Development 120,1851-1859 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

Involvement of inositol 1,4,5-trisphosphate-mediated Ca^{2+} release in early and late events of mouse egg activation

Zhe Xu[1], Gregory S. Kopf[1] and Richard M. Schultz[2,*]

[1]Division of Reproductive Biology, Department of Obstetrics and Gynecology and [2]Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA

*Author for correspondence

SUMMARY

Sperm-induced activation of mammalian eggs is associated with a transient increase in the concentration of intracellular Ca^{2+}. The role of inositol 1,4,5-trisphosphate (IP_3)-mediated release of Ca^{2+} from intracellular stores during mouse egg activation was examined in the present study by determining the effects of microinjected monoclonal antibody (mAb) 18A10, which binds to the IP_3 receptor and inhibits IP_3-induced Ca^{2+} release, on endpoints of egg activation following insemination. The antibody inhibited in a concentration-dependent manner the ZP2 to $ZP2_f$ conversion that is involved in the zona pellucida block to polyspermy, as well as the ZP2 to $ZP2_f$ conversion promoted by microinjected IP_3 in non-inseminated eggs. As anticipated, inseminated eggs that had been microinjected with the antibody were polyspermic. In addition, the antibody inhibited the fertilization-associated decrease in H1 kinase activity and pronucleus formation, and the concentration dependence for inhibition of these events was similar to that observed for inhibiting the ZP2 to $ZP2_f$ conversion. Last, the antibody inhibited the fertilization-induced recruitment of maternal mRNAs and post-translational modifications of proteins. In each case, eggs microinjected with the mAb 4C11, which also binds to the IP_3 receptor but does not inhibit IP_3-induced Ca^{2+} release, had no inhibitory effect on fertilization and egg activation. Results of these studies suggest that IP_3-mediated Ca^{2+} release is essential for both early and late events of mouse egg activation.

Key words: inositol 1,4,5-trisphosphate receptor, calcium, egg activation, H1 kinase, zona pellucida

INTRODUCTION

Sperm-induced egg activation initiates a series of responses in the egg that are temporally classified as 'early' and 'late' events. Early events include the transient rise in intracellular Ca^{2+} that subsequently leads to cortical granule (CG) exocytosis. The contents of these CGs modify the extracellular coat of the egg and results in a block to polyspermy in many species. In mouse eggs, the extracellular coat is called the zona pellucida (ZP) and is composed of three glycoproteins called ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980a; Wassarman, 1988). O-linked carbohydrates on ZP3 (Florman and Wassarman, 1985) mediate the species-specific binding of acrosome-intact sperm (Bleil and Wassarman, 1980b). ZP3 then induces the acrosome reaction of these bound sperm (Bleil and Wassarman, 1983) and it appears that both protein and carbohydrate moieties are required. Acrosome-reacted sperm, which do not interact with ZP3, then establish an interaction with ZP2 via the inner acrosomal membrane (Bleil and Wassarman, 1986; Bleil et al., 1988). Following fertilization both ZP2 and ZP3 are modified. ZP3 is modified, perhaps by a CG-derived glycosidase (Miller et al., 1993), to a form called $ZP3_f$ that can neither bind nor induce the acrosome reaction (Bleil and Wassarman, 1980b, 1983). ZP2 (M_r=120,000) is converted by proteolysis to a form called $ZP2_f$ (M_r=90,000) (Bleil and Wassarman, 1981; Moller and Wassarman, 1989) and this form cannot interact with acrosome-reacted sperm (Bleil and Wassarman, 1986). These modifications of the ZP constitute the ZP block to polyspermy and represents one of the early events of egg activation. Late events of egg activation include the emission of the second polar body, recruitment of maternal mRNAs (Cascio and Wassarman, 1982) and post-translational modifications of proteins (Van Blerkom, 1981; Endo et al., 1986; Howlett, 1986), pronucleus formation and initiation of DNA synthesis, and cleavage.

An initial transient increase in the concentration of intracellular Ca^{2+} in the egg appears to be critical for the initiation of both early and late events of egg activation (Cuthbertson et al., 1981; Cuthbertson and Cobbold, 1985; Miyazaki et al., 1986, 1992; Kline and Kline, 1992; Tombes et al., 1992; Sun et al., 1992; Fissore et al., 1992). The increase in the concentration of intracellular Ca^{2+} is observed approximately 10-30 seconds following sperm attachment (Miyazaki et al., 1986) and spreads in a wave-like fashion throughout the egg (Miyazaki et al., 1986, 1992; Kline and Kline, 1992; Tombes et al., 1992; Sun et al., 1992; Fissore et al., 1992). Treatments that increase the intracellular Ca^{2+} concentration, e.g., microinjection of Ca^{2+} (Fulton and Whittingham, 1978) or treatment with

calcium ionophores (Igusa and Miyazaki, 1983; Ducibella et al., 1988; Kline and Kline, 1992), elicit both early and late events of egg activation in the absence of sperm. In contrast, loading mouse eggs with BAPTA, which is a Ca^{2+} chelator, results in a concentration-dependent inhibition of the fertilization- or ionophore-induced Ca^{2+} transient, CG exocytosis and second polar body emission (Kline and Kline, 1992).

