The expression of calpain 1 and calpain 2 in spermatogenic cells and spermatozoa of the mouse

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Abstract

There is some evidence suggesting that Ca^{2+} is involved in processes that occur during the development and function of spermatozoa. Calcium-dependent proteins, such as calmodulin, are expressed during mammalian spermatogenesis further suggesting that Ca^{2+} takes part in its regulation. However, the precise roles of Ca^{2+} in spermatogenesis remain to be elucidated. Calpains are a family of Ca^{2+} -dependent cysteine proteases whose members are expressed ubiquitously or in a tissue-specific manner. Calpain has been demonstrated to mediate specific Ca^{2+} -dependent processes including cell fusion, mitosis and meiosis. We herein followed the expression pattern of calpain's ubiquitous isoforms, 1 and 2, throughout spermatogenesis at the RNA and protein levels by RT-PCR and Western blotting analysis. Both RNA and protein studies revealed that these isoforms are expressed in all spermatogenic cells. The expression of calpain 1 levels is slightly higher in spermatocytes entering the meiotic phase. Both calpain isoforms are also expressed in mouse spermatozoa and are localized to the acrosomal cap. Inducing capacitated spermatozoa to undergo the acrosome reaction in the presence of a selective calpain inhibitor significantly reduced the acrosome reaction rate in a dose-dependent manner. Thus, calpain, a pluripotential protease with numerous substrates, may serve as an effector in more than one pathway in the complex process of spermatogenesis and in the events preceding fertilization, such as the acrosome reaction.

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Introduction

Calcium has been shown to play a pivotal role in processes that occur during the development and function of spermatozoa. Spermatogenesis involves a series of mitotic divisions by spermatogonia that give rise to spermatocytes, and a pair of meiotic divisions by spermatocytes to yield the spermatids. Transient increases in intracellular Ca^{2+} concentration serve as a key signal for the initiation and termination of mitosis in somatic cells (Whitaker & Patel 1990) and the resumption of meiosis in mammalian oocytes (Kline & Kline 1992, Berrios *et al.* 1998). Spermatogenic cells are known to possess a variety of Ca^{2+} channels (Hagiwara & Kawa 1984). Changes in Ca^{2+} concentrations in spermatogenic cells in response to alkalization have been reported (Santi *et al.* 1998) and it has been demonstrated that Ca^{2+} signaling occurs in round spermatids (Berrios *et al.* 1998). Moreover, calcium-dependent proteins, such as calmodulin, are present during mammalian spermatogenesis (Slaughter *et al.* 1989, Watanabe *et al.* 1994), further suggesting that Ca^{2+}

is involved in the regulation of spermatogenesis. However, the precise roles of Ca^{2+} in spermatogenesis remain to be elucidated.

Binding of spermatozoa to the zona pellucida (ZP) during fertilization activates a G-protein-coupled signaling pathway. This triggers a Ca^{2+} influx and leads to an increase in the intracellular Ca^{2+} concentration that induces the acrosome reaction (AR), enabling the spermatozoon to penetrate the ZP and fertilize the egg (Bailey & Storey 1994, Abou-Haila & Tulsiani 2000). Furthermore, Ca^{2+} -mediated signaling leads to fusion of the spermatozoon plasma membrane with the oolemma (Yanagimachi 1989).

Calpains constitute a superfamily of intracellular calcium-dependent neutral cysteine proteases whose members are widely expressed in a variety of tissues and cells (Murachi *et al.* 1981, Sorimachi *et al.* 1993a,b, Carafoli & Molinari 1989). Two ubiquitous members of the calpain family, calpain 1 (CANP1) and calpain 2 (CANP2), have been well characterized in many cells. They are the large

catalytic subunits (80 kDa) that heterodimerize with calpain 4, the small regulatory subunit (30 kDa) of the holoenzyme. However, they differ considerably in the calcium concentrations required for their activation *in vitro*. Calpain 1 (μ calpain) is activated at micromolar calcium concentrations and calpain 2 (m calpain) at millimolar concentrations (Croall & Demartino 1991, Sorimachi *et al.* 1994). The need for calpain in normal development has been demonstrated in transgenic mice lacking the calpain 4 regulatory subunit. These mice die during embryonic development apparently due to defects in vascular development (Arthur *et al.* 2000, Perrin & Huttenlocher 2002). The non-ubiquitous tissue-specific calpains are most likely related to the specific functions of the organs in which they are predominantly expressed.

