Biochemical and biological effects of KN-93, an inhibitor of calmodulin-dependent protein kinase II, on the initial events of mouse egg activation induced by ethanol

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Calmodulin-dependent protein kinase II (CaMKII) is transiently activated in mouse eggs by the increase in calcium that occurs upon activation with ethanol. This study investigated the biological and biochemical effects of KN-93, a reported selective inhibitor of CaMKII, to explore the potential role of this kinase in the initial events of egg activation. Mouse eggs were incubated for 30 min in the presence of different concentrations of KN-93 and induced to activate by 7% ethanol. KN-93 elicited a dose-dependent inhibition of polar body emission that resulted from the failure of the eggs to undergo meiosis resumption and inactivation of maturation-promoting factor (MPF). Furthermore, 15 $\mu\text{mol KN-93 l}^{-1}$ produced a marked reduction in ethanol-induced loss of cortical granules. *In vivo* biochemical analysis revealed that 15 $\mu\text{mol KN-93 l}^{-1}$ was responsible for significant inhibition of ethanol-stimulated CaMKII. The activity of the enzyme remained at a resting value, in spite of the presence of a calcium signal similar to that measured in control activated eggs. The inhibitory effects of KN-93 on the parameters tested in this study could not be mimicked by the inactive analogue KN-92. These results show that in mouse eggs, when ethanol-induced CaMKII activation was prevented, cortical granule exocytosis and meiosis resumption were inhibited. This suggests that CaMKII acts as a switch in the transduction of the calcium signal triggering mammalian egg activation.

Introduction

Sperm–egg fusion in mammals results in the activation of a series of biochemical and physiological changes including cortical granule exocytosis, a process involved in blocking polyspermy, and inactivation of MPF which triggers meiosis resumption (for a review see Yanagimachi, 1994). There is considerable evidence that the principal intracellular signal responsible for the subsequent events that constitute egg activation is the increase in cytosolic Ca^{2+} that immediately follows sperm–egg fusion (for a review see Jones, 1998a). In mice, direct evidence for this role is provided by the fact that both cortical granule exocytosis and meiosis resumption are prevented when the increase in calcium triggered by sperm or by artificial activating agents is prevented (Kline and Kline, 1992; Tombes *et al.*, 1992; Xu *et al.*, 1994). However, the downstream Ca^{2+} -dependent messengers that mediate specific events in the egg activation process remain to be elucidated.

Calcium may be the trigger for a number of parallel pathways involved in organization of the early events of development. Although protein kinase C (PKC) is implicated

in aspects of mammalian egg activation (Gallicano *et al.*, 1997; Jones, 1998b), several reports suggest that other Ca^{2+} -dependent pathways might be involved in the transition from metaphase to interphase (Winston and Maro, 1995; Xu *et al.*, 1996) or in cortical granule exocytosis (Colonna and Tatone, 1993; Ducibella and LeFevre, 1997). CaMKII is a good candidate as an effector of the Ca^{2+} signal at fertilization. This enzyme is a ubiquitous serine–threonine protein kinase that is activated by calcium and calmodulin to phosphorylate diverse substrates involved in many cellular functions, including neurotransmitter release, membrane fusion and cell cycle control (Hanson and Shulman, 1992; Means, 1994). In *Xenopus laevis*, microinjection of a constitutively active form of this protein into metaphase II arrested eggs results in the destruction of cyclin and the inactivation of p34^{cdc2} kinase activity, demonstrating the active role of this kinase in the molecular mechanism underlying cell cycle resumption in amphibian eggs (Lorca *et al.*, 1993; Lorca *et al.*, 1994). In mice, CaMKII is activated transiently upon egg stimulation with ethanol and acts upstream to the microtubule-dependent cyclin destruction machinery (Winston and Maro, 1995). A putative inhibitor of calmodulin, such as W-7, causes only a transient inhibition of sperm-induced cell cycle events and fails to block exocytosis (Xu *et al.*, 1996). However, these

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earlier studies have not directly addressed the role or function of CaMKII in mouse egg activation.

