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# Calcium as a Mediator of Apoptosis in Bovine Oocytes and Preimplantation Embryos

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**Cell death pathways and their regulation during development of the preimplantation embryo are poorly understood. Our previous studies indicate that $Ca^{2+}$ is a key mediator of apoptosis in different cell models. We hypothesized that $Ca^{2+}$ signaling mediates apoptotic cell death during early embryonic development via activation of $Ca^{2+}$-dependent proteases, μ-calpain, and caspase-12. To test this hypothesis, established procedures for in vitro production of bovine embryos in concert with fluorescence, high-resolution, digital $Ca^{2+}$ imaging, detection of $Ca^{2+}$-dependent apoptotic mediators, and measurement of apoptotic cell death were used in the present studies. We found that an increase in intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in the individual embryo cells (2.6-fold) and mature oocytes (1.6-fold) was associated with activation of μ-calpain, induction of pan-caspase activity (5–10-fold), and expression of the $Ca^{2+}$-dependent caspase-12. Inhibition of calpain or caspase activity significantly (1.5–2-fold) reduced apoptotic indices in embryos treated with the mobilizer of intracellular $Ca^{2+}$ stores, thapsigargin, or the $Ca^{2+}$ ionophore, ionomycin. Taken together, these results support our hypothesis that $Ca^{2+}$ is involved in apoptosis of the preimplantation bovine embryo and that $Ca^{2+}$-dependent apoptotic proteases are μ-calpain and caspase-12.**

**Key Words:** Intracellular calcium; apoptosis; calpain; caspase-12; embryo; oocyte.

## Introduction

The potential role of apoptosis in the preimplantation embryo and oocytes remains obscure, and the nature of developmental signals, required to trigger the switch to apoptosis, is not known. Generally, apoptosis is considered beneficial during embryonic development and in adult life, but its dysregulation accompanies pathogenesis of many diseases *(1)*. In the early embryo, an increase in apoptotic cell death may serve as a mechanism for eliminating damaged or nonfunctional cells or the whole embryo, whereas a decrease in apoptosis may contribute to abnormal development during later stages. From a practical point of view, in vitro production of embryos of agriculturally important animals can be significantly improved by decreasing the death of embryos. The early embryonic mortality may directly result from the excessive induction of apoptosis in in vitro microenvironment, when survival signals are lacking and/or death signals are activated.

Calcium signaling plays a vital role in regulation of cellular processes *(2)*. Rises and oscillations of cellular $Ca^{2+}$ due to influx of $Ca^{2+}$ from the extracellular space and mobilization of $Ca^{2+}$ from intracellular stores regulate many key steps, from initiating early signaling events to causing irreversible changes, in the pathways leading to cell proliferation, differentiation, and death *(3,4)*. Precise cellular and molecular mechanisms of how $Ca^{2+}$ exerts its regulatory effects are not known, and $Ca^{2+}$-dependent targets have not been conclusively identified. Regulation of intracellular $Ca^{2+}$ underlies the temporal and spatial patterns of $Ca^{2+}$ signaling, which is crucial for functioning of the cell and determining cell fate *(4)*.

The involvement of $Ca^{2+}$ signaling occurring in the oocyte at fertilization is well characterized *(5–8)*; however, it is not known if and how $Ca^{2+}$ signals trigger or regulate events in the early embryonic development, including apoptosis *(9)*. It is reasonable to hypothesize that $Ca^{2+}$ signaling may play a significant role in development of the preimplantation embryo by interacting with $Ca^{2+}$-dependent mediators of cell death. We *(4,10–13)* and others *(3,14)* demonstrated that increase of intracellular $Ca^{2+}$ is a necessary early signaling event in triggering apoptosis in different models. Moreover, our results indicate that the $Ca^{2+}$-activated cysteine protease, μ-calpain, may take over the role of the major execution protease in apoptosis *(15)*. Disruption of the endoplasmic reticulum (ER) $Ca^{2+}$ homeostasis has been linked to activation of the ER-specific caspase-12 and induction of apoptosis *(16)*, with calpains responsible for cleaving pro-

