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Calmodulin-dependent protein kinase II triggers mouse egg activation and embryo development in the absence of Ca^{2+} oscillations

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Abstract

Fertilization in mammalian eggs is accompanied by oscillatory changes in intracellular Ca^{2+} concentration, which are critical for initiating and completing egg activation events and the developmental program. Ca^{2+}/Camodulin-dependent protein kinase II (CaMKII) is a multifunctional enzyme that is postulated to be the downstream transducer of the Ca^{2+} signal in many cell types. We tested the hypothesis that CaMKII is the major integrator of Ca^{2+}-induced egg activation events and embryo development by microinjecting a cRNA that encodes a *c*onstitutively *a*ctive (Ca^{2+}-independent) mutant form of CaMKII (CA-CaMKII) into mouse eggs. Expression of this cRNA, which does not increase intracellular Ca^{2+}, induced a sustained rise in CaMKII activity and triggered egg activation events, including cell cycle resumption, and degradation and recruitment of maternal mRNAs; cortical granule exocytosis, however, did not occur normally. Furthermore, when mouse eggs were injected with sperm devoid of Ca^{2+}-releasing activity and activated with either CA-CaMKII cRNA or by $SrCl_2$, similar rates and incidence of development to the blastocyst stage were observed. These results strongly suggest that CaMKII is a major integrator of the Ca^{2+} changes that occur following fertilization.

Keywords: Egg activation; Calmodulin-modulated protein kinase II; Calcium; Embryo development; Mouse

Introduction

Calcium released from the IP_3-sensitive pool is essential for all events of egg activation (Miyazaki et al., 1992; Xu et al., 1994) including cortical granule (CG) exocytosis, cell cycle resumption with a decrease in CDK1 and MAPK activity, and recruitment of maternal mRNA that is essential for genome activation (Aoki et al., 2003; Hara et al., 2005). Following fusion of the sperm and egg plasma membranes, diffusion of a sperm-specific phospholipase C (PLC), PLC-ζ, into the egg is essential to initiate release of intracellular Ca^{2+} (Knott et al., 2005; Saunders et al., 2002), which displays an oscillatory pattern (Swann and Lai, 1997). The Ca^{2+} oscillatory pattern is characterized by a first transient that is of longer duration than subsequent oscillations (Cuthbertson and Cobbold, 1985; Saunders et al., 2002) and whose frequency is characteristic for each species (Swann and Lai, 1997). These oscillations cease following pronucleus (PN) formation, presumably because the nuclear localization signal in PLC-ζ sequesters it from its substrate (Larman et al., 2004; Yoda et al., 2004). Previous work indicates that the egg "counts" the number of

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Ca^{2+} oscillations, summing the total amount of Ca^2 that is released (Ducibella et al., 2002; Ozil et al., 2005). Because different events of egg activation require different amounts of total Ca^{2+} released, e.g., CG exocytosis only requires a few oscillations (~4), whereas cell cycle resumption and recruitment of maternal mRNAs require more (>8), events of egg activation occur in a precise temporal order.

Ca^{2+}/Camodulin-dependent protein kinase II (CaMKII) is likely the primary integrator of the information encoded by the Ca^{2+} oscillations because its activity oscillates in synchrony with Ca^{2+} oscillations (Markoulaki et al., 2003, 2004) and expression of a constitutively active (Ca^{2+}-independent) mutant form of CaMKII (CA-CaMKII) results in PN formation in the absence of an increase in intracellular Ca^{2+} (Madgwick et al., 2005). These results suggest that early events of egg activation, in addition to cell cycle resumption, are triggered by CaMKII activation, but this was not established in that report. More important, the developmental potential of these activated eggs could not be assessed because the zygotes that were generated were parthenogenotes that cannot develop to term.

We report here a model that overcomes the use of parthenogenotes by introducing into an egg by intracytoplasmic sperm injection (ICSI) a sperm incapable of inducing Ca^{2+} oscillations. We used this model to test the hypothesis that CaMKII activity is sufficient to induce development in the absence of Ca^{2+} oscillations. We find that expression of a constitutively active CaMKII provides a sustained increase in CaMKII activity in these sperm-injected eggs. Although cortical granule exocytosis appears abnormal, changes in CDK1 and MAPK activity that precede PN formation, as well as recruitment and degradation of maternal mRNAs, occur and mimic what is observed following fertilization.

Materials and methods

Generation of mutant constructs and in vitro transcription of cRNA

Generation of a plasmid construct encoding CA-CaMKII, which is a C-terminal deletion of wild-type CaMKIIα, was described previously (Madgwick et al., 2005). This plasmid was linearized and transcribed in vitro using the T3 mMESSAGE mMACHINE® according to the manufacturer's instructions (Ambion, Austin, TX). The cRNA was purified using an RNeasy kit (Qiagen, Valencia, CA), eluted in RNase-free water and then stored at −80°C until use.

