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ORIGINAL RESEARCH

Cyclic AMP efflux, via MRPs and A1 adenosine receptors, is critical for bovine sperm capacitation

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ABSTRACT: Sperm capacitation has been largely associated with an increase in cAMP, although its relevance in the underlying mechanisms of this maturation process remains elusive. Increasing evidence shows that the extrusion of cAMP through multidrug resistance associated protein 4 (MRP4) regulates cell homeostasis not only in physiological but also in pathophysiological situations and studies from our laboratory strongly support this assumption. In the present work we sought to establish the role of cAMP efflux in the regulation of sperm capacitation. Sperm capacitation was performed *in vitro* by exposing bovine spermatozoa to bicarbonate 40 and 70 mM; cAMP; probenecid (a MRPs general inhibitor) and an adenosine type 1 receptor (A1 adenosine receptor) selective antagonist (DPCPX). Capacitation was assessed by chlortetracycline assay and lysophosphatidylcholine-induced acrosome reaction assessed by PSA-FITC staining. Intracellular and extracellular cAMP was measured by radiobinding the regulatory subunit of PKA under the same experimental conditions. MRP4 was detected by western blot and immunohistochemistry assays. Results showed that the inhibition of soluble adenylyl cyclase significantly inhibited bicarbonate-induced sperm capacitation. Furthermore, in the presence of 40 and 70 mM bicarbonate bovine spermatozoa synthesized and extruded cAMP. Interestingly, in the absence of IBMX (a PDEs inhibitor) cAMP efflux still operated in sperm cells, suggesting that cAMP extrusion would be a physiological process in the spermatozoa complementary to the action of PDE. Blockade of MRPs by probenecid abolished the efflux of the cyclic nucleotide resulting not only in the accumulation of intracellular cAMP but also in the inhibition of bicarbonate-induced sperm capacitation. The effect of probenecid was abolished by exposing sperm cells to cAMP. The high-affinity efflux pump for cAMP, MRP4 was expressed in bovine spermatozoa and localized to the midpiece of the tail as previously reported for soluble adenylyl cyclase and A1 adenosine receptor. Additionally, blockade of A1 adenosine receptor abolished not only bicarbonate-induced sperm capacitation but also that stimulated by cAMP. Present findings strongly support that cAMP efflux, presumably through MRP4, and the activation of A1 adenosine receptor regulate some events associated with bicarbonate-induced sperm capacitation, and further suggest a paracrine and/or autocrine role for cAMP.

Key words: cAMP efflux / MRP4 / sperm capacitation / A1 adenosine receptors / adenosine

Introduction

Mammalian spermatozoa are not able to fertilize an oocyte immediately upon ejaculation (Yanagimachi, 1994). They acquire fertilization competence during their transit through the female reproductive tract in a process known as capacitation. Sperm capacitation involves an increase in plasma membrane fluidity, cholesterol efflux, ion fluxes that alter sperm membrane potential, increased tyrosine phosphorylation of proteins and the appearance of hyperactivated motility (Visconti *et al.*, 2002; Visconti *et al.*, 2011).

Earlier events in sperm capacitation include intracellular calcium and bicarbonate elevation followed by an increase in cyclic adenosine monophosphate (cAMP). Protein tyrosine phosphorylation represents the hallmark of capacitation and it is modulated through cAMP-dependent pathways (Visconti *et al.*, 1995; Visconti and Kopf, 1998).

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In testes both, membrane-associated adenylyl cyclases (ACs), regulated by G-protein coupled receptors (GPCRs), and the soluble AC isoform, sensitive to calcium and bicarbonate and independent of GPCRs and forskolin regulation, have been described (Braun *et al.*, 1977; Forte *et al.*, 1983; Tresguerres *et al.*, 2011). Controversial reports exist regarding the nature of the AC involved in sperm capacitation, although increasing evidence strongly supports that the soluble isoform is the predominant source of cAMP in the spermatozoa (Esposito *et al.*, 2004; Hess *et al.*, 2005; Xie *et al.*, 2006). Blockade of soluble AC by the selective inhibitor KH7 abolishes cAMP production and protein phosphorylation associated with sperm capacitation and motility (Hess *et al.*, 2005). Furthermore, stimulation of membrane-bound AC by the non-physiological activator forskolin fails to induce capacitation-related events (Leclerc *et al.*, 1996).

The termination of cAMP signalling is achieved by cAMP degradation by the phosphodiesterase (PDE) enzyme family associated or not with desensitization of stimulatory GPCRs. Nevertheless, diverse studies have suggested that intracellular cyclic nucleotide levels are also regulated by its efflux to the extracellular compartment (Hofer and Lefkimmiatis, 2007; Russel *et al.*, 2008). Experimental evidence supports that in various cell types cyclic nucleotide efflux represents a regulatory mechanism, in addition to PDE activity, to restrict intracellular accumulation and to prevent eventual cell damage (Godinho and Costa, 2003; Rodríguez *et al.*, 2011; Duarte *et al.*, 2012). Furthermore, cAMP efflux has also been reported to play a key role in the differentiation and proliferation of human myeloid leukaemia cells (Copsel *et al.*, 2011). The extrusion of cAMP and cGMP is mediated by members of the multidrug resistance-associated proteins (MRPs) family, also known as the ATP-binding cassette (ABC) transporter subfamily C, which also actively transports other non-structurally-related compounds out of the cells (Deeley *et al.*, 2006). Although the extrusion of cyclic nucleotides has been well documented in different tissues and cell types exposed to diverse agonists signalling through Gs-coupled receptors, its physiological relevance still remains to be fully elucidated.

MRP4/ABCC4, MRP5/ABCC5 and MRP8/ABCC11 have been shown to efflux cAMP and cGMP in a large array of tissues (Jedlitschky *et al.*, 2000; Wielinga *et al.*, 2003; Guo *et al.*, 2003). Increasing evidence supports that MRP4 functions as a high-affinity efflux pump for cAMP whrn compared with MRP5, which is more sensitive for cGMP transport (Wielinga *et al.*, 2003; Russel *et al.*, 2008).

In the reproductive system MRP4 is expressed in testes (Morgan *et al.*, 2012), placenta (Azzaroli *et al.*, 2007), endometrium (Lacroix-Pépin *et al.*, 2011) and ovary (Maher *et al.*, 2005). It has been shown that in human and rodent testicular Leydig cells MRP4 plays a relevant role in testosterone production (Morgan *et al.*, 2012). MRP4 knock-out mice exhibit early gametogenesis impairment linked to reduced intratesticular testosterone (Morgan *et al.*, 2012). The presence of MRP4 has not been reported in spermatozoa but MRP4 knock-out male mice are subfertile suggesting that the transporter is expressed in these cells and further it may be involved in sperm capacitation.

