Expression and possible involvement of calpain isoforms in mammalian egg activation

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Abstract

At fertilization in mammals, the spermatozoon triggers a unique signal transduction mechanism within the egg, leading to its activation. It is well accepted that the earliest event observed in all activated eggs is an abrupt rise in intracellular calcium concentrations. However, little is known regarding the downstream proteins that are activated by this rise in calcium. Calpains constitute a family of intracellular calcium-dependent cysteine proteases whose members are expressed widely in a variety of cells. We investigated the expression and possible role of the calpain isoforms μ and m throughout egg activation. Both calpains were expressed in the rat egg and localized at the egg cortex as well as in the meiotic spindle. m Calpain translocated to the membrane and to the spindle area during parthenogenetic egg activation and during *in vivo* fertilization, upon sperm binding to the egg. The cytoskeletal protein α -spectrin (fodrin) was proteolysed by calpain during the egg-activation process, as demonstrated by specific calpain-breakdown products. Following parthenogenetic activation by ionomycin or puromycin, the calpain-selective permeable inhibitor, calpeptin, inhibited the resumption of meiosis and cortical reaction in a dose-dependent manner. Calpeptin was also effective in inhibiting *in vitro* fertilization. These results may imply a correlation between calpain activation and mammalian egg activation at fertilization and a possible role for calpain in the cascade of cellular events leading to resumption of meiosis.

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Introduction

At fertilization in mammals, the spermatozoon triggers a unique signal transduction mechanism within the egg, leading to its activation towards the development of an embryo. The foremost event observed in all activated mammalian eggs is an abrupt rise in intracellular calcium concentrations ($[Ca^{2+}]_i$) followed by a series of $[Ca^{2+}]_i$ oscillations (Miyazaki *et al.* 1993, Lawrence *et al.* 1994). Thus, the increase in $[Ca^{2+}]_i$ plays a pivotal role in the activation process, where it mediates the characteristic cellular events of egg activation, the earliest of which is exocytosis of the contents of the cortical granules, or the so-called cortical reaction (CR). The CR leads to modification of the zona pellucida and hence to the blocking of polyspermy (Ducibella *et al.* 1994, Raz & Shalgi 1998). Later events include resumption of meiosis and extrusion of the second polar body, decondensation of the sperm head, maternal RNA recruitment, formation of paternal and maternal pronuclei, initiation of DNA synthesis and mitotic cleavages.

Induction of a single $[Ca^{2+}]_i$ rise by exposure of eggs arrested at the second meiotic division (MII eggs) to a Ca^{2+} ionophore leads to both CR and resumption of meiosis, whereas employing Ca^{2+} chelators inhibits these events (Jaffe 1983, Kline & Kline 1992, Tombes *et al.* 1992, Vincent *et al.* 1992, Ducibella *et al.* 1994, Raz & Shalgi 1998, Ducibella *et al.* 2002). However, it has been suggested that the $[Ca^{2+}]_i$ oscillations are required for early embryonic development. Little is known regarding the cellular pathways by which calcium exerts its signal during egg activation. Recently, a role had been suggested for proteins such as Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and calmodulin in mediating the signal required for resumption of meiosis (Tatone *et al.* 2002, Markoulaki *et al.* 2003).

Calpains constitute a family of intracellular calcium-dependent cysteine proteases whose members are expressed widely in a variety of tissues and cells (Murachi 1989, Sorimachi *et al.* 1995, Carafoli & Molinari 1998). Two ubiquitous members of the calpain family, the μ and m isoforms, have been well characterized in many cells.

They are both heterodimers, comprised of a large catalytic subunit of 80 kDa and a small regulatory subunit of 30 kDa. However, they differ considerably in the calcium concentrations that they require for their activation *in vitro*. μ Calpain is activated at micromolar calcium concentrations and m calpain at millimolar concentrations (Croall & DeMartino 1991, Sorimachi *et al.* 1995). Calpains are pluripotential proteases that have been implicated in diverse cellular signaling pathways mediated by calcium, such as cytoskeleton remodeling, cell-cycle regulation, cell differentiation and apoptosis (Carafoli & Molinari 1998, Croall & DeMartino 1991). It had been suggested that calpain is associated with the chromosomes and with the spindle region during mitosis (Schollmeyer 1988, Lane *et al.* 1992). Calpain was also considered to participate in microtubule assembly and disassembly (Billger *et al.* 1988, 1993).

