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In guinea pig sperm, aldolase A forms a complex with actin, WAS, and Arp2/3 that plays a role in actin polymerization

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Abstract

Glycolytic enzymes have, in addition to their role in energy production, other functions in the regulation of cellular processes. Aldolase A has been reported to be present in sperm, playing a key role in glycolysis; however, despite its reported interactions with actin and WAS, little is known about a non-glycolytic role of aldolase A in sperm. Here, we show that in guinea pig spermatozoa, aldolase A is tightly associated to cytoskeletal structures where it interacts with actin, WAS, and Arp2/3. We show that aldolase A spermatozoa treatment increases their polymerized actin levels. In addition, we show that there is a direct correlation between the levels of polymerized actin and the levels of aldolase A–actin interaction. Our results suggest that aldolase A functions as a bridge between filaments of actin and the actin-polymerizing machinery.

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Introduction

Cytoskeletal organization as well as its dynamic regulation plays a fundamental role during sperm development and fertilization (Xiao & Yang 2007). For instance, actin polymerization is important for sperm motility during maturation (Lin *et al*. 2002). Interestingly, maturation and motility acquisition correlate with a decrease in polymerized actin (F-actin) levels (Azamar *et al*. 2007), whereas fertilization is impaired upon blockade of actin polymerization (Sánchez-Gutiérrez *et al*. 2002). Recent data have shown that besides its essential role in glycolysis, aldolase participates in many other important processes: disruption of the binding capability of aldolase to B subunit of the V-ATPase, without modification of its enzymatic activity, induces V-ATPase disassembly and malfunction (Lu *et al*. 2007); phospholipase D2 is inhibited upon the binding of aldolase to its PH domain (Kim *et al*. 2002), thus regulating the production of lipid second messengers and associated signal pathways; a portion of aldolase interacts with F-actin *in vivo* (Arnold & Pette 1968, Wang *et al*. 1997) and modulates actin polymerization *in vitro* (Schindler *et al*. 2001); therefore, opening the possibility of aldolase could regulate actin dynamics *in vivo*.

Aldolase exists as three well-characterized isoforms in vertebrates. Aldolase A is a ubiquitous enzyme, predominantly expressed in muscle, and has been described in the fibrous sheath of mouse and human sperm (Krisfalusi *et al*. 2006, Kim *et al*. 2007). Aldolase B is highly expressed in both kidney and liver whereas aldolase C is found mainly in the nervous system, but has been also identified in bovine spermatozoa (Fiedler *et al*. 2008). In addition, three novel aldolase A isoenzymes are expressed in mouse spermatozoa (Vemuganti *et al*. 2007).

Among the proteins that participate in the modulation of actin dynamics, our group has already reported in the guinea pig spermatozoa the presence of the Wiskott-Aldrich syndrome protein (WAS), actin-related protein (Arp) 2/3, Ras homolog gene family (Rho) member A and B, profilins I and II, as well as the small GTPase of the Rho family CDC42 (Delgado-Buenrostro *et al*. 2005).

In the context of the ability of aldolase to interact with both actin (O'Reilly & Clarke 1993) and WAS (Buscaglia *et al*. 2006, St-Jean *et al*. 2007), and considering that the function of WAS is being an actin nucleation-promoting factor via the interaction with Arp2/3, we examined, in guinea pig spermatozoa, the interaction of aldolase A with actin as well as WAS and Arp2/3. Moreover, we found a direct correlation between F-actin levels and aldolase A–actin interaction.

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Results

Aldolase A identification and localization in guinea pig spermatozoa

As a first step, we determined the expression of aldolase A in guinea pig spermatozoa in whole extracts; a single immunoreactive band of 39 kDa was detected in sperm, as well as in muscle, which was used as positive control (Fig. 1). Next, aldolase A cell distribution was analyzed by immunofluorescence (IF) in both intact and membrane-free (i.e., Brij-treated) spermatozoa. In the first case, aldolase A was found conspicuously along the flagellum and in the acrosome (Fig. 2), whereas the Brij treatment induced the loss of the acrosome and the signal in the acrosome but kept the signal in the flagellum, in addition, a pale and diffused signal was found in the head. In order to corroborate the aldolase A localization in guinea pig spermatozoa, we performed cell fractionation experiments, each fraction was subjected to Western blot analysis. Aldolase A was found in the following fractions: cytosol, plasma–acrosomal membranes, flagella, perinuclear theca (PT)–flagellum, and PT (Fig. 3).

Aldolase A is associated to cytoskeletal structures in guinea pig spermatozoa

Although glycolysis occurs in the cytoplasm, it has been reported that aldolase is associated to cytoskeletal structures (Gillis & Tamblyn 1984, Volker & Knull 1997, Schindler *et al*. 2001). Indeed, our ability to retrieve aldolase A in Brij-treated spermatozoa suggests that this could be the case in our system; to explore this possibility in more detail, we purified non-ionic detergent-insoluble proteins from Brij-treated spermatozoa suitable for proteomic analysis. Tricine–SDS–PAGE analysis resolved around 16 bands (Fig. 4A), out of these, 3 bands ranging from 30 to 50 kDa were selected for digestion with trypsin and their identity were assessed by peptide mass fingerprinting using MALDI-Tof/MS analysis. The Mascot program reported that six peptides from band 2′ have a significant match to rabbit's aldolase

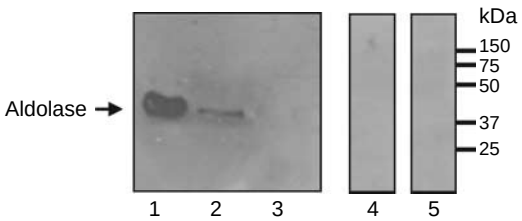


Figure 1 Aldolase A identification in guinea pig spermatozoa. Whole-cell sperm extracts obtained from mature spermatozoa were analyzed by Western blot; a single band of about 39 kDa was detected. (1) Muscle extract, used as positive control; (2) Sperm extract. The specificity of the anti-aldolase antibody was assessed, no immunoreactive band was detected when using purified actin (3), neither when first antibody was avoided (4) nor when pre-immune serum was used (5).

