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RESEARCH ARTICLE

MRP4-mediated cAMP efflux is essential for mouse spermatozoa capacitation

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ABSTRACT

Mammalian spermatozoa must undergo biochemical and structural changes to acquire the capacity for fertilization, in a process known as capacitation. Activation of PKA enzymes is essential for capacitation, and thus cAMP levels are tightly regulated during this process. Previously, we demonstrated that during capacitation, bovine spermatozoa extrude cAMP through multidrug resistance-associated protein 4 (MRP4, also known as ABCC4), which regulates intracellular levels of the nucleotide and provides cAMP to the extracellular space. Here, we report the presence of functional MRP4 in murine spermatozoa, since its pharmacological inhibition with MK571 decreased levels of extracellular cAMP. This also produced a sudden increase in PKA activity, with decreased tyrosine phosphorylation at the end of capacitation. Blockade of MRP4 inhibited induction of acrosome reaction, hyperactivation and *in vitro* fertilization. Moreover, MRP4 inhibition generated an increase in Ca^{2+} levels mediated by PKA, and depletion of Ca^{2+} salts from the medium prevented the loss of motility and phosphotyrosine inhibition produced by MK571. These results were supported using spermatozoa from CatSper Ca^{2+} channel knockout mice. Taken together, these results suggest that cAMP efflux via MRP4 plays an essential role in mouse sperm capacitation.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: cAMP efflux, ABCC4, MRP4, PKA activity, Ca^{2+}, Sperm capacitation, Mouse

INTRODUCTION

Ejaculated spermatozoa are unable to fertilize the oocyte; on entry to the female reproductive tract, maturational changes occur in sperm that render them competent for fertilization in a process known as capacitation (Stival et al., 2016; Visconti et al., 2011). Even though the molecular basis of this process remains unclear, it correlates with a series of cellular and biochemical changes including cholesterol efflux from the sperm plasma membrane (Gadella and Harrison, 2000), ion influx, membrane hyperpolarization (De la Vega-Beltrán et al., 2012; Escoffier et al., 2015), cAMP production (Buffone et al., 2014), kinase activation, and protein phosphorylation (Visconti et al., 1995).

Enzymes such as members of the PKA and EPAC families are directly regulated by cAMP (Buffone et al., 2014). Thus, this nucleotide orchestrates several downstream events with critical outcomes in cell physiology. While the role of cAMP and the enzymes in charge of its production have been extensively characterized, the mechanisms involved in terminating its effects have been less well studied. Phosphodiesterases (PDEs) are responsible for breaking down cAMP to 5′AMP, downregulating the signalling events mentioned above (Baxendale and Fraser, 2005). Another mechanism to stop cAMP signalling is the extrusion of this nucleotide from the cell to the extracellular space. In this sense, multidrug resistance-associated protein 4 (MRP4, also known as ABCC4) has been described in somatic cells as an endogenous transporter of several physiological substrates including cyclic nucleotides, with a remarkable affinity for cAMP (Wen et al., 2015). In agreement with its ubiquitous distribution, the participation of this transporter has been shown in numerous physiological and pathophysiological events (Copsel et al., 2011; Morgan et al., 2012; Sinha et al., 2015). Extrusion of cAMP by MRP4 tightly modulates the concentration of the nucleotide and the activity of its effectors while providing molecules to the extracellular space available for further signalling events (Sinha et al., 2015).

In previous work, we demonstrated the presence of MRP4 in bovine spermatozoa and established its importance in bicarbonate-induced sperm capacitation (Osycka-Salut et al., 2014). Incubation of bovine spermatozoa under capacitating conditions (40 mM bicarbonate) stimulated the efflux of cAMP, whereas blocking MRP4 with the broad-range inhibitor probenecid resulted in accumulation of the nucleotide inside the cell (Osycka-Salut et al., 2014). Probenecid was shown to inhibit capacitation-related events, but this inhibition was reversed by adding cAMP (10 nM) to the incubation media, suggesting that the role of MRP4 was not only to maintain the homeostasis of cAMP inside the cell, but also to stimulate extracellular signalling by transporting the nucleotide to the extracellular space. We have also previously examined the role of extracellular non-permeable cAMP as an inductor of bovine sperm capacitation (Alonso et al., 2017). PLC enzymes, PKC enzymes, and ERK1 and ERK2 (also known as MAPK3 and MAPK1) are activated and a Ca^{2+}-mediated PKA upregulation is observed when spermatozoa are incubated with exogenous cAMP (Alonso et al., 2017). Further, addition of cAMP to non-capacitating incubation media produced a rise in mitochondrial activity, promoted hyperactivated motility, the release of spermatozoa attached to the oviductal epithelium and supported egg fertilization (Alonso et al., 2017). These results provided strong evidence of the robust effect produced by extracellular nucleotides and nucleosides on sperm physiology (Bellezza and Minelli, 2017; Burnstock, 2014).

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Given the critical role of cAMP in mouse sperm function, and the relevance of MRP4 in cAMP-mediated events, the objective of this work was to determine whether MRP4 is present in mouse spermatozoa, and if so, to establish its role. In addition, we wanted to determine whether extracellular non-permeable cAMP was involved in signalling associated with sperm capacitation in this species.

RESULTS

Mouse spermatozoa possess functional MRP4

To test whether cAMP is extruded from mouse spermatozoa, we performed time course experiments measuring cAMP in the intracellular and extracellular space from spermatozoa incubated under capacitation conditions (represented as the pellet and the supernatant, respectively). As shown in Fig. 1A,B, cAMP is found in both cellular compartments and while intracellular levels fluctuate over time, cAMP in the supernatant reaches the highest levels at 5 min of incubation. To demonstrate whether MRP4 is responsible for cAMP efflux, we measured cAMP levels during a capacitation time course in the intracellular and extracellular space in the presence or absence of MK571 (50 µM), an MRP4 inhibitor. This inhibitor was selected over other MRP activity modulators such as probenecid, as its pharmacological properties render it more selective towards MRP4. The concentration employed was chosen by means of a concentration response curve in a functional assay measuring levels of phosphotyrosine (Fig. S1), and has been used in previous works to cause a strong inhibition of MRP4 in intact cells (Chen et al., 2018). Results showed that addition of MK571 (50 µM) to the incubation media produced a significant increase in intracellular cAMP levels at 1 min and 5 min (Fig. 1A), and a reduction of extracellular cAMP levels at 5 min (Fig. 1B), suggesting that cAMP efflux might be driven by MRP4.

To evaluate MRP4 presence in this cell type, we performed western blot and immunocytochemistry assays. A single immunoreactive band was detected at the expected molecular weight (Fig. 1C), while positive immunolabelling was found in the principal piece of the flagellum (Fig. 1D).

