Extracellular cAMP activates molecular signalling pathways associated to sperm capacitation in bovines

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The abbreviations used are: A1r: Adenosine receptor 1. BOEC Bovine Oviductal Epithelial cells. CTC: Chlortetracyclin. ERK-1/2: Extracellular Regulated Kinase. LPC: Lyso-Phosphatydil Choline. MMP: Mitochondrial Membrane Potential. MRP4: Multidrug Resistant-associated Protein 4. PDE: Phosphodiesterases. PKA: Protein Kinase A. PKC: Protein Kinase C. PLC: Phospholypase C. pTyr: Tyrosine phosphorylation. ROS: Reactive Oxygen Species. sAC: Soluble Adenylyl Cyclase. SPZ: Spermatozoa.

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AUTHOR CONTRIBUTION: CAIA performed the experiments, analyzed data statistically and prepared the figures. COS and LC performed IVF and sperm-oviduct interaction experiments, AC conducted the Ca^{2+} determinations, AJ and JMM performed and conducted FACS analysis. AM contributed with reagents/tools for IVF assays. NDS performed the

cAMP determinations. SPM and CD designed the experiments and funded the study. CAIA, CD and SPM wrote the manuscript text. All authors reviewed the manuscript.

ABSTRACT
In order to fertilise the oocyte, ejaculated spermatozoa must undergo a series of physiological changes in the female reproductive tract known as capacitation. This correlates with a number of membrane and metabolic modifications that include an increased influx of bicarbonate and Ca^{2+}, activation of a soluble adenylyl cyclase (sAC) to produce cAMP, protein kinase A (PKA) activation, protein tyrosine phosphorylation, and the development of hyperactivated motility. We previously reported that cAMP efflux by MRP4 occurs during sperm capacitation and the pharmacological blockade inhibits this process. Moreover, the supplementation of incubation media with cAMP abolishes the inhibition and leads to capacitation, suggesting that extracellular cAMP regulates crucial signalling cascades involved in this process. In the present work, we elucidate the molecular events induced by extracellular cAMP. Our results showed that external cAMP induces capacitation, through the A1 receptor, depending upon the action of its coupled PLC. Downstream this enzyme increased the ERK1-2 activation through PKC and elicited a rise of sperm Ca^{2+} levels. Moreover, extracellular cAMP-induced capacitation depends also upon the activity of sAC and PKA, increased the tyrosine phosphorylation, that showed a different kinetics respect to that observed with other canonical capacitating inducers. In addition, cAMP induced hyperactivation of the motility, and concomitantly increased the number of sperm with high mitochondrial activity. Finally, cAMP increased the *in vitro* fertilization rate respect to control conditions. All of these findings strongly suggest an important role of extracellular cAMP in the regulation of the signalling pathways involved in the acquisition of sperm fertilizing capability in bovines.

Fertilisation process in mammals is the result of a complex amount of molecular events. In order to fertilise the oocyte, sperm has to go through a series of biochemical and structural changes that begins in male epididymis with the so-called sperm *maturation* and ends in the female reproductive tract with capacitation(1–3). Sperm capacitation is correlated with an augment in membrane fluidity, hyperpolarization, rise in intracellular pH, cAMP, Ca^{2+} influx, activation of kinases, and an increase in tyrosine phosphorylation(4). Nevertheless, this process is still not totally understood at molecular level.
The classic role of cAMP is to stimulate Protein Kinase A (PKA) and other intracellular effectors such us EPAC(5). In sperm capacitation, soluble Adenilyl Cyclase (sAC, also known as ADCY10 or SACY) is the main responsible for cAMP increase in sperm, and it is stimulated by HCO_3^- and sensitized by Ca^{2+} (6, 7). Levels of these ions as well as the activity of phosphodiesterases modulate the intracellular availability of cAMP and its downstream signalling pathways. However, in other tissues and cell types, the exclusion of this molecule by Multidrug Resistant Protein family (MRPs) also regulates intracellular concentration of nucleotides and other molecules, and provides cAMP to the extracellular space, which can trigger purinergic signalling events(8, 9).
Purinergic signalling is a key component in the physiology of several tissues. By the self-production of nucleotides and nucleosides and them binding to specific receptors, a wide-broad of cellular responses is finely modulated such as cell-growth, differentiation and motility(10–12). In reproduction it has been shown that purinergic signalling is responsible of a variety of processes both in female and male side from steroidogenesis and gametogenesis to embryo development. Adenosine receptors (A1, A2a, A2b and A3) are G protein-coupled

receptors that elicit excitatory and inhibitory responses, depending on the cell-type and the type and number of receptors involved(13). Moreover the genetic lack of either MRP4 or A1r has a subfertile phenotype(9). A1r lacking mice displayed defects in Ca^{2+} levels regulation and ERK-1/2 phosphorylation in sperm(14).