It is generally accepted that extracellular cAMP is sequentially metabolized to 5′AMP and adenosine by ecto- PDEs (ecto-PDE) and ecto-nucleotidases (Chiavegatti *et al.*, 2008). Adenosine then binds to purinergic receptors (A1, A2A, A2B or A3) to activate or inhibit different signalling pathways (Lynge *et al.*, 2001; Zheng *et al.*, 2007). In mammalian spermatozoa activation of A1 adenosine receptors stimulates phospholipase C (PLC) that cleaves phosphatidylinositil 4,5 biphosphate into diacyl glicerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Minelli *et al.*, 2000; Allegrucci *et al.*, 2001; Minelli *et al.*, 2008). Adenosine was shown to promote capacitation in mouse spermatozoa by interacting with its receptors and modulating cAMP availability (Fraser and Adeoya-Osiguwa, 1999). A1 adenosine receptor knock-out mice are subfertile suggesting that the underlying mechanism may be a delay in sperm capacitation (Minelli *et al.*, 2004). Interestingly, adenosine also activates flagellar beat by a non-receptor-mediated mechanism involving extracellular calcium, soluble AC, cAMP and protein kinase A (PKA) (Schuh *et al.*, 2006, 2007).

Given that these findings open the possibility that MRP4 and A1 adenosine receptor play a role in sperm function, we sought to establish the role of cAMP efflux through MRPs and A1 adenosine receptor activation in the regulation of some of the events associated with sperm capacitation.

Present findings support that cAMP efflux and activation of A1 adenosine receptors are critical in the regulation of bicarbonate-induced sperm capacitation, and further suggest a paracrine and/or autocrine role for cAMP in the extracellular compartment.

Materials and Methods

Chemicals

2-(1*H*-benzimidazol-2-ylthio) propanoic acid 2-[(5-bromo-2-hydroxyphenyl)methylene]hydrazide (KH7), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), isobutylmethyl xanthine (IBMX), cAMP, probenecid, bovine serum albumin (BSA), chlortetracycline (CTC), *Pissum sativum* agglutinin-FITC staining (PSA-FITC), Hoescht 33258 (H258) and L-α-lysophosphatidylcholine (LPC; Sigma Chemical Co, St. Louis, MO, USA); [^{3}H]cAMP (~31 Ci/mmol; Perkin Elmer Life Sciences, MA, USA); MRP4 antibody (Santa Cruz Biotechnology, CA, USA) and Alexa-Fluor555 goat anti-rabbit IgG was (Invitrogen, Carlsbad, CA, USA). All other chemicals were of analytical grade and obtained from standard sources.

Solubilization and handling of drugs

Probenecid was solubilized in DMSO (500 mM) and further diluted in ethanol to prepare the working solution (100 mM). Working solution was added to sp-TALP to reach a final concentration of 500 μM probenecid.

KH7 was diluted in DMSO (30 mM) and further diluted in sp-TALP to reach 1 mM KH7. The final concentration of the inhibitor in the incubation media was 10 μM.

DPCPX was initially diluted in DMSO (25 mM) and further diluted in ethanol to prepare the working dilution (2.5 mM). The final concentration of the inhibitor in sp-TALP was 10 μM.

Sperm preparation and evaluation of motility

Frozen bovine semen from six bulls ($20-25 \times 10^6$ spermatozoa/0.5 ml straw), obtained from CIAVT (Artificial Insemination Center Venado Tuerto, Santa Fé, Argentina), Juan Debernardi S.R.L. (Buenos Aires, Argentina) and Cabaña Las Lilas (Buenos Aires, Argentina), was used in the experiments. Straws were thawed in a water bath at 37°C for 30 s. Spermatozoa were subjected to sperm selection using glass wool columns (Gervasi *et al.*, 2011) and washed by centrifugation at 800*g* with BSA-free Tyrode's Albumin Lactate Pyruvate (sp-TALP). Pellets were assessed for sperm concentration and motility using a haemocytometer mounted on a microscope stage heated at 38°C. Progressive motility was evaluated following each treatment by light microscopy (magnification, ×300) on a heat platen (38°C) by the same observer. In each sample, at least 200 spermatozoa were counted.

Human semen samples collected after 2 and 3 days of sexual abstinence were obtained from Center of Reproductive Medicine PROCREARTE with the approval of its Clinical Investigation Committee. Six healthy donors signed a consent form for the use of their sperm cells for research purposes. All samples used were normal according to World Health Organization criteria (World Health Organization, 2010).

Motile sperm were separated using glass wood column and washed in HAM-F10 medium (Revelli *et al.*, 1997). After that sperm pellets were processed for RT–PCR, western blot and immunohystochemistry assays.

In vitro sperm capacitation

Ten to 15 × 10^6 spermatozoa/ml were incubated in 0.3% BSA sp-TALP (99 mM NaCl; 3.1 mM KCl; 0.4 mM NaH_2PO_4; 0.4 mM $MgCl_2{\cdot}6H_2O$; 21.6 mM Lactato de Na; 10 mM HEPES; 2 mM $CaCl_2{\cdot}H_2O$; 25 mM $NaHCO_3$; 1 mM Na-Piruvate; 50 μg/ml gentamycin; pH 7.4; Parrish *et al.*, 1988) at 38.5°C and 5% CO_2 atmosphere for 45 min (Osycka-Salut *et al.*, 2012) under different experimental conditions. From a concentration response curve, 40 and 70 mM bicarbonate were chosen to induce sperm capacitation in spermatozoa pretreated with 10 μM KH7 (soluble AC inhibitor), 500 μM probenecid (MRPs general inhibitor) or 10 μM DPCPX (A1 antagonist). To evaluate the role of extracellular cAMP on sperm capacitation, 10–15 15 × 10^6 spermatozoa/ml were incubated in 0.3% BSA sp-TALP at 38.5°C and 5% CO_2 atmosphere for 45 min in the presence of 0.1, 1 or 10 nM cAMP. In all experimental conditions the capacitation media pH was adjusted to 7.4.

Sperm viability was evaluated in all experimental conditions and it always ranged between 75 and 80%.

Sperm capacitation was assessed by CTC assay and LPC-induced acrosome reaction assessed by *Pissum sativum* agglutinin-FITC staining. CTC assay was performed as previously detailed (Gervasi *et al.*, 2011). 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) (Fraser *et al.*, 1995).

The induction of acrosome reaction was performed as previously described (Osycka-Salut *et al.*, 2012). Spermatozoa were incubated for 45 min under the experimental conditions detailed above and the sample divided in two aliquots that were further incubated for 15 min at 38.5°C in the presence or absence of 100 μg/ml LPC. To assess viability and acrosome reaction, spermatozoa were incubated with H258 (2 μg/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 μg/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.

cAMP assay

Spermatozoa (7.5 × 10^6 cells/ml) were preincubated for 3 min in 0.3% BSA sp-TALP medium supplemented with 1-mM IBMX at 38.5°C ($t = 0$) followed by incubation for 5 or 45 min with 40 or 70 mM bicarbonate in the presence or absence of probenecid (500 μM). Samples were centrifuged for 5 min a 3000*g* and 1 ml ice-cold ethanol added to supernatants (extracellular cAMP) and pellets (intracellular cAMP). 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 also measured in the absence of IBMX and in the presence of KH7 (sAC inhibitor). Sperm cells were pretreated with KH7 and then incubated for 5 min in the presence or absence of bicarbonate (70 mM). Cells were then centrifuged as indicated above. Cyclic AMP levels were measured by competitive radio-binding assay to the regulatory subunit of PKA using [^{3}H] cAMP, as previously described (Davio *et al.*, 1995). Duplicate samples in at least three independent experiments were analysed.