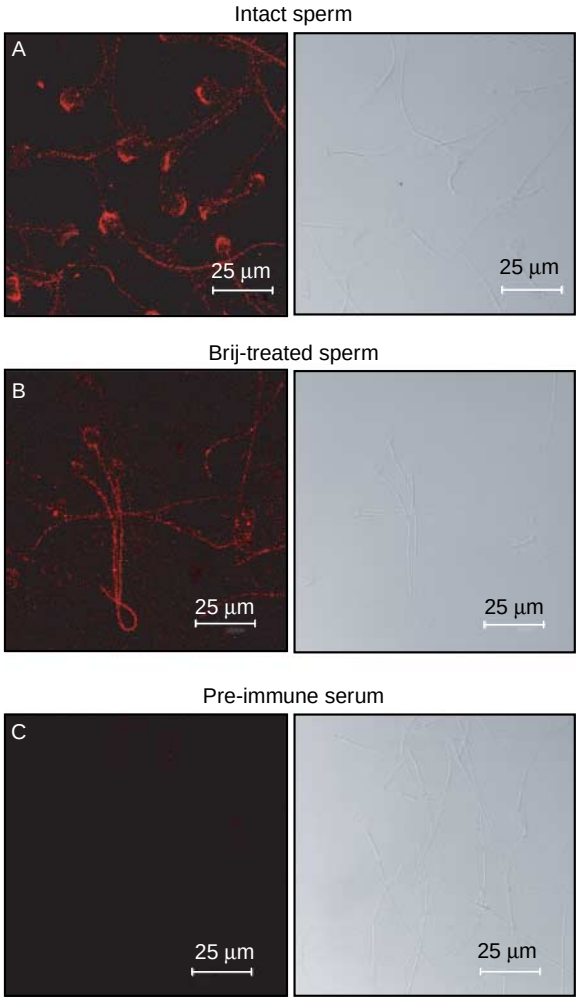


Figure 2 Aldolase A localization in guinea pig spermatozoa. Sperm cells were immunostained with anti-aldolase to determine its subcellular distribution. (A) In intact spermatozoa, aldolase A is observed along the flagellum and in the acrosome. (B) In Brij-treated spermatozoa, aldolase A remains all through the flagellum but is lost in the acrosomal region. In addition, a less intense and diffuse signal was observed in the sperm head. (C) Pre-immune serum was used as control of specificity of the first antibody. Each immunofluorescence picture is accompanied by its phase contrast image.

A sequence (Fig. 4B; Table 1) and moreover, band 2′ has an apparent molecular mass of 39 kDa (Fig. 4A), which is the same molecular weight reported for aldolase A (Sia & Horecker 1968). In addition, band 1′ was identified as PIP5K11 whereas band 3′ could not be readily identified, thus it is reported as a hypothetical protein.

Modulation of the relative levels of F-actin

The results mentioned above suggested to us that aldolase A might have an additional role in spermatozoa besides its known role in glycolysis, because of its ability to interact with cytoskeletal proteins (O'Reilly & Clarke 1993, Krisfalusi *et al*. 2006, Kim *et al*. 2007); a possibility

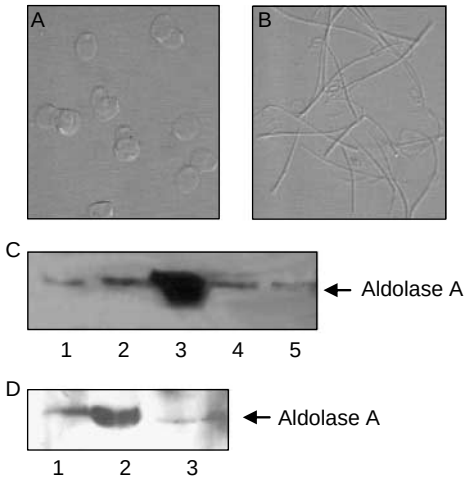
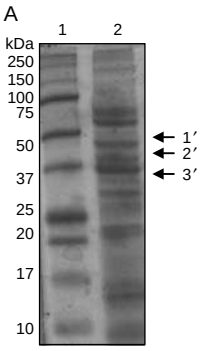


Figure 3 Aldolase A subcellular distribution. Guinea pig spermatozoa were disrupted by sonication and purified by Percoll gradients to obtain pure (A) sperm heads (>95%) or (B) sperm tails. (C) After sonication, cytosolic and plasma–acrosomal membranes fractions were obtained by ultracentrifugation. Fractions were subjected to Western blot to detect aldolase A. (1), plasma–acrosomal membranes fraction; (2), cytosolic fraction; (3), muscle extract used as positive control; (4), perinuclear theca–flagellum; and (5), flagellar fraction. (D) (1), flagellum, (2), muscle extract; and (3), perinuclear theca.