In our laboratory, we demonstrated the existence of cAMP efflux by MRP4 and studied its role in the regulation of bicarbonate-induced sperm capacitation in bovines(15). We showed that the presence of bicarbonate (40 mM) in sperm incubation media produces an early sAC-dependant rise of cAMP not only in the intracellular but in the extracellular space, suggesting an efflux of cAMP from spermatozoa. We detected, by western blot, immunocytochemistry and RT-PCR assays, the presence of MRP4, the main transporter associated to cAMP efflux. When MRP4 was inhibited with probenecid –broad spectrum MRP inhibitor- sperm did not pump out cAMP, and failed to undergo capacitation. However when incubation media was supplemented with cAMP (10 nM), sperm capacitation was restored. In addition, blockade of A1r abolished bicarbonate and cAMP-induced sperm capacitation. Those findings strongly support the hypothesis that cAMP efflux, through MRP4, and further activation of A1r regulate downstream events associated with bicarbonate-induced sperm capacitation in bovines.

In the present work, we elucidate the role of extracellular cAMP in the regulation of signalling pathways involved in bovine sperm capacitation. Our results strongly suggest that cAMP extrusion by MRP4 plays and important role in the regulation of the signalling pathways involved in the capacitation process.

EXPERIMENTAL PROCEDURES

Sperm preparation

Straws of frozen bovine semen (20-25 x10^6 SPZ/ml) were thawed in a water bath at 37°C for 30 seconds. Sperm were selected by wool glass column method(16), and washed by centrifugation in BSA-Free Tyrode's Albumin Lactate Pyruvate (sp-TALP). Pellets were resuspended to a final concentration of 7.10^7 and then diluted as described below for each treatment.

In vitro capacitation

Ten to 15 x 10^6 SPZ/ml were incubated in 0,3% BSA sp-TALP (99 mM NaCl; 3.1 mM KCl; 0.4 mM NaH2PO4; 0.4 mM MgCl2.6H2O; 21.6 mM Na-Lactate; 10 mM HEPES; 2 mM $CaCl_2.H_2O$; 25 mM $NaHCO_3$; 1 mM Na-Piruvate; 50 mg/ml gentamycin; pH 7.4; Parrish et al., 1988) at 38.5°C and 5% CO2 atmosphere for 45 min(15) under different experimental conditions. To evaluate the role of extracellular cAMP on sperm capacitation, 10 nM cAMP was added to the incubation media as well as different inhibitors of enzymes associated with signalling transduction pathways: U73122 (PLC inhibitor 10 µM), Gö6983 (PKC inhibitor 10 µM), PD98059 (ERK-1/2 inhibitor 30 µM), H89 and KT (PKA inhibitors 50 µM and 100 nM respectively), KH7 (sAC inhibitor 10 µM), BAPTA-AM (intracellular Ca^{2+} chelator, 50 µM) and Probenecid (MRPs general inhibitor 300 µM). None of the chemicals exhibited activity by its own and the concentrations used here are the result of concentration response-curve or manufacturer's indications.

Assessment of sperm capacitation

Sperm capacitation was assessed by CTC assay and LPC-induced acrosome reaction/*Pissum sativum* agglutinin-FITC staining. CTC assay was performed as previously detailed(16). Briefly, sperm capacitation was assessed by detection of CTC fluorescence in the sperm head except in the post-acrosomal region characteristic of capacitated sperm (Pattern B)(17).

The induction of acrosome reaction was performed as previously described (15). Briefly, sperm was incubated for 45 min under the experimental conditions detailed above and the sample was divided in two aliquots that were further incubated for 15 min at 38.58°C in the presence or absence of 100 mg/ml LPC. To assess viability and acrosome reaction, spermatozoa was incubated with Hoechst33258 (2 mg/ml) for 5 min, fixed with 1% w/v paraformaldehyde for 8 min at room temperature and washed with phosphate buffer solution (PBS). An aliquot was air dried onto slides and permeabilized in methanol for 10 min at 4° C. Slides were incubated for 60 min at room temperature with 50 mg/ml PSA-FITC. At least 200 stained cells/treatment were scored in an epifluorescence microscope. The percentage of capacitated spermatozoa was represented by the difference between percentages of viable-acrosome-reacted spermatozoa in LPC-treated and in non-LPC-treated samples.

Western Blot assay

Total soluble protein isolation was performed as described by (18) with slights modifications. Briefly, after the incubations, 3 to 4 x 10^6 spermatozoa were centrifuged 3 min at 10000 g, resuspended with 1 ml of PBS containing 2 mM of orthovanadate. Spermatozoa were centrifuged again 3 min 10000 g, resuspended in 15 ul of Laemmli buffer and boiled for 5 min. Samples were centrifuged 3 min 10000 g, and supernatants were supplemented with 5% B-mercaptoethanol and boiled again for five minutes. Proteins were separated in 10% SDS polyacrylamide gel and transferred to PVDF membranes that were blocked either with 5% cold fish skin gelatin (for pTyr and ERK-1/2 phosphorylation detection) or 3% skimmed milk (PKA phosphorylated substrates detection). Membranes were incubated with primary antibodies in the following conditions: pTyr antibody (Millipore 4G10), 1:5000 in TPBS (PBS 0,1% Tween20), Overnight 4°C on agitation, washed tree times with TPBS; pERK-1/2 (Cell Signalling), 1:5000 in TPBS, overnight 4°C on agitation, washed tree times with TPBS; pPKA antibody (Cell Signalling), 1:1000 in TTBS (Tris 20 mM, NaCL 150 mM, 0,1% Tween20) with 1% skimmed milk, Overnight 4°C, washed tree times with TTBS. For primary antibody detection, an anti-mouse or anti-rabbit polyclonal antibody linked to horse radish peroxidase and enhanced chemo-luminescence reagents were used following the manufacturer's instructions (Jackson).