The initial Ca^{2+} transient induced by sperm does not require extracellular Ca^{2+}, suggesting that this Ca^{2+} is released from intracellular stores (Igusa and Miyazaki, 1983). In mammalian eggs, this intracellular Ca^{2+} source appears to be derived from an inositol 1,4,5-trisphosphate (IP_3)-sensitive store (Miyazaki et al., 1992); IP_3 is generated following phospholipase C-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate. Consistent with this hypothesis is that microinjection of IP_3 results in an initial Ca^{2+} transient similar to that observed following fertilization (Fujiwara et al., 1993; Miyazaki, 1988; Kline and Kline, 1992, 1994), as well as CG exocytosis (Ducibella et al., 1993) and the full complement of ZP modifications (Kurasawa et al., 1989). Moreover, the IP_3-sensitive Ca^{2+} store appears to be the target for IP_3 action, since microinjection of hamster eggs with the mAb 18A10, which binds to the IP_3 receptor and blocks Ca^{2+} release but not IP_3 binding (Nakade et al., 1991), blocks the sperm-induced Ca^{2+} transient (Miyazaki et al., 1992). Although these results suggest a role for Ca^{2+} released from IP_3-sensitive stores in response to a fertilization-induced production of IP_3, an inhibitory effect of the microinjected 18A10 antibody on other events of egg activation, e.g., CG exocytosis, resumption of the cell cycle, was not reported (Miyazaki et al., 1992). In addition, microinjected IP_3 does not induce resumption of the cell cycle, i.e., emission of second polar body and pronuclear formation, or recruitment of maternal mRNAs and post-translational protein modifications that are associated with egg activation (Kurasawa et al., 1989). Thus, the precise role of a sperm-induced IP_3-mediated Ca^{2+} release in the early and late events of mammalian egg activation is still unresolved.

We report here that mouse eggs microinjected with mAb 18A10, which recognizes a 12 amino acid epitope of the IP_3 receptor and inhibits IP_3-induced Ca^{2+} release without effecting IP_3 binding to the receptor (Nakade et al., 1991), inhibits sperm-induced modifications of the ZP and consequently results in polyspermy. In addition, microinjection of this antibody also inhibits the fertilization-associated drop in H1 kinase activity, emission of the second polar body and pronucleus formation, and recruitment of maternal mRNAs and initiation of post-translational protein modifications.

MATERIALS AND METHODS

Collection of gametes

Sperm were collected from 12- to 24-week-old (C57BL/6J × SJL/J) F_1 males (Jackson Laboratories) as previously described (Moore et al., 1993). Metaphase-II arrested eggs were collected from superovulated CF-1 females (Harlan) as previously described (Endo et al., 1987; Moore et al., 1993) with the following modifications: Cumulus cell-enclosed eggs were collected in bicarbonate-free Waymouth medium (Gibco-BRL) supplemented with 10% fetal calf serum and 20 mM Hepes, pH 7.2. Cumulus cells were removed with 0.05% hyaluronidase in bicarbonate-free Waymouth medium. Eggs were cultured under paraffin oil in Waymouth medium supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2 in humidified air until further use. ZP-free eggs were obtained by removing ZP with acid Tyrode's solution (Bornslaeger and Schultz, 1985).

Microinjection of mouse eggs

Eggs were microinjected with ~10 pl of the appropriate antibody solution as previously described (Kurasawa et al., 1989); final intracellular concentrations are indicated in the figure legends and are based on an egg volume of 200 pl. Following microinjection of the antibody, the eggs were cultured in CZB medium (Chatot et al., 1989) at 37°C in a humidified atmosphere of 5% CO_2 in air for 1 hour prior to in vitro fertilization (see below) to allow the antibody to diffuse throughout the egg. When IP_3 was microinjected, eggs were washed in 4 drops of bicarbonate-free CZB medium (Chatot et al., 1989) supplemented with 20 mM Hepes, pH 7.2 (CZB/Hepes) to remove the fetal calf serum. Eggs were microinjected in the same medium and cultured afterwards in CZB at 37°C in an atmosphere of 5% CO_2 in humidified air. Microinjection was also performed using bicarbonate-free Waymouth medium. The 18A10 and 4C11 antibodies used in these studies were the generous gifts of Dr Katsuhiko Mikoshiba (Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo).

In vitro fertilization

In vitro fertilization (IVF) of cumulus cell-free eggs was performed as previously described (Moore et al., 1993). IVF of ZP-free eggs was performed using 10 eggs in a 10 μl drop of Whitten's medium containing BSA (15 mg/ml) and sperm (2.5×10^4/ml).