B

1 PHSHPALTPE QKKELSDIAH RIVAPGKGIL AADESTGSIA KRLQSIGTEN
51 TEENRRFYRQ LLLTADDRVN PCIGGVILFH ETLYQKADDG RPFPQVIKSK
101 GGVVGIKVDK GVVPLAGTNG ETTTQGLDGL SERCAQYKKD GADFAAWRCV
151 LKIGEHTPSA LAIMENANVL ARYASICQQN GIVPIVEPEI LPDGDHDLKR
201 CQYVTEKVLA AVYKALSDHH IYLEGTLLKP NMVTPGHACT QKYSHEEIAM
251 ATVTALRRTV PPAVTGVTFL SGGQSEEEAS INLNAINKCP LLKPWALTFS
301 YGRALQASAL KAWGGKKENL KAAQEEYVKR ALANSLACQG KYTPSGQAGA
351 AASESLFISN HAY

Figure 4 Aldolase A is associated to cytoskeletal structures in guinea pig spermatozoa. (A) Detergent-insoluble proteins obtained from membrane-free spermatozoa were separated by tricine–SDS–PAGE. The gel was stained with Coomassie Brilliant Blue and 16 bands were revealed (track 2). Bands 1′, 2′, and 3′ were chosen to be identified by mass spectroscopy: 1′, PIP5K11; 2′, aldolase A; and 3′, a hypothetical protein. Track 1 shows the molecular weight markers. (B) MALDI-Tof/MS analysis of band 2′ found six peptides that match with aldolase A sequence (red-labeled amino acids). The six peptides cover 24% of the reported sequence (Table 1).

was perhaps that aldolase A would modulate cytoskeletal dynamics in spermatozoa. To begin exploring whether aldolase A might participate in sperm actin polymerization, we permeabilized living spermatozoa with streptolysin O (SLO spermatozoa), then treated with aldolase A in Tyrode-pyruvic, lactic acid, and glucose (TPLG) medium, and interestingly, aldolase A supplementation increased the relative levels of polymerized actin (F-actin) measured by flow cytometry (FACS). On the contrary, addition of anti-aldolase A antibody to SLO-treated sperm slightly decreased the relative level of polymerized actin versus permeabilized spermatozoa (Fig. 5A). This last reduction might be the endogenous aldolase cancellation leading to direct inhibition of the actin polymerization process.

Because the effects of aldolase A in actin polymerization could be related to glycolysis, we incubated SLO spermatozoa with aldolase A in Tyrode-pyruvic and lactic acid (TPL) medium without glucose. We found that, as in the presence of glucose, aldolase A supplementation in this glucose-free medium increased the sperm relative levels of F-actin (Fig. 6A). Next, we inhibited glycolysis by the addition of 2-deoxy-D-glucose (2DG; Mújica & Valdes Ruíz 1983) under glucose-free conditions (i.e., TPL medium); we found that 2DG treatment had a negative effect on F-actin concentration (Fig. 6B). Afterwards, aldolase A supplementation to permeabilized spermatozoa incubated in TPL–2DG prevented 2DG effect on F-actin decrease (Fig. 6B). Overall, these results directly involve aldolase A in actin polymerization.

These results prompted us to determine whether aldolase A directly interacts with actin. First, we determined by IF that both actin and aldolase A are located in the same cellular compartments in spermatozoa (Fig. 7A), then their interaction was confirmed by co-immunoprecipitation assays (Figs 7B and 9C and D). Considering that sperm capacitation decreases the levels of F-actin (Sánchez-Gutiérrez *et al.* 2002, Azamar *et al.* 2007), we decided to evaluate the aldolase A–actin interaction levels in capacitated spermatozoa; as can be seen in Fig. 7, after 2.5 h of capacitation, the levels of F-actin were decreased (Fig. 7C) to a similar extent to the interaction between actin and aldolase A (Fig. 7B and B′). In order to corroborate that the aldolase A–actin interaction depends on the polymerization state of actin, we next prevented its polymerization by cytochalasin D

Table 1 Identification of aldolase A in guinea pig sperm.

Protein identified	Accession number	Score	Sequence coverage (%)	Number of matching peptides	MW	pI
Aldolase	gi|673062	71	24	6	39.586	8.2

Proteins were trypsinized and analyzed by MALDI-ToF/MS. Proteins with a value of $P<0.05$ were considered statistically significant.

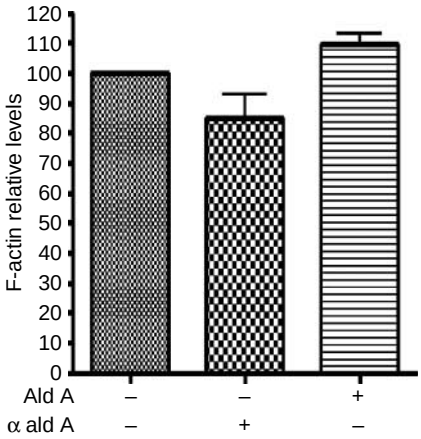


Figure 5 Effect of aldolase A on F-actin concentration. (A) Mature SLO-permeabilized spermatozoa were incubated in TPLG alone or with aldolase A (ald A) or anti-aldolase A antibody (α-ald A) for 1.5 h, and then fixed. F-actin relative levels were measured by FACS using TRITC-Ph. *$P<0.05$ versus TPLG used as control.

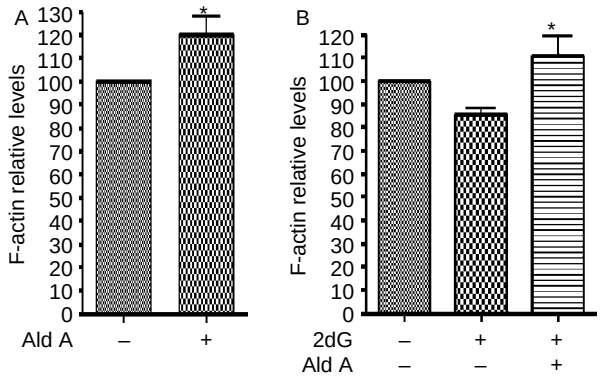


Figure 6 Effect of aldolase A on F-actin concentration with or without 2-deoxy-D-glucose. (A) SLO-permeabilized spermatozoa were incubated in glucose-free TPL with and without aldolase A for 30 min and then fixed. F-actin relative levels were measured by FACS using TRITC-Ph. *$P<0.05$ versus TPL. (B) SLO-permeabilized spermatozoa were incubated in TPL without or with 2-deoxy-D-glucose, and added or not with aldolase A for 30 min and then fixed. F-actin relative levels were measured by FACS using TRITC-Ph. *$P<0.05$ versus TPL plus 2-deoxy-D-glucose.