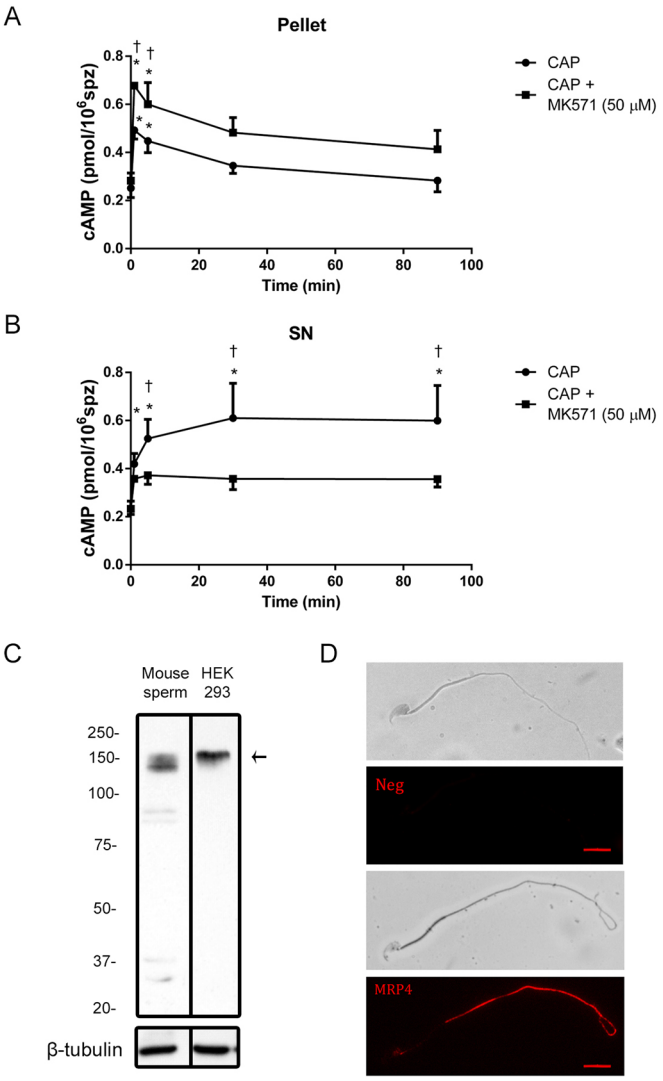

Fig. 1. MRP4 is present and functional in mouse spermatozoa. (A,B) Time course of intracellular (pellet) (A) and extracellular (SN, supernatant) (B) levels of cAMP from spermatozoa incubated in capacitation (CAP) medium in presence or absence of MK571 (50 µM), measured by radioprotein assay. Asterisks (*) indicate significant differences versus from time zero (A) P=0.05; (B) P=0.0022; n=4. Daggers (†) indicate significant differences CAP versus CAP+MK571; (A) P=0.034; (B) P=0.005; n=4. spz, spermatozoa. (C) Presence of MRP4 in mouse spermatozoa assessed by immunoblotting. Protein extract from HEK293 cells overexpressing MRP4 was used as a positive control; n=3. (D) Subcellular localization of MRP4 (red) in mouse spermatozoa; n=5. Neg, negative control. Scale bars: 10 µM. Representative images of each experiment are shown. All results are expressed as mean±s.e.m. Statistical analysis was performed by using two-way blocked ANOVA, for two-group comparison, paired t-test was assessed.

MRP4 function is necessary for adequate PKA activity and tyrosine phosphorylation

Intracellular cAMP has a vast repertoire of effectors; the PKA enzyme family is one of the most studied and its activity is directly related to tyrosine phosphorylation (Visconti et al., 2011). Therefore, we studied whether cAMP extrusion by MRP4 might modulate PKA activity and phosphotyrosine levels. Spermatozoa were incubated in the presence or absence of MK571 (50 µM) and phosphorylated substrates of PKA (pPKA-Subs) and total phosphotyrosine were evaluated at different time points. Results showed that at short incubation times (1 min) the inhibition of MRP4 by MK571 augmented the levels of pPKA-Subs, but no significant changes were detected at longer incubation periods (Fig. 2, upper panel), in accordance with the results of Fig. 1A. However, MK571-incubated spermatozoa exhibited lower levels of phosphotyrosine at 90 min (Fig. 2, lower panel). This suggests that MRP4 activity is necessary for early PKA regulation and its pharmacological inhibition may have a negative impact on the phosphorylation cascade that allows phosphotyrosine induction during capacitation.

Capacitation-relevant outcomes are impaired when MRP4 is blocked

An extensive range of articles in the literature link PKA activity with important capacitation outcomes such as the ability to undergo acrosome reaction (Romarowski et al., 2015; Stival et al., 2018). Thus, spermatozoa were incubated in the presence of MK571 (10 µM and 50 µM) under capacitating conditions, and acrosome reaction was induced with Ca^{2+} ionophore A23187. Results indicate that the percentage of acrosome reaction was significantly reduced by the addition of 50 µM MK571, in comparison with samples incubated in capacitation (CAP) medium plus ionophore in the absence of MK571. This decrease in the percentage of induced acrosome reaction could be understood as a reduction in the population of capacitated spermatozoa (Fig. 3A).

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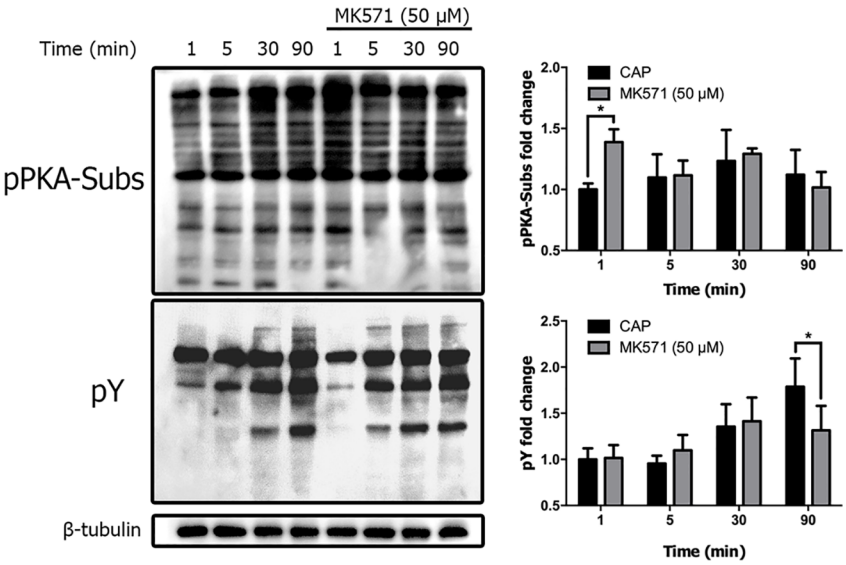

Fig. 2. MRP4 modulates PKA activity and phosphorylation of tyrosine. Time course of levels of phosphorylated protein substrates of PKA enzymes, as a marker of PKA activity (pPKA-Subs, upper panel) and phosphotyrosine (pY, lower panel) of spermatozoa incubated in CAP medium in the presence or absence of MK571 (50 µM). The asterisks (*) represent significant differences versus control; *n*=4; *P*=0.001. Representative blots from each experiment are shown. All results are expressed as mean±s.e.m. Statistical analysis was performed by paired *t*-test.

Additionally, previous reports described a correlation between motility and cAMP levels and PKA activity (Carlson et al., 2007; Lindemann et al., 1987). In order to investigate the influence of the cAMP efflux system on sperm motility, we incubated spermatozoa in the presence or absence of MK571 (10 µM and 50 µM) and evaluated motility by computer-assisted semen analysis (CASA). We observed a decrease in progressive motility and an increase in the percentage of immotile cells in spermatozoa incubated with MK571 50 µM, without affecting viability of the cells (Fig. S2). Other motility parameters such as curvilinear velocity (VCL), straight line velocity (VSL), mean path velocity (VAP), linearity (LIN), straightness (STR) and amplitude of lateral head displacement (ALH) were decreased in the presence of the inhibitor (Table 1). Additionally, the percentage of hyperactivated spermatozoa in the motile population was also lower in the presence of MK571 (Fig. 3B).

Concomitantly with these assays, the presence of 50 µM MK571 in the CAP medium resulted in a reduction in the percentage of fertilized oocytes in IVF assays (Fig. 3C). Incubation of MK571 with eggs alone showed no deleterious effect (data not shown).