Calpains have been implicated in diverse cellular signaling pathways mediated by calcium. These include cytoskeleton remodeling, cell cycle regulation, cell differentiation and membrane fusion (Croall & Demartino 1991, Carafoli & Molinari 1998, Barnoy *et al.* 1999). Calpains also participate in several phases of mitosis and meiosis (Schollmeyer 1988, Lane *et al.* 1992, Santella *et al.* 1998). They are suggested to play a role in regulating meiosis by inactivating c-mos proto-oncogene product, pp39, hence leading to resumption of the second meiosis in MII-arrested *Xenopus* eggs (Sagata *et al.* 1989, Watanabe *et al.* 1989). Injection of calpain into the nuclei of prophase-arrested starfish oocytes induced re-initiation of meiosis by mediating cytoskeletal remodeling (Santella *et al.* 1998, 2000). In the rat egg, we demonstrated a correlation between calpain activation and egg activation at fertilization, where inhibiting calpain activity impeded resumption of meiosis (I Ben-Aharon, K Haim, R Shalgi and D Ben-Yosef, unpublished observations).

In mammals, calpain 2 was localized to the acrosomal cap of porcine sperm (Schollmeyer 1986), and Rojas *et al.* (1999) demonstrated the presence of a calpain-calpastatin system in human spermatozoa. Both isoforms are expressed in spermatozoa of the *Cynomolgus* macaque, and ultrastructural studies indicated they were localized between the plasma membrane and the outer acrosomal membrane (Yudin *et al.* 2000). Calpain inhibitors used during *in vitro* fertilization impaired the ability of human sperm to fuse and penetrate the oocyte (Rojas & Morreti-Rojas 2000). The putative role of calpain in human spermatozoa prior to and during fertilization along with its possible involvement throughout meiosis in eggs had prompted us to examine further the expression of calpain ubiquitous isoforms in the spermatogenic germ line in the mouse. We have demonstrated the expression of both mRNA and protein during spermatogenesis, and localized calpain 1 and calpain 2 in mouse spermatozoa. To further elucidate calpain's possible role in spermatozoa, we introduced capacitated spermatozoa to a highly selective calpain inhibitor, calpeptin, and found a dose-dependent reduction in acrosome reaction rate.

Materials and Methods

Tissue collection

Spermatozoa

Cauda epididymides from 10-week-old C57BL/6 mice were punctured and the spermatozoa were collected into an ice-cold buffer containing 20 mM Tris, 130 mM NaCl, 2 mM EGTA, pH 7.15 (TN/EGTA; Kalab *et al.* 1994). Spermatozoa were centrifuged at 1000 rpm for 10 min (4°C) and the pellet was then re-suspended in TN/EGTA buffer. Aliquots containing 1×10^6 spermatozoa were stored at -70°C until use.

Spermatogenic cells

To obtain enriched populations of certain spermatogenic cell types we have employed the method developed by Bellvé *et al.* (1977a) for RNA and protein studies by harvesting testes at specific time points throughout the first wave of spermatogenesis. The first wave of spermatogenesis in the mouse is from day 10 to day 30 after birth. Testes were therefore collected from 6- to 30-day-old CD-1 mice and crude protein extracts were isolated by homogenizing in lysis buffer (20 mM HEPES pH 7.4, 140 mM NaCl, 0.1% Triton X-100, and Complete Proteinase Inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) at a concentration of 50 mg (wet weight)/ml. All animal procedures were approved by the Tel-Aviv University and the National Institute of Environmental Health Sciences Animal Care and Use Committees before being used.