Quantification of ZP2 to $ZP2_f$ conversion

ZP were isolated as previously described (Kurasawa et al., 1989) and the conversion of ZP2 to $ZP2_f$ in individual ZP was quantified by a biotinylation-enhanced chemiluminscent assay (Moos et al., 1994).

Histone H1 kinase assay

Histone H1 kinase activity in single eggs was assayed as follows. ZP-free eggs were washed in 4 drops of PBS supplemented with 3 mg/ml of polyvinylpyrrolidone (PBS/PVP). Single eggs were lysed in 2.5 μl of kinase buffer containing 80 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 15 mM p-nitrophenylphosphate, 10 μg/ml each of leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 2.2 μM protein kinase A inhibitor peptide (Sigma), 10 mM $MgCl_2$, 10 mM EGTA and 25 mM Hepes, pH 7.4. Egg lysates were stored at −80°C before use. Histone H1 kinase activity was assayed in a final volume of 10 μl of kinase buffer supplemented with 0.2 mg/ml histone H1 (Boehringer Mannheim), 0.1 mM ATP and 0.1 μCi/μl [^{32}P-γ]ATP (specific activity ~3000 Ci/mmol, Amersham). To determine the background level of H1 phosphorylation, 2.5 μl of kinase buffer was added instead of the egg lysate. The reaction mixture was incubated at 30°C for 30 minutes (^{32}P incorporation is linear for at least 60 minutes, Moos, Schultz and Kopf, unpublished observations) and the reaction was terminated by adding 10 μl of 2× SDS sample buffer (Laemmli, 1970). Samples were then subjected to SDS-PAGE (12% gel) and autoradiography using Kodak X-AR5 X-ray film and intensifying screens. The autoradiograms were scanned with an Image I/AT image processor (Interactive Video Systems, Inc., Concord, MA). For each experiment, the mean value of the H1 kinase activity for unfertilized eggs was arbitrarily set as 100% and the amount of activity present in the eggs following the various treatments was expressed relative to this amount.

DNA staining with DAPI (4′,6-diamidino-2-phenylindole)

ZP-free eggs were washed in PBS/PVP and fixed in 3.7% paraformaldehyde (in PBS, pH 7.4) for at least 1 hour at room temperature. Eggs were then washed in PBS/PVP, permeabilized for 30 minutes in PBS/PVP supplemented with 0.1% Triton X-100 and washed in PBS/PVP supplemented with 0.02% Tween-20

(PBS/PVP/Tween). The eggs were then stained with DAPI (1 μg/ml of PBS/PVP/Tween) in the dark for 30 minutes and washed four times, each wash for 5 minutes with PBS/PVP/Tween in the dark. The stained eggs were mounted in mounting medium (Vectashield, Vector Laboratories) and examined by fluorescence microscopy.

[^{35}S]Methionine radiolabeling of eggs and two-dimensional gel electrophoresis

Eggs were radiolabeled with [^{35}S]methionine (1 mCi/ml, specific activity ~1500 Ci/mmol, Amersham) as previously described (Poueymirou and Schultz, 1989). Radiolabeling was terminated by washing the eggs through several drops of bicarbonate-free Minimal Essential Medium containing 3 mg/ml PVP (MEM/PVP)(Moore et al., 1993) 10 hours after insemination, at which time pronuclei formed in most eggs of the control group. The eggs (about 20 for each treatment group) were transferred to lysis buffer containing 9.9 M urea, 4% NP-40, 2.2% pH 3-10 ampholytes (Millipore) and 100 mM dithiothreitol.

Two-dimensional gel electrophoresis was performed using the Investigator 2-D Electrophoresis System (Millipore) according to the manufacturer's instructions. Radiolabeled proteins were detected by fluorography (Bonner and Laskey, 1974) at −85°C using Kodak X AR5 X-ray film.

RESULTS

Effect of mAb 18A10 on the sperm-induced ZP2 to ZP2$_f$ conversion

The 18A10 mAb binds to an epitope of 12 amino acid residues at the carboxy terminus of the IP_3 receptor and blocks IP_3-induced Ca^{2+} release but not IP_3 binding (Nakade et al., 1991). This epitope is close to the proposed Ca^{2+} channel region and is absent in all ryanodine receptor subtypes and other IP_3 receptor subtypes identified thus far (Furuichi et al., 1989). 18A10 recognizes a single polypeptide of M_r=250,000 in hamster eggs (Miyazaki et al., 1992) and this corresponds to the size of the mouse IP_3 receptor (Maeda et al., 1988). As discussed above, although microinjection of hamster eggs with this antibody inhibits the fertilization-induced Ca^{2+} transient, its effect on early and late events of egg activation was not reported. Accordingly, we examined the effects of mouse eggs microinjected with 18A10 on fertilization-induced early and late events of egg activation.