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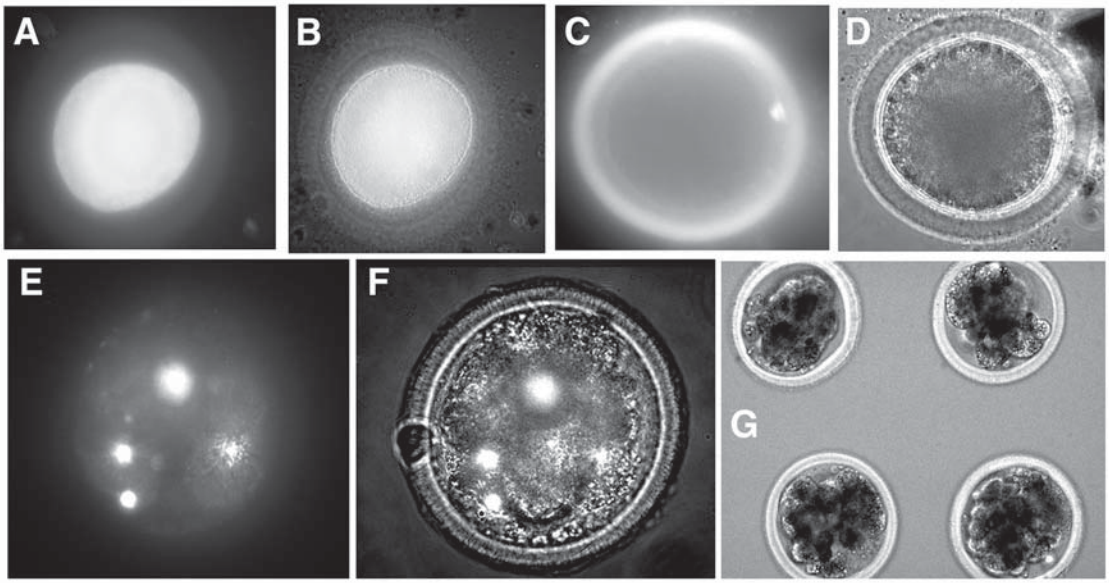


**Fig. 1.** Caspase activation in the bovine oocytes and embryos. Representative fluorescence (**A,C,E**) and simultaneously acquired fluorescence/light images (**B,D,F,G**) of mature oocytes (**A–D**) and compacted morulae (**E–F**) illustrate caspase activation. Fluorescence labeling of activated caspases in an oocyte (**A,B**) and four cells of an embryo (**E,F**) is clearly visible. Negative controls, i.e., not labeled oocytes (**C,D**) and embryos (**G**) are also shown (autofluorescence of zona pellucida is visible). Embryos and oocytes were cultured and labeled for pan-caspase activity as described in *Methods.* Here and in Fig. 2 at least five embryos per group were evaluated.

caspase-12 to generate the active caspase *(17)*. Therefore, $Ca^{2+}$ signaling may be an important mechanism for determining the fate of the embryo, and accordingly stabilization of intracellular $Ca^{2+}$ may promote early embryonic survival and normal development.

It has been demonstrated recently that apoptosis (as evaluated by DNA fragmentation with terminal deoxynucleotidyl transferase) occurs in mature bovine oocytes *(7, 18)* and early embryos, particularly at the morula and blastocyst stages *(19–21)*. Caspases have been implicated in mediating heat shock–induced apoptosis in the bovine embryo *(22,23)*; however, mechanism(s) of spontaneous apoptosis during in vitro embryo culture remains undetermined. It is noteworthy that changes in $[Ca^{2+}]_i$ in embryos and oocytes can impact processes other than apoptosis. An increase in $[Ca^{2+}]_i$ in peri-implantation blastocysts strengthens trophoblast adhesion *(24)*. $[Ca^{2+}]_i$ oscillations in fertilized oocytes after an extended residence in the oviduct or in culture can trigger their fragmentation and abnormal development *(25)*.

This study was undertaken to evaluate the signaling pathway mediating cell death in bovine oocytes and preimplantation embryos. The focus was centered on $Ca^{2+}$ signaling and identifying those pathways responsible for cell death via $Ca^{2+}$-dependent proteases. The results obtained indicate that an increase in intracellular $Ca^{2+}$ in mature oocytes and preimplantation embryos triggers apoptosis associated with activation of the $Ca^{2+}$-dependent μ-calpain, induction of pan-caspase activity and expression of the $Ca^{2+}$-dependent caspase-12.