Extracellular cAMP does not rescue the effect of MK571 on tyrosine phosphorylation and motility

When MRP4 is inhibited, lower levels of cAMP are found in the extracellular space. In our previous work, we observed that the inhibitory effect of MRP4 blockade on bull sperm capacitation was reversed by adding exogenous cAMP at nanomolar concentrations

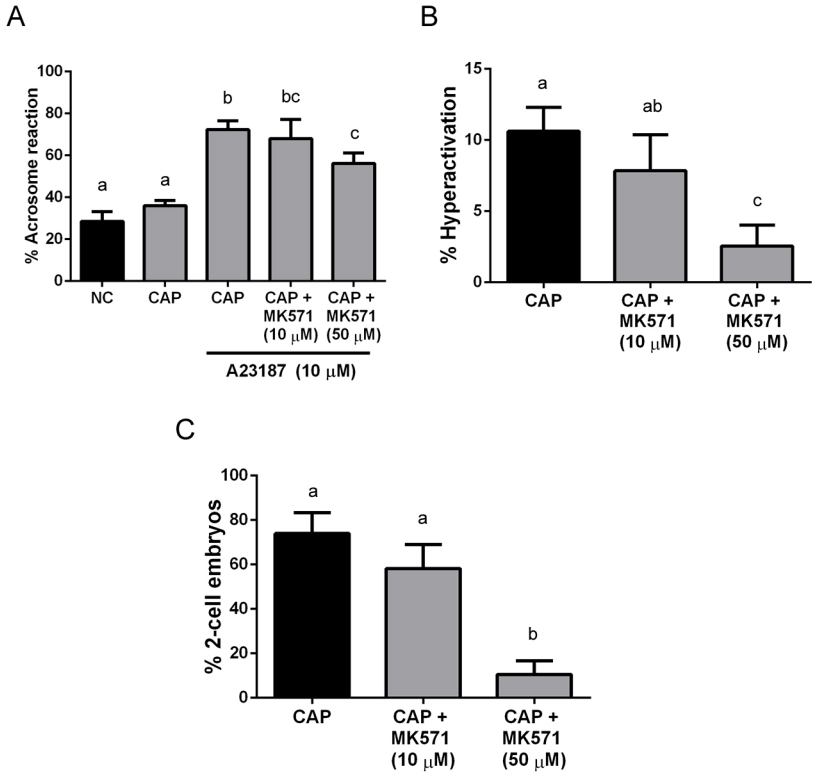

Fig. 3. Effect of MK571 on relevant capacitation outcomes. (A) Percentage of acrosome-reacted spermatozoa. Sperm was incubated in non-capacitation (NC) or CAP media alone (spontaneous acrosome reaction), or CAP medium with 10 µM A23187 (induced acrosome reaction) in the presence or absence of MK571 (10 or 50 µM); *n*=4; *P*=0.0001. (B) Percentage of hyperactivation determined by CASA in spermatozoa incubated in the presence or absence of MK571 (10 or 50 µM); *n*=4; *P*=0.003. (C) Percentage of embryo cleavage (two-celled embryo) obtained from total oocyte population in IVF assays using spermatozoa incubated in CAP medium in the presence or absence of MK571 (10 or 50 µM); *n*=4; *P*=0.011. All results are expressed as mean±s.e.m. Different letters represent statistically significant differences. Statistical analysis was performed using two-way blocked ANOVA.

Journal of Cell Science (2019) 132, jcs230565. doi:10.1242/jcs.230565

Table 1. Effect of MK571 on sperm motility parameters

	CAP	CAP+MK571 (10 µM)	CAP+MK571 (50 µM)
Progressive motility (%)	66.4±1.1[a]	60.1±6.3[a]	34.4±6.4[b]
Non-progressive motility (%)	3.8±0.5[a]	3.2±0.3[a]	16.2±3.3[b]
Immotile (%)	29.8±1.4[a]	36.7±6.1[ab]	49.4±4.25[b]
VCL (µm/seg)	185.6±3.4[a]	174.7±11.1[a]	79.1±16.5[b]
VSL (µm/seg)	74.5±1.4[a]	64.5±2.3[a]	21.0±6.0[b]
VAP (µm/seg)	102.0±2.0[a]	92.9±4.0[a]	42.8±10.6[b]
LIN (%)	38.3±0.9[a]	35.0±1.4[a]	23.2±2.5[b]
STR (%)	66.8±0.3[a]	62.7±1.5[a]	43.4±1.9[b]
WOB (%)	54.6±1.0	52.6±0.9	51.1±3.5
ALH (µm)	4.0±0.1[a]	3.81±0.2[a]	1.9±0.3[b]
BCF (Hz)	13.7±0.5[a]	13.8±0.6[a]	6.1±1.2[b]

Statistical analysis was performed by using two-way blocked ANOVA. Different letters represent statistically significant differences. *n*=4. Progressive motility, *P*=0.002; non-progressive motility, *P*=0.012; immotile, *P*=0.0095; VCL, *P*=0.0012; VSL, *P*=0.001; VAP, *P*=0.0024; LIN, *P*=0.0103; STR, *P*=0.0001; ALH, *P*=0.0017; BCF, *P*=0.0003.

(Osycka-Salut et al., 2014). Thus, in this study we evaluated whether the addition of cAMP to the incubation media rescues the altered MRP4-inhibited phenotype. As stated in the Materials and Methods, a working cAMP concentration of 1 nM was selected from an assay where tyrosine phosphorylation levels were evaluated in spermatozoa incubated in incomplete CAP medium (see Materials and Methods for details) supplemented with different concentrations of cAMP (1 nM–1 µM) (Fig. S3B). Therefore, we studied phosphotyrosine levels and motility in spermatozoa incubated with MK571 in the presence or absence of cAMP (1 nM). Extracellular cAMP was unable to restore phosphotyrosine levels (Fig. 4A) or the motility decrease elicited by 50 µM MK571 (Fig. 4B). Furthermore, addition of cAMP (1 nM) to CAP medium did not increase phosphotyrosine levels beyond CAP medium alone (Fig. 4A).

MRP4 blockade increases Ca^{2+} levels through a PKA-dependent mechanism

Since the addition of extracellular cAMP did not rescue the phenotype resulting from inhibition of MRP4, we explored other aspects of sperm physiology that may explain the effect observed on MRP4 inhibition. Because the observed motility loss and decreased phosphotyrosine levels in spermatozoa incubated with MK571 resembled the effects of abnormal Ca^{2+} signalling (Suarez et al., 1987; Tateno et al., 2013), we used flow cytometry to evaluate intracellular Ca^{2+} levels in spermatozoa capacitated in the presence or absence of MK571 (50 µM). Results showed that the presence of the MRP4 inhibitor significantly increased Ca^{2+} to levels comparable to those detected when spermatozoa were incubated with ionophore A23187 (10 µM) (Fig. 5A). In addition, incubation with EGTA (0.3 mM; a Ca^{2+}-chelating agent) prevented this Ca^{2+} increase, supporting the hypothesis that extracellular Ca^{2+} is the source of the increased intracellular Ca^{2+} (Fig. 5B). To discount non-specific effects of MK571, spermatozoa were capacitated in the presence of probenecid (500 µM), another MRP inhibitor, and a rise in Ca^{2+} levels was also observed (Fig. 5C).

Our results indicated a rapid increase in phosphorylation of PKA substrates in the presence of MK571 (Fig. 2). Recently, it has been reported that PKA activity is necessary for Ca^{2+} influx (Orta et al., 2018; Stival et al., 2018). In order to evaluate whether PKA might be the link between MRP4 inhibition and the increase of Ca^{2+} levels, we measured Ca^{2+} content in spermatozoa pre-incubated with two PKA inhibitors, H89 (10 µM) or KT5720 (0.1 µM).