Immunoblotting procedures

Aliquots of spermatozoa or spermatogenic cell lysates corresponding to 0.5 mg (wet weight) were mixed with Laemmli buffer (New England BioLabs, Beverly, MA, USA) and boiled for 5 min. Proteins were fractionated by one-dimensional sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gels at 30 mA per gel in 25 mM Tris, 192 mM glycine and 0.1% SDS (Laemmli 1970). Gels were electro-transferred overnight onto nitrocellulose membranes (Amersham, UK) or Immobilon nylon membranes (Millipore Corp., Bedford, MA, USA) at 40 mA in 25 mM Tris, 192 mM glycine and 20% methanol transfer buffer (Towbin *et al.* 1979). Approximate molecular masses were determined by comparison with the migration of prestained protein standards (Amersham, UK or BioRad, Hercules, CA, USA). Blots were blocked in 150 mM NaCl, 10 mM Tris, 0.5% Tween 20 (TBS/Tween) and 5% nonfat dry milk (referred to as 'blocking solution') for 2 h at room temperature. Membranes were then incubated for 18 h at 4°C with either a monoclonal antibody to calpain 1 (208728; Calbiochem, San Diego, CA, USA) diluted 1:100 in blocking solution, or a monoclonal antibody to calpain 2 (208729; Calbiochem) diluted 1:500 in blocking solution. These antibodies were also used in the immunofluorescence and

immunoblotting procedures described below. Following 3 washes in blocking solution, blots were incubated for 1 h in donkey anti-mouse IgG antibody conjugated to horseradish peroxidase (Jackson, Immunoresearch Laboratories, West Grove, PA, USA) diluted 1:5000 in blocking solution. The blots were washed in TBS/Tween solution and proteins detected using the Supersignal chemiluminescence detection system (Pierce, Rockford, IL, USA) and autoradiographed. Each set of experiments was repeated at least three times.

Immunofluorescence procedures

Spermatozoa collected from the cauda epididymides of 10-week-old C57BL/6 mice were suspended in Dulbecco's phosphate buffered saline (DPBS) supplemented with 3% fetal calf serum. Cells were permeabilized by slowly dripping pellets along the side of a test-tube immersed in ice. Samples were warmed to room temperature and the procedure was then repeated (Jones *et al.* 1983). Permeabilized spermatozoa were incubated for 2 h in the presence of monoclonal antibodies (diluted 1:75) to calpain 1 and calpain 2 and washed three times in blocking solution. Samples were then transferred into blocking solution containing Cy3-labeled donkey antibody to mouse IgG (1:500) and 1 µg/ml of a DNA-specific fluorochrome (Hoechst 33342; Sigma) for a further 30 min incubation in the dark. Spermatozoa were washed 3 times in blocking solution, and then mounted between a slide and a coverslip.

Spermatozoa were visualized and photographed using a 40× NA1.2 planapochromat water immersion lens with a Zeiss (Oberkochen, Germany) LSM 410 confocal laser scanning microscope (CLSM) equipped with a 25-mW Krypton-Argon laser, a 10-mW helium-neon laser (488, 543 and 633 maximum lines), and a UV laser (Coherent Inc. Laser Group, Santa Clara, CA, USA). For localization of either calpain, spermatozoa were scanned using the CLSM through the Z-axis to select a section at the equatorial plane of the spermatozoon. Data was obtained from visualizing 6 representative spermatozoa in each experiment. Each set of experiments was repeated at least three times.

Immunohistochemical procedures

Testes from juvenile and adult CD-1 mice were fixed in Bouin's solution, paraffin embedded, and sections were mounted on Superfrost/Plus slides (Daigler and Co., Wheeling, IL, USA). Deparaffinized sections were treated with 3% H₂O₂ to inactivate endogenous peroxidase, blocked for 1 h with MOM blocking reagent (Vector Labs, Burlingame, CA, USA), and then incubated with antibodies to calpain 1 or calpain 2 (1:300 in diluent reagent, Vector Labs) for 30 min. Negative controls were incubated with diluent only. Slides were then incubated with biotinylated anti-mouse IgG in diluent (1:250, Vector) for 30 min, followed by ABC reagent (Vector) for 30 min, and rinsed in water. The peroxidase activity was visualized after

exposing slides to diaminobenzidine (DAB; Sigma) for 5 min. Slides were rinsed with TBS and coverslips were mounted with glycerol. Each set of experiments was repeated at least three times.