## Results

### *Intracellular $Ca^{2+}$ and Caspase Activity*

$[Ca^{2+}]_i$ and caspase activity were measured in mature oocytes and morulae/blastocysts. Pan-caspase labeling (Fig. 1) was observed in 27% of the mature oocytes and 100% of the morulae/blastocysts (at least one positive cell). Basal $[Ca^{2+}]_i$ in oocytes and the embryo cells with detectable pan-caspase activity was higher than in nonapoptotic cells of the embryos and oocytes (Table 1). Morphological changes characteristic of the execution phase of apoptosis (i.e., blebbing, chromatin condensation, nuclear fragmentation) were observed in the cells with active caspases. These data indicate that increase in intracellular $Ca^{2+}$ is associated with caspase activation in mature oocytes, morulae, and blastocysts.

### *Calpain and Caspase-12*

$Ca^{2+}$-dependent apoptotic proteases, caspase-12 and μ-calpain, were detected with appropriate characterized antibodies. We found that caspase-12 is present in the embryo (morulae/blastocysts) and that it appears to demonstrate the staining pattern that is typical for ER (Fig. 2A). μ-Calpain (large subunit) was also present in the embryos (Fig. 2B). Calpain cleavage (i.e., presence of its small subunit, which is an indication of calpain activation) was detected in embryos with increased $[Ca^{2+}]_i$ (Fig. 2C, D). It appears that active calpain was also associated with the ER. Additionally, calpain activation was demonstrated by cleavage

**Table 1**
Caspase Activity and $[Ca^{2+}]_i$ in Bovine Oocytes and Embryos[a]

| Group/Parameter | Mature oocytes | | Morulae/Blastocysts | |
|---|---|---|---|---|
| Caspase activity | – | + | – | + |
| $[Ca^{2+}]_i$, n*M* | 148 ± 10.0 | 232 ± 18.4* | 118 ± 5.7 | 309 ± 39.1* |

[a]Mature oocytes and the embryo cells were classified as caspase-positive (+) or caspase-negative (–). The caspase-positive cells and oocytes were defined as labeled with the fluorescent probe FAM-VAD-fmk, the caspase-negative cells or oocytes did not differ from the background of fluorescence images. Pan-caspase activity was detected, $[Ca^{2+}]_i$ measured, and image analysis performed as described in *Methods*. *$p < 0.05$, as compared with the caspase-negative group. Only representative cells were analyzed in morulae and blastocysts (five to seven cells per embryo). The results are means ± SEM of at least three replicates.

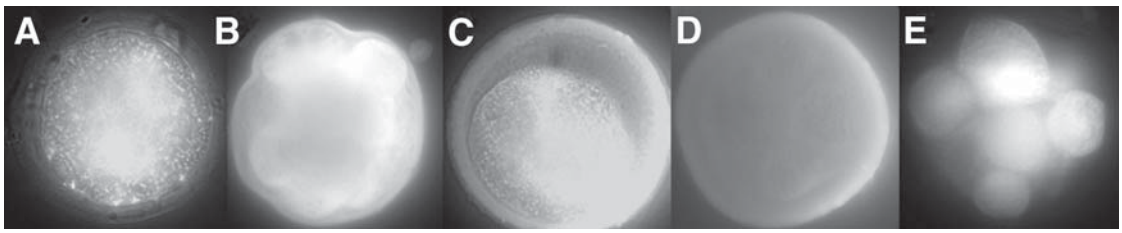


**Fig. 2.** μ-Calpain and caspase-12 in the bovine embryo. Immunofluorescence detection of caspase-12 (**A**), calpain large (**B**), and small (**C**,**D**) subunits was carried out as described in *Methods*. The calpain small subunit was detectable in apoptotic embryos with increased $[Ca^{2+}]_i$ (**C**), but undetectable in embryos with baseline $[Ca^{2+}]_i$ (**D**). Calpain activity was also observed in the embryo cells with increased $[Ca^{2+}]_i$ (**E**). The following primary and secondary antibodies were used: rat anti-caspase-12 monoclonal antibody; mouse anti-calpain small subunit (μ- or m-calpain) monoclonal antibody; mouse anti-calpain (μ-calpain) large subunit monoclonal antibody; Alexa Fluor-488 signal-amplification mouse antibodies; FITC-conjugated AffiniPure anti-rat IgG. Calpain activity in living embryos (**E**) was evaluated with fluorogenic substrate *t*-Boc-Leu-Met-CMAC (50 μ*M*). Panels **B**, **D**, and **E** show fluorescence images; panels **A** and **C** are combined fluorescence/light images. Negative fluorescence controls (i.e., without primary antibodies or the fluorogenic substrate) did not differ from the background. Embryos were at the morula/early blastocyst stage (5–6 d post-insemination).

of the cell-permeable fluorogenic peptide substrate, *t*-Boc-Leu-Met-CMAC (Fig. 2E).