Membrane permeant pharmacological compounds have been introduced as specific CaMKII inhibitors (Tokumitsu *et al.*, 1990; Sumi *et al.*, 1991; Mamiya *et al.*, 1993; Minami *et al.*, 1994). These drugs are designed to interact with the regulatory domain of the enzyme and thereby inhibit its activation, which normally occurs as a result of autophosphorylation (Hanson and Schulman, 1992). The aim of this study of CaMKII in mouse egg activation was to investigate whether the initial events of egg response to a parthenogenetic agent such as ethanol could be affected by the CaMKII inhibitor KN-93 and its inactive analogue KN-92. KN-93 is reported to elicit potent inhibitory effects on CaMKII phosphorylating activity with an inhibition constant of $0.37 \mu\text{mol l}^{-1}$, but has no significant effect on the catalytic activity of cAMP-dependent protein kinase, PKC, myosin light chain kinase and Ca^{2+} -phosphodiesterase (Sumi *et al.*, 1991). Therefore, this drug is commonly used to investigate the role of CaMKII-dependent pathways in many cellular activities in living cells, including cell cycle control (Rasmussen and Rasmussen, 1995; Tombes *et al.*, 1995; Miralem and Templeton, 1998; Morris *et al.*, 1998) and protein secretion (Mamiya *et al.*, 1993; Niki *et al.*, 1993; Fujimoto *et al.*, 1998; Waters *et al.*, 1998).

Materials and Methods

Animals

Random bred Swiss CD1 female mice (24 days old, Charles River) were superovulated by intraperitoneal injection of 5 iu pregnant mares' serum gonadotrophin (PMSG; Folligon, Oss) and 48–50 h later by injection of 5 iu hCG (Serono, Roma). 15 h after injection of hCG, metaphase II arrested eggs were released from oviducts into M2 medium (Hogan *et al.*, 1986) and cumulus cells were dispersed by brief exposure to 0.1 mol l^{-1} hyaluronidase I⁻¹ (Sigma, St Louis, MO).

Egg treatments

In all experiments, zonae pellucidae were removed with acid Tyrode's solution (Nicolson *et al.*, 1975) and eggs were cultured in M16 medium (Hogan *et al.*, 1986) under paraffin oil at 37°C in humidified air with 5% CO_2 for 1 h before use in the experimental groups. For treatment with the CaMKII inhibitor and its analogue, eggs were cultured for 30 min in M16 medium containing increasing concentrations of KN-93 or KN-92 (Calbiochem, La Jolla, CA; stock solutions 10 mmol l^{-1} in dimethyl sulfoxide stored at -20°C) ranging from 1 to $25 \mu\text{mol l}^{-1}$. The concentrations were based on those used in other studies on the inhibition of CaMKII by KN-93 in living cells (Sumi *et al.*, 1991; Mamiya *et al.*, 1993; Tombes *et al.*, 1995). Egg activation was achieved by exposure to 7% ethanol (Fluka, Buchs) in M16 medium for 6.5 min. Treated eggs were incubated in M16 medium before processing for the evaluation of the designated parameters. Polar body

emission was monitored using a phase-contrast microscope. For each treatment group, the inhibition percentage of polar body emission (polar body frequency) was calculated by comparing the KN-93 or KN-92 treated groups with control activated eggs, according to the following equation:

$$\text{percentage inhibition} = 1 - \left[\frac{\text{polar bear frequency of treated group}}{\text{polar bear frequency control}} \right] \times 100$$

Staining of chromosomes and microtubules

For double labelling of spindle and chromosomes, eggs were fixed with methanol at -20°C , washed with PBS, attached to a coverslip coated with 1 mg ml^{-1} polylysine, and then incubated for 1 h at room temperature in a 1:200 dilution of mouse monoclonal antibody against α -tubulin (Amersham; stock solution $25 \mu\text{g ml}^{-1}$). After rinsing in PBS, the samples were incubated for 1 h in a 1:500 dilution of the secondary antibody (goat anti-mouse fluorescein-conjugated IgG; Cappel, West Chester, PA; stock solution $5 \mu\text{g ml}^{-1}$), and stained with $1 \mu\text{g}$ Hoechst 33342 ml^{-1} . The eggs were then washed in PBS and examined on whole mount preparation under a microscope fitted for epifluorescence (Leitz Dialux equipped with Leitz I2 and N2 filters).

