Reproduction in Domestic Animals

Reprod Dom Anim **51**, 150–157 (2016); doi: 10.1111/rda.12659
ISSN 0936–6768

Involvement of Protein cAMP-dependent Kinase, Phospholipase A_2 and Phospholipase C in Sperm Acrosome Reaction of *Chinchilla lanigera*

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Contents
The mechanisms involved in fertilization are the centre of attention in order to determine the conditions required to reproduce *in vitro* the events that take place *in vivo*, with special interest in endangered species. Previous data from mouse sperm, where acrosome reaction (AR) occurs more often in the interstitium of the cumulus oophorus, contribute to strengthen the use of progesterone as a physiological inducer of this process. We studied the participation of protein kinase A (PKA), phospholipases A_2 and C (PLA_2, PLC) in the AR induced by progesterone from *Chinchilla* epididymal spermatozoa. The addition of db-cAMP to the incubation medium caused an increase of 58% in the AR, while the use of H89 (30 μM), a PKA inhibitor, reflected a decrease of 40% in the percentage of reacted gametes. The assays conducted with arachidonic acid showed a maximum increase of 23% in the AR. When gametes were pre-incubated with PLA_2 inhibitors, a dose-dependent inhibitory effect was observed. The addition of phorbol12-myristate13-acetate (10 μM) revealed higher percentages of AR induction (60%). When PLC was inhibited with neomycin and U73122, a dose-dependent decrease in AR percentages was observed. Combined inhibition of PKA, PLA_2 and PLC, AR values similar to control were obtained. This work shows evidence, for the first time in Chinchilla, that progesterone activates the AC/cAMP/PKA system as well as sperm phospholipases and that these signalling pathways participate jointly and cooperatively in AR. These results contribute to the understanding of the complex regulation that is triggered in sperm after the effect of progesterone.

Introduction

Sperm capacitation and the subsequent acrosome reaction (AR) are essential prerequisites for successful fertilization (Yanagimachi 1994). Even more, only those sperm that have undergone acrosome exocytosis have the capacity to fuse with the oocyte plasmatic membrane and fertilize it.

Until recently it was assumed that sperm had to reach the zona pellucida (ZP) with an intact acrosome and interact in the ZP with the glycoprotein ZP3 in order to undergo AR. However, the findings of Jin et al. (2011) provided a different picture of the process, showing that AR in transgenic mice takes place before contact with the ZP in the interstitium of the cumulus oophorus. Besides, the above authors reported that reacted gametes were able to become attached, pass through the ZP and fertilize the egg. Obviously, this fact has important implications in the molecular study not only of the AR but also in the sperm–egg interaction in eutherian mammals.

In view of the above findings, it may be supposed that substances present in the cumulus oophorus might act as physiological inducers of acrosomal exocytosis. In this sense, progesterone, a steroid hormone secreted by the cumulus cells, has been extensively studied in several mammalian species such as humans (Osman et al. 1989), hamsters (Llanos and Anabalon 1996), mice (Roldan et al. 1994a), dogs (Brewis et al. 2001), horses (Meyers et al. 1995) and bulls (Thérien and Manjunath 2003) because of its ability to induce AR in capacitated sperm.

The action of progesterone on surface receptors causes a non-genomic effect (Meizel and Turner 1991) resulting in an increase in intracellular calcium, stimulation of the phospholipase (PL) activity and phosphorylation of proteins with tyrosine residues (Baldi et al. 2002). Previous studies in human sperm (De Jonge et al. 1989) showed the participation of the AC/AMPc/PKA system when the AR is induced with progesterone, but the molecular mechanisms of this signalling pathway have not been sufficiently clarified yet.

Among the sperm PLs, we find phospholipase A_2, which hydrolyses phosphatidylcholine, a membrane phospholipid, originating lysophosphatidylcholine and free fatty acids. This enzyme would play a key role during the AR of guinea pig, hamster, bull and human sperm treated with progesterone (Llanos et al. 1982; Lax et al. 1990; Domínguez et al. 1996; Chen et al. 2005).

Other PLs such as PLC constitute an important signal transduction mechanism in somatic cells (Berridge 1987) and belong to a group of hydrolases that produce 1,2-diacylglycerol (DAG) from membrane phospholipids. DAG generation plays a key role in the regulatory mechanisms of acrosomal exocytosis. Besides, it is known that this metabolite is capable of activating PKC (O'Toole et al. 1996) and PLA_2 (Roldan et al. 1994b) and even further, evidence has been shown that it may have an effect of positive feedback with PLC specific for phosphatidylcholine (Roldan and Murase 1994).

The mechanisms involved in fertilization are still the focus of study in numerous species in order to define the conditions required to reproduce *in vitro* the events that

take place *in vivo*. This goal is particularly important in the case of endangered species.

Chinchilla sp., included in Appendix I of the CITES, represents a challenge to reproductive biology due to its seasonal reproductive periods and its low reproductive rate in captivity. In previous studies, we showed that progesterone induces *in vitro* AR of sperm of *Chinchilla lanigera*, a domestic species bred in captivity (Gramajo-Bühler et al. 2012). However, there are no reports for this species concerning the signalling pathways involved in the process.