### *Apoptosis*

To investigate the involvement of caspase-12 and μ-calpain in apoptotsis signaling, we employed calpain and caspase inhibitors. Embryos were treated with the mobilizer of the ER $Ca^{2+}$ stores, thapsigargin, or the $Ca^{2+}$ ionophore, ionomycin, with and without calpain inhibitor PD 150606, which blocks $Ca^{2+}$ binding sites of calpain, or the pancaspase inhibitor z-VAD-fmk, which also inhibits caspase-12. The results (Fig. 3) demonstrate that calpain and caspase inhibitors significantly reduce apoptotic indices in embryos with the thapsigargin- or ionomycin-induced increase in $[Ca^{2+}]_i$. Importantly, the calpain inhibitor decreased caspase activation in thapsigargin- and, particularly, ionomycin-treated embryos. This observation implies that the calpain-sensitive, $Ca^{2+}$-activated caspase is important in the bovine embryo. A less efficacy of the calpain inhibitor in decreasing apoptosis and caspase activity may indicate that caspase-12 is located downstream of μ-calpain in the $Ca^{2+}$-mediated apoptosis pathway. The trend to a decreased level of spontaneous apoptosis was also observed in the control embryos treated with pan-caspase inhibitor, which suggests a broader involvement of caspases in the apoptosis of the embryo.

## Discussion

A sustained increase of intracellular $Ca^{2+}$ level induces apoptosis in various cell models *(3,4,14)*. Here we report that such an increase triggers apoptosis in both the preimplantation embryo and mature oocytes. In vitro culture of bovine embryos in the presence of the $Ca^{2+}$ ionophore, ionomycin, or the selective mobilizer of the ER $Ca^{2+}$ stores, thapsigargin, induced a marked increase in basal $[Ca^{2+}]_i$, associated with induction of apoptosis. Importantly, "spontaneously" elevated $[Ca^{2+}]_i$ was observed in some cells of the control embryos (Table 1), and those cells demonstrated evidence of apoptosis, as evaluated by DNA fragmentation and morphological criteria (not shown). These observations