Histone H1 assay

MPF activity in mouse eggs was evaluated by assessing the protein kinase activity of p34^{cdc2} -cyclin B towards histone H1 according to the procedure of Gallicano *et al.* (1997). The autoradiograms were scanned and analysed using image analysis software (Imaging Densitometer GS-670, Molecular Analyst v.1.0, Bio-Rad Laboratories, Hercules, CA). After quantitation, histone H1 activity in the different experimental groups was expressed as a percentage of activity in metaphase II arrested eggs.

CaMKII assay

CaMKII activity in mouse eggs was measured according to Winston and Maro (1995). Briefly, immediately after ethanol treatment, eggs were collected in $2 \mu\text{l}$ medium and added to $25 \mu\text{l}$ assay buffer containing 70 mmol l^{-1} Hepes pH 7.4, 4 mmol l^{-1} MgCl_2 , $6 \mu\text{Ci}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ml^{-1} (Amersham), 1 mg syntide 2 peptide ml^{-1} (Sigma, St Louis, MO) and anti-proteases ($1 \mu\text{g}$ leupeptin ml^{-1} , $1 \mu\text{g}$ pepstatin ml^{-1} , $1 \mu\text{g}$ aprotinin ml^{-1} , $200 \mu\text{mol}$ 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF) l^{-1}). After 10 min at 25°C , assays were stopped by adding $27 \mu\text{l}$ 20% (w/v) TCA and the samples were spotted onto P81 phosphocellulose filters (Whatman International Ltd, Maidstone), air dried for 1 min, and then washed with 1% (v/v) phosphoric acid for 20 min. After five cycles of washing, the radioactivity retained on the filters was quantified in a liquid scintillation counter. Sample blanks of $2 \mu\text{l}$ containing medium were assayed in parallel

with each group. In a given experiment, all samples contained the same number of eggs (20–30) and each treatment was performed in triplicate. CaMKII activity in the different experimental groups was expressed as a percentage of activity in control ethanol-activated eggs.

Cortical granule staining and quantitation

Eggs were fixed in 3.7% (w/v) paraformaldehyde. After permeabilization with Triton-X100, the eggs were incubated with *Lens culinaris* agglutinin (LCA) coupled to biotin and then with Texas red–streptavidin, according to Ducibella *et al.* (1988). The eggs were then mounted on slides in 50% (v/v) glycerol and granules in the cortex were visualized by a Leitz Dialux microscope equipped with a Leitz filter block M2. The density of cortical granules per 100 μm^2 was determined using a $\times 100$ objective in flat optical fields of cortex resulting from partial compression of the egg by the coverslip. The density of cortical granules for each egg was computed as the mean of the counts from three equal areas of cortex containing cortical granules, according to Ducibella *et al.* (1988). For each group, the percentage loss of cortical granules from the cortex was calculated by comparing the mean density of cortical granules of the treated group with the mean density of cortical granules of the untreated control group (Ducibella and Buetow, 1994), according to the following equation:

$$\text{percentage loss of cortical granules} = 1 - \left[\frac{\text{density of cortical granules in treated group}}{\text{density of cortical granules in untreated group}} \right] \times 100$$