The aim of this work is to study the signal transduction pathways involved in the acrosome reaction in epididymal sperm of Chinchilla lanigera grey under the stimulus of progesterone. The present study was conducted in order to determine the possible role of PKA, PLA_2 and PLC in this process.

Materials and Methods

Animals

The animals used in this study were provided by a commercial hatchery enabled for production skin. We selected 9 sexually mature male specimens from 11 to 12 months of age with a body mass of 600–650 g. All animals were housed under a 12-h dark–light cycle at a constant temperature of 22 ± 1°C and received water and food *ad libitum*. The animals were sacrificed by cervical dislocation and immediately taken to the laboratory in thermal containers to maintain constant temperature.

Reagents and incubation media

PBS 1X (Dulbecco's phosphate-buffered saline solution) Irvine Scientific® (Santa Ana, CA, USA). Dimethyl sulfoxide (DMSO), dibutyryl cyclic AMP, H89 dihydrochloride hydrate**,** arachidonic acid (AA), quinacrine, aristolochic acid (ATA), U73122 hydrate, phorbol12-myristate13-acetate (PMA) and progesterone from Sigma Aldrich. Co. St. Louis, MO, USA). Coomassie Brilliant Blue G250 from Biopack® (Autónoma de Buenos Aires, Argentina).

Modified HTF medium hepes

To 750 ml of purified water, add 5.931 g of NaCl, 0.35 g of potassium chloride (KCl), 0.05 g of magnesium sulphate heptahydrate (MgSO4.7H2O), 0.05 g of potassium dihydrogen phosphate(KH2PO4), 0.366 g of sodium bicarbonate (NaHCO3), 0.5 g of d-glucose, 0.036 g of sodium pyruvate, 0.3 g of calcium chloridedihydrate (CaCl2.2H2O) and 4.0 g of sodium dl-lactate (60%(v/v) syrup). To 1 ml of the above medium, add 10 μg phenol red, 100 U penicillin and 50 μg streptomycin sulphate. Adjust the pH to 7.4 with 1 mol/l hydrochloric acid (HCl). Add 20 mmol/l Hepes (Na salt: 5.21 g/l).

Collection and preparation of spermatozoa

Immediately after slaughtering, spermatozoa were collected by puncture from the cauda epididymis. The samples were washed twice in PBS and centrifuged at 1000 × ***g*** for 10 min. All materials used for the sample collection and subsequent trials were under strict temperature control (37 ± 0.5°C).

We only used samples with a vitality percentage equal to or >85%, using the eosin test 0.5% (w/v) with an inverted microscopy at 400×. Counts were performed on 200 cells per replicate.

Capacitation of spermatozoa

The washed spermatozoa were separated into aliquots (final concentration 4–5 × 10^6 cells/ml) and incubated with HTFm (modified human tubal fluid) supplemented with 2.5 g/l albumin fraction V for 2.30 h at 37°C in an atmosphere containing 5% CO2.

Determination of downstream signalling components activated with progesterone mediated induction of acrosomal exocytosis

In order to determine whether the activation of cAMP-dependent PKA has a role in the AR, we used an analog of cAMP mimicking activation of AC/cAMP system. The dibutyryl-cAMP (db-cAMP) was used, which is able to pass easily through the plasma membrane. Aliquots of the sperm suspensions capacitated were incubated with increasing db-cAMP concentrations (0–1 mM) for 15 min (Moseley et al. 2005).

In the same way, to analyse the participation of phospholipase A_2 in this process, aliquots of capacitated sperm were co-incubated with increasing concentrations (12.5–100 μM) of arachidonic acid (AA) for 15 min, a metabolite of the hydrolysis of membrane phospholipids (PL) by the action of PLA_2.

Following the same schemes, to study the involvement of PLC, we first activated the signalling cascade with a metabolite or a product of the PLC. We added to the culture medium a synthetic analog of DAG, phorbol 12-myristate 13-acetate (PMA), at increasing concentrations (0.05–10 μM) to which the capacitated spermatozoa were exposed for 15 min.

To understand the mechanism of action progesterone-mediated acrosome reaction, capacitated spermatozoa were pre-treated with various pharmacological inhibitors of this key enzymes such as H89 (0–30 μM) – PKA inhibitor, 30 min; aristolochic acid (ATA) at increasing concentrations (0–320 μM); and quinacrine (0–40 μM) – PLA_2 specific inhibitors, 5 and 15 min, respectively; neomycin (0–800 μM) and U73122 (0–20 μM) – PLC inhibitors. After incubation with the inhibitors, AR was induced with progesterone 20 μM (Gramajo-Bühler et al. 2012).

When the protocol included the addition of two or more drugs, each of them was left alone the time

specified above, the sperm before the addition of the following one after washing in HTFm with centrifugation. The control experiments were run for the same time periods. Controls were performed adding the corresponding solvent.

The doses and incubation times used in this study were selected from the literature and/or from preliminary experiments and the same final concentration was always used.