Microinjection of 18A10 inhibited the sperm-induced ZP2 to ZP2$_f$ conversion in a concentration-dependent manner (Fig. 1A). Although 18A10 at a final concentration of 1 μg/ml had no significant inhibitory effect on the ZP2 conversion, 15 μg/ml resulted in a modest and significant (P<0.01, t-test) inhibition. Concentrations greater than 30 μg/ml totally inhibited the ZP2 to ZP2$_f$ conversion. It should be noted that this concentration of antibody (30 μg/ml) blocks the increase in intracellular Ca^{2+} elicited in response to IP_3 delivered by iontophoresis, as well as the increase in intracellular Ca^{2+} following fertilization in the hamster (Miyazaki et al., 1992). Last, note that PBS-injected eggs that were not inseminated did not display this conversion (Fig. 1A).

The inhibition observed in response to 18A10 antibody injection appeared specific for several reasons. First, no inhibition of the ZP2 to ZP2$_f$ conversion was observed following fertilization of eggs injected with IgG (60 μg/ml, final concentration) (Fig. 1A). Second and more important, eggs microinjected with the mAb 4C11, which recognizes the amino terminus of the IP_3 receptor but does not block either IP_3 binding or IP_3-induced Ca^{2+} release (Nakade et al., 1991), underwent a similar extent of the ZP2 to ZP2$_f$ conversion following fertilization as did control eggs (Fig. 1A). Injection of 4C11 into hamster eggs does not block the Ca^{2+} transient induced by either sperm or injected IP_3 (Miyazaki et al., 1992).

The inhibition observed following 18A10 microinjection on the ZP2 to ZP2$_f$ conversion was likely due to its ability to inhibit IP_3-induced Ca^{2+} release, since the IP_3-induced ZP2 to ZP2$_f$ conversion of unfertilized eggs was markedly inhibited

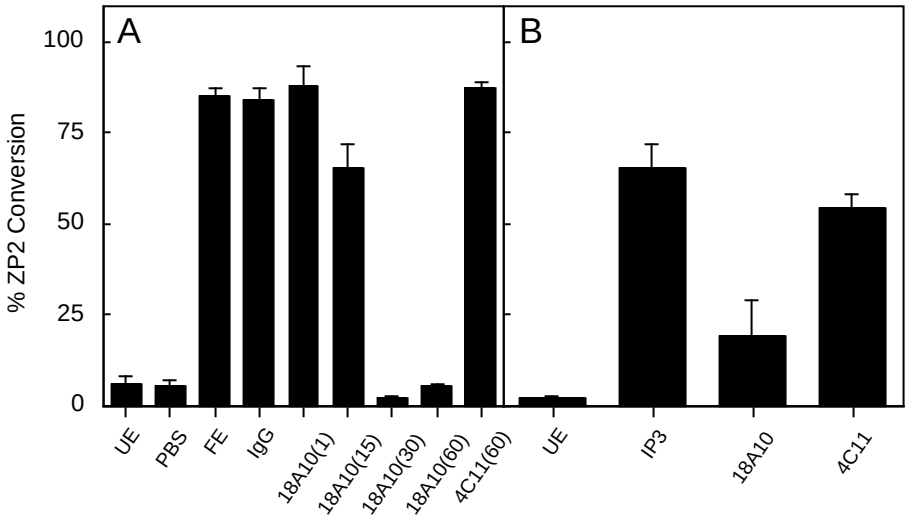

Fig. 1. (**A**) Effect of microinjected monoclonal antibodies (mAb) 18A10 and 4C11 on the ZP2 to ZP2$_f$ conversion of inseminated eggs. Eggs were microinjected with the indicated protein, inseminated and the extent of ZP2 to ZP2$_f$ conversion quantified as described under Materials and Methods. UE, unfertilized egg; PBS; eggs injected with PBS but not fertilized; FE; fertilized egg; IgG, eggs injected with IgG and fertilized; 18A10, eggs injected with the mAb 18A10 and inseminated, the numbers in parentheses indicates the final concentration of the antibody in μg/ml in the egg; 4C11(60), eggs injected with the mAb 4C11 to a final concentration of 60 μg/ml. The experiment was performed 4 times and similar results were obtained in each case. The data were pooled and are expressed as the mean ± s.e.m. The number of eggs analyzed for each treatment group was about 17. (B) Effects of monoclonal antibodies 18A10 and 4C11 on the ZP2 to ZP2$_f$ conversion induced by microinjected IP_3. Eggs were microinjected with either IP_3 (final concentration, 250 nM; column 2) or first with either 18A10 or 4C11, each at a final concentration of 60 μg/ml, prior to a subsequent injection with IP_3 (final concentration, 250 nM; columns 3 and 4). The experiment was performed 3 times and similar results were obtained in each case. The data were pooled and are expressed as the mean ± s.e.m. The number of eggs analyzed for each treatment group was about 12.