Measurement of intracellular Ca^{2+} changes

An Acas 570 System (Meridian Instrument Ltd, Okemos, MI) was used for fluorescence recordings. Eggs were incubated at 37°C with 20 μmol Indo-1 acetomethyl ester (Indo-1 AM) l^{-1} and 0.02% (w/v) Pluronic (Molecular Probes, Eugene, OR) for 30 min and then subjected to KN-93 or KN-92 treatments. Eggs were extensively washed in BSA-free M2 medium and transferred on a polylysine precoated coverslip which formed the base of a chamber containing 1 ml BSA-free M2 medium. The assembly was positioned on the warming stage (37°C) of an Olympus inverted microscope, equipped with the filter sets required to record the two emission wavelengths of Ca^{2+} -free (490 nm) and Ca^{2+} -bound (405 nm) Indo-1. Confocal scans of the eggs were taken at the equatorial level at 50 s intervals. The signal was displayed as fluorescence ratio for the two emissions after background subtraction. At the end of stimulation, ethanol was removed through repeated cycles of washing with M2 medium. At the end of some experiments, 1 μmol ionomycin l^{-1} was added to verify whether the dye was reporting in its dynamic range. After the recording, the chamber containing the eggs stimulated by ethanol was maintained at 37°C for 2 h to assess meiotic stage. Eggs attached to the coverslip were fixed by a 20 min incubation in 3.7% (w/v)

paraformaldehyde in PBS, stained with 1 μg Hoechst 33342 ml^{-1} and examined on whole mount preparation under a microscope fitted for epifluorescence.

Statistical analysis

Experiments were repeated at least three times and the effect of replicates was examined by analysis of variance (ANOVA). Since the analysis did not show replicate effects, data were pooled and expressed as mean $\pm$ SEM. Statistical significance was analysed using Student's *t* test. Differences associated with a *P* value lower than 0.05 were considered statistically significant.

Results

KN-93 inhibition of ethanol-induced cell cycle resumption

The aim of this part of the study was to determine whether cell cycle resumption induced in mouse eggs by ethanol (Cuthbertson, 1983) could be affected by a specific CaMKII inhibitor. Polar body emission was monitored before ethanol treatment in eggs exposed for 30 min to increasing concentrations of KN-93 or KN-92, its inactive analogue. KN-93 elicited a dose-dependent inhibition of polar body emission over a concentration range from 7.5 to 15 $\mu\text{mol l}^{-1}$ (Fig. 1). Further inhibition of polar body emission was not achieved when the inhibitor was used at concentrations higher than 15 $\mu\text{mol l}^{-1}$. The inactive derivative KN-92 had no significant effect at any concentration tested. At 2 h after ethanol stimulation, KN-93-treated eggs that did not undergo polar body emission were double labelled for chromatin and tubulin. These eggs displayed condensed chromosomes arranged on the metaphase plate of normal bipolar spindles

Fig. 1. Dose-dependent KN-93 inhibition of ethanol-induced polar body emission in mouse eggs. Untreated eggs and eggs treated with increasing concentrations of (■) KN-93 and (□) KN-92 were exposed to ethanol and monitored for polar body emission after 2 h. The experiments were repeated at least three times and data (mean $\pm$ SEM) are expressed as percentage inhibition of polar body emission. The number of eggs examined in each treatment group ranged between 56 and 264.

(Fig. 2). This finding illustrates that the lack of polar body emission in KN-93-treated eggs was due to failure of release from metaphase arrest. Furthermore, this inhibitor did not damage the meiotic apparatus, the integrity of which is necessary for meiosis resumption (Kubiak *et al.*, 1993; Winston *et al.*, 1995).

Histone H1 kinase activity in KN-93-treated eggs was determined 1 h after ethanol exposure to investigate whether KN-93 inhibited cell cycle resumption in ethanol-stimulated eggs by preventing MPF inactivation (Winston *et al.*, 1995). Eggs treated with 15 $\mu\text{mol l}^{-1}$ KN-93 had high histone H1 activity similar to the control metaphase II arrested eggs (Fig. 3). This is consistent with the presence of condensed chromosomes aligned on a metaphase plate. In contrast, in KN-92-treated eggs, histone H1 activity decreased after activation, to a value similar to that of eggs treated with ethanol ($P > 0.5$, Student's *t* test).

These results demonstrate the ability of KN-93 to prevent ethanol-induced cell cycle resumption, as determined by release from metaphase arrest, polar body emission and histone H1 kinase activity.