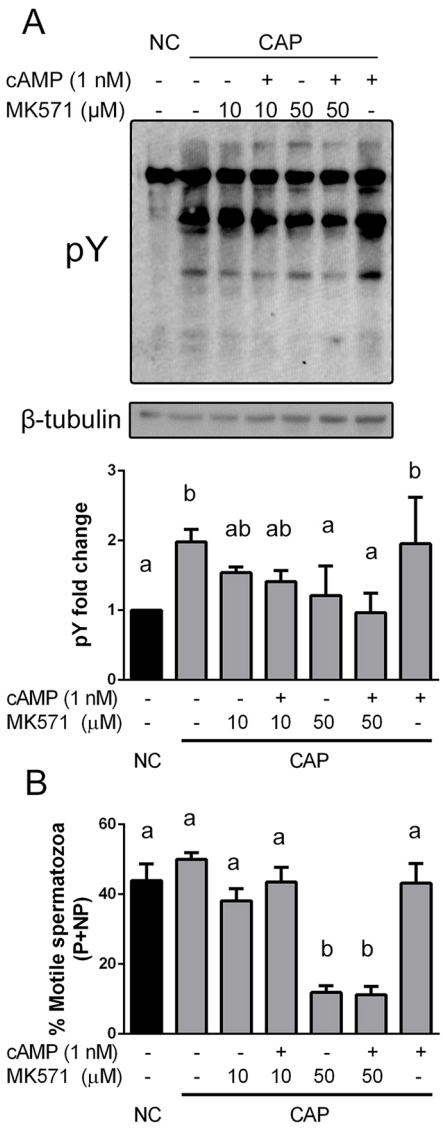

Fig. 4. Extracellular cAMP does not rescue MRP4 inhibition. Spermatozoa were incubated with MK571 (0, 10 or 50 µM) in the presence or absence of cAMP (1 nM). (A) Sperm proteins from each treatment were isolated and phosphotyrosine (pY) levels were detected. Optical density of each lane was measured and normalized to β-tubulin and the NC condition (lower panel); *n*=3; *P*=0.042. Representative blot is shown. (B) Percentage of motile spermatozoa (both progressive and non-progressive) from each sample was assessed using CASA; *n*=3; *P*=0.001. Different letters represent statistically significant differences. All bars indicate the mean±s.e.m. of medians of independent experiments. Statistical analysis was performed by using two-way blocked ANOVA.

Concentrations used were selected from previous reports (Alonso et al., 2017) and did not affect cellular viability (data not shown). Pre-incubation with either inhibitor prevented MK571-induced Ca^{2+} elevation (Fig. 5D).

Next, we aimed to determine the relationship between the elevated Ca^{2+} levels detected in spermatozoa incubated with MK571, and the observed phenotype (lower phosphotyrosine levels and impaired motility). Previously, Navarrete et al., (2015) demonstrated that Ca^{2+} has a biphasic behaviour in mouse sperm physiology. They observed that intracellular Ca^{2+} stimulates not only soluble adenylyl cyclase (sAC) but also PDEs through the calmodulin (CaM) signalling pathway. This depends on the amount

Journal of Cell Science (2019) 132, jcs230565. doi:10.1242/jcs.230565

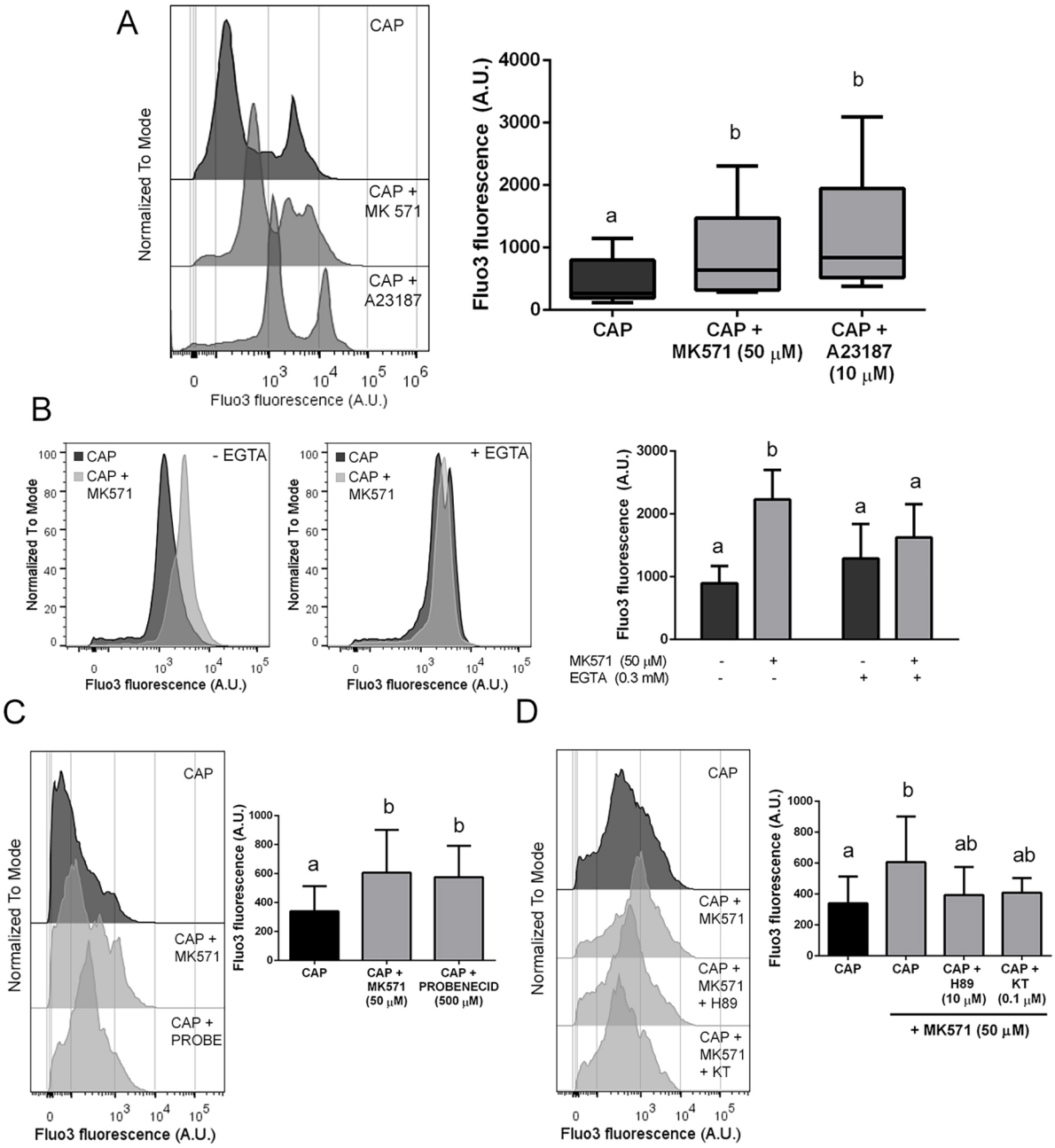

Fig. 5. Inhibition of MRP4 increases Ca^{2+} levels. (A) Intracellular levels of Ca^{2+} in spermatozoa incubated in CAP medium in the presence or absence of MK571 (50 µM) or A23187 (10 µM). After treatment, spermatozoa were loaded with Fluo3-AM and PI and the fluorescence of each probe was assessed by flow cytometry. Only the fluorescence of fluo3-AM in living spermatozoa (PI negative) was plotted in histograms (left panel), and the mean±s.e.m. of medians is represented in a box plot (right panel); n=6; P=0.005. (B) Ca^{2+} levels of spermatozoa incubated in presence or absence of MK571 (50 µM) in complete CAP medium (left histogram) or in CAP medium devoid of Ca^{2+} salts (without added salts and supplemented with 0.3 mM EGTA); n=4; P=0.005. (C) Ca^{2+} levels of spermatozoa incubated in the presence or absence of MK571 (50 µM) or probenecid (500 µM); n=4; P=0.0021. (D) Ca^{2+} levels of spermatozoa incubated in CAP medium in the presence or absence of MK571 (50 µM) and PKA inhibitors (H89, 10 µM; KT5720, 0.1 µM); n=3; P=0.0022. Representative histograms of each experiments are shown. In box plots, the box represents the 25–75th percentiles, and the median is indicated. The whiskers show the 5–95th percentiles. Different letters indicate statistically significant difference. All bars indicate the mean±s.e.m. of medians of independent experiments. Statistical analysis was performed by using two-way blocked ANOVA.