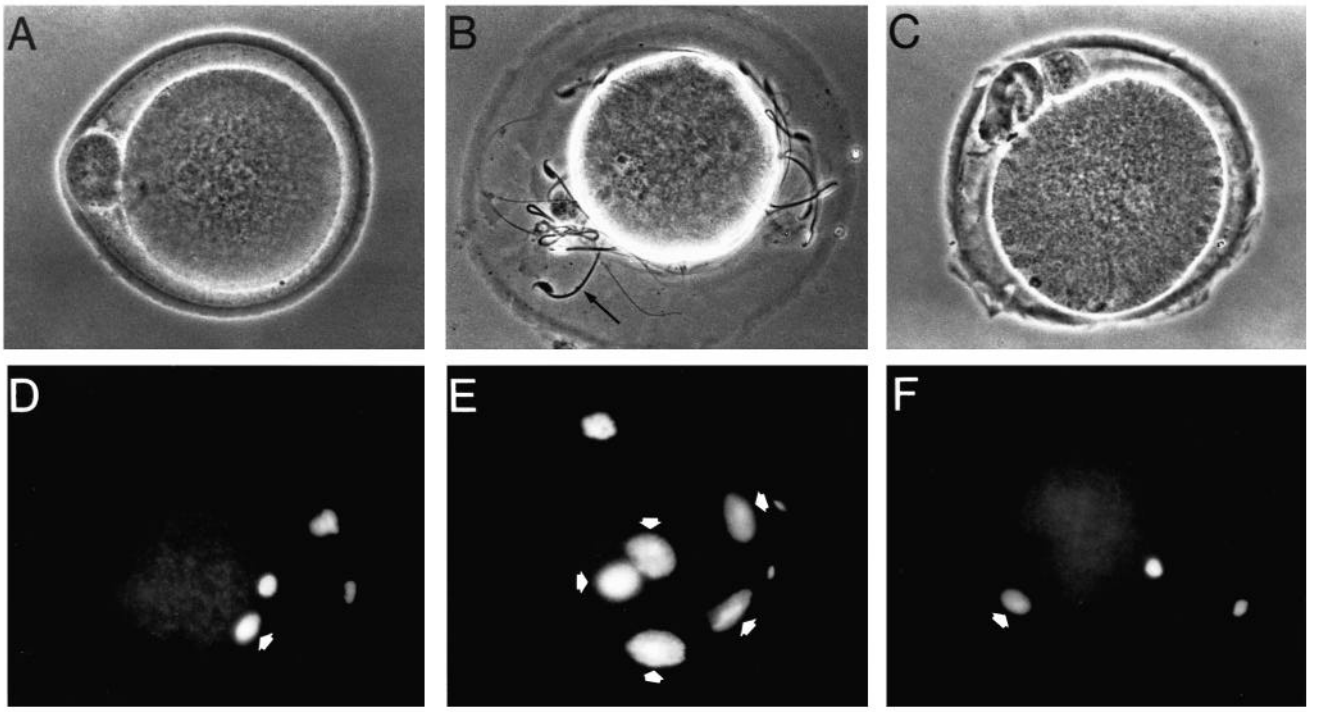

Fig. 2. Photomicrographs of fertilized eggs and eggs microinjected with either monoclonal antibodies 18A10 or 4C11 and then inseminated. (A-C) Photomicrographs of representative inseminated eggs and (D-F) photomicrographs of eggs stained with DAPI to visualize the sperm chromatin. (A,D) Buffer-injected inseminated eggs (control); (B,E) eggs injected with 18A10 (final concentration, 60 μg/ml) and then inseminated; (C,F) eggs injected with 4C11 (final concentration, 60 μg/ml) and then inseminated. (B) The arrow points to a sperm that is within the perivitelline space. (D-F) The white arrowheads point to sperm-derived DNA that is present within the egg cytoplasm. The other DAPI-staining DNAs are due to maternal DNA present in either the egg cytoplasm or polar body, or sperm that have not penetrated the egg's plasma membrane.

when the eggs were microinjected with 18A10 prior to being microinjected with IP_3 (Fig. 1B). This inhibition was not complete and may be due to the fact that relatively high concentrations of IP_3 (250 nM) were used in these studies to induce the ZP2 to $ZP2_f$ conversion. Consistent with this interpretation is that the suppressive effect of 18A10 at 60 μg/ml on Ca^{2+} release is overcome by increasing concentrations of microinjected IP_3 (Miyazaki et al., 1992). As anticipated, eggs microinjected with 4C11 and subsequently with IP_3 underwent a similar extent of ZP2 conversion as IP_3-injected eggs (Fig. 1B).

