syk/ZAP70 families) are

phosphorylable proteins that either have been involved in integrin signaling, fertilization associated signaling or both (Clark & Brugge 1995, Kinsey 1997, Giusti *et al.* 1999, Sato *et al.* 2002, Ulanova *et al.* 2005, McGinnis *et al.* 2007). Syk is a 72 kDa PTK that only recently has been detected in non-hematopoietic cells and has been involved in $\beta 1$ -integrin signaling (Ulanova *et al.* 2005). Its participation in fertilization has not been deeply assayed with the exception of starfish eggs (Giusti *et al.* 1999). The Src family, on the contrary, has been studied more deeply in fertilization signaling (Sato *et al.* 1996, 2002, Kinsey 1997, Giusti *et al.* 1999, Mammadova *et al.* 2009). Recently, a PTK (p57 Xyk, related to Src-family protein kinases) has been purified from *X. laevis* oocytes (Sato *et al.* 1996). The kinase activity of Xyk is elevated several-fold upon fertilization, concomitant with its translocation from the oocyte cortex to the cytosolic fraction (Sato *et al.* 1999). Additionally, RGDS peptide was able to induce Xyk activation and translocation (Sato *et al.* 1999). The phosphorylated band that we detect at 58 kDa in cytosols from 10 min post-fertilization and RGDS-treated eggs could be the *B. arenarum* homolog of this PTK. Moreover, a detailed inspection of the PMs obtained 1 min post-fertilization shows bands with similar electrophoretic mobility. This last fact could be interpreted as a phenomenon of activation at the PM, followed by a re-localization to the cytosol minutes later. We did not detect a similar band in PMs from RGDS-treated eggs. This might be a difficult task considering that RGDS-sustained stimulation probably narrows the chance of detecting the pre-translocated phosphorylated form, without the aid of further enrichment (e.g. immunoprecipitation).

There are two classes of hypothesis to explain how sperm activates eggs. The first class imagines that the sperm activates a signal transduction receptor, much as a hormone might; the second class is based around the idea that sperm-egg fusion is the event that initiates the fertilization. Whatever the model to activate eggs, undoubtedly, both require cell-cell recognition and attachment. These two tasks should be performed by sperm receptors that may or may not transduce a transmembrane signal. Certainly, integrin receptors can transduce such a signal to generate a calcium response in general (Hynes 1992, Whitaker 2006). But also, they can act as co-receptors participating and helping (e.g. making it faster or more efficient) without being sufficient for a full activating response. Our results point to such a role for integrins in fertilization in *B. arenarum*. As far as we know, the oocyte integrin-associated proteins activated due to sperm attachment, if any, have not been reported. This knowledge would certainly help in clarifying integrin role in fertilization.

In conclusion, our results suggest that RGDS-binding proteins (integrins) have a role in fertilization as attachment proteins. Although peptide application has some effects over protein tyrosine phosphorylation, these

were not accompanied with activation or MAPK dephosphorylation. As has been suggested elsewhere (Whitaker 2006), there may not be a single, unique biochemical mechanism that operates at fertilization. Our findings suggest that different amphibians could have different activation mechanisms.

Materials and Methods

Materials

The following antibodies were used: anti-human β 1-integrin (M-106, sc-8978), anti-human β 3-integrin antibody (H-96, sc-14009), anti-human α V antibody (H-75, sc-10719), anti-phosphotyrosine antibody (PY-350, cat. sc-18182), anti-phospho-extracellular regulated kinase (p-ERK 1/2 Thr202/Tyr204, sc-16982) and anti-actin (sc-1616) were from Santa Cruz Biotechnology. Polyclonal antibody to the rabbit kidney Na^+/K^+ -ATPase α subunit (cat. 119115) was from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). Anti- α -tubulin (T5168) was from Sigma-Aldrich. MAB 8C8 against the β 1-integrin of *X. laevis* was developed by Gawantka *et al.* (1992) and D71E2 against chicken α 5-integrin was developed by Muschler & Horwitz (1991). These antibodies were obtained from Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, USA. Goat anti-mouse IgG (H+L)-HRP Conjugate and Precision Plus Protein Standards Dual Color were from Bio-Rad and donkey anti-rabbit HRP-conjugated F(ab)2 was from Amersham. 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.