of Ca^{2+} available in the cytoplasm that may favour one pathway or the other: (i) when Ca^{2+} is chelated from the medium, meaning a low intracellular Ca^{2+} concentration, or when the CaM kinase pathway is pharmacologically inhibited, phosphotyrosine levels are high; (ii) when Ca^{2+} is chelated from the medium or cannot enter through CatSper proteins (CatSper-null mice) resulting in low intracellular Ca^{2+}, as well as when the CaM kinase pathway is pharmacologically inhibited, phosphotyrosine levels are high; (iii) when spermatozoa are incubated with a Ca^{2+} ionophore, causing a high intracellular Ca^{2+} concentration, phosphotyrosine levels are low. Additionally, Ca^{2+} homeostasis is critical for proper flagellar beating, and previous reports indicate that incubation with a Ca^{2+} ionophore immobilizes spermatozoa (Suarez et al., 1987). Therefore, we hypothesize that in control CAP medium, MK571,

Journal of Cell Science (2019) 132, jcs230565. doi:10.1242/jcs.230565

through PKA upregulation, induces an exacerbated Ca^{2+} influx mimicking the effect of a Ca^{2+} ionophore.

Thus, we examined the effect of MK571 (50 µM) on phosphotyrosine levels and motility in the absence of Ca^{2+} salts in the incubation medium (ØCa^{2+}). As expected, the absence of Ca^{2+} ions resulted in increased phosphotyrosine levels when spermatozoa were treated with MK571 (Fig. 6A). Moreover, in ØCa^{2+} medium, the decrease of sperm motility produced by MK571 was not observed (Fig. 6B).

Recent reports propose CatSpers as the main protein family responsible for the entry of Ca^{2+} mediated by PKA activation (Orta et al., 2018; Stival et al., 2018). Based on this, we hypothesize that CatSpers, upon PKA activation, are the channels responsible for Ca^{2+} influx elicited by MK571. We evaluated the effect of MK571 on spermatozoa from mice deficient for the *Catsper1* gene (*CatSper*$^{-/-}$). First, we examined motility by CASA in spermatozoa from *CatSper*$^{-/-}$ and from wild-type (WT) mice, incubated in presence or absence of MK571. Results showed that, unlike spermatozoa from WT mice, incubation with MK571 did not affect the percentage of motile cells in samples from *CatSper*$^{-/-}$ mice (Fig. 6C). Along the same line of thought, we evaluated the effect of MK571 on phosphotyrosine levels in spermatozoa from *CatSper*$^{-/-}$ mice. As observed in spermatozoa incubated in ØCa^{2+}, the addition of MK571 to CAP medium increased phosphotyrosine levels in *CatSper*$^{-/-}$ spermatozoa (Fig. 6D). These results suggest that Ca^{2+} is indeed the mediator between MRP4 inhibition and the decreased phosphotyrosine–impaired motility phenotype observed upon incubation with MK571.

DISCUSSION

Regulation of cAMP levels is a critical process in cellular physiology since it orchestrates several events (Buffone et al., 2014). Particularly in spermatozoa, binding of cAMP to PKA upregulates phosphorylation of a certain subset of proteins that ensures correct flux of ions (Visconti et al., 2011), lipid remodelling (Harrison and Miller, 2000), development of hyperactive motility (Baker et al., 2006) and changes in intracellular pH and membrane potential (Escoffier et al., 2012; Puga Molina et al., 2017) that allow spermatozoa to undergo acrosome reaction (Romarowski et al., 2015; Stival et al., 2018). Therefore, the enzymes that regulate cAMP availability play a critical role in male gamete physiology. In this context, MRP4 emerges as a novel regulator of PKA activity since it

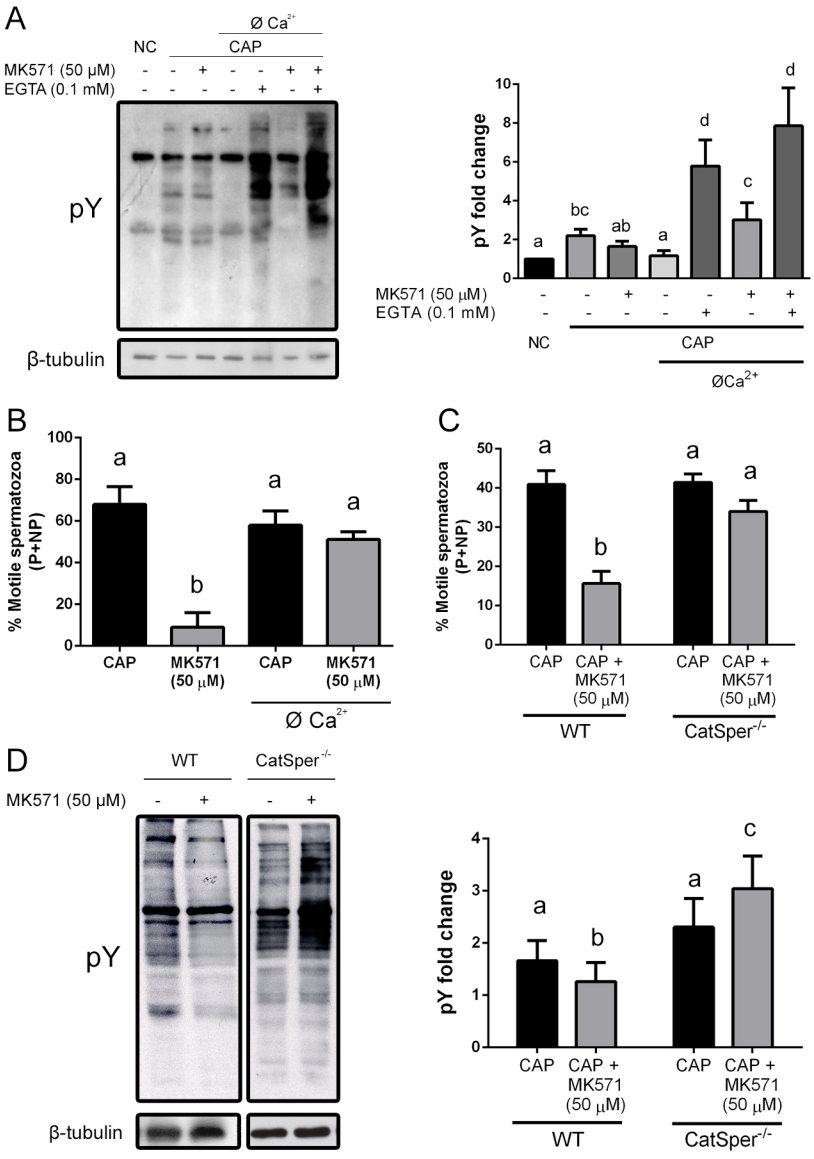

Fig. 6. Absence of Ca^{2+} salts in the medium prevents MK571-induced inhibition of motility and phosphotyrosine. (A) Levels of phosphotyrosine (pY) detected by western blot in spermatozoa incubated with MK571 (50 µM) in CAP medium with or without added Ca^{2+} salts (ØCa^{2+}) and/or supplemented with EGTA (0.1 mM). Optical density of each lane was measured and normalized to β-tubulin and NC condition (right panel); n=4; P=0.0001. (B) Percentage of motile spermatozoa (progressive+non-progressive) incubated in presence or absence of MK571 (50 µM) in control CAP medium or ØCa^{2+} was assessed by subjective motility. (C) Percentage of motile spermatozoa (progressive+non-progressive) from WT and *CatSper*$^{-/-}$ mice, incubated in presence or absence of MK571 (50 µM) in control CAP medium, assessed by CASA; n=4; P=0.0008. (D) Levels of phosphotyrosine detected by western blotting in spermatozoa from WT and *CatSper*$^{-/-}$ mice incubated with MK571 (50 µM) in CAP medium. Optical density of each lane was measured and normalized to β-tubulin and CAP condition; n=4; P=0.0094. Representative blot is shown. All bars indicate the mean±s.e.m. of medians of independent experiments. Different letters represent statistically significant differences. Statistical analysis was performed using two-way blocked ANOVA. Two-way nested ANOVA was used in experiments performed with WT and *CatSper*$^{-/-}$ mice.