RT–PCR assay

Total RNA was isolated from spermatozoa using TRIzol reagent following the manufacture's instructions (Invitrogen, Carlsbad, CA, USA). For the first-strand cDNA synthesis, 3 μg total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega) with random primers. Two microlitres of the resulting cDNA was amplified at 40 cycles for 30 s at 95°C, 30 s at melting temperature (54°C) and 1 min at 72°C, followed by a final amplification step for 10 min using 1.6 U of Taq DNA Polymerase and 200 μM of the following primers: human MRP4 forward 5′-GGACAAAGACAACT GGTGTGCC-3′ and reverse 5′-AATGGTTAGCACGGTGCAGTGG-3′ and human β-actin (Actin) forward 5′- GCTACGAGCTGCCTGACGG -3′ and reverse 5′- GAGGCCAGGATGGAGCC -3′. The PCR products were analysed by 2% agarose gel electrophoresis and visualized with ethidium bromide. Sperm cells showed no contamination with ribosomal RNA (18S and 28S) (Supplementary data, Fig. S1).

Western blot assay

Spermatozoa membrane vesicles were diluted with sample buffer and boiled for 5 min (Copsel *et al.*, 2011). Sperm proteins (5–25 μg) were separated by 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The residual-binding sites were blocked with 5% non-fat powdered milk in PBS containing 0.05% Tween-20, and membranes were incubated with 3 μg/ml rat monoclonal anti-MRP4 M4I-10 (Alexis Biochemicals) in TBS containing 0.05% Tween-20. All subsequent washes were performed with the same buffer. Reactivity was developed using an anti-rat polyclonal antibody linked to horseradish peroxidase and enhanced chemiluminescence reagents, following the manufacturer's instructions (Amersham Biosciences, UK).

Immunohystochemistry

Spermatozoa were fixed (5 min, room temperature, 0.2% w/v paraformaldehyde), immobilized on slides and permeabilized with cold methanol (Gervasi *et al.*, 2009). Non-specific binding sites were blocked (60 min, 10% v/v normal goat serum) and incubated with 10 μg/ml primary MRP4 antibody. Samples were then further incubated with Alexa555-conjugated goat anti-rabbit IgG (1:500). Specificity of the immunodetection was assessed by omitting the first antibody or by the replacement of specific primary antibody by serum from non-immunized rabbits at the same concentration (MRP4). Sperm cells were mounted and examined under a confocal laser imaging system (Nikon C1; Plan Apo 60/0.95, Japan).

Statistical analysis

Data were analysed by GLM procedures of one-way ANOVA (Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., Tablada M., Robledo C.W. InfoStat version 2010. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Raw data were analysed by Shapiro–Wilks and Levene tests to assess normality of data distribution and variance homogeneity, respectively. These procedures were applied in all variance analyses. Pairwise comparisons of means were made with Tukey or Fisher honestly significant differences. Results are expressed as mean ± SEM of at least three independent determinations.

Results

Bovine spermatozoa fail to undergo capacitation when exposed to 25 mM bicarbonate. In accordance, a recent study by Breininger *et al.* (2010) showed that bicarbonate acts as a capacitating inducer at

concentrations >20 mM. Therefore, a concentration–response curve was performed in order to evaluate bicarbonate concentrations that induced sperm capacitation in our experimental conditions. Given that sperm capacitation is a multievent process and in the present study only some of events were investigated, the terms 'capacitated spermatozoa' and 'sperm capacitation' refer to an increase in Pattern B or the percentage of LPC-acrosome-reacted spermatozoa. CTC assay showed that the percentage of capacitated bovine sperm (Pattern B) increased in a bicarbonate concentration-dependent fashion as previously reported (Breininger *et al.*, 2010).

We next evaluated whether soluble AC was involved in bicarbonate-induced bovine sperm capacitation. Blockade of soluble AC by KH7 inhibited bicarbonate-induced sperm capacitation supporting that this enzyme mediates bicarbonate response in cryopreserved bull sperm cells (Fig. 1a).

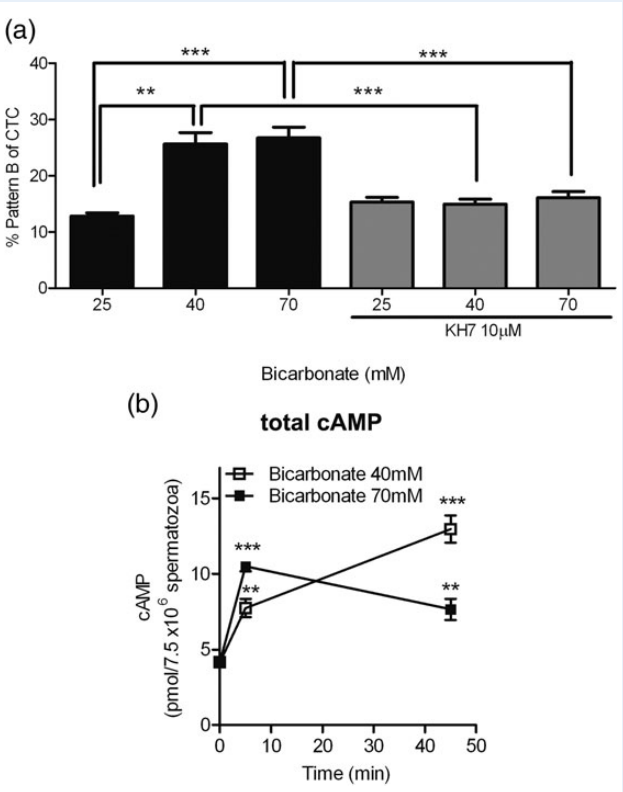

Figure 1 (**a**) Blockade of soluble AC on bicarbonate-induced sperm capacitation. Bovine spermatozoa pre-treated with 10 μM KH7 (soluble AC inhibitor) were incubated for 45 min at 38.5°C with 40 and 70 mM bicarbonate concentrations as detailed in the section Materials and methods and sperm capacitation assessed by CTC assay (Pattern B). Results are expressed as percentage Pattern B of CTC; $^{**}P < 0.01$ versus 25 mM bicarbonate; $^{***}P < 0.001$ versus 25 mM bicarbonate; versus 40 or 70 mM bicarbonate + 10 μM KH7 ($n = 7$). (**b**) Total cAMP levels in bovine spermatozoa. Sperm cells were incubated with 40 mM (open squares) and 70 mM (closed squares) bicarbonate and cAMP assessed by radioimmunoassay as detailed in the section Materials and methods. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus time 0 min ($n = 5$). All results are expressed as mean ± SEM.