(CytD) sperm treatment and evaluated their physical interaction by co-immunoprecipitation. In line with previous reports (Sánchez-Gutiérrez *et al*. 2002), we detected a CytD-induced decrease in F-actin levels (Fig. 8A) of a similar extent than the decrease in the aldolase A–actin interaction (Fig. 8B and C).

Interaction of aldolase with actin, WAS, and Arp2/3

The involvement of aldolase A in F-actin levels raised the question of whether aldolase A would directly induce polymerization or would function as a recruiting element for proteins directly involved in modulating actin dynamics, so we decided to explore the latter hypothesis. Two natural candidates were WAS and Arp2/3 that have been previously described in our system (Delgado-Buenrostro *et al*. 2005). We found by IF that both WAS and Arp2/3 colocalize with aldolase A (Fig. 9A and B respectively) likewise, F-actin; remarkably, actin, WAS and Arp2/3 are located along the flagellum. These findings were corroborated by co-immunoprecipitation using dithiothreitol (DTT)–cetyl trimethyl ammonium bromide (CTAB) extracts (i.e., nuclei-free cell extracts), as depicted in Fig. 9, by pulling down aldolase A we retrieved actin, WAS, and Arp2/3 (section C) and vice versa (section D), therefore showing that actin, aldolase A, WAS, and Arp2/3 are part of the same complex.

Discussion

Although glycolysis is an essential biochemical process, recent evidence shows that glycolytic enzymes are multifaceted enzymes participating in additional functions, for instance, in transcriptional regulation, cell motility stimulation, and in apoptosis regulation (Kim & Dang 2005). Aldolase is a glycolytic enzyme that because of its ubiquity interacts with many proteins to either modulate (S100) or distribute its enzymatic activity (dynein LC8, erythrocyte band 3; Zimmer & Van Eldik 1986, Navarro-Lerida *et al*. 2004, Campanella *et al*. 2005). Aldolase A has been also demonstrated in spermatozoa to have a glycolytic role. Thus, the aim of this work was to identify aldolase A in guinea pig sperm and to determine whether it participates in the modulation of actin polymerization.

Actin polymerization is a process that regulates many cellular functions in mammalian cells including cell cycle, cell morphogenesis, and cell migration (Jaffe & Hall 2005). In spermatozoa, actin polymerization is (i) fundamental during both spermatogenesis and on sperm transit through the male and female reproductive tracts (Xiao & Yang 2007), (ii) necessary for the motility acquisition during the gamete maturation (Lin *et al*. 2002) and (iii) essential for sperm incorporation into the egg's cytoplasm (Sánchez-Gutiérrez *et al*. 2002). Furthermore, actin localization in the acrosomal region of several mammalian species suggests a role for it in sperm capacitation and acrosome reaction (Hernández-González *et al*. 2000, Cabello-Agüeros *et al*. 2003, Breitbart *et al*. 2005). In line with previous reports (Krisfalusi *et al*. 2006, Kim *et al*. 2007) that found aldolase A tightly associated with the fibrous sheath of mouse spermatozoa, we show here that a fraction of aldolase A is associated to the spermatic cytoskeleton, and that the enzyme remains associated with structural sperm components even after treatment with the non-ionic detergents (i.e., Brij 36-T, Triton X-100, and Tween 20) we used here, and still after extraction with minimal culture medium (MCM) solution (data not shown). We hypothesized that because of its localization in the