Semi-quantitative reverse transcriptase PCR (RT-PCR)

Primers specific for mouse *Canp1*, *Canp2*, and β-actin were designed (Table 1). Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) from testes collected on postnatal days 6 through 34. The RNA (2 µg) was treated with amplification grade DNase I (Invitrogen) prior to addition of 200 U SuperScript II RNase H⁻ reverse transcriptase (Invitrogen) according to the supplier's recommendations. The RT products (1 µl) were then used as PCR templates. The PCR cycle parameters were 30 s at 94°C, then 20 s at 94°C, 20 s at 58°C, and 20 s at 72°C for 30 cycles using AmpliTaq polymerase. These reactions were repeated three times and the average concentration (ng/µl) determined (see Fig. 3). Samples were analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), a microfluidic system using microfabricated chip technology. This method normalizes the mean values obtained for calpain isoforms for each age group by using β-actin values as standard, and depicts a densitometric presentation for the results.

Effect of calpain inhibitor on the acrosome reaction

Cauda epididymides and part of the vas-deferens were collected from 10-week-old C57BL/6 mice, and minced in 1 ml modified Krebs-Ringer bicarbonate medium (HM; Visconti *et al.* 1995). Spermatozoa were discharged from the epididymal lumen for 15 min at 37°C, washed, and left for 'swim-up' for an additional 20 min at 37°C. Spermatozoa were collected, counted and resuspended (10⁶ cells/ml) in capacitation medium (HMB; Visconti *et al.* 1995) for 1.5 h at 37°C; 5% CO₂ in air. Spermatozoa were incubated in the presence of the calpain inhibitor, calpeptin (03340051; Calbiochem) for 10 min. Spermatozoa were then exposed to 10 µM Ca²⁺ ionophore (A23187; Calbiochem), for the induction of the acrosome reaction, for 20 min in the presence of calpeptin. An aliquot of spermatozoa (10⁶ cells) was smeared on a glass slide and allowed to air dry. Spermatozoa were then permeabilized by methanol for 15 min at room temperature, washed three times at 5-min intervals with 25 mM Tris-buffered saline pH 7.6 (TBS) and air dried. FITC-conjugated *Pisum sativum* agglutinin (PSA;

Table 1 Primers specific for calpain 1, calpain 2 and β-actin.

Primer		cDNA (bp)	Genomic (bp)
Calpain 1	5' cgggttgaggagggtgatga 3' atcacgcttcaagtgacag	189	~2980
Calpain 2	5' caccctcacctgtgactctataa 3' atcctcctcatcttctt	163	~2310
Mouse β-actin	5' ttccgatgccctgaggctcttc 3' ctgctgatccacatctgctggaa	314	~525

Sigma) (Mendoza *et al.* 1992) was used on air-dried spermatozoa smears to trace microscopically acrosome-reacted spermatozoa. Cells were incubated with FITC-PSA (6.25 µg/ml in TBS) for 0.5 h, washed with H₂O, and mounted with FluoroGuard Antifade (Bio-Rad Lab).

Data analysis

Spermatozoa which exhibited green staining over the acrosomal cap were considered as acrosome intact whereas those with only equatorial green staining (i.e. no tri-dimensional green structure) or no staining at all were considered acrosome reacted. A total of three experiments were performed. For each experiment at least 100 cells per slide were evaluated. Acrosome reaction rate was determined by calculating the acrosome reacted spermatozoa/total spermatozoa observed; we then excluded the spontaneous acrosome reaction (AR) rate at the end of capacitation (control without ionophore). The significance of differences between experimental groups was determined by paired *t*-test. *P* < 0.01 was considered significant.

Results

Calpain 1 and calpain 2 proteins are expressed in mouse spermatozoa

Western blot analysis was performed using monoclonal antibodies specific for calpain 1 or calpain 2 on extracts of spermatozoa from the cauda epididymis. An 80 kDa band was seen for each isoform (Fig. 1A,B), consistent with the reported molecular mass in somatic cells and in macaque spermatozoa (Yudin *et al.* 2000). These antibodies were also used for immunofluorescence confocal microscopy to determine the localization of calpain 1 and calpain 2 in mouse spermatozoa. Both calpain 1 and calpain 2 were shown to be localized to the acrosome (Fig. 2A,B).