Cyclic AMP Radio binding assay

Spermatozoa (7.5 × 10^6 cells/ml) was incubated for 7 min in 0.3% BSA sp-TALP medium stimulated with 10 nM cAMP alone or preincubated 3 min with and KH7 (10 μM) and then stimulated. Samples were centrifuged thrice for 5 min 3000 g and 1 ml ice-cold ethanol were added to pellets. Ethanol was evaporated and residues were resuspended in 50 mM Tris–HCl, pH 7.4, 0.1% BSA for cAMP determination. Cyclic AMP levels were measured by competitive radio-binding assay to the regulatory subunit of PKA using [^{3}H] cAMP, as previously described (19). Duplicate samples in at least three independent experiments were analysed.

Mitochondrial Membrane Potential assessment

Mitochondrial membrane potential (MMP) of sperm cells was measured by using the lipophilic cationic probe JC-1, according to the method described by (20). Briefly, 10 μM JC-1 was added to sperm aliquots 5 min after the in vitro capacitation has started, and incubated at 38°C 40 min. The stained samples were analysed with a BD LSR flow cytometer (Beckon Dickinson). Excitation of stained cells was obtained by the instrument's argon-ion laser (488 nm). Emitted fluorescence was detected using both FL1 (530/30 nm) and FL2 (585 nm) filters. Green emission was analyzed in FL1 and greenish-orange in FL2. A total of 30,000 cells were evaluated and classified as percentages (CellQuest, version 3.3; Beckon

Dickinson) of 3 distinct groups: sperm cells with high respiratory activity (orange fluorescence) and those with low respiratory activity (green fluorescence). We reported the changes in the population with high respiratory activity.

CASA

Computer-assisted sperm analysis was performed using a SpermVisionanalyzer (Minitüb GmbH), connected to an Olympus BX 51 microscope (Olympus, Tokyo, Japan) with a heated stage (38°C). Aliquots (6 μL) of sperm from different treatments were pipetted on to a warm glass slide and 18 × 18 mm coverslip was placed on top. Sperm motility was analyzed using the SpermVision software program for bull spermatozoa following manufacturer's instructions. For the purposes of this study, only hyperactivated motility is reported.

Bovine oviductal epithelia cell cultures and sperm co-cultures

Bovine oviducts were obtained as donation from Rio de la Plata slaughterhouse (Buenos Aires, Argentina). Cultures of oviductal epithelia were prepared as described previously by (21). Briefly, oviducts were collected at the time of slaughter, transported at 4°C, cleaned of surrounding tissues and washed three times in sterile PBS at 4 °C. After that, the oviducts were cut, flushed with sterile PBS and squeezed by pressure with tweezers.

Laminae of bovine oviduct epithelial cells (BOEC) from ampulla and isthmus were recovered from different animals and pools of epithelial cells from 6 oviducts were collected together.

Different pools of BOEC were washed by centrifugation at 1500 g for 5 min and incubated in M199 medium supplemented with 10% of FBS (M199 medium + FBS), gentamicin (0.1 mg/ml) and fungizone (1 μg/ml) at 38.5 °C in a 5% CO2 atmosphere. Incubations were performed in six-well tissue culture dishes with 12 mm round cover slips on the well bottom. After 48 h, BOEC were washed by centrifugation (1500 g for 5 min) and replaced in the tissue dishes. Medium was changed every 48 h. Bovine OEC in culture displayed a characteristic epithelial polygonal shape and rarely overlapped; confluence was achieved around 7 days after starting the culture in the round cover slip. Finally, the oviductal monolayers were washed three times in BSA-free sp-TALP and left in this medium for 60 min until co-culture with spermatozoa.

Within each experiment, confluent BOEC monolayers from different pool of oviducts were inseminated with sperm suspensions (0.5 x10^6 sperm/ml of BSA-free sp-TALP/well) for 60 min at 38.5 °C in a 5% CO2 atmosphere. After that, unbound sperm were removed by washing three times with BSA-free sp-TALP and the cAMP was added for 15 min. After that, control and treated wells were washed three times with BSA-free sp-TALP to remove released sperm, fixed in glutaraldehyde 2.5% v/v for 60 min at room temperature, extensively washed and the round cover slips containing the cocultures were mounted on a glass slide. The number of bound sperm was determined analyzing 20 fields of 0.11 mm^2/cover slip under a phase contrast microscope (Olympus) in blinded experiments. The results were expressed as the media of the average of bound spermatozoa in a 0.11 mm2 area. A replicate (n) in these experiments was defined by the co-culture of 1 pool of BOEC inseminated with sperm from 1 bull. All the treatments (including the control) were performed for each replicate. The number of spermatozoa bound to the BOEC in the controls depends on the replicate and it ranged between 30 and 80 per 0.11 mm^2 area.