Evaluation of acrosomal status by Coomassie Blue stain

In all incubations, the reactions were stopped with the addition of buffered formalin (final concentration 4%), samples being fixed at 4°C for 10 min. Then they were washed twice with a solution of 0.1 M ammonium acetate, pH 9, followed by centrifuging for 5 min at 1200 × ***g***. The smears were air-dried and hydrated in distilled water for 5 min, dyed with Coomassie Blue G250 solution and finally washed with distilled water for 5 min.

A total of 200 cells were analysed by light microscopy at 1000×, taking as a positive response the absence of acrosome, and as a negative response the presence of the acrosomal vesicle, which is evident in the brilliant blue band in the anterior region of the head.

Statistical analysis

Means and standard errors were calculated for all data sets. Differences between groups were evaluated using one-way analysis of variance (ANOVA). A p value of <0.05 was considered as statistically significant.

Results

Participation of PKA in acrosomal exocytosis

We have studied the participation of PKA in the process of epididymal sperm acrosome reaction. In a first stage, the sperm suspensions were incubated with an analog cAMP. The dose–response curve (Fig. 1) shows a significant increase in the acrosome reaction percentages in the samples incubated with 0.250 mM of db-cAMP, the highest increase in the AR (58%) being reached with a concentration of 0.500 mM. This effect was not modified when the concentration was doubled.

Previously capacitated sperm suspensions were pre-incubated with H89, a PKA inhibitor prior to addition progesterone. The Fig. 1 showed a decrease in the percentage of sperm that underwent total acrosome exocytosis, observed significantly lower values compared with a control group from a concentration of 5 μM of H89. The highest inhibitory effect of H89 was obtained in sperm treated with concentrations of 30 μM. This effect is shown in a decrease of approximately 40% in AR with respect to the control.

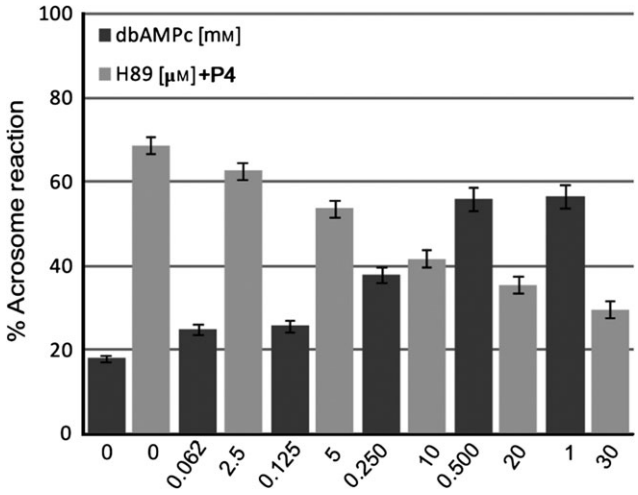

Fig. 1. Effect of increasing concentrations (0.062; 0.125; 0.250; 0.500; 1 mM) of db-cAMP on the acrosome reaction in capacitated sperm and effect of the PKA inhibitor (H89) on sperm later stimulated with progesterone 20 μM. The controls have the same volume of vehicle and represent spontaneous acrosome reaction. The results are the mean ± SEM of four experiments with different animals

Assessment of the role of PLA_2 in the acrosome reaction

Capacitated spermatozoa exposed to different concentrations of exogenous AA showed a significant increase in AR (Fig. 2a) at all concentrations assayed.

However, no significant variations were observed between the doses tested, maximum response of 35% being found in the samples treated with 50 μM of AA, showing an increase in AR of 23% compared with the spontaneous acrosome reaction (SAR).

The role of PLA_2 activation in the AR was analysed using two specific inhibitors of the enzyme, aristolochic acid (ATA) and quinacrine. After incubation with the inhibitors, AR was induced with progesterone.

Figure 2b shows the effect of PLA_2 inhibition on progesterone-induced AR. Remarkably low AR values were obtained in samples pre-incubated with concentrations of 80 μM of ATA; this inhibitory effect remained with no changes at higher concentrations. With respect to quinacrine, a significant inhibitory effect was found at the lowest concentration assayed (5 μM), while values obtained at higher concentrations, of up to 8 times the initial one, caused no significant changes compared with those obtained at the dose of 5 μM.

Participation of PLC in the acrosome reaction

When is added to the culture medium, a mimetic of DAG (PMA) at increasing concentrations are recorded increases in dose-dependent manner of the percentages of AR at all concentrations assayed. The results obtained are shown in Fig. 3a. The 15% increase obtained with the lowest concentration assayed is significant and concentrations of 10 μM caused maximum response (60%).