The dominant rise in $[Ca^{2+}]_i$ following fertilization may also imply calpain involvement during re-initiation of meiosis. Injection of calpain into the nuclei of prophase-arrested starfish oocytes induced re-initiation of meiosis, reflected by germinal vesicle breakdown, as occurs in oocyte maturation (Santella *et al.* 1998). Thus, calpain may participate in the breakdown of cytoskeletal protein during the disassembly of the nuclear envelope (Santella *et al.* 2000). In MII-arrested *Xenopus* eggs, calpain might play a role in meiosis regulation by inactivating the *c-mos* proto-oncogene product, pp³⁹, thus leading to resumption of the second meiosis (Watanabe *et al.* 1989, Sagata *et al.* 1989). However, this theory has been controversial since it has been implied that calpain is capable of degrading the *c-mos* product only *in vitro*, at supraphysiological calcium concentrations (Lorca *et al.* 1991). In an earlier study, we demonstrated calpain expression in rat eggs (Malcov *et al.* 1997). We have recently displayed the expression of the calpain-calpastatin system in human oocytes as well (Ben-Aharon *et al.* 2005).

In our present study, we focused on the calpain isoforms μ and m, and demonstrated their expression and localization in the rat egg throughout egg activation. We further examined the role of calpain in egg activation during *in vitro* fertilization and during parthenogenetic activation by a calcium ionophore.

The appearance of breakdown products of α -spectrin (fodrin), a known substrate of calpain, following egg activation served as a marker for calpain activation. In order to explore a possible role for calpain in mammalian egg activation, we introduced either one of calpain inhibitors, calpeptin or calpain inhibitor III (MDL28170), to MII eggs and then activated them. Inhibition of resumption of meiosis was detected in a dose-dependent manner. Inhibition of cortical granule exocytosis was also detected to a lesser extent.

Materials and Methods

All studies were carried out according to an approved protocol as dictated in Tel Aviv University guidelines of the institutional animal care and use committee.

Collection of eggs

MI eggs

For ovulation induction, 23–26-day-old immature Wistar-derived female rats were injected with 10 IU human chorionic gonadotropin (hCG; Sigma, St Louis, MO, USA) 48–54 h after administration of 10 IU pregnant mares' serum gonadotropin (Syncro-part, Sanofi, France). Rats were killed 14 h after hCG administration. Cumulus-enclosed MII eggs were isolated from the oviductal ampullae in Toyoda Hepes (TH) medium (Ben-Yosef *et al.* 1995) supplemented with 0.4% BSA (for immunofluorescence) or 0.1% BSA (for immunoblotting). Cumulus cells were removed by a brief exposure to 400 IU/ml highly purified hyaluronidase (H-3631; Sigma) in TH medium. The temperature of the medium was kept at 37°C for all experiments.

Fertilized eggs

Immature female rats were allowed to mate after an injection of hCG with males of proven fertility. The females were killed 15 h after hCG administration. Eggs were isolated from the oviductal ampullae at different stages of fertilization, as described for the hamster (Eliyahu & Shalgi 2002). Only early stages of fertilization were assessed for the experiments: sperm binding, 0–15 min after sperm attachment; fertilization cone, 15–60 min after sperm attachment.

Parthenogenetic activation

MI ovulated eggs were activated parthenogenetically in the dark by 2 μ M calcium ionophore (ionomycin; Calbiochem, San Diego, CA, USA) for 3–5 min followed by an additional 0 or 20 min of incubation in fresh TH medium lacking the activator. We also looked at egg activation by 20 μ M puromycin (Sigma) for 3 h.

Immunofluorescence staining and laser-scanning confocal microscopy

Eggs at the appropriate developmental stages were fixed for 10 min at room temperature in 3% paraformaldehyde (Merck, Gibbstown, NJ, USA) in Dulbecco's PBS (DPBS; Biological Industries, Kibbutz Beit Ha'emek, Israel), supplemented with 0.01% glutaraldehyde (Polysciences, Warrington, PA, USA). The eggs were then washed in a solution of 3% fetal calf serum (Biological Industries) in DPBS solution, which served as a blocking solution. Zonae pellucida (ZP) were removed using 0.25% Pronase (Sigma). Zonae pellucida-free eggs were permeabilized for

10 min in 0.05% Nonidet P-40 (NP-40; Sigma) in blocking solution and washed in 0.005% NP-40 in blocking solution. Permeabilized eggs were incubated for 2 h in the presence of a primary antibody, and washed before transfer to the fluorescently labeled secondary antibody (30 min in the dark).