In vitro fertilization assay

Bovine ovaries were obtained also as donation from Rio de la Plata slaughterhouse (Buenos Aires, Argentina). Ovaries were collected at the time of slaughter, transported at 4 °C, and washed three times in sterile PBS supplemented with streptomycin (0,1 g/lt). Right after, follicles ranging from 2 to 7 mm were punctured with 21 G needles and follicular fluid was

mixed with recollection medium (M199 balanced with Hank's salts and supplemented with 0,1 mg/ml heparin, 1 µl/ml gentamycin and 1 mg/ml BSA). COCs were selected under magnification by the presence of 2 or 3 continuous layers of cumulus cells and homogenous cytoplasm of the oocytes. After selection 50-100 COCs were washed 3 times in recollection medium, and incubated in Maturation Medium (M199 balanced with Earle's salts, supplemented with 10 mg/ml L-glutamine, 11 mg/ml Pyruvate, 10% FBS, 1 µl/ml Gentamicin and 100 U hrFSH) in an atmosphere of 5% CO2 for 18 h at 38,5° C. Matured oocytes were washed in IVF SOF and subsequently inseminated with a final concentration of1-2x10^6cell/ml previously Percoll selected cryopreserved sperm in 400 µl of IVF-SOF supplemented with 10 mg/ml BSA, and 50 µg/ml heparin or 10 nM cAMP. Inseminated oocytes were cultured at 38.5 °C in in an atmosphere of 5% CO2 in air for 6 h. On-cell-stage prospective embryos were isolated from cumulus cells by exposure to IVF-SOF supplemented with 1 mg/ml hyaluronidase and gently agitation. Then they were incubated 10 min with Hoechst (2 mg/ml), fixated with 1% paraformaldehyde 10 min at RT and examined under fluorescence microscope (Nikon) to evaluate the no. of 2-pronuclei zygotes of each treatment.

RESULTS

Extracellular cAMP elicits capacitation-like responses

Cyclic AMP is not permeable to biological membranes given its structural and chemical properties. Therefore, diverse permeable analogues like db-cAMP o 8Br-cAMP, have been developed in order to evaluate cAMP intracellular effects. However, our previous results using non-permeable cAMP at concentrations found in the extracellular space indicated that the cyclic nucleotide increased sperm capacitation suggesting its involvement in this process possibly by interacting with extracellular effectors.

In order to confirm the relevance of the extracellular cAMP (10nM) in sperm capacitation (Supplementary Figure 1), we evaluated the effect of this nucleotide and bicarbonate at 40 mM –known capacitating concentration- on hyperactivation of the motility, process associated to capacitation. Results indicated that extracellular cAMP induces a significant increase in hyperactivated motility (Fig. 1*A*). In addition, it is described that capacitation correlates with an augment in mitochondrial activity probably to support the energetic demand produced by exacerbated flagellum movement. We evaluated whether bicarbonate or cAMP could elicit changes in the mitochondria membrane potential as an indirect way to measure its activity. Results showed that 24 ± 4,3% of sperm displayed an elevated mitochondrial activity in the presence of cAMP respect to 11.5 ± 3,2% in control conditions (Fig. 1*B*).

On the other hand, one of the most important changes in sperm capacitation is the increase of protein phosphorylation in tyrosine residues (pTyr). This event takes place in the later stages of the capacitation process and is the result of a complex signalling network activation. In the next experiment, we incubated sperm with cAMP for different times and then we evaluated pTyr by western blot. Our results showed that cAMP produced an augment in sperm pTyr that increased over the time (Fig. 1, *C* and *D*).

Cyclic AMP stimulates the release of sperm from Bovine Oviductal Epithelial Cell (BOEC) and promotes fertilising ability

Taking into account that sperm release from the oviduct is correlated with sperm capacitation, we evaluated the ability of sperm to release from oviductal cells using different stimuli. We first performed co-cultures of BOEC and sperm cells as described in the

experimental procedures and evaluated the role of MRP4 in the regulation of sperm release. Co-cultures were incubated with bicarbonate in capacitating and non-capacitating concentrations with or without probenecid, a general MRP inhibitor. Results shown in Fig. 2 indicated that bicarbonate at 40 mM produced sperm release from oviductal cells and that probenecid inhibited the effect. Then, the role of the cyclic nucleotide in the sperm release from oviductal epithelia was evaluated (Fig. 2*A*). Results indicated that the incubation of co-cultures with cAMP (10 nM) induced sperm release from the oviductal cells, as shown in Fig. 2*B*. Concomitantly, we performed *in vitro* fertilization assays, using cAMP as the capacitation inductor or heparin (known capacitating inductor). The number of 2-pronuclei oocyte after the incubation with sperm was similar either these were incubated with cAMP or heparin (50 µg/ml), and significantly greater in both cases than control conditions, supporting that extracellular cAMP may act as a capacitating agent (Fig. 3*C*).