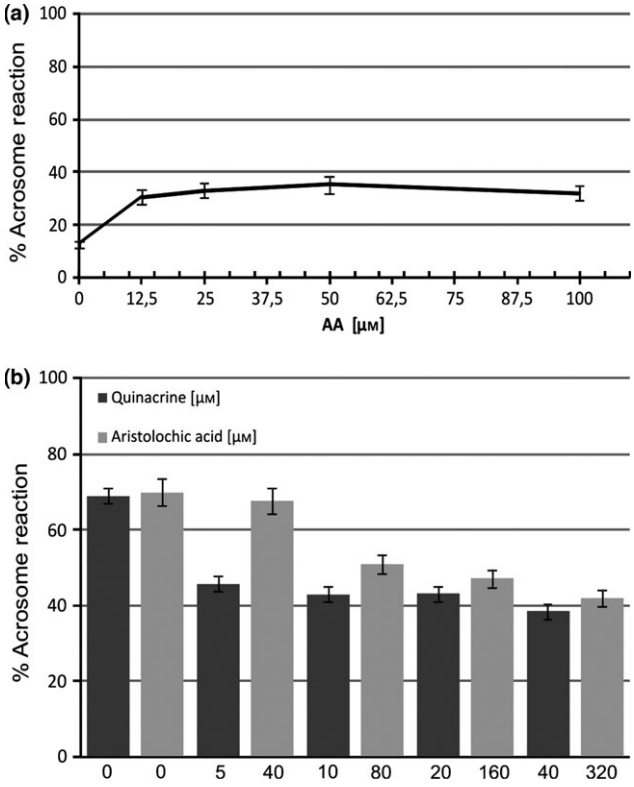

Fig. 2. (a) Effect of different concentrations of arachidonic acid (AA) (12.5; 25; 50; and 100 μM) on capacitated sperm. The control has DMSO and its AR value corresponds to SAR (12.5%). Results are expressed as mean± SEM of 4 experiments carried out with con different animals. (b) Effect of increasing concentrations of aristolochic acid (ATA) and quinacrine on the AR of capacitated epididymal sperm stimulated with progesterone (20 μM). Data represent the mean ± SEM of 5 experiments with different animals. SAR of 14.1% (data not shown)

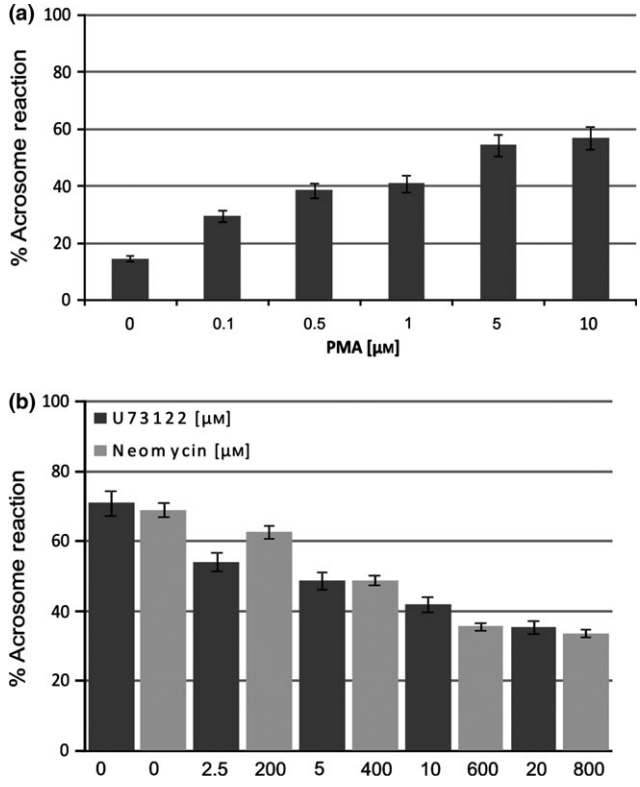

Fig. 3. (a) Effect of increasing concentrations of phorbol 12-myristate 13-acetate (PMA) on capacitated sperm. The control has the same volume of DMSO and its value corresponds to SAR. Results are expressed as the mean± SEM of 5 experiments with different animals. (b) Effect of PLC inhibitors, neomycin (200–800 μM) and U73122(2.5–20 μM), on progesterone-induced AR in capacitated sperm. Values represent the mean± SEM of 5 experiments with different animals

Together with the data from the previous assay, we might infer the participation of PLC in the AR.

The role of PLC in this progesterone-induced process was studied by pre-incubating the capacitated sperm in the presence of inhibitors of the enzyme, neomycin and U73122.

In both cases, we observed an inhibitory effect of the exocytosis of the acrosome compared with the controls.

The assays conducted with neomycin at different concentrations showed significant differences since 400 μM, maximum inhibitory effect being reached with 600 μM (Fig. 3b). Doses of 20 μM of U73122 produced similar results. However, at the lowest concentration assayed (2.5 μM), a significant decrease in sperm with total acrosome exocytosis was observed compared with the control.

Study of interrelations between PKA, PLA_2 and PLC 2

PKA activation – inhibition of PLA_2 and PLC

Capacitated spermatozoa were pre-treated with inhibitors of PLA_2 (ATA-160 μM) and PLC (U73122-20 μM) separately. The cAMP analog was added to the culture medium and incubation continued for 15 more min in order to activate PKA.

The data obtained from the samples pre-incubated with the inhibitors of the PLs show lower percentages of AR induced compared with the samples stimulated only with db-AMPc (Fig. 4). However, the inhibition isolated from the PLs shows a sperm population (30%) in which PKA activation still triggers the AR.