Calpain labeling

Monoclonal anti-(μ calpain) or anti-(m calpain) antibody (1:50 in blocking solution; Calbiochem) served as the primary antibody. Fluorescently labeled donkey anti-mouse IgG Cy3 served as a secondary antibody (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Assessment of CR

Cortical granule exudate was detected by labeling fixed eggs with lens culinaris aectin-biotin (Vector Laboratories, Burlingame, CA, USA; 5 μ g/ml in DPBS supplemented with 1% BSA), which interacts with cortical granule content (Ducibella *et al.* 1988). The eggs were then washed and stained with Texas Red–streptavidin (Vector Laboratories; 1 μ g/ml in DPBS supplemented with 1% BSA).

Assessment of the meiotic stage

The DNA-specific fluorochrome Hoechst 33342 (1 μ g/ml; Sigma), which served to mark the chromatin stage, was added either to the secondary antibody solution while performing calpain labeling or to the Texas Red solution while assessing CR. Resumption of meiosis was documented by monitoring the separation of the chromosomal dyads and the polar body II (PBII) extrusion.

Confocal microscopy

DNA for calpains μ and m and cortical granule exudates were visualized and photographed by a Zeiss (Oberkochen, Germany) confocal laser-scanning microscope (LSM). The Zeiss LSM 410 is equipped with a 25 mW krypton/argon laser, a 10 mW helium/neon laser (488, 543 and 633 maximum lines) and an UV laser (Coherent Laser Group, Santa Clara, CA, USA). A 40 $\times$ numerical aperture/1.2 planapochromat water-immersion lens (Axiovert 135 M; Zeiss) was used for all imaging. The eggs were scanned using the LSM through the z-axis to perform a section at the equatorial plane of the egg for localization and possible translocation of each calpain. Each experiment was repeated at least three times and at least 20 eggs were examined in each experimental group, of which three or four were analyzed densitometrically. The labeling intensity was measured using the corrected mean density values obtained by the LSM software. The fluorescence intensity was expressed relative to values obtained for MII eggs, which were set to 1.0 (arbitrary units). Calpain translocation was evaluated by calculating

the ratio between calpain staining at the egg membrane and in the cytosol.

Immunoblotting

Calpains

Samples of 300 cumulus-free MII eggs were collected in 7–10 μ l TH medium mixed with 7 μ l lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4 , 5 mM NaF and 10 μ g/ml aprotinin (Sigma)). The protein extracts were kept at -70°C until use. Lysates of oocytes were mixed with Laemmli buffer (New England Biolabs, Beverly, MA, USA) and boiled for 5 min. Proteins were separated by SDS/PAGE on 10% gels at a constant current of 30 mA alongside marker proteins of known molecular masses (Amersham Bioscience). The gels were electro-transferred onto a nitrocellulose membrane (Amersham Bioscience) for 18 h at 40 mA. Following electro-transfer, the blots were blocked in 5% nonfat dry milk in TBS/Tween (150 mM NaCl, 10 mM Tris and 0.5% Tween 20) for 2 h at room temperature and incubated overnight at 4°C with either monoclonal anti-(μ calpain) (1:100) or anti-(m calpain) (1:150) antibody in blocking solution. The secondary antibody used was donkey anti-mouse antibody (IgG) conjugated to horseradish peroxidase (1:5000). The blots were processed by the chemiluminescence detection system (Supersignal; Pierce, Rockford, IL, USA) and autoradiographed.

Calpain-specific α -spectrin-breakdown products

Samples of either 300 MII eggs or 300 ionomycin-activated eggs were collected as described above, but egg lysates were separated on a 7.5% gel and then electro-transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). Following 2 h of blocking (5% nonfat dry milk in TBS/Tween), the membranes were incubated overnight with anti-(α -spectrin) monoclonal antibody (AFFINITI Research Products, Exeter, UK; 1:4000). The secondary antibody used was goat anti-mouse antibody (IgG) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories; 1:10 000). The blots were processed as described for calpains.