PKC and ERK-1/2 are involved in cAMP-induced capacitation

As we previously demonstrated, cAMP is extruded from sperm when exposed to 40 mM bicarbonate(15). In addition, cAMP in the extracellular space reach levels around 10nM, and this concentration of cAMP induces capacitation when added to in vitro capacitation media. We also described that a selective antagonist for purinergic receptor A1r blocks cAMP effect. A1r receptor stimulates phospholipase C (PLC) and its activity is associated with an intracellular calcium release, an event strongly related to capacitation(22, 23). Thus we investigated whether PLC may be involved in cAMP-induced capacitation. Sperm were incubated with cAMP in the presence or absence of U73122, a PLC inhibitor. Results showed that the inhibitor reversed the capacitating effect of cAMP, abolishing the increase of hyperactivated sperm percentages and the rise of pattern B of the CTC assay (Fig. 3 *A* and *B*). The inhibitor effect was specific as its non-active analogue did not prevent cAMP capacitation (data not shown).

By the cleavage of PIP3 by PLC, there is a production of DAG that activates the traditional isoforms of PKC. Also, in several cell types, the induction of PKC triggers MAPK cascade(24, 25).There are increasing reports of the critical role of ERK-1/2 in motility, capacitation and acrosomal reaction(14, 26, 27). Therefore we tested whether PKC, and ERK-1/2 were also involved in cAMP induced capacitation. We incubated sperm with cAMP in presence or absence of Gö6983 and PD98059 (canonical PKC isoforms and ERK-1/2 inhibitors respectively) and evaluated events associated to capacitation. Results showed that the presence of Gö6983 or PD98059 reversed the capacitating effect of cAMP (Fig. 4, *A* and *B*) suggesting that cAMP-induced capacitation requires PKC and ERK-1/2 activities. However, the incubation with PKC but not ERK-1/2 inhibitor abolished the pTyr induction by cAMP (Fig. 4*C*).On the other hand, cAMP upregulated the levels of pERK-1/2, and this stimulation was reversed by the PKC inhibitor (Fig. 4*D*), suggesting that the activity of this member of the MAPK cascade is modulated upstream by PKC. As ERK-1/2 localization in bovine sperm has not been studied before, immunocytochemistry assays were carried to detect its subcellular distribution. Post-acrosomal and equatorial segment were positive for this immunolabeling (Supplementary figure 2).

Ca^{2+} movement stimulated by cAMP

The activation of PLC is also associated to Ca^{2+} augment by the triggering of IP3r coupled to intracellular Ca^{2+} deposits(28, 29). We wanted to evaluate Ca^{2+} dynamics in the sperm population when exposed to cAMP. Fluorescence analysis of the whole sperm population showed a significant increase of intracellular Ca^{2+} (Fig. 5, *inset*). To confirm this result, single cell Ca^{2+} measurements were performed and results showed that only 19± 5% of sperm

suffered a fast rise in the ΔF/F0 ratio in the presence of cAMP in the incubation media (Fig. 5).

SAC/PKA pathway is indirectly activated by external cAMP.

During capacitation, PKA activation is required to trigger a variety of processes such as change in the motility pattern, activation of kinases cascade and the reorganization of cytoplasm(30, 31). Previous results indicated that extracellular cAMP produces an increase, time dependent in sperm pTyr; thus, it is possible that cAMP modulates PKA activity along the incubation time. The presence of sAC and PKA inhibitors (KH7, and H89 respectively) inhibited events associated to cAMP-induced sperm capacitation such as the rise of CTC B pattern (Fig. 6, *A* and *B*) and the induction of hyperactivated motility (Fig. 6*C*). Confirmatory assays using an alternative PKA inhibitor (KT, 100 nM) were conducted, obtaining similar results (Data not shown). Moreover, we observed that cAMP incubation produces a significant increase of PKA phosphorylated substrates at 15 min which is maintained over the time (Fig.7, *A* and *B*). The use of sAC and PKA inhibitors decreased the above mentioned phosphorylated residues (Fig. 7, *C* and *D*), as expected. Sub-cellular localization of PKA activity was analysed and a rise in the midpiece of sperm was detected (Fig. 7*E*).

These results suggest that external cAMP may modulate cAMP production and therefore intracellular cAMP levels and the cAMP/PKA pathway. To support this hypothesis, we performed in vitro capacitation in the presence of extracellular cAMP and measured intracellular cAMP concentrations. Results, as shown in Figure 8*A*, indicated that extracellular cAMP increased the concentration of intracellular cAMP and the presence of KH7 inhibited the induction, suggesting that the increase of intracellular levels induced by extracellular cAMP is mediated by sAC activity (FALTA RIA AMPC). Finally, to evaluate a possible link between Ca^{2+} movements and sAC/PKA signalling pathways, we determined PKA substrates in sperm pre-loaded with BAPTA-AM and stimulated with extracellular cAMP (Fig. 8*B*). The induction of PKA substrates did not occur in the presence of the intracellular Ca^{2+} chelator, suggesting that Ca^{2+} is involved in the stimulation of sAC/PKA pathway by the extracellular nucleotide.