Cyclic AMP time course

Experiments were conducted in order to evaluate temporal total cAMP changes in bovine spermatozoa incubated in sp-TALP containing 40 and 70 mM bicarbonate.

Time course of total cAMP in bovine spermatozoa exposed to 40 and 70 mM bicarbonate showed that the levels of the cyclic nucleotide were significantly increased in a concentration-dependent manner (Fig. 1b). At 5 min the increase in cAMP was higher in spermatozoa exposed to 40 mM bicarbonate than in those capacitated with 70 mM bicarbonate. However, at 45 min the total cAMP content was higher in cells incubated with 40 mM bicarbonate.

Evaluation of cAMP extrusion during sperm capacitation

Diverse studies support that cAMP extrusion in various cell types is a mechanism which regulates cyclic nucleotide intracellular levels. Therefore, it was investigated whether cAMP extrusion occurred in bovine spermatozoa by incubating them in capacitating conditions and measuring intracellular and extracellular cAMP levels.

When bovine spermatozoa were capacitated with 40 and 70 mM bicarbonate and cAMP was measured in cells (intracellular cAMP) and in the incubation media (extracellular cAMP), results showed that bicarbonate at both concentrations rapidly increased intracellular cAMP (left panel) but it also augmented the content of the cyclic nucleotide in the extracellular media (right panel) suggesting that the second messenger was extruded from the cells (Fig. 2a). Intracellular cAMP gradually increased and peaked at 5 min in response to both bicarbonate concentrations. Extracellular cAMP also gradually increased and at 5 min a considerable amount of the second messenger was detected. No changes were observed in either intracellular or extracellular cAMP in basal conditions (25 mM bicarbonate).

In addition, results showed a positive correlation ($r^2 = 0.9975$; $n = 3$) between the number of sperm cells used and the level of cAMP reached in the extracellular compartment following bicarbonate stimulation at 5 min (peak of production/extrusion kinetic) (data not shown).

Extracellular cAMP reached a mean value of ~10 nM in all experimental conditions (Fig. 2b). The appearance of cAMP in the extracellular media suggested that the cyclic nucleotide was extruded by MRPs.

In order to evaluate whether cAMP extrusion occurred in the presence of active PDEs, intracellular and extracellular cAMP levels were measured in the absence of IBMX. Results showed that the efflux of the cyclic nucleotide was evident in spermatozoa incubated with spTALP with or without 70 mM bicarbonate suggesting that the process occurs even in the presence of PDEs activity (Fig. 2c). Furthermore, cAMP production and extrusion were inhibited in the presence of KH7 supporting the involvement of soluble adenylate cyclase. These findings are in agreement with the observation that sperm capacitation was also inhibited by KH7.

In order to confirm that the efflux of cAMP was mediated by MRPs, bovine spermatozoa were pre-treated with probenecid (general MRPs inhibitor) and exposed to bicarbonate (40 and 70 mM). Blockade of MRPs resulted in enhanced intracellular cAMP accumulation and significantly reduced extracellular cyclic nucleotide suggesting that in bovine spermatozoa bicarbonate induces cAMP efflux through MRPs (Fig. 3a). Furthermore, probenecid also prevented bicarbonate-induced sperm

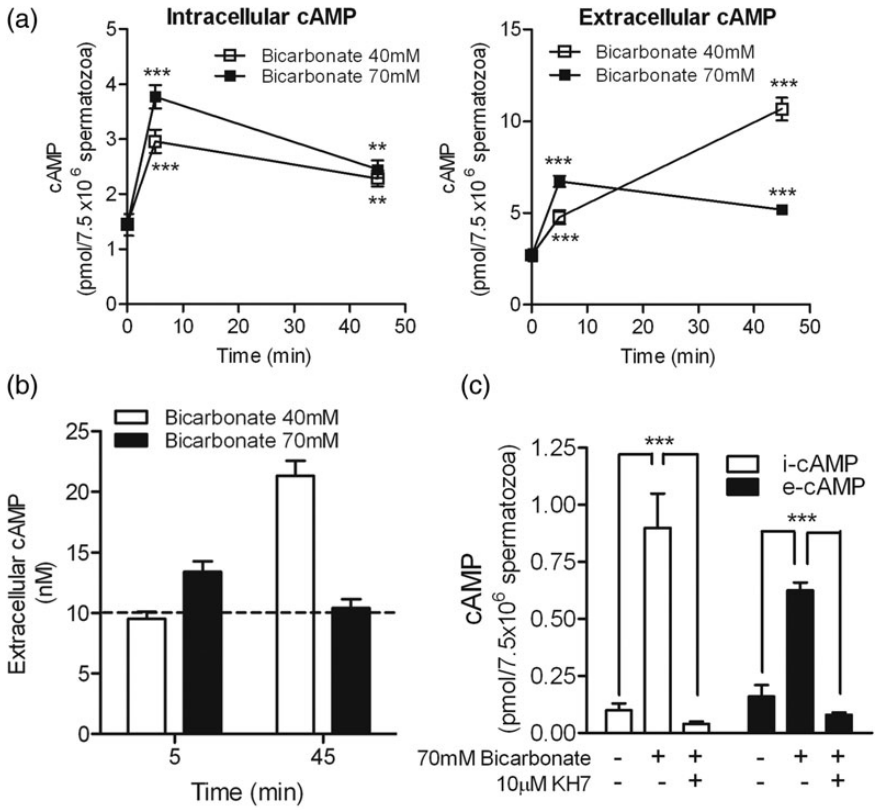

Figure 2 (**a**) Time course of intracellular and extracellular cAMP levels in response to bicarbonate. Spermatozoa were preincubated for 3 min in 0.3% BSA sp-TALP medium supplemented with 1-mM IBMX (PDE inhibitor) at 38.5°C ($t = 0$) followed by incubation with 40 or 70 mM bicarbonate. Intracellular (right panel) and extracellular (left panel) cAMP was measured at 0, 5 and 45 min by radioimmunoassay as described in the section Materials and Methods. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus cAMP concentration at time 0 min ($n = 5$). Results are expressed as mean ± SEM. (**b**) Extracellular cAMP at 5 and 45 min. Dotted line represents the mean concentration achieved in all experimental conditions. *** $P < 0.001$ versus cAMP concentration at time 0 ($n = 5$). Results are expressed as mean ± SEM. (**c**) Spermatozoa were incubated in the absence of IBMX (PDE inhibitor) and pre-treated with or without 10 μM KH7 (sAC inhibitor) followed by incubation with sp-TALP (control) or bicarbonate (70 mM). Intracellular and extracellular cAMP was assessed by radioimmunoassay. $^{***}P < 0.001$ ($n = 3$). i-cAMP, intracellular cAMP; e-cAMP, extracellular cAMP.

capacitation supporting that cAMP efflux is intimately involved in this reproductive mechanism (Fig. 3b and c).

Effect of extracellular cAMP on bovine sperm capacitation

Cyclic AMP is not permeable to biological membranes given its structural and chemical properties. Therefore, diverse permeable analogues like db-cAMP have been developed in order to evaluate cAMP intracellular effects. In the present study we used cAMP at the concentration found in the extracellular space in order to investigate its ability to interact with extracellular effectors.