Effect of mAb 18A10 on the block to polyspermy

The ZP2 to $ZP2_f$ conversion constitutes part of the ZP block to polyspermy. The observation that microinjected 18A10 inhibited the sperm-induced ZP2 to $ZP2_f$ conversion implied that eggs treated in this manner and then inseminated should contain multiple sperm in the perivitelline space, as well as being penetrated by several sperm. This in fact was the case (Fig. 2). Compared to control eggs (Fig. 2A,D), eggs microinjected with 18A10 (60 μg/ml, final concentration) contained many sperm within the perivitelline space (Fig. 2B) and multiple sperm heads were observed within the egg cytoplasm (Fig. 2E); typically 2-6 partially decondensed sperm heads were observed. In contrast, eggs microinjected with 4C11 (60 μg/ml, final concentration) and then inseminated contained few sperm within the perivitelline space (Fig. 2C) and were monospermic (Fig. 2F). The incidence of polyspermy increased as a function of 18A10 concentration (Fig. 3) and this concentration-dependent increase was inversely related to the inhibitory effect of the antibody on the fertilization-induced ZP2 to $ZP2_f$ conversion.

Effect of mAb 18A10 on pronucleus formation

Pronucleus formation is a late event of egg activation and is observed starting 5-7 hours postfertilization. To ascertain if IP_3-induced Ca^{2+} release initiated by the fertilizing sperm was also involved in pronucleus formation, the effect of microinjected 18A10 on this event was examined. As observed for both ZP2 to $ZP2_f$ conversion and polyspermy, this antibody inhibited pronucleus formation in a similar concentration-dependent manner (Fig. 4). Moreover, this inhibitory effect was specific to 18A10, as microinjection of 4C11 (60 μg/ml, final concentration) had no inhibitory effect on pronucleus formation (Fig. 4). The inhibition caused by 18A10 was not transient since injected eggs incubated for up to 18 hours postinsemination did not form pronuclei. It should also be noted that ionophore A23187 treatment of unfertilized eggs that were first injected with 18A10 (60 μg/ml, final concentration) resulted in the emission of the second polar body (data not shown). This suggests that the inhibitory effect of 18A10 occurred through blocking the release of intracellular Ca^{2+} and that injection of 18A10 did not cause damage to the intracellular Ca^{2+} stores.

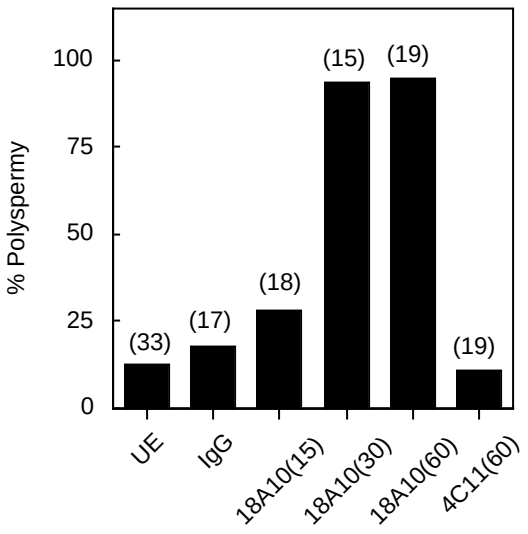

Fig. 3. Effect of microinjected mAbs 18A10 and 4C11 on the incidence of polyspermy. Eggs were microinjected with the indicated proteins, inseminated and the incidence of polyspermy, i.e., more than two sperm present within the cytoplasm of the egg, was determined. The experiment was performed three times and the numbers in parentheses indicate the number of eggs examined. The abbreviations are as described in the legend to Fig. 1.

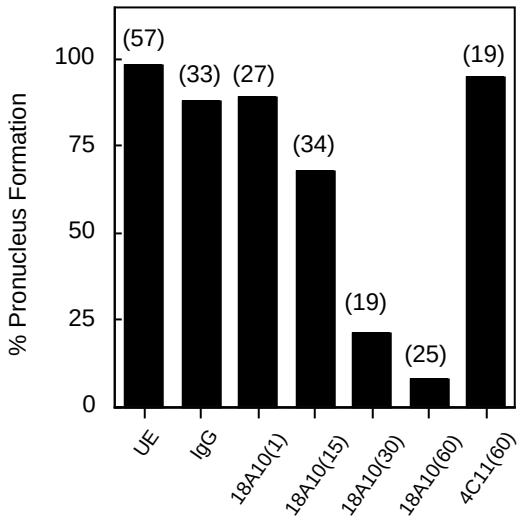

Fig. 4. Effect of microinjected mAbs 18A10 and 4C11 on the incidence of pronucleus formation. Eggs were microinjected with the indicated protein, inseminated and the incidence of pronucleus formation was determined 10 hours following insemination. The experiment was performed three times and the numbers in parentheses indicate the number of eggs examined. The abbreviations are as described in the legend to Fig. 1.