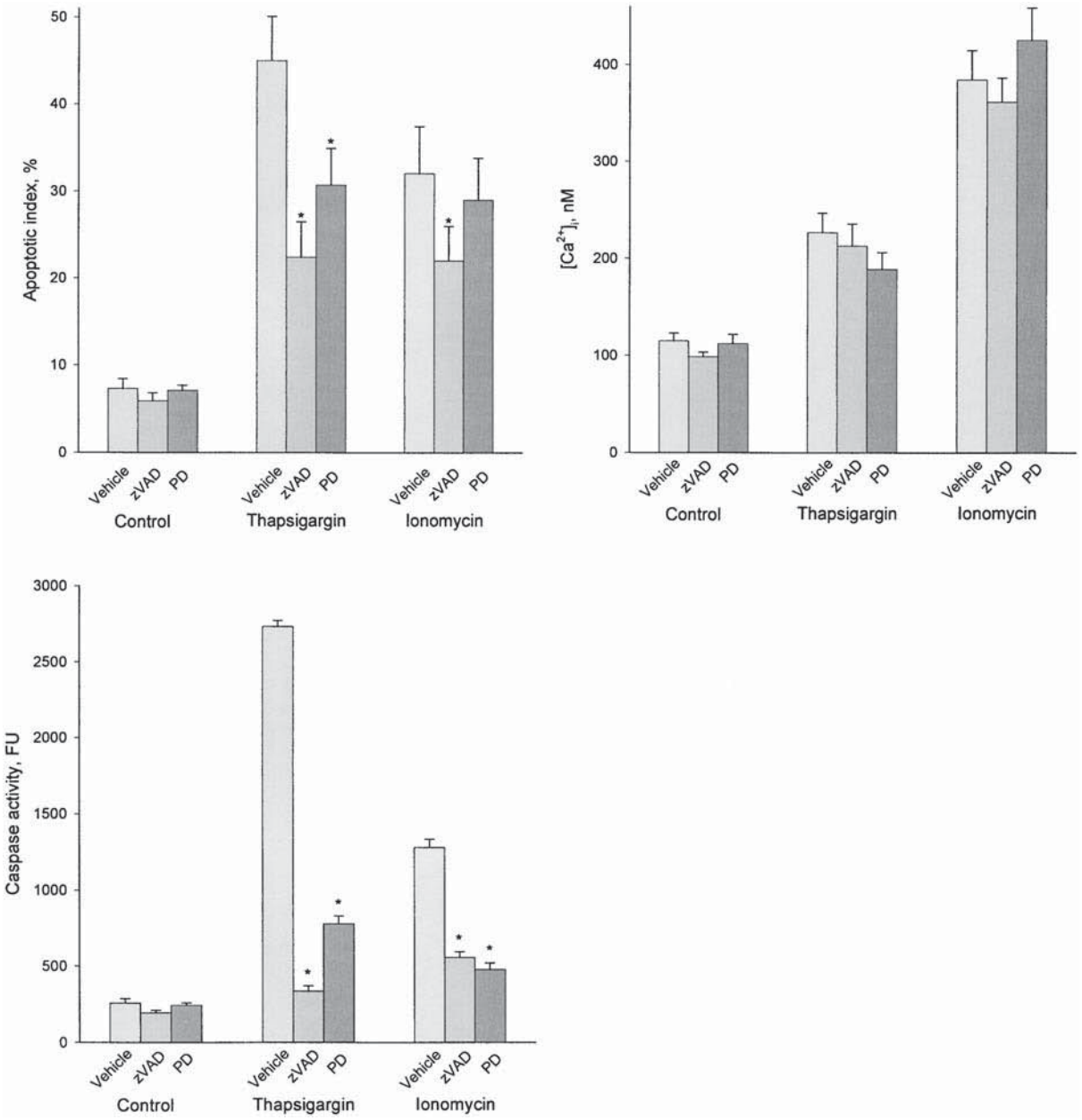


**Fig. 3.** Apoptotic indices, $[Ca^{2+}]_i$ and caspase activity in bovine embryos treated with pan-caspase or calpain inhibitor. Embryos (d 5 post-insemination) were treated with thapsigargin (0.5 μ*M*) or ionomycin (1 μ*M*) for 2 d in the presence of vehicle, pan-caspase inhibitor z-VAD-fmk (80 μ*M*), or calpain inhibitor PD 150606 (50 μ*M*). The embryo $[Ca^{2+}]_i$ was measured with fura-2. Apoptosis was detected by TUNEL, as described in *Methods*; nuclei were counterstained with Hoechst 33258. Apoptotic indices were calculated by dividing the number of apoptotic cells by the total number of cells. Baseline $[Ca^{2+}]_i$ was measured in the whole embryo. Fluorescence intensity of the pan-caspase inhibitor was also measured in the whole embryo and expressed in fluorescence units (FU). At least five embryos per group/treatment were evaluated; means ±SEM; $*p < 0.05$, as compared with the corresponding vehicle subgroup in the same group.

clearly indicate that an increase in intracellular $Ca^{2+}$ induces cell death in the embryo.

Next, we aimed to identify $Ca^{2+}$-activated mediators in the apoptosis pathway. Intracellular proteolytic enzymes, caspases and calpains, are responsible for limited cleavage of endogenous proteins. It is well established that caspases are involved in, and are often essential for, apoptosis *(1)*, whereas the role of calpains is less clear *(26)*. Remarkably, ablation of the common 30K subunit of calpains causes embryonic lethality in mice *(27)*. Possible interaction between calpain- and caspase-mediated signaling in apoptosis remains controversial. Several studies demonstrated that calpain activation

precedes onset of apoptosis and that calpains can utilize existing apoptosis pathways, e.g., via activation of caspase-12 *(17)*. It was also suggested that activation of caspase-3 may depend on increase in $[Ca^{2+}]_i$ *(28)*. We have previously shown that μ-calpain can execute apoptosis, induced by the $Ca^{2+}$-regulating hormone 1,25-dihydroxyvitamin $D_3$, without apparent activation of known caspases *(15)*. Recently, it was suggested that calpains are not capable of directly activating caspases, but rather act via apoptosis regulators of Bcl-2 family *(29)*. Noteworthy, different cell models were used in the mentioned above studies.