DISCUSSION

The role of cAMP and its effectors have been largely studied since the beginning of the description of the signalling pathways that rule capacitation(32). Several reports describe its involvement in critical capacitation outcomes such as progressive motility, hyperactivation and the ability to undergo the acrosome reaction (33–35). However, lesser efforts have been directed to elucidate the tight regulation of cAMP levels in sperm (15, 36). In our previous work we characterised the cAMP exclusion system and reported its important contribution to bovine sperm capacitation, but we also reported that providing the extracellular space with this nucleotide might have a further role in sperm physiology. Interestingly, we observed that extracellular non-permeable cAMP reversed the probenecid-inhibition of capacitation(15). This strongly suggested that the nucleotide exert responses from the extracellular space that resembled a capacitation-like state.

In this work we sought to deepen in this novel role of cAMP as a paracrine/autocrine factor examining what happened to sperm when we incubated them with a concentration of cAMP that was physiological and easily achievable by its own production and extrusion (15). In this sense we performed a series of experiments to test whether the incubation with cAMP elicited events associated with sperm capacitation acting as a signal-triggering factor from the extracellular side as was proposed in other systems (37). Cyclic AMP as well as 40 mM

bicarbonate augmented the percentage of hyperactivated sperm, supporting our previous results. In addition, cAMP increased the sperm mitochondrial activity, an event that might be needed to provide sperm the ATP to sustain sperm exacerbated flagellar beat during hyperactivation(38, 39). Our results also indicated that cAMP rendered sperm a rise in tyrosine phosphorylation over the time, a largely studied hallmark of capacitated cells. In addition, the nucleotide resembled the activity of positive control in releasing sperm from CEOs. Our lab has developed CEO sperm co-culture as a technique not only to study interaction but also as a method to assess capacitation. Reorganisation of sperm membrane surface and flagellum hyperactivation impact over the ability of sperm to bind to CEO, making this assay a reliable and physiological way to study capacitation (21). Finally, after its exposure to cAMP, sperm reached fertilising rates of the positive control, heparin, in IVF assays, implying that they underwent all changes needed for this task. All these results strongly support cAMP as capacitating agent in bovines.

It is surprising that such low concentration of the nucleotide exerted this robust response in sperm. Instead, other capacitating agents previously identified for bovine sperm require higher concentrations to carry the same response (40). However, there are two possible explanations. The first one is that the media used in these experiments have a non-capacitating yet basal concentration of bicarbonate and BSA, which might facilitate the activation of different signaling pathways. Second, it has been shown that sperm does not travel alone in the female reproductive tract, but are more likely to gather in groups(41). In this sense, the concentration of nucleotide (10 nM) measured in the whole supernatant, might be higher in the sperm surroundings in an in vivo situation, similarly to other paracrine factors (42, 43).

Regarding the question of how these important fertilization outcomes take place, in our previous report we stated that A1r might be the receptor involved in the sperm capacitation induced by cAMP. It has been shown that this receptor could trigger a broad spectrum of responses in different types of cells (13, 44–46). However, reports showed that several adenosine receptors may participate in the acquisition of fertilising ability (47, 48), and other nucleotides and nucleosides receptors like P2X ATP-gated ion-channels might be also involved in this process (49). Even more, we cannot discard that the described extracellular-cAMP effects could be mediated by a still unknown specific receptor, which has been previously postulated in the literature. However we have previously shown that A1r specific antagonist inhibits cAMP-elicited capacitation, indicating its critical contribution to this process (15). A1r might be involved in Ca^{2+} uptake, ERK1/2phosphorylation and rendering of capacitation, and A1r gene lacking mice showed reduced pup litters by deregulation of sperm fertilising ability (14, 50). In addition, depending on the agonist, different G-protein heterotrimers couple to A1r (51) and probably, interacting promiscuously with Gq/11 and Gai2 (14). In both cases by the alpha or the beta-gamma subunit of each heterotrimer respectively, these complexes are linked to certain subtypes of PLC(52). An increase in PLC activity and the importance of IP3 receptors in the acquisition of sperm capacitation in bovines has been previously demonstrated (23, 28).Consistently, our results show the involvement of PLC and the triggering of downstream effectors. We confirmed the participation of ERK pathway in cAMP-induced capacitation and reported some of its extent in bovine sperm physiology. Our results showed that the activity of this kinase is necessary for certain capacitation outcomes such as CTC patterns changes and in the LPC-induced acrosomal reaction. However, we did not detect modifications of pTyr levels as reported by other authors in the presence of ERK-1/2 inhibitors (26).Nevertheless, this might be one of the first reports of ERK-1/2 in bull sperm and further studies might be needed in order to clarify its role in this species. On the other hand, the localization of this enzyme was similar to that reported in other sperm species and was concomitant to the A1r immuno-labelling

reported by Alegrucci and col. 2001 (22).Our results pointed out that ERK-1/2 phosphorylation depends on PKC activity. Canonic PKCa and PKCb isoforms have been identified specifically in bovine (53), and the localization was also also consistent with A1R. The implication of this protein in capacitation has been lately studied and many evidences indicate that its activity is necessary for acrosome reaction (AR)(53–55). The involvement of ERK-1/2 has also been demonstrated in AR (27, 56, 57), suggesting that their activation by A1r, might be preparing spermatozoa for this process.