Figure 1 Expression of calpain 1 and calpain 2 in mouse spermatozoa. Sperm proteins were resolved by SDS-PAGE analysis and transferred onto PVDF membrane. The blots were incubated with monoclonal antibodies to either calpain 1 (1:100; A) or calpain 2 (1:100; B), followed by secondary donkey anti mouse antibody (1:10 000) and ECL detection. The arrow indicates calpain bands at 80 kDa, as calculated from the migration of known protein standards.

Figure 2 Localization of calpain 1 and calpain 2 in mouse spermatozoa. Cold permeabilized epididymal spermatozoa were labeled with monoclonal antibodies to either calpain 1 (1:75; A, B) or calpain 2 (1:75; C, D), followed by a Cy3-labeled secondary antibody (1:500). Spermatozoa stained only with secondary antibody and Hoechst served as controls (E, F). The localization of calpain 1 is shown at B and of calpain 2 at D. Phase light micrographs of the same spermatozoa are shown at A and C. Bar = 10 µm.

Canp1 and Canp2 mRNAs are present during spermatogenesis

Using gene-specific primers, RT-PCR generated 189 bp products for *Canp1*, 163 bp products for *Canp2*, and 314 bp products for β -actin (Fig. 3). We had employed the Agilent 2100 Bioanalyzer, a microfluidic system that provides highly reproducible quantification and sizing, which replaces slab gel electrophoresis (Gottwald *et al.* 2001). This system was used, following the manufacturer's protocol, to analyze the double-stranded cDNA products essentially to replace the need for Northern blotting experiments. Nonetheless, Northern blotting procedures have been employed as well (data not shown). Thus, although the results were concomitant with the RT-PCR results, the signal of the Northern blotting appeared faintly, and therefore could not be used as the sole method. Although it appears visually that *Canp1* and *Canp2* are expressed at a relatively constant level throughout spermatogenesis, the mean values of three experiments calculated from band densities compared with the β -actin controls suggested that a minor increase in *Canp1* levels occurs during post-natal days 18 to 20 (data not shown).

Calpain 1 and calpain 2 protein expression in adult and juvenile testes

Immunohistochemistry was used to determine the expression patterns of calpain 1 and calpain 2 in the adult testis. Both isoforms were seen to be expressed throughout spermatogenesis (Fig. 4A,B). However, a higher level of

Figure 3 Expression of *Canp1* and *Canp2* mRNA throughout spermatogenesis. Semi-quantitative RT-PCR amplicons of *Canp1*, *Canp2* and β -actin (control) were detected using the Agilent 2100 instrument. Extracts of total RNA from testes of mice from 6 to 34 days of age served as template for synthesizing first-strand cDNA.

Figure 4 Expression of calpain 1 and calpain 2 in adult and juvenile mice testes. Immunohistochemistry of sections of testes from adult (A–C) and 10-day-old (C–E) mice was performed using monoclonal antibodies to calpain 1 (1:300; A, D), calpain 2 (1:300; B, E) or no primary antibody (C, F) followed by peroxidase-labeled secondary antibody and DAB substrate. The arrows point to the intensely stained cells. Bar = 100 μ m.

calpain 2 labeling was noted in specific cells at the periphery of the tubule (Fig. 4B). The location and stage of spermatogenesis suggested that these cells were at the beginning of the meiotic phase. To further investigate the meiotic stage, we used immunohistochemistry on testes collected daily from juvenile mice of 6 to 12 days of age. Prior to day 10, the immunostaining was homogenous. However, on day 10 there was enhanced immunostaining of some cells (Fig. 4E) and on days 11 and 12, the number of strongly stained cells increased. Calpain 1 also showed a slight increase in staining in cells corresponding to zygotene spermatocytes on day 10 (Fig. 4D).