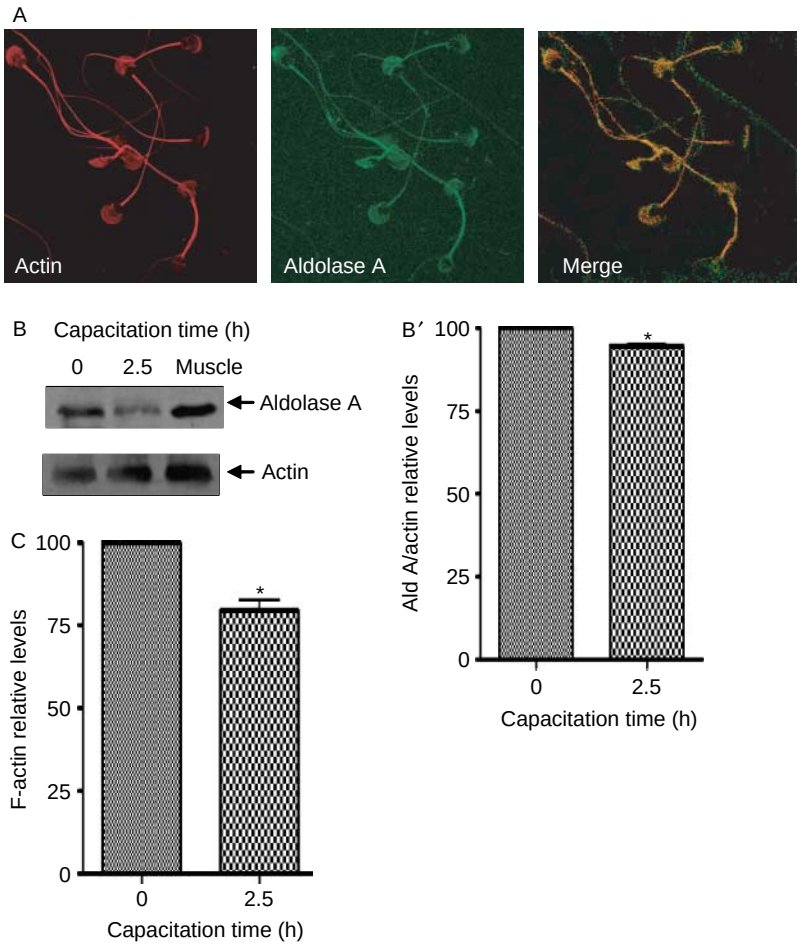


Figure 7 Aldolase A interacts with actin guinea pig spermatozoa. (A) In order to determine any co-localization between actin and aldolase A, fixed spermatozoa were immunostained against aldolase A and stained with TRITC-Ph to detect F-actin; preparations were analyzed by confocal-scanning microscopy. Aldolase A is present within the acrosome and in the entire flagellum and actin was found likewise. (B) The physical interaction of aldolase with actin was determined in both capacitated and non-capacitated spermatozoa, DTT–CTAB extracts were immunoprecipitated with anti-actin antibody, and the precipitates were immunoblotted against aldolase A. Muscle extracts were used as positive control. (B′) The relative amount of aldolase A was evaluated by densitometric analysis to determine aldolase A–actin interaction. The interaction was found to be lower after 2.5 h of capacitation than in non-capacitated spermatozoa (0 h). (C) The levels of F-actin were determined in parallel by FACS. *$P<0.05$ versus control (0 h).

flagellum (where the motor organelle is present) and its tight interaction to the cytoskeleton, aldolase A might participate in sperm motility by modulating actin dynamics. In line with our hypothesis, we found a direct relationship between F-actin concentration and aldolase A–actin interaction.

Because of its enzymatic nature, it could be argued that any possible role of aldolase A in actin polymerization can be attributed to energy production by glycolysis; this is because of the production of local ATP immediately available, for instance, to increase local concentration of G-actin–ATP ready to polymerize. Addition of exogenous aldolase A to SLO spermatozoa had a similar effect on F-actin levels regardless of the presence or absence of glucose. Moreover, the blockade of glycolysis with 2DG in the glucose-free medium did not prevent the effect of aldolase A supplementation on actin polymerization. On the other hand, if we consider that under control conditions (a) the basal ATP concentration in the sperm under TPLG conditions is twice that in TPL glucose-free medium (Mújica *et al.* 1991) and (b) the F-actin relative levels are higher in TPL than in TPLG up to 1 h incubation (data not shown), then differences in ATP levels do not account for our observations on F-actin levels and clearly indicate that actin polymerization might be an independent process of glycolytic ATP. In the same way, the decrease in F-actin levels upon anti-aldolase A antibody treatment, although not significant, is likely to be the effect of the blockade of endogenous aldolase A–actin-polymerizing machinery interaction.

In support of our hypothesis of the role of aldolase A in actin polymerization processes in sperm, we not only found that aldolase A and actin directly interact with each other, (Figs 7 and 9); but we also found that aldolase A interacts with polymerized actin rather than with actin monomers, because CytD treatment decreased the aldolase A–actin interaction (Fig. 8); thus, their interaction depends on the polymerization state of actin. It has been reported that actin polymerization occurs during capacitation (Castellani-Ceresa *et al.* 1993, Brener *et al.* 2003, Dvoráková-Hortová*et al.* 2008). However, we have consistently reported in guinea pig spermatozoa a significant decrease in the levels of polymerized actin within at least 90 min of capacitation (Hernández-González *et al.* 2000, Sánchez-Gutiérrez *et al.* 2002,

Azamar *et al.* 2007); we thus consider this decrease as a species specific mechanism of partial actin depolymerization. Nonetheless, we have reported the formation of a post-acrosomal cone that involves actin polymerization at the boundary of capacitation/acrosomal reaction events (Sánchez-Gutiérrez *et al.* 2002). Hence, we consider that polymerization processes occur in different subcellular compartments during sperm capacitation.