In order to confirm the relevance of extracellular cAMP in bicarbonate-induced capacitation, bovine spermatozoa were incubated with increasing concentrations of cAMP in 0.3% BSA sp-TALP medium. The cyclic nucleotide dose dependently increased sperm capacitation suggesting that extracellular cAMP is involved in this process (Fig. 4a and b). In addition, this assumption was further confirmed when bovine spermatozoa were pre-treated with probenecid and incubated with 10 nM cAMP. Results showed that cAMP reversed probenecid effect on bicarbonate-induced capacitation (Fig. 5). Concomitant addition of bicarbonate and cAMP did not further increase Pattern B as measured by CTC assay when compared with bicarbonate or cAMP alone, indicating that no additive effect occurs (Fig. 5).

Participation of adenosine receptors

Different studies report that extracellular cAMP is metabolized to adenosine which then binds to adenosine receptors to modulate different intracellular signalling. As A1 adenosine receptors regulate sperm capacitation, the participation of this receptor subtype was investigated. Blockade of A1 adenosine receptor inhibited both bicarbonate and cAMP-induced sperm capacitation supporting that A1 adenosine receptors are activated by the presence of extracellular cAMP and/or its metabolites (Fig. 6).

Expression and localization of MRP4 in mammalian spermatozoa

As cAMP efflux occurred in bovine spermatozoa we investigated whether MRP4 was expressed in these cells since given that this

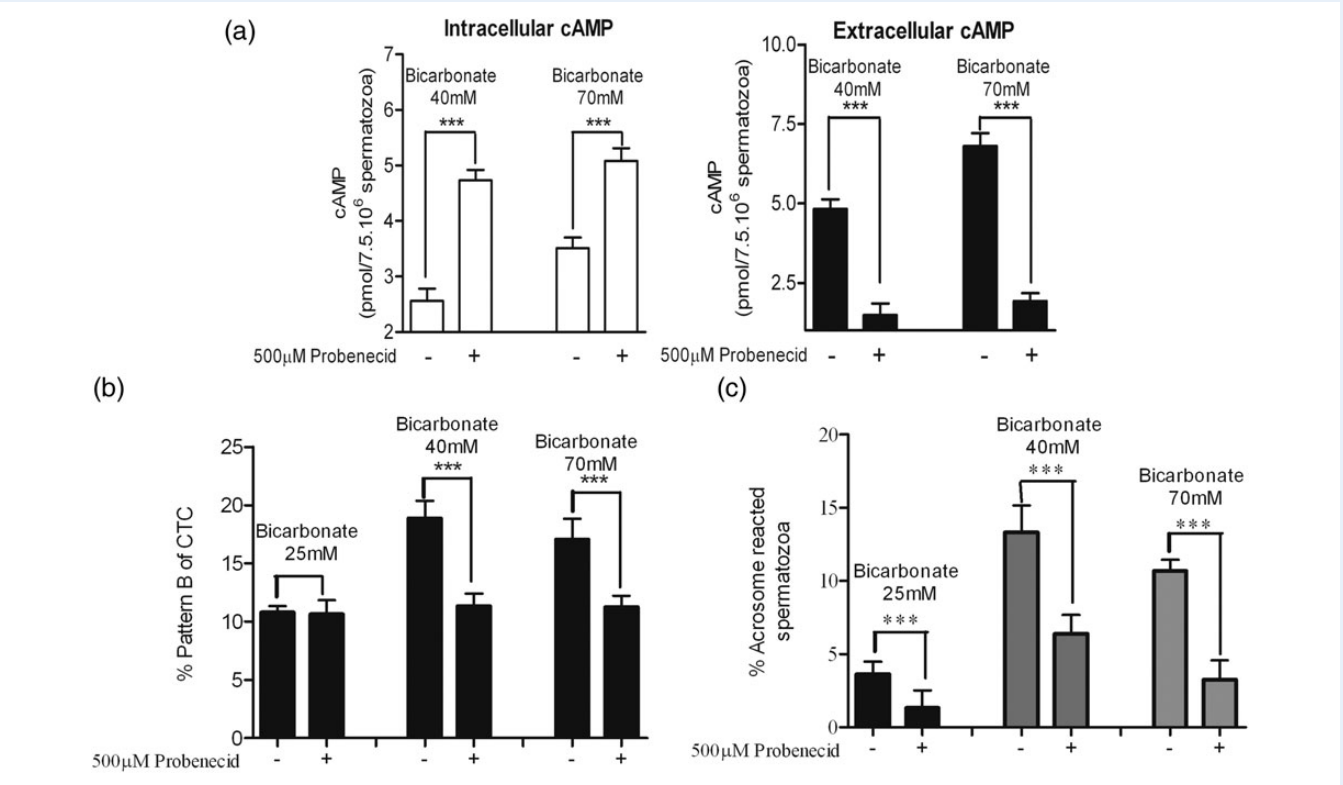

Figure 3 Effect of MRPs inhibition on cAMP levels (**a**) and sperm capacitation (**b** and **c**). Spermatozoa were pre-treated with 500 μM probenecid (MRPs inhibitor) followed by incubation with bicarbonate (40 and 70 mM). Intracellular and extracellular cAMP (a) was assessed by radioimmunoassay as detailed in the section Materials and methods. ***$P < 0.001$ versus control (without probenecid) ($n = 5$). Results are expressed as mean ± SEM. Sperm capacitation was evaluated by CTC (b) and LPC-induced acrosome reaction (c) as described in the section Materials and methods. Bars in 'c' represent the difference (%) of spermatozoa that underwent LPC-induced acrosome reaction and spontaneous acrosome reaction. ***$P < 0.001$ versus control (without probenecid) ($n = 6$). Data represent mean ± SEM.

transporter has emerged as a high-affinity efflux pump for this cyclic nucleotide. Western blot and immunohistochemical assays revealed that MRP4 was expressed in bovine spermatozoa and localized to the midpiece of the tail in sperm cells (Fig. 7a and b). Furthermore, human spermatozoa expressed mRNA and protein MRP4 (Fig. 7c; Supplementary data, Fig. S2) and the localization of the transporter was similar to that of bovine sperm (Fig. 7d).

Discussion

Cyclic AMP is critical for mammalian spermatogenesis (Sassone-Corsi, 1998), and for capacitation, a maturational process that sperm undergo prior to fertilization (Hess *et al.*, 2005; Visconti *et al.*, 2011). Furthermore, cAMP appears to be intimately involved in other sperm processes such as the regulation of motility (Hess *et al.*, 2005) and the acrosome reaction (De Jonge *et al.*, 1991; Leclerc and Kopf, 1995; Garde and Roldan, 2000). Early events in sperm capacitation are associated not only with changes in bicarbonate and calcium but also with the activation of soluble AC and increased cAMP intracellular levels although, the precise mechanisms underlying cAMP contribution remain poorly understood. In the present study we provide evidence that cAMP efflux through MRPs is critical for the regulation of bovine bicarbonate-induced sperm capacitation through the activation of A1 adenosine receptors.