Collection of eggs and embryos

CF-1 female mice between 6 and 8 weeks of age (Harlan, Indianapolis, IN) were superovulated by sequential IP injections of 5 IU eCG followed by 5 IU hCG 48 h later. Metaphase II (MII)-arrested eggs were collected 13.5 h post-hCG administration into HEPES-buffered Whitten's medium (Whitten, 1971) containing 0.01% polyvinyl alcohol (PVA, average MW 30,000–70,000; Sigma) (Whittens/PVA) and cumulus cells were removed by a brief hyaluronidase treatment. Eggs were cultured in 50 μl drops of Whittens/PVA under mineral oil at 37°C in a humidified atmosphere of 5% CO_2 in air. Fertilized eggs were obtained by mating superovulated CF-1 females with B6D2F1 males (Jackson Laboratory; Bar Harbor, ME); the resulting one-cell embryos were collected 15 h post-hCG as described above.

Preparation of sperm heads devoid of Ca^{2+} releasing activity for intracytoplasmic sperm injection (ICSI)

Sperm were collected from FVB/NJ males (Jackson Laboratory). Caudae epididymides were transferred into 900 μl of Whitten's medium containing 0.01% PVA and incubated for 15 min at 37°C to allow sperm to swim out. Sperm were pelleted and then resuspended in nuclear isolation medium (NIM) containing PVA and briefly sonicated to remove the sperm tails (Kimura and Yanagimachi, 1995). To inactivate the Ca^{2+}-releasing activity, sperm heads were subjected to alkaline carbonate extraction (100 mM Na_2CO_3, pH 11.5) for 10 min at 4°C as previously described (Kurokawa et al., 2005). Sperm were washed two times in NIM/PVA and resuspended in a solution of NIM/PVA containing 50% glycerol. Sperm heads were stored at −20°C until use. ICSI using these treated sperm demonstrated that they failed to induce any increase in intracellular Ca^{2+} concentration over the period of 3 h (data not shown).

Microinjection, ICSI, and embryo culture

All micromanipulations were carried out in 5 μl drops of Whittens/PVA. For microinjection, needles were filled with 1.0 μg/μl CA-CaMKII cRNA and 5–10 pl was injected into the cytoplasm of MII eggs by pneumatic pressure using a Picoliter Injector Microinjection System (Harvard Apparatus, Holliston, MA). Following injection, eggs were cultured in either Whittens/PVA or in KSOM medium with amino acids (Specialty Media) (Ho et al., 1995).

For ICSI sperm heads were transferred into NIM/PVA and washed two times to remove glycerol. Sperm heads were resuspended in NIM medium containing 6% PVP and stored on ice until use. Co-injection of CA-CaMKII cRNA and sperm heads was carried out as follows. Three to 5 sperm heads were picked up at a time in a sperm drop and then transferred into a second drop containing 0.5 μg/μl CA-CaMKII cRNA. Sperm heads were then drawn back up into the ICSI needle and injected singularly into the cytoplasm of MII eggs using a PrimeTech piezo-impact drive (PrimeTech, Ibaraki, Japan). Groups of 10–15 eggs were quickly injected and then transferred back into KSOM medium. Control eggs were injected with sperm heads alone and then artificially activated with 10 mM $SrCl_2$ in Ca^{2+}/Mg^{2+}-free CZB for 2 h. Following activation, eggs were washed and cultured in KSOM medium with amino acids for 5 days at 37°C in a humidified atmosphere of 5% CO_2, 90% N_2 and 5% O_2. We initially activated the eggs by injecting a sperm extract containing PLC-ζ activity but settled on strontium because we obtained more consistent results.

CaMKII, histone H1 and MAP kinase assays

Measurement of CaMKII activity was carried out essentially as described previously (Markoulaki et al., 2003). In brief, eggs were washed through one drop of cold Ca^{2+}/Mg^{2+}-free PBS containing 0.05% PVA, transferred to 2 μl cold lysis buffer in a 500 μl thin-walled PCR tube, snap-frozen in dry ice and stored at −80°C until use. Egg lysates were assayed for autonomous CaMKII activity using the SignaTECT CaMKII assay system (Promega, Madison, WI), 2.5 mCi [γ-^{32}P]ATP (Perkin Elmer; Boston, MA), and a biotinylated CaMKII substrate. The autonomous activity assay measures the amount of autophosphorylated CaMKII that is active in the absence of additional Ca^{2+} or calmodulin in the reaction buffer. The samples were incubated at 37°C for 30 min, and the reaction was terminated by adding 5 M guanidine hydrochloride. Each reaction was spotted onto a SAM biotin Capture Membrane (Promega), and the membrane was washed and dried according to the manufacturer's directions. The amount of [γ-^{32}P]ATP incorporated into the CaMKII substrate was quantified by scintillation counting.

Histone H1 kinase and MAP kinase activities were each measured in single eggs, as previously described, by using histone H1 and a peptide substrate containing the MAP kinase consensus phosphorylation sequence found in myelin basic protein, respectively (Svoboda et al., 2000).

Cortical granule labeling and quantification

CGs were stained using *Lens culinaris* agglutinin–biotin (10 μg/ml, Sigma) followed by avidin–FITC (2 μg/ml, Molecular Probes) as previously