Evidence for further role of this protein has been found. An old report in hamster sperm show that PMA stimulation, a PKC inducer, produces an intracellular cAMP augment, indicating a possible link between these signalling pathways (58). Indeed, crosstalk between PKC and PKA has been reported before(55, 59, 60). However some authors point out that a previous activation of PKA is necessary for PKC activation(61, 62). This does not disagree with our findings, given the fact that we also detect a rise in sAC/PKA pathway activity induced by extracellular cAMP. The enhancement in the activity of this pathway by an IP3-Ca^{2+} increase seems possible given the allosteric properties of sAC(6, 7, 63). In fact, in control conditions we detect PKA-driven Ser-Thr phosphorylation, indicating a basal activity of this kinase, and it is possible that a new wave in the activity of this kinase, driven by the extracellular cAMP, might be necessary for all the mentioned process to take place.

Another possible hypothesis on how extracellular cAMP is orchestrating this broad spectrum of responses is through the activity of reactive oxygen species (ROS). Later studies show the importance of these ROS regulating proteins like MEK, isoforms of PKC and even PKA, proposing a more intricate crosstalk between these signalling members(39, 64, 65). In our study we analysed the subcellular localization of PKA activity and a rise in middle piece PKA-phosphorylation was detected. It has been reported that an augment of AMP/ATP ratio as well as PKA and PKC activity upregulate mitochondria membrane potential through AMPK(66). This gives more substance to the hypothesis that a rise in mitochondrial activity enables a ROS level needed for trigger or maintain sperm capacitation.

It is also worth noting that micromolar concentrations of nucleotides and nucleosides might be found in reproductive fluids like seminal plasma or oviductal fluid(67, 68). Moreover, a recent report point that a presence of extracellular soluble PDEs are found in seminal plasma, so that their activity could modulate the availability of different nucleotides for sperm (69). It is not surprising then, that presence of probenecid in the incubation media caused a significant decrease in the percentage of capacitated sperm, whereas MRP4-lacking mice only showed a subfertile phenotype(9). However, multiple mechanisms might be compensating this protein loss to guarantee fertility, but its importance in the regulation of intracellular cAMP as well as the supply of the nucleotide to the extracellular space have been evidenced in this work.

Data presented in this work along with the literature point that not only a rise but also a regulation of cAMP levels are necessary to ensure sperm fertilising ability. Thus, exclusion of the nucleotide to the extracellular space might be essential to guarantee the achievement of a cAMP tone, needed for all capacitation-associated events to take place. Moreover, the ability of cAMP to trigger such broad and complex signalling events allows us to hypothesize the cAMP as a self-produced autocrine/paracrine factor, and supports the emerging paradigm that sperm does not compete but communicate with each other.

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Figure Legends

Figure 1. Effect of cAMP as a capacitating agent.

Bovine cryopreserved sperm was incubated in control sp-TALP or medium supplemented with high bicarbonate concentrations (40 mM) or cAMP (10 nM). Aliquots were taken to asses hyperactivation of the flagellum by Computer Assisted Semen Analysis (n=4) (A) or stained with JC-1 to asses mitochondrial membrane potential (MMP) (n=4) (B). Results are expressed as the % of sperm displaying hyperactivation or high MMP. Different letters indicate statistically significant differences (A) a≠b: $p=0,005$; B) a≠b: $p<0,05$). In another set of experiments, sperm was incubated in control sp-TALP or supplemented with cAMP (10 nM). Aliquots were taken at different times and proteins were extracted and subjected to Western Blot analysis using α-pTyr antibody (n=5). β-tubulin was used as the protein loading control. Densitometry of the lanes was performed and pTyr/β-tubulin and cAMP/Control ratios were calculated. All results are expressed as mean ± SEM. (C) Asterisk (*) indicates statistically significant difference respect to control conditions ($p<0,05$). Representative blot is shown (D).

Figure 2. Physiological relevance of cAMP extrusion system in the fertilisation process.

BOEC cultures were inseminated with spermatozoa and co incubated for 1h. After that time, SPZ release was induced with high bicarbonate concentrations (40 mM) in the presence or absence of Probenecid (300 µM) (n=8) (A). In another set of experiments, sperm release was induced with cAMP (10 nM) (n=8) (B) After fixation, cocultures were mounted and examined under phase contrast microscope to count the number of remaining sperm bound to BOEC. Results are expressed as the N° of sperm bound/0,11 mm^2 BOEC culture. In another set of experiments, in vitro matured oocytes were inseminated with sperm in control IVF-SOF media or supplemented with heparin (50 µg/ml) or cAMP (10 nM). After 6 hours, prospective embryos were stained with Hoechst 33258 and examined under fluorescence microscope to assess the n° of 2-pronuclei zygotes. Results are expressed as the % of 2-pronuclei zygotes (C). All results are expressed as mean ± SEM. Different letters indicate statistically significant differences. (a≠b : A, $p=0,0144$; and B, $p=0,0144$, $C<0,001$).