Animals, gametes and eggs

Sexually mature *B. arenarum* specimens 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, deposited 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 $0.1 \times$ Ringer solution. Sperm at a final concentration of at least 5×10^5 cells/ml were used to fertilize 600–800 oocytes/dish. Fertilization was stopped at the desired times with ice-cold $1 \times$ 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. Under these conditions, 0, 4, 43, 93.5 and 100% eggs underwent cortical contraction/rotation after no insemination and 1, 5, 10 and 30 min of insemination, respectively.

Assay for activation of *B. arenarum* oocytes

About eight dejellied, unfertilized oocytes were placed in a well made of polystyrene filled with 100 μ l of 50% SB (58 mM NaCl, 0.67 mM KCl, 0.34 mM CaCl_2 , 0.85 mM MgSO_4 , 4.6 mM Tris-HCl, pH 7.4). After removing as much solution as possible, 100 μ l of the peptide (1.4 mM dissolved in 50% SB) were added. Since about 40 μ l of 50% SB were left in the well, final concentration of the peptides in the well was estimated to be about 70% of that of the peptides added. Activation of the oocytes can be detected in the live egg by movement of pigments, cortical contraction and formation of a fertilization coat. The oocytes were examined 10, 20, 30 and 60 min after treatment. At the end of the experiment, calcium ionophore was added to check that oocytes were able to undergo activation upon stimulation.

Fertilization inhibition studies

Peptides

Groups of 4–6 jelly-intact oocytes were placed in wells filled with 100 μ l of RGDS (Arg-Gly-Asp-Ser) solution in 50% SB. After 20 min, the lack of activation was visualized under the stereoscopic magnifier and 40 μ l of egg water were added. Egg water was supplemented to eliminate any dilution of the factors that are necessary for successful fertilization (Arranz & Cabada 2000). Fertilization was carried out adding 5 μ l of sperm stock solution to reach the desired final sperm concentration. After 25 min, the eggs were washed three times with 100 μ l 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.

To set up the conditions, initial experiments were performed varying the sperm final concentration (1×10^4 , 5×10^4 , 1×10^5 , 3.3×10^5 and 1×10^6 cells/ml) and final (in the well) RGDS peptide concentration (1, 0.5 and 0.25 mM). The chosen sperm working concentration was 3.3×10^5 cells/ml and the peptide concentration was 1 mM. Using these concentrations, controls with RGES (Arg-Gly-Glu-Ser) and AAA (Ala-Ala-Ala) peptides were performed.

For certain controls, sperm were pre-incubated with 1 mM RGDS peptide, then washed twice with a 20-fold excess of Ringer solution before addition to eggs that had not been exposed to peptide.

Antibodies

Groups of 4–6 jelly-intact oocytes were placed in wells filled with 100 µl of anti-β1 integrin rabbit polyclonal antibody (M-106) at a final concentration of 50 µg/ml in 50% SB. After 20 min, the oocytes were fertilized as previously described. For specificity controls, normal rabbit IgG or anti Na⁺/K⁺-ATPase α subunit was used at the same final concentration.

Sperm extracts for immunoblotting

Sperm obtained as described above were lyophilized and resuspended in 20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% w/v SDS pH 7.5 plus protease inhibitors 5% v/v (Sigma cocktail). The extract was incubated 2 h at 37 °C with occasional vortexing. After centrifugation, the supernatant was boiled in electrophoresis sample buffer with or without 0.1 M DTT.

Subcellular fractionation of oocytes and eggs

Total membranes and cytosol isolation

Subcellular fractionation was performed according to the procedure described by Sato *et al.* (1999) with modifications. All procedures were carried out at 0–4 °C. Dejellied oocytes or eggs were mixed (1 ml in a packed volume) with 8 ml of buffer A (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄) added with protein inhibitor cocktail 1% v/v (Sigma), phenylmethylsulphonyl fluoride (PMSF) 1 mM, tosyl phenylalanyl chloromethyl ketone (TPCK) 14 µg/ml, NaF 10 mM and homogenized in a Teflon-glass homogenizer. The homogenate was centrifuged 10 min at 1000 g to remove cellular debris and yolk platelets (total extract). The supernatant was collected and centrifuged at 150 000 g for 1 h. After centrifugation, the clear supernatant was set aside as the cytosolic fraction. The pellet and fluffy part of the pellet were rediluted with buffer A and recentrifuged at 150 000 g for 30 min. The pellet fraction obtained was suspended in 1 ml of buffer A plus 1% v/v Triton X-100 and sonicated (TM fraction). By this procedure, 1 ml of packed volume eggs yielded 12–13 mg of cytosolic protein and 3–4 mg of membrane protein.