Journal of Cell Science (2019) 132, jcs230565. doi:10.1242/jcs.230565

has been demonstrated that its blockade promotes focal PKA stimulation by punctual cAMP accumulation (Sinha et al., 2015). The importance of MRP4 has been established in cattle spermatozoa (Osycka-Salut et al., 2014; Alonso et al., 2017), but no further reports have been published in other mammalian species, this being the first work to address the role of MRP4 in murine sperm physiology.

Fig. 7 summarizes the results of this work in a proposed model. Here, we have shown that spermatozoa possess functional MRP4, and that cAMP is to be found in the intracellular and extracellular spaces. The localization of the transporter is concomitant with the subcellular distribution of several enzymes related to the cAMP–PKA signalling pathway (Wertheimer et al., 2013). The blockade of this transporter produced a rapid increase in cAMP levels inside spermatozoa while diminishing extracellular levels of the nucleotide, indicating specificity in the extrusion process. Accumulation of cAMP promoted an increment in PKA activity that, contrary to our expectations, was rapidly normalized and resulted in lower phosphotyrosine levels at the end of capacitation. This was accompanied by a significant loss in general motility and compromised fertilizing ability (Fig. 7B).

Several reports support that in various cell types cAMP extrusion is a mechanism that regulates cyclic nucleotide intracellular levels (Decouture et al., 2015; Copsel et al., 2011; Godinho and Costa, 2003; Osycka-Salut et al., 2014). The experimental observation that at early times of incubation in the CAP medium extracellular cAMP levels were higher than intracellular levels suggests that extrusion of the cyclic nucleotide also operates under basal conditions in mouse spermatozoa, as has been well documented in bovine spermatozoa (Osycka-Salut et al., 2014), and in other cell types (Godinho and Costa, 2003; Rodríguez et al., 2011). This difference was even greater when bicarbonate and BSA were added to the incubation media to induce capacitation, indicating that both intracellular and extracellular levels of cAMP are modulated. Levels of cAMP detected at each point are the result of interaction between synthesis by adenylyl cyclases, extrusion and degradation by PDEs. In this context, it is possible that extrusion of the cyclic nucleotide operates under basal conditions, whereas in the presence of capacitating molecules, the extrusion mechanism predominates with respect to the other two, in order to maintain a stable intracellular level of cAMP. We were not able to place the role of extracellular cAMP in murine spermatozoa, and so further studies are required to establish or discard its significance.

An important feature of cAMP extrusion in this species is the fact that spermatozoa incubated with MK571 presented elevated Ca^{2+} levels. This offers a suitable explanation for the broad effect that blockade of MRP4 had on sperm physiology. The requirement of Ca^{2+} for proper sperm function has been extensively demonstrated (Darszon et al., 2005; Gualtieri et al., 2005; Carlson et al., 2007; Costello et al., 2009; Li et al., 2016), but fewer works have shown the deleterious effect that an excessive concentration of Ca^{2+} may have. Baker and collaborators (Baker et al., 2004) showed a negative relationship between Ca^{2+} content and the availability of ATP in mouse spermatozoa. They state that substantial levels of ATP are needed for maintaining Ca^{2+} homeostasis, therefore depriving other functions. Additionally, it is known that incubation of spermatozoa with a Ca^{2+} ionophore immobilizes spermatozoa, and after washing or quenching the ionophore, spermatozoa recover motility (Suarez et al., 1987; Tateno et al., 2013). However, spermatozoa from PMCA4 (also known as ATP2B4, the principal ATP-dependent pump that extrudes Ca^{2+}) knockout (*Pmca4*$^{-/-}$) mice are not able to recover motility after removing the ionophore from the medium (Navarrete et al., 2016). The authors propose that spermatozoa from WT mice achieve Ca^{2+} clearance through PMCA4 in an ATP-dependent process, whereas spermatozoa from *Pmca4*$^{-/-}$ mice cannot accomplish this, supporting the dynamic relationship between Ca^{2+} and ATP, thus supporting Baker and colleagues' hypothesis (Baker et al., 2004). It is possible that incubation of spermatozoa with MK571, which highly elevates Ca^{2+} levels, also disrupts normal ATP physiology in order to achieve Ca^{2+} clearance, to the detriment of motility and regular phosphotyrosine levels. By contrast, Navarrete and collaborators (Navarrete et al., 2015) showed the dual role of Ca^{2+} that controlled sAC activity at the same time as the CaM kinase–calmodulin pathway. This hypothesis is supported by differential affinity of these enzymes for Ca^{2+}, resulting, as seen in other cell types, in a finely regulated balance that under certain stimuli may tip the scales towards a phosphorylated or dephosphorylated state of sperm proteins. In our study, the elevated Ca^{2+} levels determined under MRP4 blockade may result in over-stimulation of the CaM kinase–calmodulin

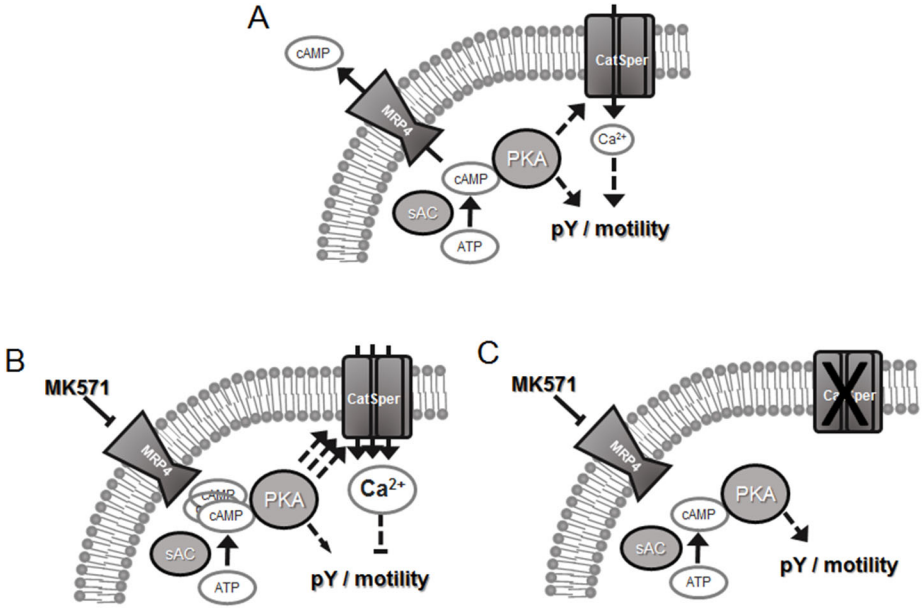

Fig. 7. Proposed model of events related to MRP4 inhibition. (A) Schematic summary of molecular events in murine spermatozoa incubated in CAP medium. The process of cAMP efflux through MRP4 contributes to control of PKA activity, allowing proper Ca^{2+} influx, phosphotyrosine (pY) increase and regular motility. (B) When murine spermatozoa are incubated with MK571, PKA activity is upregulated, which associates with higher Ca^{2+} levels, low phosphotyrosine levels and impaired motility, thus compromising fertilizing competence. (C) This panel illustrates sperm motility or phosphotyrosine levels in spermatozoa from *CatSper*$^{-/-}$ incubated with MK571. Continuous arrows indicate direct interaction; discontinuous arrows indicate indirect interaction.

pathway, raising the activity of PDEs that catalyse cAMP breakdown, as well as Ser–Thr dephosphorylation, resulting in diminished phosphotyrosine levels.