Inhibition of PKA-PLA_2-PLC

In order to analyse the process when progesterone acts as the trigger of the AR, epididymal spermatozoa capacitated *in vitro* were subjected to inhibition of only PKA (H89-30 min), PLA_2 (ATA-5 min), PLC (U73122-30 min) or jointly (PKA-PLA_2-PLC) and later stimulated with progesterone. The data obtained (Fig. 5) revealed the following: First, when each enzyme was inhibited individually, significant decreases in AR were found in all cases assayed comparing the results with the samples stimulated with progesterone without the addition of inhibitors, a greater inhibitory effect being found when PKA was blocked (31.1%).

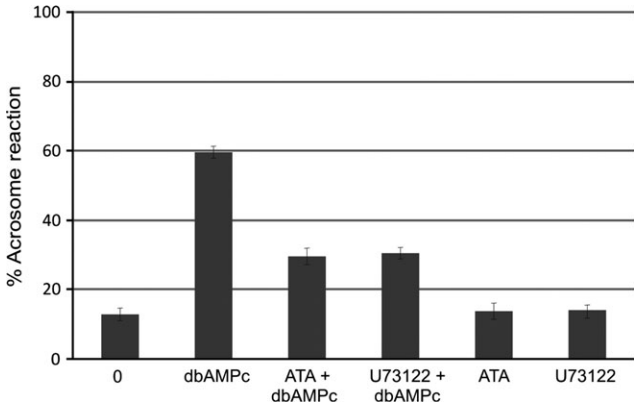

Fig. 4. Effect of the activation of PKA (db-cAMP) and inhibition of PLA_2 (ATA) and PLC (U73122) on the AR in capacitated epididymal sperm. The control corresponds to samples with the same volume of DMSO and its value represent to spontaneous acrosome reaction. The data are the mean± SEM of 4 experiments with different animals

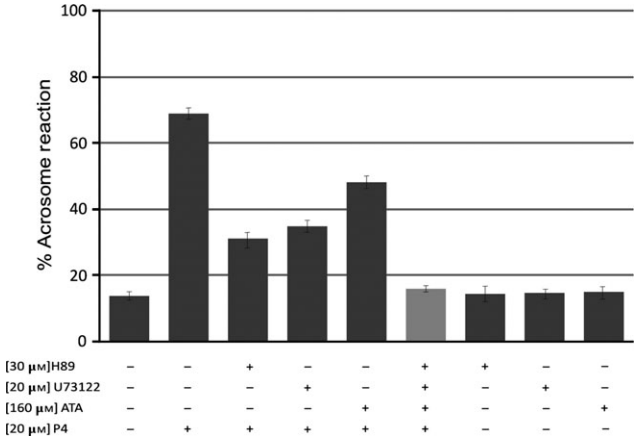

Fig. 5. Effect of PKA (H89), PLC (U73122) and PLA_2 (ATA) inhibitors on the AR, added in combination or singly to capacitated epididymal sperm stimulated with progesterone. The samples without inhibitors or progesterone have the same volume of vehicle. The negative control represents SAR (13.9%). Results are expressed as the mean ± SEM of 5 experiments with different animals

Second, when the enzymes were inhibited jointly, the values obtained were similar to those of the controls, which showed the effect of spontaneous AR (Fig. 6).

Discussion

In a biological response, the specific signal transduction pathway or one of its components is often multifactorial and in the specific case of the actions of progesterone, the evidence shows that it is not an exception.

The inductor role of the AR *in vitro* has been demonstrated in eutherian mammals. In spite of the large number of investigations to determine the signal transduction pathways involved in this process, they have not been completely elucidated yet.

Mammalian spermatozoa have a single soluble form of AC besides the enzyme with transmembrane domains. Its function is limited to cAMP formation, which exerts an allosteric effect on PKA regulatory subunits, producing their activation.

In Chinchilla epididymal spermatozoa, we demonstrated that treatment with a cAMP analog (db-AMPc) results in an increase in AR percentages, a maximum response of 58% being obtained with a 0.5 μM concentration. Even further, when PKA activity was prevented by the addition of H89 before progesterone stimulation, an inhibitory effect on AR was evidenced. The above results allow us to suggest the participation of the AC/AMPc/PKA system in the progesterone-induced acrosomal exocytosis in this species.

In agreement with our results, the studies conducted in humans by Liu et al. (2013) show that in normospermic individuals, the prevalent cause of infertility is a failure in signal transduction when PKA is defective, reinforcing the key role played by cAMP-dependent kinase.

Over 40 years ago, Allison and Hartee (1970) discovered a phospholipase A_2, an phospholipase present in the acrosome of mammalian sperm, and its participation in the process of membrane fusion has been suggested.

PLA_2 produces the hydrolysis of membrane lipids, releasing lyso-phosphatidylcholine (LPC) and fatty acids. Numerous investigations were conducted bypassing PLA_2, adding its metabolites to the culture media, in order to verify their participation, and were reported as effective fusogenic agents (Creutz 1981). LPC was tested in guinea pig epididymal sperm (Fleming and Yanagimachi 1981) and ejaculated sperm of hamsters (Ohzu and Yanagimachi 1982) and humans (Kyono et al. 1984).

The results presented in this work show that the addition of arachidonic acid (AA) exogenous to the culture medium is capable of triggering AR in capacitated epididymal sperm of *Chinchilla lanigera*, obtaining a response of 30%, with low concentrations (12.5 μM). Similar observations were reported by Meizel and Turner (1983) in hamster sperm.