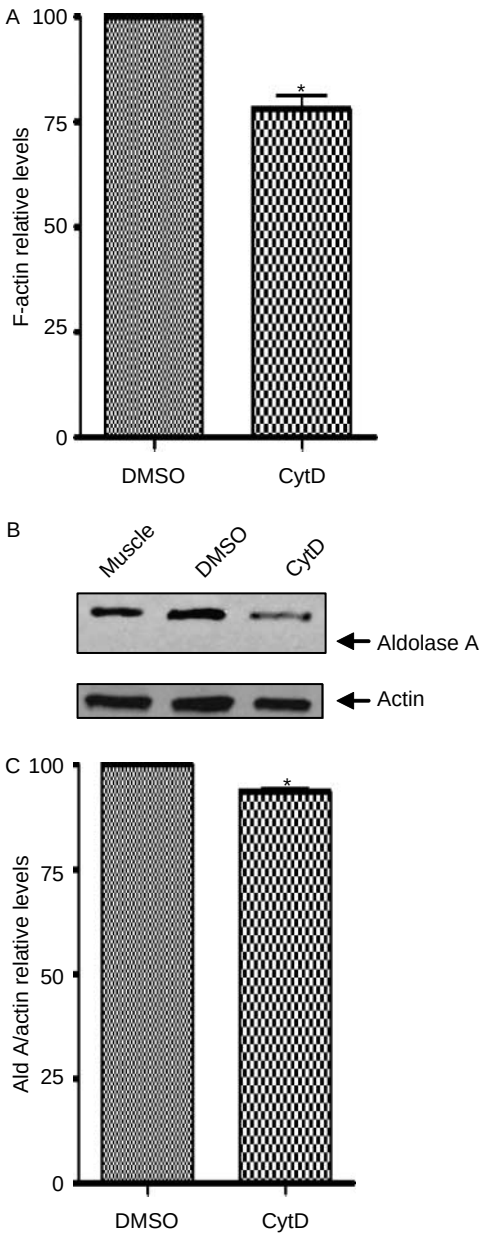


Figure 8 The interaction of aldolase A with actin depends on the polymerization state of actin. Spermatozoa were capacitated in the presence of CytD 50 μM or its vehicle (DMSO) for 2.5 h at 37 °C. The effects of CytD on the levels of F-actin (A) and on the interaction of aldolase A with actin (B) were evaluated. (A) One portion of the CytD-treated sample was fixed to be stained with TRITC-Ph to determine the relative concentration of F-actin by FACS. The graph shows that CytD treatment induced a decrease in polymerized actin levels comparison with control (DMSO). (B) The remaining sample was processed to obtain DTT–CTAB extracts, subjected to immunoprecipitation with anti-actin antibody, and immunoblotted against aldolase A. (C) The graph represents the aldolase A relative levels measured by densitometry to determine aldolase A–actin interaction. *$P<0.05$ versus control (DMSO).

Based on the above results, we attempted to define the possible mechanism by which aldolase A modulates actin polymerization. WAS, Arp2/3, prophilins, CDC42, and RHOA/B regulate actin dynamics, modulate cell motility, and recently were identified in guinea pig spermatozoa (Delgado-Buenrostro *et al.* 2005). Because aldolase A is reported to have the ability to interact with WAS (Buscaglia *et al.* 2006, St-Jean *et al.* 2007), we decided to explore the possibility that aldolase A recruits WAS and therefore promotes actin polymerization. We found that indeed aldolase A is located in the same compartments as WAS and Arp2/3 and that it interacts physically with them as a complex in the sperm actin cytoskeleton. Likewise, it was described previously that the interaction domain of aldolase A for actin is located within the 32–52 residues region of aldolase A (O'Reilly & Clarke 1993), whereas the interaction domain of WAS for aldolase A is probably located within the C terminus of WAS (Buscaglia *et al.* 2006).

Aldolase A is a tetrameric enzyme that has one binding site per monomer for actin (Wang *et al.* 1997), giving it the ability to cross-link proteins, and it also has the role of *in situ* energy production, which can be used by axoneme structures. It is possible that aldolase function as scaffold between actin and the actin-polymerizing machinery, and the binding of aldolase to cytoskeletal structures provokes conformational changes (Heyduk *et al.* 1991) to fit WAS to actin in complex.

We propose here that aldolase A may have a dual role: on one hand, to ensure proper flagellar rigidity guarantying the stabilization of the flagellar cytoskeleton, on the other hand, to guarantee the energy provision for motility to sustain a propel movement. It is very likely that aldolase A, after recruiting the actin-polymerizing machinery, remains attached to the cytoskeleton to avoid any further changes in energy supply, even though mitochondrial ATP is present. In addition, aldolase A might indirectly regulate signaling pathways (for instance, by producing new docking sites for other proteins, producing new microdomains or modulating the local availability of ATP for the activity of several kinases). The latter indeed may affect capacitation and/or the acrosomal reaction. Cabello-Agüeros *et al.* (2003) showed actin cytoskeletons in the outer acrosomal and plasma membranes, which were disrupted during capacitation. We proposed that the aldolase A–actin interaction might work to stabilize the membrane's cytoskeletons, because this interaction decreases with capacitation. Currently, in our laboratory, we are studying the non-glycolytic role of aldolase A in the acrosomal reaction.