KN-93 inhibition of ethanol-activated CaMKII

The effect of KN-93 on the transient activation of CaMKII that occurs immediately after ethanol exposure was

investigated (Winston and Maro, 1995). CaMKII activity was measured in eggs stimulated with ethanol after a 30 min incubation in 5 or 15 $\mu\text{mol l}^{-1}$ KN-93. Eggs exposed to the inhibitor under conditions that were ineffective in causing cell cycle arrest (5 $\mu\text{mol l}^{-1}$) displayed increased CaMKII activity (Fig. 4). However, when the inhibitor was used at a concentration that causes significant inhibition of polar body emission (15 $\mu\text{mol l}^{-1}$), the activity of the enzyme stimulated by ethanol decreased to a value similar to that in untreated metaphase II arrested eggs ($P > 0.5$, Student's *t* test) (Fig. 4). In contrast, 15 $\mu\text{mol l}^{-1}$ KN-92 was ineffective at inhibiting CaMKII. These findings indicate that 15 $\mu\text{mol l}^{-1}$ KN-93 causes significant inhibition of ethanol-induced CaMKII activation in mouse eggs.

KN-93 inhibition of cortical granule exocytosis

The effects of the CaMKII antagonist KN-93 on cortical granule exocytosis were examined to investigate further the role of CaMKII in egg activation. Eggs were incubated for 30 min in medium containing 15 $\mu\text{mol l}^{-1}$ KN-93 and then exposed to ethanol. The same concentration of the inactive analogue KN-92 was used to control the specificity of KN-93. The eggs were assayed for density of cortical granules 1 h after ethanol treatment, and release of cortical granules was quantified as cortical granule loss (see Materials and Methods). Control eggs activated by ethanol underwent a considerable loss of cortical granules (Fig. 5). In contrast, eggs treated with 15 $\mu\text{mol l}^{-1}$ KN-93 before ethanol exposure maintained a cortical granule pattern similar to that observed in metaphase II arrested eggs. Furthermore, loss of cortical granules in these eggs was very low compared with either control activated eggs or eggs exposed to 15 $\mu\text{mol l}^{-1}$ KN-92, which did not interfere with the marked release of cortical granules induced by ethanol. The difference in loss of cortical granules between these two experimental groups was not significant ($P > 0.5$; Student's *t* test).

Fig. 2. Effect of KN-93 on metaphase arrest and meiotic spindle in mouse. Eggs exposed to 15 $\mu\text{mol l}^{-1}$ KN-93 were double labelled for (a) chromatin and (b) tubulin 2 h after ethanol treatment. Scale bars represent 10 μm .

Fig. 3. Effect of KN-93 on ethanol-induced histone H1 kinase activity in mouse eggs. The experiment was performed three times and data (mean $\pm$ SEM) are expressed as the amount relative to that present in untreated metaphase II arrested eggs. For each treatment group a total of 30 eggs was analysed. Histone H1 kinase activity was monitored in untreated metaphase II (MI) arrested eggs, control eggs activated by ethanol, and in eggs treated with 15 $\mu\text{mol l}^{-1}$ KN-93 or 15 $\mu\text{mol l}^{-1}$ KN-92 before ethanol treatment.

Fig. 4. KN-93 inhibition of ethanol-induced calmodulin-dependent protein kinase II (CaMKII) activation. CaMKII activity was monitored in untreated metaphase II (MI) arrested eggs, control eggs activated by ethanol, and in eggs treated with 5 or 15 µmol KN-93 l⁻¹ or 15 µmol KN-92 l⁻¹ before ethanol treatment. The experiment was performed five times and data (mean ± SEM) are expressed as CaMKII activity relative to that present in control activated eggs.