Although the molecular basis for sperm capacitation is not well defined, the elevation of bicarbonate is considered as one of the key initiators in fertilization competence acquisition. In the epididymis bicarbonate concentration is actively maintained around 5 mM but upon ejaculation, in the female reproductive tract sperm are exposed to levels of bicarbonate >25 mM (Johnson, 1998). In the present work we show that bicarbonate dose dependently enhanced sperm capacitation assessed by CTC assay and LPC-acrosome-induced reaction and also increased total cAMP in spermatozoa. However, blockade of soluble AC, the major source of cAMP in these cells, inhibited bicarbonate response. A previous study showed that blockade of soluble AC abolishes cAMP production and protein phosphorylation associated with sperm capacitation and motility (Hess *et al.*, 2005). Our findings further confirm that bicarbonate-induced sperm capacitation is mediated by soluble AC activation and cAMP production. Compelling evidence supports that membrane-bound AC isoforms, although expressed in germ cells and spermatozoa, do not participate in bicarbonate sperm capacitation given that these isoforms are not regulated by bicarbonate and further a well-characterized activator of the catalytic subunit of these isoforms fails to induce capacitation-related events (Forte *et al.*, 1983; Leclerc *et al.*, 1996; Defer *et al.*, 1998; Baxendale and Fraser, 2003).

Several reports support that in various cell types cAMP extrusion is a mechanism that regulates cyclic nucleotide intracellular levels. Present

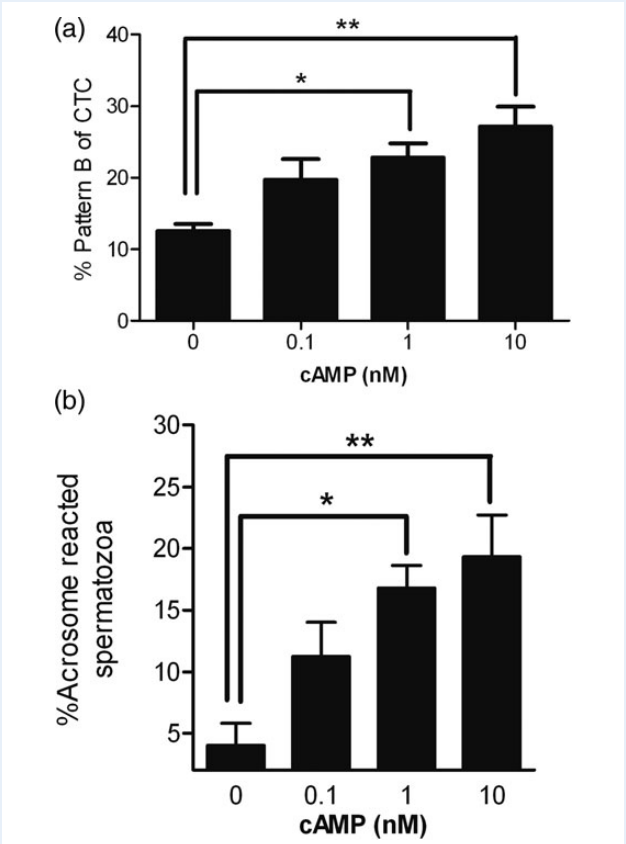

Figure 4 Effect of extracellular cAMP on bovine sperm capacitation. Spermatozoa were incubated in the presence of increasing concentrations of cAMP for 45 min at 38.5°C as detailed in the section Materials and methods and sperm capacitation evaluated by CTC assay (Pattern B) (**a**) or LPC-induced acrosome reaction (**b**). Bars in 'b' represent the difference (%) of spermatozoa that underwent LPC-induced acrosome reaction and spontaneous acrosome reaction. *$P < 0.05$ versus 0 nM cAMP and **$P < 0.01$ versus 0 nM cAMP ($n = 6$). Data are expressed as mean ± SEM.

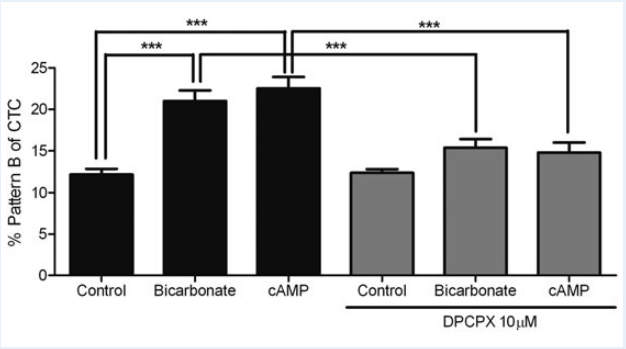

Figure 6 Blockade of A1 adenosine receptors on sperm capacitation. Spermatozoa were pretreated with 10 μM DPCPX (selective A1 adenosine receptor antagonist) followed by incubation with 40 nM bicarbonate or 10 nM cAMP and sperm capacitation evaluated by the CTC assay (% Pattern B). ***$P < 0.001$ versus control; versus bicarbonate 40 mM + DPCPX 10 μM; versus cAMP 10 nM + DPCPX 10 μM ($n = 7$). Data are expressed as mean ± SEM.

results show that cAMP extrusion occurred in spermatozoa incubated in capacitating conditions. Extracellular cAMP levels were not detected without cell exposure to bicarbonate and the point '$t = 0$' in Figs. 1 and 2 corresponded to bicarbonate concentrations which do not induce sperm capacitation. The experimental observation that at time 0 extracellular cAMP levels were higher than intracellular levels suggests that the extrusion of the cyclic nucleotide also operates in basal conditions as it has been well documented in other cell types (Godinho and Costa, 2003; Rodríguez *et al.*, 2011). Given that various studies support that cAMP efflux is a mechanism which operates when PDE activity is limited or inhibited (Reid *et al.*, 2003), experiments were conducted in the presence of active PDEs (without IBMX). Results showed that the mechanism still operated in the presence of active PDEs suggesting that the exclusion of the cyclic nucleotide is physiologically involved in sperm capacitation and it would be a complementary mechanism to PDEs activity.

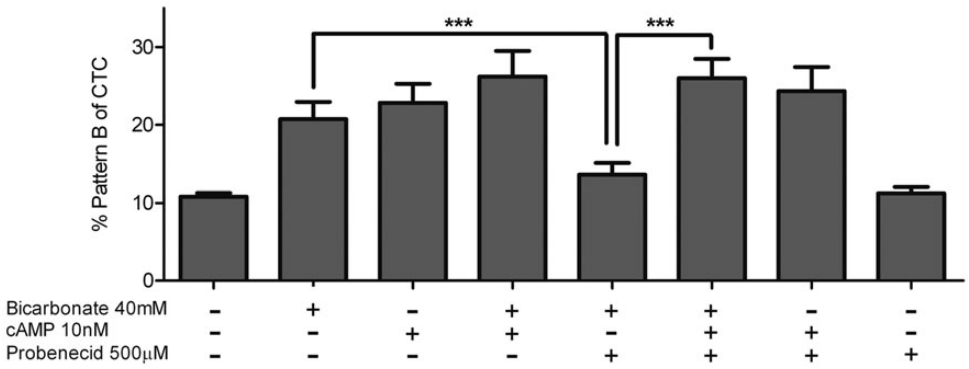

Figure 5 Effect of cAMP on probenecid-induced inhibition of sperm capacitation. Bovine spermatozoa were incubated in the presence of 500 nM probenecid, 40 mM bicarbonate and/or 10 nM cAMP for 45 min at 38 C as detailed in the section Materials and methods. Sperm capacitation was evaluated by the CTC assay (Pattern B). ***$P < 0.001$ versus bicarbonate 40 mM and versus bicarbonate 40 mM + probenecid 500 μM + cAMP 10 nM ($n = 6$). Data are expressed as mean ± SEM.