Inhibition of calpain activity by calpain synthetic inhibitors

Parthenogenetic activation

The eggs were incubated in TH medium containing 0.4% BSA for 30 min in the presence of either calpeptin (Calbiochem; 25–125 μ M) or calpain inhibitor III (MDL28170; Calbiochem; 25–50 μ M) as employed to successfully inhibit calpain in other studies (Forsythe and Befus 2003, London 2003, Spira *et al.* 2003). They were then exposed to parthenogenetic activation by ionomycin, followed by 22 min of incubation at 37°C in the presence of the inhibitor. The eggs were then fixed and labeled for calpain

isoforms, cortical granule exudate and chromatin, and visualized as described in the section on immunofluorescence, above. Both inhibitors were dissolved in 100% DMSO, which also served as a control. MII eggs that had been exposed to either calpain inhibitor without any activation were also assessed, to detect any effect of the inhibitor itself on the egg. The effect of calpain inhibitors on egg activation was explored by evaluating chromatin status as a marker for resumption of meiosis and the degree of CR, both analyzed by immunofluorescence confocal microscopy. For CR assessment, eggs were classified in one of three groups – no CR, weak CR or strong CR – based on their fluorescence intensity. The labeling intensity was measured using the corrected mean density values obtained by the LSM software.

In vitro fertilization

Sperm were collected from the uteri of superovulated rats soon after mating and diluted in modified rat fertilization medium (Shalgi 1991) to a final concentration of $(7-10) \times 10^5$ spermatozoa/ml. Aliquots of the sperm suspension (100 μ l) were incubated for 5 h in Petri dishes (Corning, Corning Acton, MA, USA) under mineral oil (Sigma) at 37°C, 95% humidity and 5% CO₂ in air to allow capacitation. MII eggs were collected as described above. The eggs were exposed to 125 μ M calpeptin 30 min before they were introduced into the sperm suspension (20 eggs per 100 μ l capacitated sperm). Eggs exposed to DMSO alone served as a control. The eggs were examined 2 h later by Nomarski interference-contrast microscopy to determine fertilization. Only eggs containing a sperm tail in the vitellus were classified as fertilized.

Data analysis

For analyzing the inhibition effect on resumption of meiosis, data were expressed as the fraction of treated eggs successfully reaching each developmental stage divided by the fraction of control eggs reaching the same developmental stage at each time point. The data were evaluated by analysis of variance (ANOVA) to determine whether differences between treated eggs and control eggs were significant. The significance of differences between experimental groups was determined by ANOVA with repeated measures (for calpain inhibitor III, since two concentrations of the inhibitor were compared) or paired *t*-test (for calpeptin, since three concentrations of the inhibitor were compared); $P < 0.01$ was considered significant.

For evaluating calpain translocation, ratios of calpain staining at the egg membrane and the cytosol were compared by ANOVA test. The inhibition effect on CR was presented as the number of eggs displaying strong versus weak CR. For each CR pattern, either strong or weak, data were calculated as the ratio between the intensity of the cortical granule exudates and the cortical area of the egg.

Student's *t*-test was applied to compare data; $P < 0.01$ was considered significant.

Results

Detection of calpain isoforms in the rat egg

Western-blot analysis was performed on extracts of 300 rat eggs arrested at the metaphase of the second meiosis. Using two monoclonal antibodies for either μ or m calpains, we could demonstrate a single band of 80 kDa for each isoform (Fig. 1), which conforms to the reported molecular masses of μ or m calpains in other cells.

Localization of calpain isoforms throughout egg activation

To determine the localization of calpain isoforms in the egg at different stages of egg activation, we artificially raised the $[Ca^{2+}]_i$ by ionomycin, thus inducing parthenogenetic activation. We followed the dynamics of calpain activation by performing immunofluorescence confocal microscopy using calpain-isoform-specific antibodies. The eggs were also labeled for chromatin to distinguish the different stages of egg activation: eggs that had been fixed immediately after ionomycin stimulus still had MII configuration whereas eggs fixed 20–25 min after ionomycin stimulus had an anaphase configuration.

m Calpain was distributed homogeneously throughout the ooplasm in the MII unfertilized egg, with a more distinct labeling of the egg membrane–cortex area as well as the meiotic spindle (Fig. 2A–C; mean ratio value, 1.24 ± 0.14). Immediately after exposure of the eggs to ionomycin, a significantly marked accumulation of m calpain was observed at the membrane area, as was an enhancement of the spindle staining (Fig. 2D–F; mean ratio value, 1.58 ± 0.29 , $P < 0.01$). The faded staining of the spindle area at later stages of development (Fig. 2G–I) can be attributed to the disassembly of the spindle

Figure 1 Expression of calpain isoforms in MII rat eggs. Proteins of 300 egg lysates were resolved by SDS/PAGE analysis and transferred onto nitrocellulose paper. Blots were incubated with either anti- μ calpain (1:100) or anti-m calpain (1:150) monoclonal antibodies (IgG), followed by secondary goat anti-mouse antibody conjugated to horseradish peroxidase (1:5000) and visualized by enhanced chemiluminescence detection. The arrow points to the calpain bands at 80 kDa, as calculated from the migration of known protein standards.