Stimulation of capacitated gametes with progesterone showed changes in membrane lipids, indicating PLA_2 participation in humans (Baldi et al. 1993) and guinea pigs (Shi et al. 2005). On the basis of these findings, we inhibited directly PLA_2 with quinacrine and aristolochic acid and obtained AR percentages in *Chinchilla lanigera*, which suggests the participation of this pathway when sperm are stimulated with progesterone. As in hamster, Lui and Meizel (1979) proposed its key role in the acrosome reaction by demonstrating that blocking the enzyme with quinacrine or mepacrine would cause a decrease in the percentage of cells with total exocytosis of their acrosome.

On the other hand, 1,2-diacylglycerol (DAG), a product of the hydrolysis of PLC, has been studied in several experimental models in order to determine its role in the AR. The stimulation with different inducers showed an increase in DAG concentration in human sperm treated with progesterone, calcium ionophore

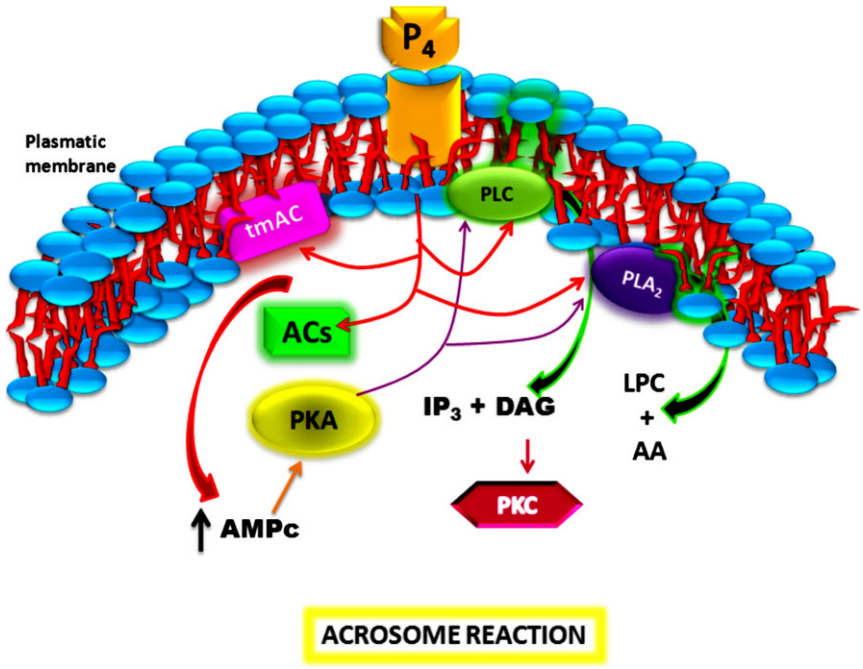

Fig. 6. Diagram of the signalling pathways that participate in the progesterone acrosome reaction-induced in epididymal sperm of *Chinchilla lanigera*. P_4: progesterone; tmAC: transmembrane adenylatecyclase; ACs: soluble adenylatecyclase; LPC: lysophosphatidylcholine; and AA: arachidonic acid

A23187 and ZP (Roldan and Murase 1994), in mouse (O'Toole et al. 1996), in ram (Roldan and Harrison 1992) and in boar (Vázquez and Roldan 1997).

This metabolite plays a key role as a second lipidic messenger and it is known to be an effective PKC activator (Roldan and Fragio 1994). Besides, studies conducted by Roldan and Murase (1994) reported a positive feedback with PLC specific for phosphatidylcholine.

In *Chinchilla lanigera*, we demonstrated an increase in AR percentages when capacitated spermatozoa were coincubated with PMA, a DAG analog, triggering AR in a dose-dependent manner.

From the above results, we could infer the participation of PLC in this process. Even further, when capacitated spermatozoa previously treated with two PLC inhibitors (neomycin and U73122) were stimulated with progesterone, in both cases a decrease similar to the inducer effect of the hormone was observed, verifying the activation of this pathway.

We have shown evidence that AR process induced by progesterone all the pathways studied here are activated to transduce the signal, but we are wondering if the pathways function independently one another or whether interaction occurs between them. Leclerc and Kopf (1999) assert that in mouse sperm, the simple stimulation with cAMP triggers the direct activation of PLA_2. In this sense, we studied the different inducers and inhibitors of the pathways in a combined way.

When analysing the assays in which, in separate experiments, a PKA such as db-cAMP was combined with inhibitors of the enzymes PLC and PLA_2; significant decreases in AR induction were observed.

These assays strongly suggest a relationship or points in common, in the transduction of the signal to trigger exocytosis. However, a sperm population of approximately 30% remains that, despite the inhibition of the PLs, continues to respond to the effect of db-cAMP in both treatments. However, when all the inhibitors of the key enzymes were conjugated, an almost total inhibition of the inducer effect of progesterone was observed, values similar to those corresponding to the spontaneous acrosome reaction of the controls. This is why we might infer that there is total inhibition of the stimulation caused by progesterone, indicating the participation in the process of the pathways studied.