Effect of mAb 18A10 on histone H1 kinase activity

Fertilization of metaphase II-arrested eggs triggers the resumption of the cell cycle (i.e., meiosis) that will result in the formation of a pronucleus. The inhibitory effect of microinjected 18A10 on pronucleus formation could, therefore, be due to any one of a number of steps proximal to pronucleus formation. A very early event following fertilization that is involved in resumption of meiosis is a reduction of the activity of cdc2 kinase, which can be detected as a decrease in H1 kinase activity (Choi et al., 1991). Thus, we examined the effect of microinjected 18A10 on the fertilization-induced decrease in H1 kinase activity in single eggs.

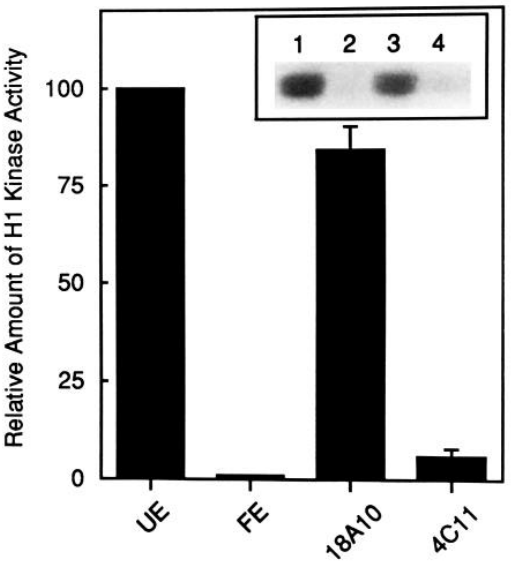

Fig. 5. Effect of microinjected mAbs 18A10 and 4C11 on histone H1 kinase activity following insemination. Eggs were microinjected with the indicated protein, inseminated and the amount of H1 kinase activity present in individual eggs was assayed 1 hour after insemination as described under Materials and Methods. The experiment was performed 4 times and data (mean ± s.e.m., where at least 15 eggs were assayed) are expressed as the amount relative to that present in the unfertilized egg. The final concentration of 18A10 or 4C11 was 60 μg/ml. The inset shows a region of the autoradiogram to demonstrate the phosphorylation of H1; there were no other visible bands on the autoradiogram. Lane 1, unfertilized egg; lane 2, fertilized egg; lane 3, egg microinjected with 18A10 (final concentration, 60 μg/ml) and then inseminated; lane 4, egg microinjected with 4C11 (final concentration, 60 μg/ml) and then inseminated.

The decrease in H1 kinase activity occurs within 30-60 minutes of fertilization or egg activation (Moos, Schultz and Kopf, unpublished observations). In order to synchronize the in vitro fertilization of the microinjected eggs so that H1 kinase could be reliably measured in single eggs, ZP-free eggs were inseminated. The fertilization-induced decrease in H1 kinase was essentially blocked by microinjected 18A10, but not microinjected 4C11 (Fig. 5). Thus, the ability of 18A10 to inhibit pronucleus formation was likely due to its ability to inhibit the resumption of the cell cycle following insemination.

Effect of mAb 18A10 on fertilization-associated changes in the protein synthesis

Fertilization results in the recruitment of maternal mRNAs (Cascio and Wassarman, 1982) as well as post-translational protein modifications (van Blerkom, 1981; Endo et al., 1986;