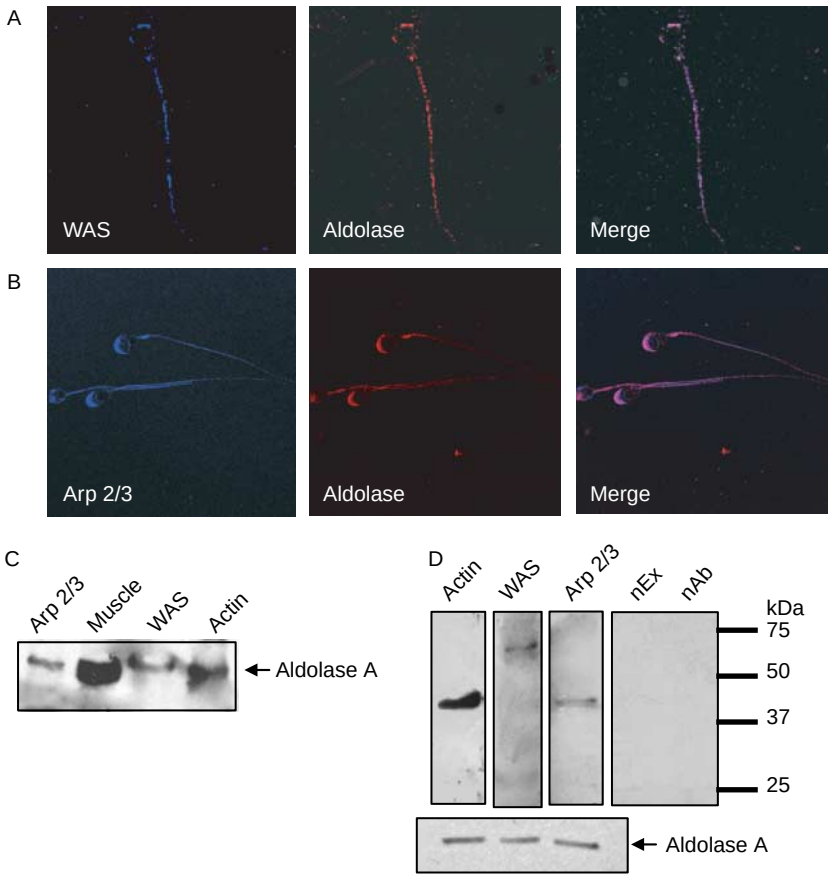


Figure 9 Aldolase A interacts with WAS and Arp2/3 in addition to actin. Co-localization of aldolase A with WAS (A) and Arp2/3 (B) in fixed spermatozoa immunostained against aldolase A. Preparations were analyzed by confocal-scanning microscopy. Aldolase A is present within the acrosome and in the entire flagellum, Arp2/3 and WAS likewise. The physical interaction of aldolase A with WAS and Arp2/3 was determined by co-immunoprecipitation. (C) DTT–CTAB extracts were immunoprecipitated with anti-actin, anti-WAS, or anti-Arp2/3 antibodies. The precipitates were immunoblotted against aldolase A. Aldolase A was found to be present in all immunoprecipitates tested. Muscle extracts were used as positive control. (D) Inverse co-immunoprecipitation was performed to confirm aldolase A interactions. DTT–CTAB extracts were immunoprecipitated using anti-aldolase A. The precipitates were immunoblotted to detect actin, WAS, and Arp2/3. Two internal controls were used: immunoprecipitation reaction without DTT–CTAB extracts (nEx) and reaction without precipitating antibody (nAb). Homogenous loading was corroborated by measuring aldolase A levels in the immunoprecipitates.

Materials and Methods

Chemicals and antibodies

Unless otherwise stated, all the reagents used here were from Sigma-Aldrich. Brij 36-T was obtained from PROTINUS (México City, México). Polyclonal antibodies (anti-WAS, anti-Arp2/3, and anti-aldolase) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin monoclonal antibody (Moreno-Fierros *et al*. 1992, Rosas *et al*. 2007) was provided by Dr JM Hernández. All secondary antibodies (HRP, TRITC, Cy5, and FITC conjugated) were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA). ECL kit was purchased from Amersham Biosciences. Protein A agarose, protease inhibitors mix (Complete), and purified aldolase A were purchased from Roche.

Isolation of guinea pig spermatozoa

Sexually mature guinea pigs were handled following the ethical regulations established under NOM-062-ZOO-1999 for animal care, management, and treatment emitted by the Mexican government. Animals were anesthetized and killed by cervical dislocation. Mature spermatozoa were obtained by perfusion of the lumen of both *vas deferens* and *cauda epididymis* with 154 mM NaCl at 37 °C, and then rinsed as previously described by Mújica & Valdes Ruíz (1983).

Identification of aldolase A in sperm extract

In order to obtain membrane-free spermatozoa with the cellular structures exposed (PT and flagellum), 1.2% Brij 36-T detergent was added up to acrosome solubilization and rinsed (de Lourdes Juárez-Mosqueda & Mújica 1999). Then actin-associated proteins were destabilized using MCM solution as follows: 35×10^6 Brij-treated cells were incubated at 37 °C for 10 min in 1 ml of MCM solution (105.8 mM NaCl, 1.71 mM $CaCl_2$, and 25.07 mM $NaHCO_3$, pH 7.8; de Lourdes Juárez-Mosqueda & Mújica 1999) containing the mix of protease inhibitors 'Complete' (used following the manufacturer's instructions) and 1 mM phenylmethylsulphonyl fluoride (PMSF). The sample was centrifuged at 5000 ***g*** for 3 min and the supernatant was collected and concentrated by filtered centrifugation using Amicon Ultra tubes and centrifugation at 3220 ***g*** at 4 °C for 50 min. Protein concentration was determined by Bradford assay. The extracts obtained in this way were separated by electrophoresis in 12% tricine–SDS–PAGE (Schagger & von Jagow 1987) and the gel was stained with Coomassie Brilliant Blue to reveal the protein bands. The selected bands (from 25 to 50 kDa) were excised from the gel and processed for proteomic analysis.

Mass spectroscopy and protein identification

Proteins selected for proteomic analysis were digested with 25 mM trypsin solution in the minimum volume rqeuired to

cover the gel pieces and incubated at 37 °C overnight; digested peptides were prepared as previous reports (Shevchenko *et al.* 1996). Briefly, digested peptides were first mixed (v/v) with a matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid and 0.1% trifluoroacetic acid (TFA) in 1:1 H_2O/acetonitrile), then loaded into a well of the target plate and dried. Peptide mass fingerprinting was performed in the Ettan MALDI-Tof Pro (Amersham Biosciences). MASCOT search program (www.matrixscience.com) was used to identify peptide sequences from the mass spectra. $^*P<0.05$ was regarded as a significant difference.

Indirect immunofluorescence

Spermatozoa were fixed in 1.5% formaldehyde–PBS and washed with PBS. One drop of the fixed cells was spread on glass slides, permeabilized with Triton X-100 0.1% in PBS for 30 min, and incubated with the primary antibody diluted in 1% BSA–PBS in a humid chamber overnight at 4 °C. After extensive PBS rinses, slides were incubated with the appropriated secondary labelled antibody diluted in 1% BSA–PBS. When F-actin determination was performed, it was revealed by using TRITC-labeled phalloidin (TRITC-Ph). Slides were washed and then mounted. The cells were visualized using a Leica TCS SP2 confocal microscope.

Subcellular fractionation

Freshly collected spermatozoa were centrifuged and resuspended in lysis buffer (LB: 50 mM Tris–HCl, pH 7.4, 1 mM EGTA, 1 mM PMSF, Complete, 10 mM sodium orthovanadate, 25 mM sodium fluoride, and 0.05% Triton X-100), then sonicated using a Dagger 130 Watt model Ultrasonic Processor set at an amplitude of 40 for 30 s at 4 °C. The sperm suspension was centrifuged for 15 min at 5000 ***g*** at 4 °C to separate heads and flagella (pellet) from cytosol and membranes (supernatant). The supernatant was ultracentrifuged to separate cytosol from membranes at 100 000 ***g***, whereas heads and flagella were separated as follows: the pellet was washed three times with LB and layered on an isotonic discontinuous Percoll gradient (90, 80, 70, and 50%), centrifuged at 3220 ***g*** for 50 min at 4 °C. Pure flagella were obtained from the 70–80% interface of the gradient and the head fraction with PT was localized at the bottom, subsequently, the PT and flagella were separated and solubilized with DTT–CTAB treatment as described bellow.