In our studies, using preimplantation bovine embryos, we have shown that both μ-calpain and caspases are necessary for execution of apoptosis. μ-Calpain and caspases were activated after addition of $Ca^{2+}$ modulators, which increase $[Ca^{2+}]_i$, ionomycin and thapsigargin, and calpain and pan-caspase inhibitors prevented induction of apoptosis. Although we did not investigate the role of specific caspases in $Ca^{2+}$-mediated apoptosis, caspase-12 seems to be a most promising candidate because of its $Ca^{2+}$/calpain dependency for activation. Therefore, these findings suggest the $Ca^{2+}$-induced, calpain-mediated activation of caspase-12 occurs in the embryo.

Taken together, our results support the hypothesis that cellular $Ca^{2+}$ is involved in apoptosis essential for the early bovine embryo development in vitro and that the $Ca^{2+}$-dependent apoptotic mediators are μ-calpain and caspase-12. For the first time, a correlation between increase in intracellular $Ca^{2+}$, activation of μ-calpain and caspases, and apoptosis of the embryo has been demonstrated.

## Materials and Methods

### *In Vitro Production of Embryos*

Established procedures for in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC) of bovine embryos were utilized *(30)*. Briefly, cumulus–oocyte complexes (COCs) (Bomed) were matured (20–24 h) in HEPES-buffered M-199 supplemented with bovine LH and FSH, sodium pyruvate, 10% fetal calf serum, and antibiotics at 38.5°C. Motile spermatozoa were selected by the swim-up technique using HEPES-buffered Tyrode's albumin–lactate–pyruvate solution (Sperm-TALP). Mature oocytes (groups of 20–30 COCs) were fertilized with $1–1.5 \times 10^6$ spermatozoa/mL for 16–18 h in microdrops of IVF-TALP supplemented with heparin and the motility-stimulating mixture (penicillamine–hypotaurine–epinephrine). After fertilization, presumptive zygotes were denuded of cumulus cells, transferred to 50 μL microdrops (10–20 embryos/drop) of the in vitro embryo culture medium, Potassium Simplex Optimized Medium (KSOM) supplemented with essential and nonessential amino acids (GIBCO), 3 mg/mL fatty acid–free BSA (Sigma), and 5 μg/mL gentamicin. Embryos were cultured in an atmosphere of 5% $CO_2$ in air at 38.5°C. Mature oocytes and embryos at the morula/early blastocyst stage (5–6 d post-insemination) *(31)* were used in experiments.

### *Intracellular Calcium*

Concentration of intracellular free $Ca^{2+}$ ($[Ca^{2+}]_i$) was measured using $Ca^{2+}$ indicator fura-2, as described previously *(11,32)*. Embryos or oocytes were transferred onto round UV-transparent plastic coverslips and loaded with the cell-permeant form (AM) of fura-2 at concentration of 1 μ*M* in Tyrode's salt solution supplemented with 0.1% dimethylsulfoxide (vol/vol) and 0.01% Pluronic F-127 (w/v) (Molecular Probes) for 30 min at 38.5°C. After incubation with the $Ca^{2+}$ indicator, embryos or oocytes were washed, placed in a microscope stage, temperature-controlled (38.5 ± 0.2°C) microincubation chamber and used for $Ca^{2+}$ imaging after 10 min preincubation period. We found that zona pellucida adheres tightly to coverslips during loading with fura-2/AM and that the adhesion survives changes in the solution.