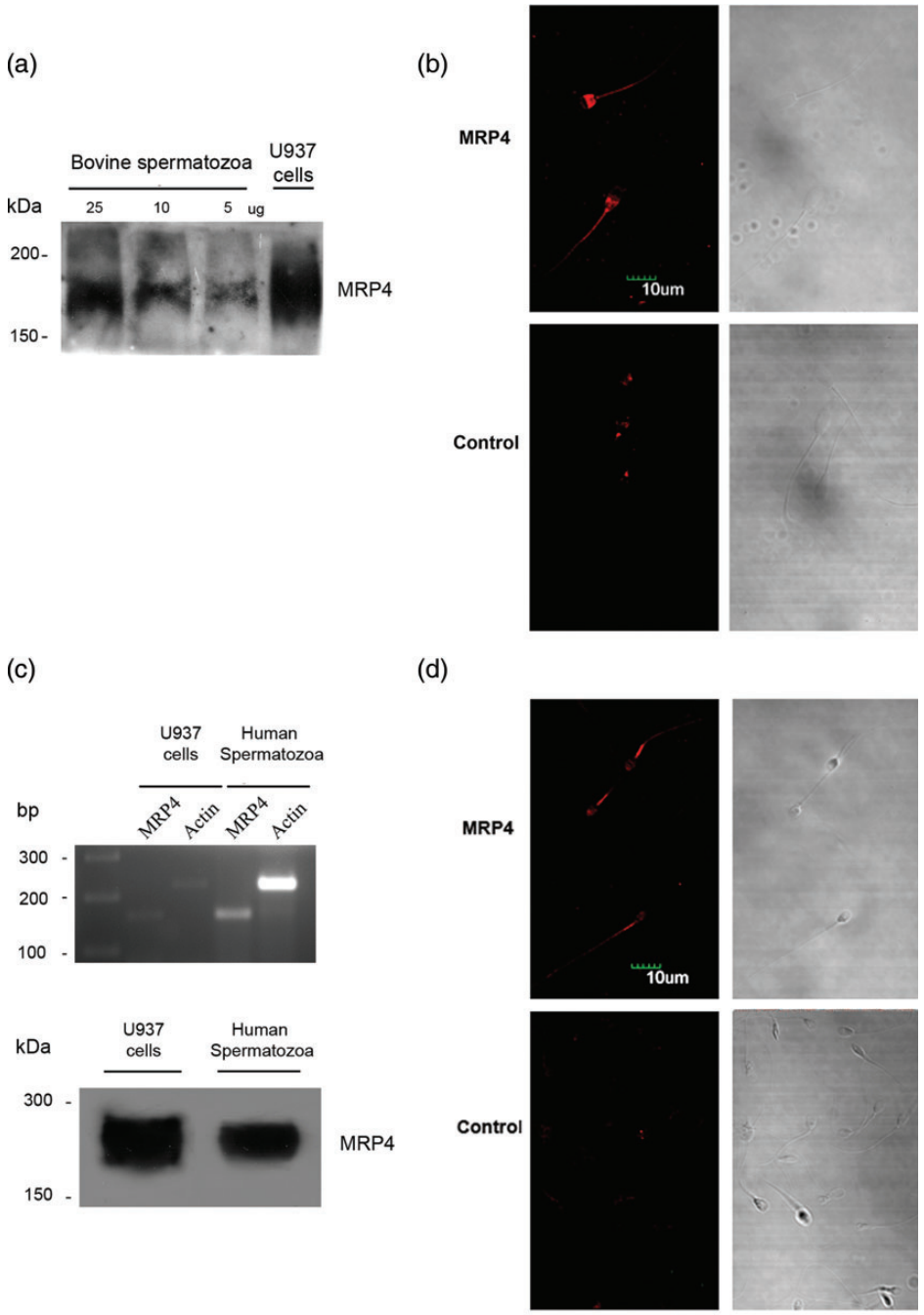

Figure 7 Expression and localization of MRP4 in bovine and human spermatozoa. Expression and localization of MRP4 in bovine spermatozoa assessed by immunoblotting (**a**) and immnunohystochemistry (**b**) (magnification X600; bar scale: 10 μm), respectively. Expression of mRNA (upper panel) and protein MRP4 (lower panel) in human spermatozoa assessed by immunoblotting and RT–PCR, respectively. (**c**) Localization of MRP4 in human spermatozoa by immunohistochemistry (**d**) (magnification ×600; bar scale: 10 μm). Immunoblotting and immunohistochemistry shown are representative of at least three independent experiments. Whole western blot was shown as Supplementary data, Fig. S2.

In addition, it is important to point out that the levels of intracellular and extracellular cAMP reached during capacitation would occur in response to soluble adenylate cyclase activation given that selective inhibition of the enzyme blunted cAMP levels. These findings support that cAMP source during capacitation results from soluble adenylate cyclase activation.

Enhanced bicarbonate-evoked sperm capacitation was also associated with an increase in extracellular cAMP, which supports efflux of the cyclic

nucleotide to the extracellular compartment through MRPs. This was further confirmed by pretreatment of bovine spermatozoa with the MRP inhibitor probenecid. Blockade of MRPs resulted in enhanced accumulation of cAMP within the cell and reduced extracellular cyclic nucleotide levels. However, inhibition of MRPs by probenecid abolished not only cAMP efflux as expected, but also bicarbonate-induced sperm capacitation, suggesting that the extrusion of the cyclic nucleotide was intimately involved in this maturational process.

Although cAMP efflux through MRPs has been reported in diverse cell types, to our knowledge this is the first report showing that this event occurs in spermatozoa and it is intimately associated with a physiological event in these cells. Sperm capacitation is a very complex process involving diverse events aiming to prepare spermatozoa for fertilization. In the present study we clearly show that cAMP extrusion plays a significant role in some of the relevant events associated with sperm capacitation.