Western blot analysis of lysates from testes isolated at two-day intervals, from day 6 to day 30 after birth, which is already post-pubertal, indicated that total calpain 2 protein levels were relatively uniform as the first wave of spermatogenesis progressed throughout spermatogenesis and spermiogenesis (Fig. 5B). However, the level of calpain 1 protein increased significantly on days 20 and 22

(Fig. 5A), correlating with the increased level of mRNA seen on days 18 and 20.

Effect of calpain inhibitor on the acrosome reaction

To further explore calpain's possible involvement in the acrosome reaction (AR), we examined the acrosome reaction rate in the presence of several concentrations of the calpain inhibitor, calpeptin. As described in the Materials and Methods section, capacitated spermatozoa were incubated with calpeptin for 10 min at the end of the capacitation period. They were then exposed to calcium ionophore for 20 min in the presence of the inhibitor. We used the immunofluorescence method described by Mendoza *et al.* (1992) to distinguish between acrosome-reacted and acrosome-intact spermatozoa, as depicted in Fig. 6A. Inhibition of AR rate was detected in a dose-dependent manner. Ionophore-induced AR rate reached 33.9% in the control group (we excluded the spontaneous AR rate at the end of capacitation; Fig. 6B). Calpeptin significantly reduced the AR in a dose-dependent manner, whereas a concentration of 125 μ M gained the maximal inhibitory effect. The same assay performed in bovine spermatozoa as a control, produced correlating results to the assay carried out in mice spermatozoa. At a concentration of 125 μ M, calpeptin inhibited the AR rate by 71% compared with control values (data not shown).

Figure 5 Expression of calpain 1 and calpain 2 throughout spermatogenesis. Testes were collected from 6- to 30-day-old mice. Crude protein extracts were prepared by homogenization in SDS lysis buffer, subjected to PAGE analysis, and transferred onto an Immobilon membrane. The blots were incubated with monoclonal antibody to either calpain 1 (1:100; A) or calpain 2 (1:100; B), followed by ECL detection. The arrows point to bands at 80 kDa, as calculated from the migration of known protein standards.

Discussion

Our study presents, for the first time, an explorative view of calpain ubiquitous isoforms, 1 and 2, beginning with the early course of spermatogenesis to spermatozoa, and leading to fertilization.

In preceding studies performed in rat eggs, we have established a correlation between calpain activation and egg activation at fertilization, while the inhibition of calpain activity hindered resumption of the second meiosis as occurs during fertilization (I Ben-Aharon, K Haim, R Shalgi and D Ben-Yosef, unpublished observations). To further explore this facet of calpain in the male germ line we examined the expression pattern of calpain 1 and

Figure 6 Effect of calpeptin on the acrosome reaction. (A) Acrosome-intact spermatozoa (solid arrow) compared with acrosome-reacted spermatozoa (dashed arrow). (B) Spermatozoa were incubated in HMB for 1.5 h to allow capacitation. Calpeptin was added at 25, 50, 100, 125, 200 μ M for 10 min followed by exposure to 10 μ M A23187 for 20 min. The percentage of acrosome-reacted cells (AR%) was determined as described in Materials and Methods. The data represent the means $\pm$ S.E. of duplicates from three experiments. $2\mu^* P < 0.01$ compared with control.

calpain 2 throughout spermatogenesis. The genomic sequences for *Canp1* and *Canp2* contain multiple transcription start sites and GC rich regions, features common to housekeeping genes (Hata *et al.* 1989, Suzuki 1990). Accordingly, we showed that the RNAs for both isoforms are expressed during the whole of spermatogenesis, as expected. This was confirmed by the Western blotting results indicating that both protein isoforms are present in all the phases of the process. Calpain 1 protein levels displayed a slight increase 20 to 22 days after birth. The most advanced germ cells present during this period include late spermatocytes and early spermatids. This period encompasses the first wave of meiotic divisions (Bellvé *et al.* 1977a). Our results correlate with the up-regulation of *Canp1* (μ calpain) mRNA levels observed in rat testis during this same period (Sultana *et al.* 2003). Immunohistochemistry revealed positive staining of calpain 2, as compared with the control, at all cells and also at the Leydig cells, in which both calpains were formerly detected (Sultana *et al.* 2003). A more distinct staining of calpain 2 was observed at the periphery of the seminiferous tubules. Enhanced cytoplasmic labeling for calpain 2 was also observed in particular cells from the testes of 10-day-old mice. The number of intensely stained cells