Figure 3. Role of PLC in cAMP-induced capacitation.

Spermatozoa were incubated in control sp-TALP or medium supplemented with cAMP (10 nM) alone or with U73122 (10 µM, PLC inhibitor). Aliquots were taken to asses hyperactivation of the flagellum by CASA (n=3) (A), or stained with CTC to asses B pattern associated to capacitation (n=3) (B). Results are expressed as the % of sperm displaying hyperactivation or CTC B pattern. All results are expressed as mean ± SEM. Different letters indicate statistically significant differences. (a≠b≠c: A, $p<0,02$; and B, $p<0,001$).

Figure 4. Participation of PKC and ERK-1/2 in cAMP-induced capacitation.

Spermatozoa were incubated in control sp-TALP or medium supplemented with cAMP (10 nM) alone or with Gö6976 (10 µM, PKC inhibitor) or PD98053 (30 µM, ERK-1/2 inhibitor). Alicuots were taken to asses LPC-induced acrosomal reaction (n=3) (A); stain with CTC to asses B pattern associated to capacitation (n=4) (B). Results are expressed as the % of reacted sperm or CTC B pattern, respectively in A) and B). All results are expressed as mean ± SEM. Different letters indicate statistically significant differences. (a≠b: A) and B) $p<0,005$). Sperm proteins were analysed by Western blot using an α-pTyr antibody (n=3) (C), or α-pERK-1/2 (n=4) (D). β-tubulin was used as the protein loading control. Representative blot of each experiment is shown.

Figure 5. Ca^{2+} movements stimulated by cAMP.

Spermatozoa were pre-loaded with Fluo3-AM, adhered to the bottom of 96-well plates and examined under fluorescent confocal microscope to evaluate the kinetic of Ca^{2+} dynamics. Basal fluorescence was obtained (F_0) and cAMP (10 nM) was added after 10 seconds of incubation (F) (black arrow). Only 19% of sperm showed a two fold increase in F/F_0 (responsive sperm), while others showed lesser or no F/F_0 increase (non-responsive sperm). Inset: Sperm were pre-loaded with FURA2-AM, adhered to the bottom of 96-well plates and to evaluate the long-term Ca^{2+} response. After 45 min of incubation in control sp-TALP or supplemented with cAMP (10 nM), plates were examined in fluorometer (n=4). Results show 360/380 ratio of each treatment. Results are expressed as mean ± SEM. Different letters indicate statistically significant differences; a≠b ($p<0,01$).

Figure 6. Role of sAC/PKA pathway in cAMP-induced capacitation.

Sperm was incubated in control sp-TALP or medium supplemented with cAMP (10 nM) alone or with H89 (50 µM, PKA inhibitor). Aliquots were taken to asses hyperactivation of the flagellum by CASA (n=3) (A), or stained with CTC to asses B pattern associated to capacitation (n=4) (B). In other set of experiments sperm was incubated in control sp-TALP or medium supplemented with cAMP (10 nM) alone or with KH7 (10 µM, sAC inhibitor). CTC staining was performed to asses B pattern (n=4) (C). Results are expressed as the % of sperm displaying hyperactivation or CTC B pattern. All results are expressed as mean ± SEM. Different letters indicate statistically significant differences. (a≠b; A: $p<0,01$; B: $p<0,002$; and C: $p<0,001$).

Figure 7. sAC/PKA pathway activation by cAMP.

Spermatozoa were incubated in control sp-TALP or medium supplemented with cAMP (10 nM) alone (A, n=4) or cAMP (10 nM) + H89 (50 µM, PKA inhibitor) (C, n=3) cAMP (10 nM) + KH7 (10 µM, sAC inhibitor) (D, n=3). Aliquots were taken at different time for A and at 45 min for C and D, and proteins were extracted and subjected to Western Blot analysis using α-pSer-Thr PKA substrates antibody. β-tubulin was used as the protein loading control. Densitometry of the lanes was done and pSer-Thr/β-tubulin and cAMP/Control ratios were

calculated (B). Results are expressed as mean ± SEM. Different letters indicate statistically significant differences. Representative blot from each experiment is shown. Subcellular localization of this PKA activity was analysed by immunocytochemistry using α-pSer-Thr PKA substrates antibody in sperm incubated alone or with cAMP (10 nM) (E). White arrows mark the middle piece with positive immuno-labeling. Representative picture of three independent assays is shown.

Figure 8. Ca^{2+} effect on sAC/PKA pathway.

Spermatozoa were incubated in control sp-TALP or medium supplemented with cAMP (10 nM) alone or pre-incubated with KH7 (10 µM). Aliquots were taken to perform intracellular cAMP quantification as described in materials and methods section (A). Results are expressed as pmol of cAMP/7,5x10^6 cells. Bars indicate mean ± SEM (n=3). Different letters indicate statistically significant differences. In another set of experiments spermatozoa were incubated with cAMP (10 nM) alone or pre-incubated with BAPTA-AM (30 µM). Proteins were extracted and subjected to Western Blot analysis using α-pSer-Thr PKA substrates antibody. β-tubulin was used as the protein loading control. Representative blot is shown (n=3) (B).