The dynamics of intracellular $Ca^{2+}$ was assessed with embryos or oocytes in the microincubation chamber on a Nikon Eclipse TE-300 inverted microscope equipped for fluorescence, high-resolution, ratiometric, digital imaging (Fryer Co.) *(11,32)*. Briefly, images were captured using a Nikon Super Fluor 40× oil-immersion objective and CoolSnapFX digital CCD camera (Photometrics), averaged and ratioed (340/380 nm excitation, 510 nm emission) on a pixel-by-pixel basis, and stored for analysis. The interval between the successive ratio of image recordings was 1–5 s. For image analysis, the commercial software MetaFluor 5.0 (Universal Imaging) was used. A mouse-driven pointer was employed to define, on the phase-contrast or fluorescent image, regions of interest for analysis of intracellular $Ca^{2+}$ (five to seven individual cells of the embryo or approximately midsection of the oocyte). Background and movement corrections were applied during data analysis. The fluorescence signal of intracellular $Ca^{2+}$ was calibrated at the end of experiments *(32)*. $Ca^{2+}$ saturation is achieved by adding 5 μ*M* ionomycin in the presence of 5 m*M* $Ca^{2+}$, and virtually zero $Ca^{2+}$ by further addition of 10 m*M* EGTA; $MnCl_2$ (5 m*M*) is subsequently added to obtain background fluorescence. $Ca^{2+}$ calibration was not performed in preparations subsequently used for calpain or caspase fluorescence imaging. The concentration of vehicles, ethanol or DMSO, never exceed 0.2%. Our findings indicate that the vehicles at this concentration do not affect concentration of intracellular $Ca^{2+}$.

### *Caspase and Calpain Activity and Immunofluorescence Labeling*

Caspase activity was detected in living cells of the embryo with the fluorochrome-labeled pan-caspase inhibitor FAM-VAD-fmk (Intergen), which binds to active caspases *(33)*. The inhibitor was used at concentration of 10 μ*M*;

loading time was 1 h. Calpain activation in the embryo cells was evaluated using the cell-permeable fluorogenic substrate *t*-BOC-Leu-Met-CMAC (Molecular Probes) *(34)* at concentration of 50 μ*M*. If embryos were used for $Ca^{2+}$ imaging, loading with the caspase inhibitor or the calpain substrate started after completion of fura-2 extrusion by the embryo cells and verifying absence of the fura-2 fluorescence (usually, 1 h). Additionally, absorption maxima of the caspase inhibitor and the fluorescent product of the calpain substrate differ from that of fura-2/$Ca^{2+}$. After measurements of $[Ca^{2+}]_i$ and caspase or calpain activity, embryos were fixed for evaluation of apoptosis or immunofluorescence detection of caspases and calpains. Monoclonal antibodies directed against caspase-12 (a gift from Dr. J. Yuan), which is associated with ER-specific apoptosis and activated by $Ca^{2+}$, and μ-calpain large and small subunits (Chemicon) were used to confirm presence/activation of these proteases. Calpain inhibitor, interacting with $Ca^{2+}$ binding sites of the protease, was PD 150606 (Calbiochem).

For immunofluorescence labeling, fixed and permeabilized in Histochoice (Amresco) embryos or oocytes were preincubated with 10% nonspecific (goat or donkey) serum for 20 min, incubated for 2 h at room temperature or overnight at 4°C with the primary antibodies, and 2 h at room temperature with secondary antibodies [Alexa Fluor-488 signal-amplification mouse antibodies (Molecular Probes) or FITC-conjugated AffiniPure anti-rat IgG (Jackson ImmunoResearch)]. Fluorescence microscopy of embryos was carried out as described for $Ca^{2+}$ imaging. Image analysis and measurement of fluorescence intensity were carried out using MetaMorph 5.0 software (Universal Imaging), as we described *(35)*.

### *Measurement of Apoptosis*

Apoptosis was measured in mature oocytes and embryos at the morula/blastocyst stage (5–7 d pi) by detection of DNA fragmentation. Treatment of embryos with ionomycin (1 μ*M*) and thapsigargin (0.5 μ*M*) with or without caspase and calpain inhibitors was used to evaluate the role of increased $[Ca^{2+}]_i$ and the proteases in apoptosis. The DNA fragmentation in apoptotic embryos or oocytes was measured with terminal deoxynucleotidyl transferase and BrdUTP (TUNEL method) *(13)*. Embryos were fixed immediately after $Ca^{2+}$ imaging in 4% paraformaldehyde (pH 7.4), permeabilized and analyzed using the *In situ* Apoptosis Detection Kit (Trevigen) or APO-BrdU TUNEL Assay Kit (Molecular Probes). Negative (in absence of labeling enzyme) and positive (treated with DNase) controls were included. Hoechst 33258 dye was used in concert with TUNEL assay to count the total number of nuclei.

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