increased during the following two days. Germ cells of the first wave of spermatogenesis enter the meiotic phase around 10 days after birth (Bellvé *et al.* 1977b). These results may imply the upregulation of calpain 2 expression at the onset of development of primary spermatocytes. However, since this increment was not observed in Western blotting, we cannot rule out the possibility that the intense labeling is due to a conformational change that increases the degree or amount of antibody binding. The potential roles of calpains in mitosis and meiosis and in cell differentiation have already been suggested (Schollmeyer 1988, Malcov *et al.* 1997, Carafoli & Molinari 1998) and the present results indicate a possible involvement of calpain at the entry into meiosis.

Previous studies had reported that calpain 1 and calpain 2 are confined to the head of mammalian spermatozoa, either in the acrosomal cap (Schollmeyer 1986, Rojas *et al.* 1999) or at the outer acrosomal membrane (Yudin *et al.* 2000). Using confocal immunofluorescence microscopy, we detected both isoforms at the acrosomal cap of the mouse spermatozoa. The acrosome is formed during the early period of spermiogenesis and represents one of the defining features of spermatozoan development. During this process, various proteins are synthesized and incorporated into the acrosome, where they undergo extensive modifications. One of the mechanisms proposed to be involved in these modifications is selective proteolysis, a process that converts an enzymatically inactive precursor form to an enzymatically active mature form (Abou-Haila & Tulsiani 2000). The localization of calpain 1 and calpain 2 strongly implies their possible participation in the development and function of the acrosome as well.

Aoyama *et al.* (2001) demonstrated a slight reduction in the acrosome reaction rate of human spermatozoa when inhibiting calpain by the use of calpain inhibitor I, calpain inhibitor II and Z-Leu-Leu-H, which are cysteine proteases inhibitors. To further explore calpain's role in mammalian spermatozoa, we used the selective cell-permeable calpain inhibitor, calpeptin, while performing the assay in bovine spermatozoa as a positive control. Calpeptin significantly impeded acrosome reaction in a dose-dependent manner, as determined by an immunofluorescence assay. Similar inhibition rates were obtained in a control assay performed in bovine spermatozoa.

Cytoskeletal components such as α -spectrin, actin and filamin have been localized to the anterior head of mammalian spermatozoa (Yagi & Paranko 1995, Yudin *et al.* 2000). These cytoskeletal proteins are known to have undergone remodeling by calpain in many cell types (Potter *et al.* 1998, Spira *et al.* 2003). Actin filament breakdown represents a key regulatory pathway, in which calpain may participate as a potential participator in the process, as previously known from other cell types (Carraher & Frame 2002). Diverse cytoskeletal remodeling such as actin filament breakdown, also occurs during the cascade of events leading to membrane fusion through the

acrosome reaction (Breitbart 2002, Brenner *et al.* 2003). In order to study calpain's possible role during fertilization, we also performed an *in vitro* fertilization assay in rats while exposing the gametes to calpeptin. Fertilization rate was drastically reduced compared with controls (I Ben-Aharon, K Haim, R Shalgi and D Ben-Yosef, unpublished observations). Using calpain inhibitors, Rojas and Moretti-Rojas (2000) demonstrated a reduced sperm-egg fusion rate in zona-free hamster eggs and the human spermatozoa system. Thus, our results may imply that calpain has a role in the calcium-mediated processes involving membrane fusion i.e. the acrosome reaction and/or sperm-egg fusion at fertilization.

There is some evidence suggesting that Ca^{2+} is involved in processes that occur during the development of spermatozoa and their function. Furthermore, it has been well documented that calcium is a key player in the cascade of events leading to successful fertilization. Since calpains, the Ca^{2+} -dependent cysteine proteases, are pluripotential with numerous substrates, it is possible that they may serve as effectors in more than one pathway in the intricate process of spermatogenesis and the functioning of the mature spermatozoa.

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