KN-93 and ethanol-induced cytosolic Ca²⁺ increase

An increase in cytosolic Ca²⁺ is necessary for egg activation (Kline and Kline, 1992) and CaMKII stimulation (Hanson and Shulman, 1992). Therefore, the possibility was eliminated that KN-93 inhibition of meiosis resumption, cortical granule exocytosis and CaMKII activation is due to the effect of this compound on the ethanol-induced increase in Ca²⁺ (Cuthbertson *et al.*, 1981). Intracellular Ca²⁺ concentrations were determined in eggs exposed to ethanol after a standard treatment with 15 µmol l⁻¹ of either KN-93 or KN-92. Application of 7% ethanol to Indo-1 loaded eggs caused a single large increase in Ca²⁺ in eggs treated with KN-93. In all eggs, after exposure to ethanol, the increase in Ca²⁺ began without a detectable delay and rapidly reached a peak. There was no difference in the average fluorescence peak ratio in eggs treated with KN-92 compared with those treated with ethanol alone (Table 1). KN-93-treated eggs monitored for changes in Ca²⁺ were found arrested at metaphase.

Table 1. Peak fluorescence ratio recorded in mouse eggs incubated in the presence or absence of KN-93 or KN-92 after exposure to ethanol

Treatment groups	Number of eggs examined	Peak ratio (405:490)
Control	20	1.54 ± 0.05
KN-93	23	1.38 ± 0.08*
KN-92	10	1.43 ± 0.07*

Values indicate mean ± SEM.

The mean ratio for basal Ca²⁺ in these experiments was 0.5 ± 0.02 (*n* = 59). The peak ratio recorded after stimulation with 1 µmol ionomycin l⁻¹ ranged between 1.5 and 1.8.

*Values are not significantly different from control eggs; *P* > 0.1, Student's *t* test.

Discussion

In the present study, the effects of a specific CaMKII antagonist on biological and biochemical events that constitute the initial phases of mammalian egg activation were investigated. Treatment of mouse eggs with KN-93 prevented cell cycle resumption and cortical granule exocytosis induced by ethanol. There is much evidence to support the idea that these inhibitory effects are specific, that is, they are not mediated by mechanisms other than the inhibition of CaMKII. The results of the present study demonstrate that eggs treated with concentrations of KN-93 known to inhibit CaMKII in mammalian somatic cell lines (Tombes *et al.*, 1995) react to ethanol exposure in a dose-dependent manner, displaying considerable inhibition of polar body emission at 15 µmol KN-93 l⁻¹. At this con-

Fig. 5. Effect of KN-93 on ethanol-induced loss of cortical granules. Loss of cortical granules was monitored in control eggs activated by ethanol, and in eggs treated with 15 µmol KN-93 l⁻¹ or 15 µmol KN-92 l⁻¹ before ethanol treatment. The experiments were repeated three times and a total of at least 50 eggs was examined per experimental group. Data are mean percentages ± SEM.

centration, ethanol-induced MPF inactivation was prevented and cortical granule exocytosis was markedly reduced. *In vivo* biochemical analysis of these eggs showed that the inhibitory effects of KN-93 on the early events of egg activation were accompanied by significant inhibition of CaMKII activation induced by ethanol. Furthermore, on the basis of Indo-1 imaging experiments, ethanol induced similar cytosolic Ca^{2+} changes in control eggs and eggs exposed to the inhibitor. This demonstrates that failure of KN-93-treated eggs to undergo CaMKII activation, cell cycle resumption and cortical granule exocytosis after ethanol exposure was not due to absence of a normal Ca^{2+} signal. The inhibitory action of KN-93 on all the parameters tested in this study could not be mimicked by the analogue KN-92, which does not inhibit CaMKII. In addition, the presence of a normal bipolar spindle in eggs treated with the inhibitor eliminates the possibility that the protracted metaphase arrest was due to KN-93-related damage of the meiotic apparatus. These findings indicate that the effects of KN-93 are specific. In conclusion, although KN-93 may have effects on parameters not investigated in this study, this compound is a useful pharmacological tool for further investigation of CaMKII-dependent steps in mammalian eggs. This is the first study to indicate that CaMKII has a key regulatory role in egg activation in mice.