As mentioned previously, a recent report has confirmed the regulation of CatSper channel activation by PKA (Orta et al., 2018). In the light of our results, it is possible that the upregulation of PKA observed in Fig. 2 might lead to an exacerbated Ca^{2+} influx via CatSper. This is further supported by results from the experiments indicating that PKA inhibitors partially prevented the MK571-induced Ca^{2+} elevation (Fig. 5D), and that the presence of MK571 in CAP medium increased phosphotyrosine levels in *CatSper*$^{-/-}$ spermatozoa (Fig. 6D, Fig. 7C). Although the CatSper family are one of the most-studied Ca^{2+} channels involved in sperm function in mouse, we cannot rule out the participation of other important channels also regulated by PKA, such as Cav1.2, Cav1.3 (also known as Cacna1c and Cacna1d) and TRPV1 (Jeske et al., 2008; Darszon et al., 2005), in the molecular events related to cAMP–PKA and MRP4 described in this work.

Nonetheless, it is still unclear how a brief cAMP accumulation and/or PKA upregulation produced such a robust Ca^{2+} influx as that observed at the end of the incubation with MK571. In this regard, it is worth noting that cAMP is only one of the many substrates that MRP4 exports, and accumulation of other molecules such as cGMP, prostaglandins and leukotrienes (Wen et al., 2015), whose roles in sperm physiology are still elusive, may also contribute to the altered phenotype observed in MRP4-blocked spermatozoa. In this sense, other authors have previously reported that cGMP accumulation leads to Ca^{2+} influx (Brenker et al., 2012) and this may explain why incubation with PKA inhibitors partially mitigated MK571-induced Ca^{2+} influx. In agreement with this, it is also important to remark that *Mrp4*$^{-/-}$ mice are subfertile, indicating that loss of this transporter has a deleterious effect on reproductive outcome (Morgan et al., 2012).

Reported differences between bovine and murine spermatozoa might result from the different physiological and technical processes they are put through prior to their examination. However, the role and importance of MRP4 and purinergic signalling might be different between these species regardless of their model differences (e.g. ejaculated versus not ejaculated and cryopreserved versus fresh spermatozoa). This is evidenced by the fact that inhibition of capacitation-associated events by MRP4 blockade is overcome when cAMP is added to the incubation media in bovine (Osycka-Salut et al., 2014) but not in murine spermatozoa. This suggests that the purpose of nucleotide extrusion in bovine spermatozoa is primarily to provide cAMP to the extracellular space, whereas in murine spermatozoa cAMP efflux through MRP4 might be a key regulator of cAMP levels and/or the PKA signalling pathway. This is also supported by the difference in MRP4 localization between these species, which may result in association with different molecules and signalling pathways (Osycka-Salut et al., 2014).

Taken together, the results of this work support that cAMP efflux by MRP4 has a critical role in controlling the activity of PKA and its effectors, thus affecting fertilizing ability in mouse spermatozoa. In this sense, MRP4 emerges as a novel molecular target for the treatment of subfertility and infertility, as well as the development of contraception.

MATERIALS AND METHODS

Reagents

MK571 (M7571), propidium iodide (PI, P4170), H89 (B1427), cAMP (A9501), bovine serum albumin (BSA, A7906), probenecid (P8761), calcium ionophore A23187 (C7522) and EGTA (E4378) were purchased from Sigma-Aldrich (MO, USA). KT5720 (1288) was purchased from Tocris Bioscience (Bristol, UK). Fluo3-AM (F1242) was purchased from Molecular Probes (Life Technologies, NY, USA). [^{3}H]cAMP 31 Ci/mmol was purchased from Perkin Elmer Life Sciences (MA, USA). All other chemicals were of analytical grade and obtained from standard sources.

Animals

CD1 mature (10–12-week-old) male mice (*Mus musculus*) were maintained at 24°C with a 12:12 h light:dark cycle. The experimental procedures reported here were reviewed and approved by the Institutional Committee for Care and Use of Laboratory Animals from the School of Medicine, University of Buenos Aires (resolution number 1162/2016), and the Institutional Animal Care and Use Committee from the University of Massachusetts. All experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals (NIH). For *in vitro* fertilization (IVF) assays, hybrid F1 (Balb/C×C57BL/6) breed mice were used following the same care conditions as CD1 mice. Homozygous male mice lacking CatSper1 (Ren et al., 2001) were produced in a C57BL/6 background and provided by Dr Pablo Visconti (University of Massachusetts, USA).

Sperm preparation and capacitation

Epididymes from mature male mice were minced, placed in 500 µl of Whitten's HEPES-buffered (WH) medium (100 mM NaCl; 4.4 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 5.4 mM glucose; 0.8 mM pyruvic acid; 4.8 mM lactic acid; 2.4 mM Ca^{2+}; 20 mM HEPES, pH 7.4), and then maintained at 37°C for 20 min to allow spermatozoa to swim out. This medium does not support capacitation (NC) unless supplemented with bovine serum albumin (5 mg/ml BSA, fatty acid-free) and bicarbonate (15 mM) (CAP). After swim-out, spermatozoa concentration was assessed with a Neubauer chamber and adjusted to 16×10^6 cells/ml with WH. Cells were pre-incubated with reagents indicated in each experiment for 10 min and then diluted two times in the appropriate medium depending on the experiment performed (final concentration 8×10^6 cells/ml). Spermatozoa were incubated at 37°C for 90 min unless indicated the contrary.

To assess whether non-permeable cAMP induces tyrosine phosphorylation, standard WH NC and an incomplete capacitation media were used. This incomplete CAP medium (1/3C) consists of WH supplemented with 1.67 mg/ml BSA and 5 mM bicarbonate.

Western blotting

Total protein isolation was performed as described previously (Visconti et al., 1995) with slight modifications. Briefly, after capacitation, ∼2×10^6 spermatozoa were centrifuged for 3 min at 10,000 ***g*** and resuspended with 1 ml of phosphate buffer saline (PBS) containing 2 mM of orthovanadate. Spermatozoa were centrifuged again for 3 min at 10,000 ***g***, resuspended in 15 µl of Laemmli buffer and boiled for 5 min. The samples were centrifuged 3 min at 10,000 ***g***, and the supernatants were supplemented with 5% β-mercaptoethanol before boiling again for 5 min. Proteins were separated in 10% SDS polyacrylamide gels and transferred to PVDF membranes that were blocked either with 5% cold fish skin gelatine (for phosphotyrosine detection) or 3% skimmed milk (for MRP4, pPKA-Subs and β-tubulin detection). Membranes were incubated with primary antibodies in the following conditions: anti-phosphotyrosine antibody (clone 4G10, 05-321 Merck-Millipore), 1:5000 in TPBS (PBS 0.1% Tween 20), overnight at 4°C; anti-MRP4 antibody, 1:500 in TPBS (12857, Cell Signaling Technology) or 1:200 (F-6, Santa Cruz Biotechnology) in TTBS (TBS 0.05% Tween20), overnight at 4°C with agitation; anti-pSer/Thr PKA substrate (pPKA-Subs) antibody (9621, Cell Signaling Technology), 1:2000 in TTBS, and β-tubulin antibody (ab131205, Abcam), 1:5000 in TPBS both 1 h at room temperature (RT). After incubation, membranes were washed three times with TPBS or TTBS. For primary antibody detection, anti-mouse or anti-rabbit polyclonal antibodies linked to horse radish peroxidase (323-005-021, Jackson ImmunoResearch) and enhanced chemo-luminescence reagents were used following the manufacturer's instructions.