Figure 2 Localization of m calpain during egg activation. MII eggs (A–C); eggs treated for 3 min with ionomycin, washed and fixed immediately (D–F) or 20 min after activation (G–I; chromosomes at anaphase stage). Primary antibody (anti-m calpain) (1:50) was detected by a fluorescently labeled Cy3 secondary antibody (1:250; red). DNA was labeled with Hoechst (blue). Eggs stained only with secondary antibody and Hoechst served as controls (J–L). At least three independent experiments were performed involving three or four eggs for each group on each experimental day. Cross-sections through the egg meiotic spindle are shown in B, E, H and K. Cross-sections at the equatorial plane of the egg are shown in C, F, I and L. C', F', I' and L' depict the cortical area by $\times 4$ magnification. Light microscopy is shown in A, D, G and J. Scale bar, 10 μ m.

structure. To further support the aforementioned findings of calpain translocation to the membrane and the spindle area during egg activation, we followed the localization of m calpain during *in vivo* fertilization. Due to the fact that calpain is activated shortly after the first $[Ca^{2+}]_i$ rise, we examined early stages of fertilization (i.e. before and after sperm binding) according to the stages described previously by Eliyahu & Sagata & Watanabe (1989). Eggs at early stages of fertilization were labeled with anti-(m calpain) antibody and Hoechst stain. A relatively uniform distribution with a delicate labeling of the cortex–membrane area was observed throughout the ooplasm of MII-arrested eggs (Fig. 3A–C). m Calpain exhibited marked translocation to the membrane area as well as to the spindle in

eggs examined following sperm fusion (Fig. 3D–F). These results correlate to the findings obtained by parthenogenetic activation as described above.

μ Calpain displayed no remarkable changes in its cellular localization following egg activation (Fig. 4). It was distributed homogeneously throughout the cytosol in MII eggs, with distinct staining of the membrane and the spindle without any significant change during or after egg activation.

Calpain activation throughout egg activation

Calpain-mediated cleavage of the cytoskeletal protein nonerythroid α -spectrin (fodrin), which results in a set of

Figure 3 Localization of m calpain during fertilization. MII (A–C) and *in vivo*-fertilized (D–F) eggs were stained with anti-(m calpain) antibody (1:50) and Cy3 secondary antibody (1:250). m Calpain (red) was visualized by confocal laser-scanning microscopy. DNA was labeled by Hoechst (blue). Localization of m calpain during the sperm-fusion phase is seen in E and F. Cross-sections through the egg's meiotic spindle are shown in B and E. Cross-sections at the equatorial plane of the egg are shown in C and F. Light microscopy is shown in A and D. At least three independent experiments were performed involving three eggs for each group on each experimental day. Scale bar, 15 μ m.

large breakdown products, serves as a marker for calpain activation. α -Spectrin hydrolysis was determined by immunoblotting extracts of MII eggs and parthenogenetically activated eggs by means of a monoclonal antibody which recognizes the two calpain-mediated breakdown products of 145 and 150 kDa. As depicted in Fig. 5, no breakdown products were found in the lysates of MII eggs, whereas

both 145 and 150 kDa breakdown products were detected, representing calpain activity, in the lysates of eggs activated by ionomycin.

The role of calpain during egg activation

To examine the role of calpain during egg activation, the percentage of eggs undergoing resumption of meiosis

Figure 4 Localization of μ calpain during egg activation. MII eggs (A–C). Eggs treated for 3 min with ionomycin, washed and fixed immediately (D–F) or 20 min after activation (G–I; chromosomes at the anaphase stage). Primary antibody (anti-(μ calpain) (1:50)) was detected by a fluorescently labeled Cy3 secondary antibody (1:250; red). DNA was labeled with Hoechst (blue). At least three independent experiments were performed involving three or four eggs for each group on each experimental day. Cross-sections through the egg meiotic spindle are shown in B, E and H. Cross-sections at the equatorial plane of the egg are shown in C, F and I. Light microscopy is shown in A, D and G. Scale bar, 15 μ m.