In amphibian eggs, CaMKII is responsible for the ubiquitin-dependent destruction of cyclin and thus for release from metaphase arrest (Lorca *et al.*, 1993, 1994). In the present study it was demonstrated that the inhibition of CaMKII maintains eggs in metaphase by preventing ethanol-induced MPF inactivation. This finding suggests that in mouse eggs, the Ca^{2+} -sensitive mechanism underlying meiosis resumption involves a CaMKII-dependent pathway, and supports other studies showing that W-7, a putative calmodulin antagonist, negatively affects sperm-induced cell cycle resumption (Xu *et al.*, 1996). In mouse eggs, MPF inactivation after parthenogenetic activation requires activation of PKC (Colonna *et al.*, 1997). This enzyme, which is active 15–20 min after fertilization or artificial activation and maintains this active state for some hours (Gallicano *et al.*, 1997), is present in mouse eggs in two detectable isoforms (PKC- δ and - λ) that do not have a Ca^{2+} -binding domain (Gangeswaran and Jones, 1997). Since CaMKII activation occurs as a result of calmodulin activation (Hanson and Schulman, 1992), it is possible that in mouse eggs, an increase in Ca^{2+} activates CaMKII directly and PKC indirectly, switching on diacylglycerol generating mechanisms (Billah and Anthes, 1990). These observations, together with the finding that CaMKII is activated upstream to the microtubule-dependent destruction machinery (Winston and Maro, 1995), suggests that in mouse eggs, CaMKII functions as a primary effector of the Ca^{2+} signal and targets MPF destruction through a pathway involving a PKC-dependent step.

It is well established that an increase in Ca^{2+} is essential for cortical granule exocytosis (Whitaker, 1987; Kline and Kline, 1992), but little is known about the intracellular signalling mechanism underlying this event. Recent evidence suggests that the protein machinery of membrane fusion during cortical granule exocytosis is similar to that in neuronal

exocytosis (Masumoto *et al.*, 1996; Avery *et al.*, 1997; Ikebuchi *et al.*, 1998; Masumoto *et al.*, 1998). In neuronal and neuroendocrine cells, secretion involves many kinases including PKC and CaMKII (Trifaro *et al.*, 1992; Sudhoff, 1995). In mouse eggs, failure of PKC inhibitors to block sperm-induced cortical granule exocytosis and the atypical exocytosis with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) (Ducibella and LeFevre, 1997) support the idea that Ca^{2+} -dependent effectors other than PKC are involved in exocytosis (Colonna *et al.*, 1989; Colonna and Tatone, 1993). However, a putative antagonist of calmodulin, a Ca^{2+} sensor for exocytosis in sea urchin eggs (Steinhardt and Alderton, 1982), did not affect sperm-induced exocytosis in mouse eggs (Xu *et al.*, 1996). Nevertheless, a calmodulin-dependent step may be involved in this process. Indeed, Xu *et al.* (1996) reported that W-7 results in a significant decrease in density of cortical granules, and suggested that prolonged stimulation of calmodulin by sperm-induced persistent Ca^{2+} oscillations interferes with the inhibitory action of W-7. Data from the present study show that when CaMKII activation occurring downstream to ethanol-induced Ca^{2+} increase is inhibited, cortical granule exocytosis is markedly reduced, suggesting that in mouse eggs, calmodulin is involved in the mechanism that controls exocytosis through the activation of CaMKII. This hypothesis is supported by the observation that CaMKII catalyses the phosphorylation of Rabphilin-3A (Kato *et al.*, 1994), a protein implicated in neurotransmitter release and in sperm-induced cortical granule exocytosis downstream to the increase in Ca^{2+} (Masumoto *et al.*, 1996). Therefore, it is reasonable to hypothesize that CaMKII is one of the effectors through which Ca^{2+} controls cortical granule exocytosis, a multistep process that probably requires multiple modulators.

This study of parthenogenetically induced events in mammalian egg activation provides an initial framework for identifying the physiological role of CaMKII in this process. The results represent preliminary evidence that this kinase has a role in mouse egg activation, dispersing Ca^{2+} signals to elements that control exocytosis and the cell cycle. In addition, the study provides an evaluation of KN-93 as a CaMKII inhibitor, which will be useful for future investigation of the role of CaMKII in the signalling pathway of spermatozoa.

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