These results reflect the coordinated participation of these signalling pathways and contribute to the global understanding of the complicated network of regulation or modulation that takes place in male gametes after the effect of progesterone.

Numerous publications have revealed that, at a certain DAG concentration, PKC is activated, and studies performed by Cobb et al.(1991) suggest that MAP kinase activation can occur through a PKC-dependent mechanism. The activated MAP kinase can then phosphorylate PLA_2 at Ser-505, causing an increase in PLA_2 activity (Lin et al. 1992, 1993). In other reports, DAG itself would act directly on this enzyme with no PKC participation (Kramer et al. 1987). Consequently, DAG is capable of modulating these activations via PKC (O'Toole et al. 1996) and MAPK (De Lamirande and Gagnon 2000; Du Plessis et al. 2001); since these elements of signal transduction are presents in the sperm, they could also regulate signalling through PKA/MAPK/PLA_2-PLC.

In view of the numerous possibilities that gamete interaction presents in the complex process of sexual reproduction, knowledge is still far from complete. In particular, the success of the acrosome reaction depends

on a strict sequence and coordination of multiple signals, so that it can take place at the right moment, thus ensuring successful fertilization and consequently the possibility of perpetuating the species.

Acknowledgements

This work was supported by a grant from the Science Council of the National University of Tucumán (CIUNT) and the National Agency for Promotion of Science and Technology (FONCYT).

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

Dr. Gramajo-Bühler performed the research design; Dr. Zelarayán was responsible for the analusis and interpretation of data and finally, Dr. Sánchez-Toranzo revising critically.

References

Allison AC, Hartee EF, 1970: Lysosomal enzymes in the acrosome and the possible rate in fertilization. J Reprod Pertil **21**, 501–515.

Baldi E, Falsetti C, Krausz C, Gervasi G, Carloni V, Casano R, Forti G, 1993: Stimulation of platelet-activating factor synthesis by progesterone and A23187 in human spermatozoa. Biochem J **292**, 209–216.

Baldi E, Luconi M, Bonaccorsi L, Forti G, 2002: Signal transduction pathways in human spermatozoa. J Reprod Immunol **53**, 121–131.

Berridge MJ, 1987: Inositol triphosphate and diacylglycerol: two interacting second messengers. Annu Rev Biochem **56**, 159–193.

Brewis IA, Morton IE, Moore H, England GC, 2001: Solubilized zona pellucida proteins and progesterone induce calcium influx and the acrosome reaction in capacitated dog spermatozoa. Mol Reprod Dev **60**, 491–497.

Chen WY, Ni Y, Pan YM, Shi QX, Yuan YY, Chen AJ, Mao LZ, Yu SQ, Roldan ERS, 2005: GABA, progesterone and zona pellucida activation of PLA_2 and regulation by MEK-ERK ½ during acrosomal exocytosis in guinea pig spermatozoa. FEBS Lett **579**, 4692–4700.

Cobb MH, Boulton TG, Robbins DJ, 1991: Extracellular signal-regulated kinases: ERKs in progress. Cell Regul **2**, 965–978.

Creutz CE, 1981: Cis-unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synexin. J Cell Biol **91**, 247–256.

De Jonge CJ, Mack SR, Zaneveld LJ, 1989: Inhibition of the human sperm acrosome reaction by proteinase inhibitors. Gamete Res **23**, 387–397.

De Lamirande E, Gagnon C, 2000: The extracellular signal-regulated kinase (ERK) pathway is involved in human sperm function and modulated by the superoxide anion. Mol Hum Reprod **8**, 124–135.

Domínguez L, Yunes R, Fornés MW, Mayorga L, 1996: Acrosome reaction stimulated by the GTP non-hydrolizable analogue GTPgS is blocked by phospholipase A2 inhibitors in human spermatozoa. Int J Androl **19**, 248–252.

Du Plessis SS, Page C, Franken DR, 2001: The zona pellucida-induced acrosome reaction of human spermatozoa involves extracellular signal regulated kinase activation. Andrologia **33**, 337–342.

Fleming AD, Yanagimachi R, 1981: Effects of various lipids on the acrosome reaction and fertilizing capacity of guinea pig spermatozoa with special reference to the possible involvement of lysophospholipids in the acrosome reaction. Gamete Res **4**, 253–273.

Gramajo-Bühler MC, Zelarayán L, López LA, Sánchez-Toranzo G, 2012: Acrosome reaction in the epididymal sperm of *Chinchilla lanigera*. Effect of progesterone, A23187 and stimulation of a GABAA-like receptor. J Exp Zool **317**, 259–265.

Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N, 2011: Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during *in vitro* fertilization. Proc Natl Acad Sci USA **108**, 4892–4896.

Kramer RM, Checani GC, Deykin D, 1987: Stimulation of Ca2 + -activated human platelet phospholipase A2 by diacylglycerol. Biochem J, **248**, 779–783.

Kyono K, Hoshi K, Saito A, Oshiai TH, Uzuki M, 1984: Effects of phospholipase A2, lysophosphatidyl choline, and fatty acid on the acrosome reaction of human spermatozoa Tohoku. J Exp Med **144**, 257–263.