Figure 5 Detection of α -spectrin-breakdown products in activated eggs. 300 MII eggs were incubated with ionomycin for 3 min, washed and further incubated for 7 min in TH medium. Treated eggs were lysed, and proteins were resolved by 7.5% SDS/PAGE and transferred onto a PVDF membrane. 300 MII-arrested eggs served as controls. Blots were incubated with anti- α -spectrin (fodrin) antibody (1:4000), followed by goat anti-mouse horseradish peroxidase (1:20 000) and enhanced chemiluminescence detection. The arrows point to the 145 and 150 kDa breakdown products, which are known as α -spectrin (fodrin)-breakdown products cleaved by calpain.

and/or cortical granule exocytosis were examined after parthenogenetic activation had been induced in the presence of one of two calpain-selective permeable inhibitors – calpeptin or calpain inhibitor III.

Both resumption of meiosis and CR were affected by each calpain inhibitor (Figs 6 and 7) with a dose-dependent inhibition response. The inhibitory effect of calpeptin was evident with all concentrations tested (Fig. 6A and B). At a concentration of 25 μ M calpeptin, 56.8% of eggs resumed meiosis, a value that was significantly lower than the 79.8% of control eggs lacking the inhibitor ($P < 0.01$). Higher concentrations of the inhibitor (i.e. 75 and 125 μ M) yielded a more pronounced inhibition (only 29.3 and 14.0% of eggs resumed meiosis, respectively, $P < 0.01$; paired t -test; Fig. 6A). Whereas 51.4% resumed meiosis with calpain inhibitor III at 25 μ M, only 28.6% underwent resumption of meiosis with calpain inhibitor III at 50 μ M ($P < 0.01$; ANOVA; Fig. 7A).

When parthenogenetic activation was induced in the presence of calpeptin, the magnitude of CR was also affected. All activated eggs, in both the control group (ionomycin only) and in the presence of any of the calpain inhibitors, displayed CR. However, activated eggs displayed two degrees of cortical granule exudates, classified as weak or strong labeling and assessed using the LSM software (Fig. 6C). Eggs that had been stained strongly attained a mean density value of 180.88 ± 15.44 units whereas weakly stained eggs were scored with 44.84 ± 4.8 units ($P < 0.01$). Exposure to calpeptin reduced the rate of eggs with strong CR to 84.52% at 75 μ M and 48.3% at 125 μ M, as compared with controls (Fig. 6B). When we examined the effect of calpain inhibition in ionomycin- versus puromycin-activated eggs, resumption of meiosis was impeded in both sets (Fig. 6A and D). Calpain inhibitor III was also effective in reducing the CR extent but only at its higher concentration (Fig. 7B).

To exclude a possible effect of the inhibitors *per se* on MII eggs, they were incubated with the higher concentration of each inhibitor devoid of any activation stimulus.

No change in the meiotic status, in the CR or in localization of calpain isoforms could be detected.

Effects of calpeptin on fertilization

For the *in vitro* fertilization assay, eggs were incubated in TH medium alone (control) or in the presence of calpeptin, transferred into sperm suspension in the presence of the inhibitor for *in vitro* fertilization and assessed for fertilization as described in the Materials and Methods section. In three separate experiments, the majority of control eggs were fertilized *in vitro* (sperm penetrated and PBII emitted in $75.0 \pm 7.3\%$ of the 110 eggs). Of the 128 eggs incubated in the presence of 125 μ M calpeptin, only $24.6 \pm 11.9\%$ were fertilized (i.e. PBII emitted; Fig. 6E).

Discussion

The earliest event observed following sperm–egg interaction in all mammalian eggs is an abrupt rise in $[Ca^{2+}]_i$. This study was designed initially to investigate the calpain system in mammalian eggs as part of the search for the downstream factors activated by this calcium rise within the egg at fertilization. The calpain system was chosen based on the published data and as a continuation of our previous study, which demonstrated calpain expression in the rat egg (Malcov *et al.* 1997). In our current work, the expression of ubiquitous calpain isoforms in the rat egg as well as calpain function were studied during parthenogenetic activation and at fertilization using two specific calpain inhibitors. Calpain involvement during resumption of meiosis and during cortical granule exocytosis has been demonstrated, apparently via cytoskeleton remodeling. Calpains represent a well-conserved family of cysteine proteases activated by calcium. Two ubiquitous calpains, μ and m , were named according to their relative requirement for calcium concentrations *in vitro*, micromolar and millimolar respectively, to elicit proteolytic activity (Croall & DeMartino 1991). At fertilization, $[Ca^{2+}]_i$ ascends to about 10^{-6} M. Since calpain requires supraphysiological Ca^{2+} levels *in vitro*, several alternative or complementary mechanisms of activation and regulation have been suggested to explain the lower Ca^{2+} requirements in physiological conditions. Among those are: binding of calpain to membrane phospholipids, autolysis, escape from its endogenous inhibitor calpastatin, binding of coactivators and calpain phosphorylation (Michetti *et al.* 1991, Kuo *et al.* 1994, Arthur & Carafoli, 1996, Melloni *et al.* 1998, Suzuki & Sorimachi 1998, Barnoy *et al.* 1999, Tullio *et al.* 1999, Glading *et al.* 2002). Calpains have been defined as biomodulators since calpain-mediated proteolysis often modulates its substrate activity by releasing a co-factor.