Oocyte plasma membrane isolation

PMs were separated by ultracentrifugation on a discontinuous sucrose gradient, following a procedure that was previously carried out to isolate light and heavy PMs on *X. laevis* oocytes (Luria *et al.* 2002) and already adapted by us (Coux & Cabada 2006). Briefly, 300 oocytes were lysed by repeatedly pipetting in ice-cold 2× TNE buffer (10 mM Tris–HCl, 150 mM NaCl and 5 mM EDTA, pH 7.4) with 1 mM PMSF, Protease Inhibitor Cocktail 1% v/v, 10 µg/ml TPCK, 1 mM orthovanadate and NaF 15 mM. Oocyte lysates were sonicated for 1 min in a cold bath at 13% watt scale (Vibra Cell; Sonics & Materials, Inc., Newton, CT, USA). The lysates were added to an equal volume of 80% w/v sucrose to give a final equivalent density of 40% w/v sucrose. They were then overlaid with 3 ml each of sucrose at concentrations of 35, 22.5 and 10% w/v. The sucrose gradients were centrifuged 3 h at 100 000 g (SW40 rotor; Beckman

Instruments, Fullertown, CA, USA). The PMs in the 10–22.5 and 22.5–35% interface were obtained, diluted with Tris 20 mM with PMSF 1 mM and 1 mM orthovanadate, and centrifuged 1 h at 150 000 g (Ti80 rotor, Beckman Instruments). The pellets were resuspended in buffer A plus protease and phosphatases inhibitors, pooled and sonicated 20 s at 6% watt scale.

Indirect immunofluorescence and confocal microscopy

Paraffin-embedded sections of eggs were treated as described by Cabada *et al.* (1996). Samples were blocked in TBS supplemented with 0.025% Triton X-100, 5% BSA, 10% fetal bovine serum and 10% ram serum for 2 h. The first antibody (M-106, anti-β1 integrin) was added at a 1:50 dilution in TBS with 0.025% Triton X-100 and 1% BSA and was left overnight at 4 °C. After washing, coverslips were incubated with Cy3-conjugated goat anti-rabbit secondary antibody (Chemicon International, Temecula, CA, USA) at a 1:1000 dilution in TBS with 3% BSA for 1 h. Coverslips were washed and mounted with ProLong (Molecular Probes, Eugene, OR, USA). Fluorescence localization was detected by laser scanning confocal microscopy (Nikon Eclipse TE-2000-E2). Images were collected with the same confocal settings in each set of experiments. Nikon confocal software was used for acquisition of the data and merging of the digital images. Controls were performed without primary antibody. For the sake of clarity, contrast and brightness adjustments were equally applied to the entire images via Nikon software.

Electrophoresis, electrotransfer and western blot analysis

PAGE under denaturing conditions (SDS-PAGE) was performed essentially according to the method of Laemmli (1970). Samples were diluted with an appropriate volume of 6× sample buffer with or without DTT, boiled for 5 min, loaded onto 8, 10 or 12% polyacrylamide mini-gels with 5% stacking gel and electrophoresed at 20 mA/gel (MiniProtean II Gel System, Bio-Rad). The apparent molecular masses were estimated with molecular mass standards (Precision Plus Protein Standard Dual Color, Bio-Rad). Gels were processed for Coomassie Brilliant Blue staining or electrotransferred to nitrocellulose membranes by the method of Towbin *et al.* (1979).

Membranes were washed twice with TBS–Tween-20 0.1% v/v and then blocked with TBS buffer supplemented with the appropriate blocker (5% w/v nonfat dry milk for 8C8, M-106, H-96, D71E2, H-75, tubulin, actin; 1% w/v gelatine for PY-350 and p-ERK 1/2). Membranes were washed with TBS–Tween-20 0.1% v/v and incubated with the primary antibody dilution (8C8: 1/1000, M-106: 1/500, H-96: 1/750, H-75: 1/750, D71E2: 1/250, PY350: 1/500). After washing, membranes were incubated with the appropriate HRP-conjugated antibody (1/8000–1/10 000 dilution), washed and developed using chemiluminescence and X-ray films.

When membrane re-probing was necessary, stripping was performed by incubation in 0.2 M NaOH at room temperature for 5 min, followed by two washings with distilled water.

Protein assays

Protein concentrations were determined according to Sedmak & Grossberg (1977), using BSA as standard.

Statistical analysis

Results are expressed as mean $\pm$ s.e.m. Data were analyzed using Student's *t*-test. The 0.05 level of probability was used as the criterion of significance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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