Immunocytochemistry

Spermatozoa were fixed (20 min, RT with 0.2% w/v paraformaldehyde), immobilized on slides and permeabilized with TPBS–Triton X-100 0.5% for 20 min at RT. Non-specific binding sites were blocked (60 min, TPBS

with 3% w/v BSA) and then slides were incubated with anti-MRP4 (1:250; Santa Cruz Biotechnology) antibody overnight at 4°C in blocking buffer. Samples were then washed and further incubated with Alexa Fluor 555-conjugated anti-rabbit IgG (1:500; ab150074, Abcam) for 60 min at RT. Specificity of the immunodetection was assessed by omitting the primary antibody. Sperm cells were mounted with DABCO (SIGMA) and examined under a fluorescence microscope (Nikon Eclipse E100) with UV lamp (510 nM) and respective emission filters with a 100× objective. Images were captured using a Nikon DS-V1 coupled camera and NIS Elements Advanced Research software.

Measurement of cAMP

Spermatozoa (6×10^6 cells) were incubated for 0, 5, 30 or 90 min in WH CAP medium, or for 5 min in the presence or absence of MK571 (50 µM) in the same medium. Samples were centrifuged for 5 min at 3000 ***g***, supernatant was separated from pellets, and 1 ml ice-cold ethanol was added to both fractions. The ethanol was then 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 (Davio et al., 1995). Duplicate samples in at least three independent experiments were analysed.

Acrosome reaction induction and assessment

After 60 min of capacitation, spermatozoa were divided in two aliquots that were further incubated in the presence or absence of 10 µM Ca^{2+} ionophore A23187 for 30 min at 37°C. Then, spermatozoa were fixed with 4% w/v paraformaldehyde for 10 min at RT and washed twice with a 100 mM pH 9 ammonium acetate solution. Spermatozoa were resuspended in 100 µl of the ammonium acetate solution, and a 10 µl aliquot for each condition was air-dried onto slides. Permeabilization was performed with ice-cold methanol for 5 min and slides were further washed with bi-distilled water. Staining was done with Coomassie solution (0.22% m/v Coomassie G-250 in 50% methanol and 10% acetic acid) for 2 min. The slides were then gently washed and mounted with 1:9 glycerol–PBS. At least 400 stained cells/treatment were analysed in a Nikon Eclipse E200 microscope with a 100× objective in clear field. The percentage of acrosome reaction was calculated as the percentage of acrosome-reacted spermatozoa out of total population spermatozoa. Samples incubated in NC or CAP media alone represent the spontaneous acrosome reaction, whereas samples incubated in CAP medium plus 10 µM of A23187 represent the induced acrosome reaction.

Ca^{2+} measurement by flow cytometry

After incubation with the appropriate medium, samples were centrifuged at 400 ***g*** for 5 min and were resuspended in 200 µl of WH medium with 10 µM fluorescent calcium indicator Fluo3-AM. After 20 min incubation at 37°C samples were centrifuged again at 400 ***g*** for 5 min and resuspended in 200 µl of WH medium. Cells were maintained at 37°C and 2 ng/µl propidium iodide (PI) was added upon acquisition in a BD Accuri C6 Plus flow cytometer. Side-scatter area (SSC-A) and forward-scatter area (FSC-A) data were collected from 20,000 events per sample in order to define sperm population. Singlets were selected from a forward-scatter height (FSC-H) versus FSC-A dot plot. Events with dim fluorescence in the FL3 channel (equipped with a 670 nM LP filter for PI detection) were gated as the living cell population and were replotted in a FL1 histogram (equipped with a 540/20 nm filter for FITC/Fluo3 detection). The two indicators had minimal emission overlap, but compensation was still applied. Results show a representative histogram as well as the mean of Fluo3 median fluorescence of each sample.

Egg collection and *in vitro* fertilization assay

Superovulated 8- to 12-week-old F1 female mice were obtained by stimulation with PMSG (5 IU) and hCG (5 IU) intraperitoneal injection protocol, separated by 48 h. 13 h after the hCG injection, animals were killed and ampullas (containing cumulus oocyte complex, COC) were dissected and placed in pre-equilibrated TYH medium (119.3 mM NaCl; 4.7 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 5.6 mM glucose; 0.5 mM sodium pyruvate; 1.7 mM Ca^{2+}; supplemented with 4 mM BSA and 25 mM bicarbonate). COCs were collected and placed in TYH fertilization drops (90 µl), covered with mineral oil and maintained at 37°C in 5% CO_2 atmosphere. Spermatozoa from mature F1 male mice were capacitated as previously described (Visconti et al., 1995) but with TYH medium supplemented with 20 mM HEPES at 37°C in the presence or absence of MK571. Sperm concentration was calculated with a Neubauer chamber and at least 15–40 oocytes were inseminated with 10^5 spermatozoa in the fertilization drops. After 4 h coincubation at 37°C in 5% CO_2 atmosphere, cumulus cells were dispersed and oocytes were washed and placed in fresh TYH drops. After overnight incubation in conditions as above, drops were examined and the number of two-cell embryos was scored. Only those experiments with control condition fertilization rates greater than 60% were included in the results.

Sperm motility analysis

Sperm suspensions were loaded on a 46 µm deep slide and placed on a microscope (Nikon Eclipse E200) stage at 37°C coupled to a BASLER acA780-75gc camera. Sperm movements were examined using a computer-assisted semen analysis (CASA) system (Sperm Class Analyzer: SCA evolution, Microptic). Parameters used were as follows: 30 frames acquired, frame rate of 60 Hz, and cell size of 30–170 µm^2. At least 20 microscopy fields corresponding to a minimum of 200 spermatozoa were analysed in each experiment. The following parameters were measured: mean path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), linearity (LIN), amplitude of lateral head displacement (ALH), and straightness (STR). Spermatozoa were considered hyperactivated when presenting VCL≥271 µm/s, LIN<50%, and ALH≥3.5 µm. Experiment shown in Fig. 6B was performed counting spermatozoa manually, analysing at least 200 cells and classifying cells as motile or immotile spermatozoa.

Sperm viability

After incubation, spermatozoa were loaded with 0.002 mg/ml propidium iodide and fluorescence was assessed by flow cytometry. Cells presenting dim fluorescence on the FL3 channel (670 nM LP filter) were taken as viable.

Statistical analysis

Data were analysed mainly by two-way blocked analysis of variance (ANOVA) using the software InfoStat (http://www.infostat.com.ar). For experiments where WT and *CatSper*$^{-/-}$ mice were used, a two-way ANOVA with the treatment nested in mouse genotype was performed. Raw data were analysed by Shapiro–Wilks and Levene tests to assess normality of data distribution and variance homogeneity, respectively. Pairwise comparisons of means were made with Tukey or Fisher honestly significant differences. For two-group comparison, paired *t*-test was assessed. Whenever either normality or homoscedasticity were not achieved, transformation of the data was applied. All results are expressed as mean±s.e.m. of at least three independent determinations. A probability (*P*) value of $P<0.05$ was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.A.I.A., M.G.B., C.D., S.P.-M.; Methodology: C.A.I.A., R.L.-L., G.M.L., Z.J.V., N.D.S., M.G.G.; Validation: C.A.I.A., R.L.-L., G.M.L.; Formal analysis: C.A.I.A.; Investigation: C.A.I.A., R.L.-L., G.M.L., Z.J.V., N.D.S., M.G.G.; Resources: M.G.G., M.G.B., C.D.; Data curation: C.A.I.A., M.G.G., C.D., S.P.-M.; Writing - original draft: C.A.I.A., C.D., S.P.-M.; Writing - review & editing: C.A.I.A., G.M.L., M.G.G., M.G.B., C.D., S.P.-M.; Visualization: C.A.I.A., C.D., S.P.-M.; Supervision: M.G.G., C.D., S.P.-M.; Project administration: M.G.G., M.G.B., C.D., S.P.-M.; Funding acquisition: M.G.B., C.D., S.P.-M.

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Supplementary information

Supplementary information available online at
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