The role of calpain at fertilization has been studied in a few species. It was implied that calpain may participate in the regulation of meiosis at fertilization in *Xenopus laevis* eggs (Watanabe *et al.* 1989). Injection of calpain into the

Figure 6 Inhibition of calpain by calpeptin. (A and B) MII eggs were incubated for 30 min in the presence of calpeptin or DMSO (control), activated by $2 \mu\text{M}$ ionomycin for 3 min and further incubated in the presence of the inhibitor for 22 min. The eggs were then fixed and labeled for cortical granule content, as described in the text. The chromatin stage was evaluated by Hoechst labeling (* Student *t*-test

nuclei of prophase-arrested starfish oocytes induced resumption of meiosis, probably by proteolysis of cytoskeletal proteins during the disassembly of the nuclear envelope (Santella *et al.* 2000). We have previously demonstrated calpain expression during rat egg activation (Malcov *et al.* 1997). In the present study, we focused on calpain ubiquitous isoforms, μ and m , in terms of their localization, activation and possible role during egg activation. We demonstrated the expression of both isoforms in the rat egg as 80 kDa proteins, similar to what had been reported in somatic cells. Immunofluorescence combined with confocal microscopy depicted a relatively homogenous distribution of m calpain in the cytoplasm of MII eggs, with a subtle marking of the membrane–cortex area and the meiotic spindle apparatus. Shortly after increasing the $[\text{Ca}^{2+}]_i$ by either ionophore (Raz & Shalgi 1998) or at the actual physiological process of fertilization, a marked accumulation of m calpain was observed at the membrane and at the spindle area. Similar results of calpain localization were observed during the mitotic division (Schollmeyer 1988, Lane *et al.* 1992).

On the other hand, μ calpain did not show any significant change in its localization during egg activation. We may therefore speculate that μ calpain does not play an active role during this process but rather functions as a bystander or a backup system for m calpain. As a house-keeping gene product, μ calpain is expressed within the egg. It is quiescent during the early phase of fertilization, but may participate later during early cleavages.

The cytoskeletal protein α -spectrin (fodrin) is regulated by calpain-mediated cleavage to produce a set of large breakdown products. We herein displayed α -spectrin degradation during parthenogenetic egg activation using a calpain-specific α -spectrin-breakdown products antibody. α -Spectrin has been detected previously in the cortical area of mouse eggs (Bonder *et al.* 1989, Bonder & Fishkind 1995) and adjacent to the meiotic spindle (Schatten *et al.* 1986). Correspondingly, both calpain isoforms appeared to be localized to the same area. Taken together, according to known features of calpain activation, both translocation of m calpain and the presence of breakdown products may indicate a correlation between mammalian egg activation and calpain activation.

To examine the role of calpain during egg activation, resumption of meiosis and cortical granule exocytosis were examined following parthenogenetic activation in the presence of calpain inhibitors. Both calpeptin and calpain inhibitor III dose-dependently impeded resumption of

($P < 0.01$). (C) Assessment of the CR. Labeling of cortical granule exocytosis was classified as strong (I) or weak (II). (D) MII eggs were incubated for 30 min in either 125 μM calpeptin or control medium. Eggs from each of the two groups were then incubated for another 3 h with or without 20 μM puromycin. (E) *In vitro* fertilization of eggs that had been incubated in the presence of 125 μM calpeptin 30 min prior to introduction to sperm suspension. Eggs containing a sperm tail in the vitellus were classified as fertilized.