Lax Y, Grossman S, Rubinstein S, Magid N, Breitbart H, 1990: Role of lipoxygenase in the mechanism of the acrosome reaction in mammalian spermatozoa. Biochem Biophys Acta, **1043**, 12–18.

Leclerc P, Kopf GS, 1999: Evidence for the role of heterotrimeric guanine nucleotide-binding regulatory proteins in the regulation of the mouse sperm adenylyl cyclase by the egg's zonapellucida. J Androl, **20**, 126–134.

Lin LL, Lin AY, Knopf JL, 1992: Cytosolic phospholipase A2 is coupled to the hormonally regulated release of arachidonic acid. Proc Natl Acad Sci USA **89**, 6147–6151.

Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A, Davis RJ, 1993: cPLA2 is phosphorylated and activated by MAP kinase. Cell **72**, 269–278.

Liu DY, Liu ML, Baker HW, 2013: Defective protein kinase A and C pathways are common causes of disordered zona pellucida (ZP)-induced acrosome reaction in normozoospermic infertile men with normal sperm-ZP binding. Fertil Steril **99**, 86–91.

Llanos MN, Anabalon MC, 1996: Studies related to progesterone-induced hamster sperm acrosome reaction. Mol Reprod Dev **45**, 313–319.

Llanos MN, Lui CW, Meizel S, 1982: Studies of phospholipase A2 related to the hamster sperm acrosome reaction. Biol Reprod **221**, 107–117.

Lui CW, Meizel S, 1979: Further evidence in support of a role for hamster sperm hydrolytic enzymes in the acrosome reaction. J Exp Zool **207**, 173–185.

Meizel S, Turner KO, 1983: Cis-unsaturated fatty acids stimulate the golden hamster sperm acrosome reaction. Fed Proc **42**, 1823.

Meizel S, Turner KO, 1991: Progesterone acts at the plasma membrane of human sperm. Mol Cell Endocrinol **71**, R1–R5.

Meyers SA, Overstreet JW, Liu IK, Drobnis EZ, 1995: Capacitation *in vitro* of stallion spermatozoa: comparison of progesterone-induced acrosome reaction in fertile and subfertile males. J Androl **16**, 47–54.

Moseley FLC, Jha KN, Lars B, Brewis IA, Publicover SJ, Barrat CLR, Lefièvre L, 2005: Protein tyrosine phosphorylation, hyperactivation and progesterone-induced acrosome reaction are enhanced in IVF media: an effect that is not associated with an increase in protein kinase A activation. Mol Hum Reprod **11**, 523–529.

Ohzu E, Yanagimachi R, 1982: Acceleration of acrosome reaction in hamster spermatozoa by lysolecithin. J Exp Zool **244**, 259–263.

Osman RA, Andria ML, Jones AD, Meizel S, 1989: Steroid induced exocytosis: the human sperm acrosome reaction. Biochem Biophys Res Commun **160**, 828–833.

O'Toole CMB, Roldan ERS, Fraser LR, 1996: Protein kinase C activation during progesterone-stimulated acrosomal exocytosis. Mol Hum Reprod **2**, 921–927.

Roldan ERS, Fragio C, 1994: Diradylglycerols stimulate phospholipase A2 and subsequent exocytosis in ram spermatozoa. Evidence that the effect is not mediated via protein kinase C. J Biochem **297**, 225–232.

Roldan ERS, Harrison RAP, 1992: The role of diacylglycerol in the exocytosis of the sperm acrosome.Studies using diacylglycerol lipase and diacylglycerol kinase inhibitors and exogenous diacylglycerols. Biochem J **281**, 767–773.

Roldan ERS, Murase T, 1994: Polyphosphoinositide derived diacylglycerol stimulates the hydrolysis of phosphatidylcholine by phospholipase C during exocytosis of the ram sperm acrosome. J BioChem **269**, 23583–23589.

Roldan ERS, Martinez-Dalmau R, Mollinedo F, 1994a: Diacylglycerol and alkylglycerol stimulate ram sperm phospholipase A2. Int J Biochem **26**, 951–958.

Roldan ERS, Murase T, Shi QX, 1994b: Exocytosis in spermatozoa in response to progesterone and zonapellucida. Science **266**, 1578–1581.

Shi QX, Chen WY, Yuan YY, Ni Y, Mao LZ, Yu SQ, Chen AJ, Roldan ERS, 2005: Progesterone primes zonapellucida-induced activation of phospholipase A2 during acrosomal exocytosis in guinea pig spermatozoa. J Cell Physiol **205**, 344–354.

Thérien I, Manjunath P, 2003: Effect of progesterone on bovine sperm capacitation and acrosome reaction. Biol Reprod **69**, 1408–1415.

Vázquez JM, Roldan ERS, 1997: Diacylglycerol species as messengers and substrates for phosphatidylcholine resynthesis during Ca2 + -dependent exocytosis in boar spermatozoa. Mol Reprod Dev **48**, 95–105.

Yanagimachi R, 1994: Mammalian fertilization. In: Knobil E, Neil JD (eds), The Physiology of Reproduction, Vol **1**. Raven Press, New York, pp. 189–318.

Submitted: 3 Aug 2015; Accepted: 18 Nov 2015

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