DTT–CTAB extracts

The DTT–CTAB extraction solubilizes proteins of acrosome, PT, and flagellum (Hernández-Montes *et al.* 1973). Spermatic suspension (35×10^6 cell/ml) was treated with DTT 25 mM for 20 min at 4 °C in constant agitation in the presence of complete protease inhibitor, then 1.2% CTAB (a cationic detergent) was added after about 30 min, and centrifuged at 5000 ***g*** for 15 min. The pellet was discarded and the supernatant was concentrated as mentioned above and stored frozen until use.

Electrophoresis and western blotting

SDS–PAGE was performed using 12% poly(acrylamide) gels. The separated proteins were electrotransferred onto nitrocellulose membranes (Towbin *et al.* 1979) to be identified by immunoblotting: membranes were blocked with a suspension of 5% skimmed milk in TBS/T (50 mM Tris, 104 mM NaCl, pH 7.6, and 0.1% Tween 20) for 1 h; then, membranes were incubated with the primary antibody overnight at 4 °C, washed with TBS/T, and then incubated at 37 °C for 1 h with HRP-conjugated appropriated secondary antibody. Primary and secondary antibodies were diluted in blocking solution following the manufacturer's instructions. Once the membranes were washed, the bands were developed by chemiluminescence's ECL kit.

Immunoprecipitation assay

Stated antibody (anti-WAS, anti-Arp2/3, anti-aldolase, or anti-actin) and protein A agarose (3 and 7 µl respectively) were incubated at 4 °C for 5 min, then mixed with 3 mg protein of DTT–CTAB extracts, and kept overnight in agitation at 4 °C. The immune complexes were collected by centrifugation at 5000 ***g*** for 10 min and washed extensively with buffer solution (50 mM Tris–HCl, pH 7.5, 0.1% Tween 20, and protease inhibitors). The precipitates were processed by Western blotting. Interactions were confirmed by inverse immunoprecipitation using anti-aldolase antibody and revealed with each of the three other antibodies. We used muscle or brain extracts as positive controls for aldolase.

Aldolase and anti-aldolase antibody sperm treatment

Sperm treatment with aldolase or the anti-aldolase antibody was performed in SLO-permeabilized spermatozoa (Hernández-González *et al.* 2000). Mature spermatozoa were suspended in TPLG (116.73 mM NaCl, 2.8 mM KCl, 11.9 mM $NaHCO_3$, 0.25 mM sodium pyruvate, 20 mM lactic acid, and 5.56 mM glucose, pH 7.6; Rogers & Yanagimachi 1975) and permeabilized with 400 UI SLO/10×10^6 spermatozoa/ml. The sample was divided into three fractions as follows: (1) maintained in TPLG (control), (2) added with 50 µg/ml aldolase, and (3) treated with 5 µg/ml anti-aldolase antibody. After 1.5 h incubation, in each sample, the relative concentration of F-actin was evaluated.

Aldolase and 2DG sperm treatment in TPL

Sperm treatment with aldolase or 2DG was performed in SLO-permeabilized spermatozoa (see above). Mature spermatozoa were suspended in TPL and permeabilized. The sample was divided into four fractions as follows: (1) maintained in TPL, (2) added with 50 µg/ml aldolase A, (3) in TPL plus 5 mM 2DG, and (4) in TPL with 5 mM 2DG plus aldolase A. After 30 min, aliquots were fixed to evaluate the relative concentration of F-actin.

CytD sperm treatment and sperm capacitation

Sperm capacitation was performed in MCM with pyruvate and lactate (105.8 mM NaCl, 1.71 mM $CaCl_2$, 25.07 mM

$NaHCO_3$, 0.25 mM sodium pyruvate, and 20 mM lactic acid, pH 7.8; Rogers & Yanagimachi 1975). Capacitating sample was divided in four parts: (1) used for incubation with 50 μM CytD in DMSO, (2) incubated with 0.0025% DMSO (Ctl), (3) taken at the beginning of capacitation and referred as time 0 (Ctl), and (4) capacitated for 2.5 h. All samples were handled for both DTT–CTAB extraction and F-actin determination.

Quantification of F-actin by flow cytometry

Polymerized actin staining was performed as previously reported (Azamar *et al*. 2007), briefly, cell suspensions were fixed and then rinsed. Cells were centrifuged at 600 ***g*** for 3 min and the pellet was resuspended in 50 mM NH_4Cl, incubated for 10 min, rinsed once with PBS, and permeabilized with 0.05% Tween-20 in PBS during 7 min. The permeabilized cells were washed twice in PBS quantified and adjusted to 88.8×10^6 cells/ml in PBS. Triplicate sample aliquots (2×10^6 cells/22.5 μl) were incubated at room temperature with 7.6 μM TRITC-Ph under dark conditions for 30 min. Cells were diluted with 1 ml of PBS, centrifuged and then resuspended in 0.5 ml PBS, and analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA). We used cells without staining as a negative control.

Statistical analysis

Data shown throughout are mean ± S.E.M. of at least three individual experiments. Data were normalized to their respective control experiments. Statistically significant differences among conditions were analyzed by Student's *t*-test (Figs 6A, 7B′ and C and 8A and B) and one-way ANOVA using Dunnett's test (Figs 5A and 6B).

Declaration of interest

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

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