Figure 7 Inhibition of calpain by calpain inhibitor III. MII eggs were incubated for 30 min in the presence of calpain inhibitor III or DMSO (control), activated by 2 μM ionomycin for 3 min and further incubated in the presence of the inhibitor for 22 min. The eggs were then fixed and labeled for cortical granule content, as described in the text. The chromatin stage was evaluated by Hoechst labeling (* ANOVA with repeated measures $P < 0.01$).

the meiosis induced by ionomycin. The chromosomes of the activated eggs in the presence of the inhibitor were aligned accurately as they were in MII-arrested eggs (the control). Lower concentrations of the inhibitor resulted in a minimal separation of the chromatids. The chromatid segregation is induced by a process named biorientation. Thus, the pole-ward movement of the kinetochore in the metaphase-anaphase transition is driven by disassembly of microtubules, and by the function of mechanochemical motor proteins, such as dynein and kinesin, on the one hand and the anaphase-promoting complex/cyclosome (APC/C) on the other (Wittmann *et al.* 2001, Terret *et al.* 2003). Microtubule dynamics seem to be regulated mainly by microtubule-associated proteins, which are known as favorable calpain substrates (Fischer *et al.* 1991, Johnson & Foley 1993, Alexa *et al.* 1996). Calpain was shown to mediate disassembly of the microtubule lattice as well (Billger *et al.* 1988, Pettigrew *et al.* 1996). Therefore, both microtubule-associated proteins and the microtubules can serve as potential targets for calpain isoforms during resumption of the second meiosis.

Little is known about the cellular pathways by which calcium signals egg activation at fertilization. A role had recently been suggested for proteins, such as CaMKII and calmodulin, in mediating the signal required for the

resumption of meiosis (Tatone *et al.* 2002, Markoulaki *et al.* 2003). In a study exploring the role of CaMKII during activation of mouse eggs, Markoulaki *et al.* (2004) demonstrated that CaMKII antagonist inhibits cell-cycle progression in fertilized, but not cycloheximide-treated, eggs. When we examined the effect of calpain inhibition in ionomycin- versus puromycin-activated eggs, resumption of meiosis was impeded in both sets. Cycloheximide-induced activation of mouse eggs, as well as puromycin, was shown to have resulted in a decrease in both cdc2/cyclin B and mitogen-activated protein kinase (MAP kinase) activities (Zernicka-Goetz & Maro 1993, Moos *et al.* 1996). Cell-cycle arrest throughout the first and second meiosis is known to depend on MAP kinase activity (Kishimoto 2003, 2004). Furthermore, MAP kinase contributes to the prevention of parthenogenetic activation (Picard *et al.* 1996). Calpain, which is also a calcium-dependent enzyme, has not yet been positioned in the cascade of events leading to egg activation. Investigation of a calpain signal transduction pathway during fibroblast motility, however, revealed that calpain activity is regulated downstream of MAP kinase (Glading *et al.* 2000). We can therefore contend that inhibition of calpain in puromycin-treated eggs does not differ from its inhibition in ionomycin-treated eggs since the inhibition effect probably occurs downstream in the cascade of events leading to resumption of meiosis. Nevertheless, we cannot exclude that calpeptin may have more than one mode of action. It may inhibit the proteasome as well as calpain activation. On the contrary, the results obtained by Markoulaki *et al.* (2004) may suggest that CaMKII is involved upstream at the level of cdc2 and cyclin B and thus inhibition of CaMKII in the presence of an activator, such as cycloheximide, varies in its effect in comparison to fertilization-/Ca²⁺-induced activation.

Both inhibitors also affected a second feature of egg activation, the CR, but to a lesser extent than inhibition of the resumption of meiosis. Thus, calpain may possibly impinge on cytoskeletal elements in the egg cortex. The cortical region in various cells contains numerous components of the cytoskeleton, such as microtubule-associated proteins, actin and α -spectrin, which are all known as calpain's substrates *in vivo* (Carafoli & Molinari 1998, Potter *et al.* 1998). These cytoskeleton components have been shown to be localized to the cortical region of the mammalian egg as well (Bonder *et al.* 1989, Bonder & Fishkind 1995).

A role has been implied for calpain in membrane fusion in myoblasts (Barney *et al.* 1999), in the transformation of axonal vesicles in regenerating neurons (Spira *et al.* 2003) and in platelets (Potter *et al.* 1998, Fox 2001). Thus, calpain might be involved in the cascade of events leading to cytoskeleton remodeling at the egg cortex in order to facilitate fusion of cortical granules. Being a pluripotent protease which participates in diverse cellular processes, calpain's role may be attributed to several facets of the signal transduction that occurs during egg activation at

fertilization. Our finding in which calpain inhibition hinders resumption of meiosis lends credence to a model in which calpain activation is required for the cytoskeletal elements of the meiotic spindle to allow segregation of chromatids. However, it is important to point out that resumption of meiosis is a complex network of signal transduction events and that a direct correlation between the aforementioned potential substrates and calpain should be explored further.

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