It is generally accepted that the regulation of cAMP is mediated by degradation of the cyclic nucleotide by PDE associated or not with Gs-coupled receptor desensitization. However, in recent years increasing evidence strongly supports that the efflux of cAMP through MRP4 represents an additional physiological mechanism in the control of cAMP intracellular levels. Studies from our laboratory show the efflux of cAMP through MRP4 in leukaemic cells and the key role of the cyclic nucleotide in cell differentiation and proliferation providing the first evidence that MRP4 may represent a new potential target for leukaemia differentiation therapy (Copsel *et al.*, 2011). Furthermore, we also reported that in pancreatic acinar cells the egression of cAMP through MRP4 stimulated by atrial natriuretic factor may attenuate the development of acute pancreatitis (Rodríguez *et al.*, 2011). Other authors showed that in cardiac myocytes, cAMP extrusion via MRP4 acts together with PDE to control cAMP levels and further MRP4-deficient mice display enhanced cardiac myocyte cAMP formation, contractility and cardiac hypertrophy (Sassi *et al.*, 2012). Collectively, these studies in different cell types strongly support that cAMP extrusion through MRP4 is a physiological mechanism involved in the maintenance of cell homeostasis. In the present study we provide further evidence by showing that cAMP efflux is intimately associated with bicarbonate-induced sperm capacitation.

Although bicarbonate-induced bovine sperm capacitation was clearly abolished by probenecid, this drug is a general inhibitor for all MRPs and so it is not possible to identify which of the MRPs involved in cyclic nucleotide efflux was responsible for the effect observed on sperm capacitation. However, MRP4 has emerged as the major high-affinity efflux pump for cAMP when compared with MRP5 which is more sensitive for cGMP transport (Wielinga *et al.*, 2003; Russel *et al.*, 2008). The presence of MRP4 has not been reported in spermatozoa but MRP4 knock-out male mice are subfertile suggesting that the transporter is expressed in these cells and further it may be involved in sperm capacitation (Morgan *et al.*, 2012). The analysis of western blot assay revealed that MRP4 was expressed in bovine spermatozoa and that the transporter is localized to the midpiece of the tail, the anterior part of the flagellum characteristically containing all of the sperm's mitochondria. Interestingly, MRP4 localization was coincident with that previously reported for soluble AC (Hess *et al.*, 2005). Our findings suggest that cAMP efflux in spermatozoa is mediated by MRP4 and further it is associated with sperm capacitation. Therefore, it is possible to assume that failure to extrude cAMP may be one of the underlying mechanism leading MRP4 knock-out male mice to be subfertile. In addition, we showed that MRP4 is also expressed in human spermatozoa and localized in the same region of the cell, supporting that the reported findings in bovine spermatozoa may also occur in human sperm. This assumption opens the promising possibility to consider MRP4 as a target to improve the diagnosis and/or therapeutics of male infertility.

In order to further confirm that extracellular cAMP was involved in sperm capacitation, bovine spermatozoa were incubated in the presence of cAMP. Sperm capacitation was stimulated in a concentration-dependent fashion with increasing concentration of the cyclic nucleotide strongly supporting the critical contribution of extracellular cAMP to sperm capacitation. Results further show that in the presence of a non-capacitating bicarbonate concentration (25 mM) cAMP induced sperm capacitation in bovines. These finding suggest that extracellular cAMP is certainly having a role outside the cell and that bicarbonate-medicated cAMP extrusion is crucial in the capacitating events studied in the present work.

Different reports show that extracellular cAMP is metabolized to adenosine by ecto-PDE. Circulating adenosine has a short half-life (less than 1 s) and so cAMP that is stable in plasma may serve as a prohormone/factor for adenosine. Once produced either locally or at a distant site adenosine binds to different receptor subtypes, A1, A2A, A2B and A3 activating distinct intracellular signalling. Interestingly, A1 and A3 activation reduces intracellular cAMP since they are GPCRs coupled to Gαi/Gα0, whereas A2 serve to increase cAMP given that A2 subtypes (A2A and A2B) are coupled to Gs. Therefore, cAMP released from cells can trigger cAMP signalling in neighbouring cells or suppress cAMP signalling depending on the cell adenosine receptor distribution. In mammalian spermatozoa activation of A1 adenosine receptors located mainly in the acrosome, equatorial segment and midpiece of the tail also activate PLC increasing DAG and inositol IP3 (Allegrucci *et al.*, 2001; Minelli *et al.*, 2008). Adenosine promotes capacitation in mouse sperm by modulating cAMP availability (Fraser and Adeoya-Osiguwa, 1999). Interestingly, A1 adenosine receptor knock-out mice are subfertile suggesting a delay in sperm capacitation (Minelli *et al.*, 2004). In order to confirm the contribution of A1 adenosine receptors to sperm capacitation, we pre-treated bovine spermatozoa with a selective A1 antagonist and further incubated them with bicarbonate or cAMP. We found that blockade of A1 resulted in the inhibition of sperm capacitation supporting that this physiological sperm event is mediated by this receptor subtype presumably activated by extracellular conversion of cAMP to adenosine. These findings suggest that a cAMP/adenosine pathway controls sperm capacitation. This pathway has been previously shown to explain glucagon renal response (Bankir *et al.*, 2002). Glucagon leads to a substantial efflux of cAMP into the circulation and an important target of this circulating cAMP is the kidney. It was shown that glucagon causes a marked increase in phosphate and sodium excretion mediated by the conversion of cAMP to adenosine (Jackson *et al.*, 2007). Furthermore, the extracellular cAMP-adenosine pathway has also been shown to regulate β(2)-AR signalling in the skeletal muscle. In this cell type the increment of intracellular cAMP induced by β(2)-AR/G(s) is followed by cAMP efflux and extracellular generation of adenosine which through A1 regulates the β(2)-AR/Gi/0 signalling that is associated with inotropic response. The involvement of the extracellular cAMP-adenosine pathway in β(2)-AR signalling would provide a negative feedback loop that may limit stimulatory G protein-coupled receptor positive inotropism and potential deleterious effects of excessive contractile response (Duarte *et al.*, 2012). Nevertheless, in our study the apparent existence of cAMP/adenosine pathway controlling sperm capacitation does not preclude the possibility that

cAMP may exert direct actions on cells in addition to indirect effects via the adenosine production.

Present findings strongly support that cAMP efflux through MRPs, likely MRP4, plays a critical role in bicarbonate-induced sperm capacitation through the activation of A1 adenosine receptors presumably through the metabolization of cAMP to adenosine. Our study provides additional evidence that strongly supports the physiological relevance of cAMP efflux in the regulation of cell homeostasis and further validates the role of extracellular cAMP as a paracrine/autocrine factor. A precise understanding in the functional competence of mammalian spermatozoa is essential to generate clinical advances in the treatment of infertility and novel contraceptive strategies

Supplementary data

Supplementary data is available at http://molehr.oxfordjournals.org/online.

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Authors' roles

The author(s) have made the following declarations about their contributions: C.O.S., F.D., C.D., S.P.-M. conceived and designed the experiments. C.O.S., F.D., J.B. performed the experiments. M.G.G., C.O.S., F.D., C.D., S.P.-M. analysed and interpreted the data. A.F., L.B., C.D. and S.P.-M. wrote and revised the paper.

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Conflict of interest

None of the authors have conflict of interest to disclosure.

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