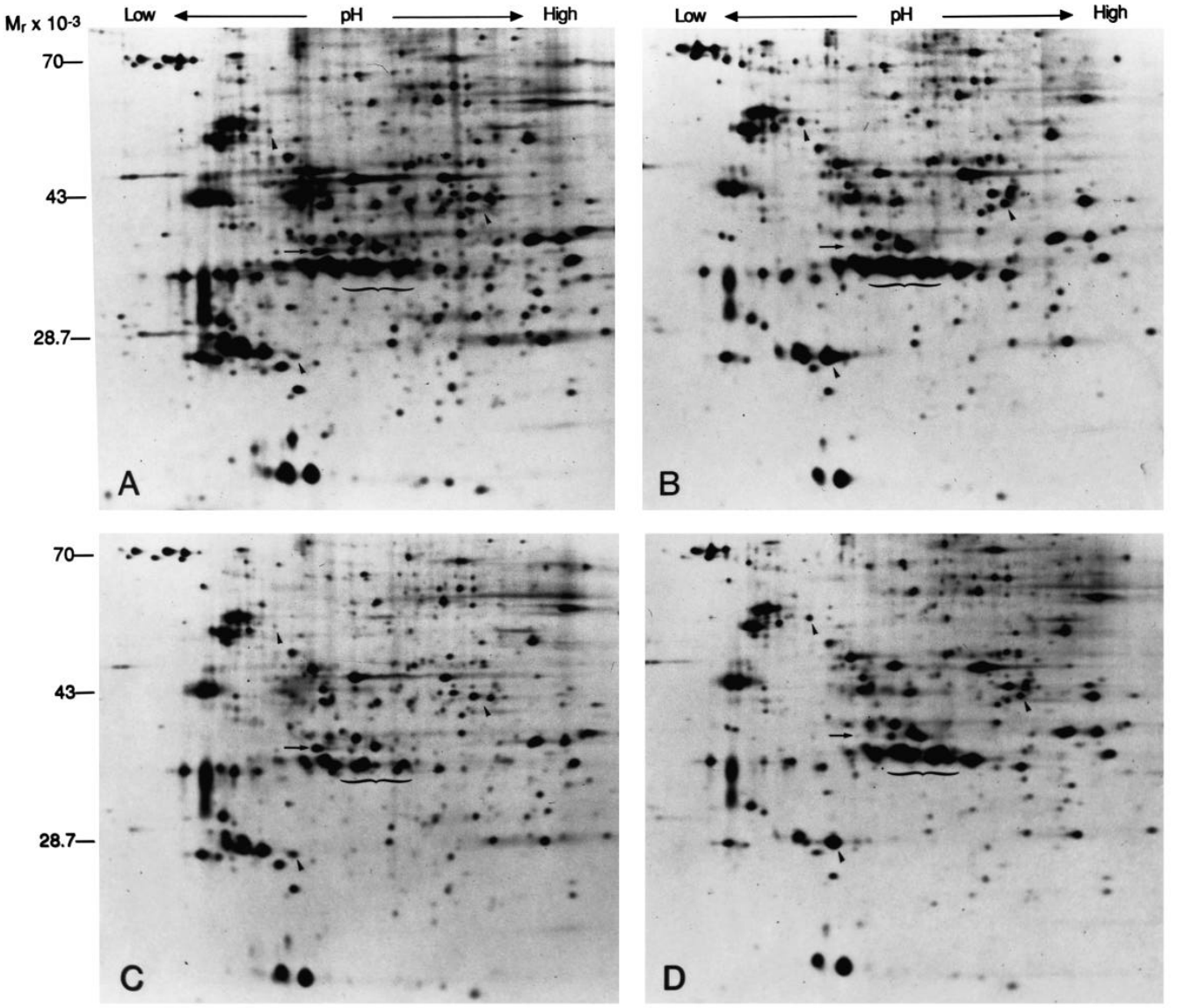

Fig. 6. Fluorograms of [^{35}S]methionine radiolabeled proteins in unfertilized and fertilized eggs, and inseminated eggs that had been microinjected with either mAb 18A10 or 4C11. The experiment was conducted as described under Materials and Methods. (A) Unfertilized eggs; (B) fertilized eggs; (C) Inseminated eggs that had been injected with 18A10, final concentration, 60 μg/ml; (D) Inseminated eggs that had been injected with 4C11, final concentration, 60 μg/ml. The chevrons indicate proteins whose synthesis increases following egg activation. The arrow points to a protein whose synthesis decreases following egg activation. The parentheses indicate a set of phosphoproteins whose synthesis changes during the first cell cycle (Howlett, 1986). The experiment was performed two times with similar results; shown are the results of one experiment.

Howlett, 1986). These changes give rise to a host of fertilization-associated changes in the pattern of protein synthesis. In order to determine if microinjected 18A10 inhibited these events, we compared the protein synthetic profiles of unfertilized and fertilized eggs to those of eggs microinjected with either 18A10 or 4C11 and then inseminated (Fig. 6). As previously reported, fertilization gives rise to increases and decreases in the synthesis of a number of proteins (Fig. 6A,B). Microinjected 18A10 blocked these fertilization-associated changes (Fig. 6C) such that the pattern of protein synthesis was similar to that of the unfertilized egg. In contrast, microinjected 4C11 did not inhibit these changes (Fig. 4D).

DISCUSSION

The results of experiments described here strongly implicate sperm-induced IP_3-mediated Ca^{2+} release in the complete activation of mouse eggs. This conclusion is based on the observations that the mAb 18A10, which inhibits IP_3-mediated Ca^{2+} release and the sperm-induced Ca^{2+} increase in hamster eggs (Miyazaki et al., 1992), inhibits (1) the ZP2 to $ZP2_f$ conversion and consequently results in polyspermy, (2) the drop in histone H1 kinase activity and hence resumption of the cell cycle, e.g., pronucleus formation and (3) the recruitment of maternal mRNAs and initiation of post-trans-

lational modifications of proteins that occur in response to egg activation.

As mentioned in the Introduction, a transient increase in the concentration of intracellular Ca^{2+} is a response common to sperm-induced activation of both non-mammalian and mammalian eggs and is essential for egg activation. There are two likely sources for this Ca^{2+}, namely the IP_3-sensitive and IP_3-insenstive Ca^{2+} stores, e.g., ryanodine-sensitive Ca^{2+} stores. During the course of fertilization of sea urchin eggs, both IP_3- and ryanodine-sensitive stores are used (Galione et al., 1993; Lee et al., 1993); inhibiting Ca^{2+} release for either store does not inhibit the fertilization-associated increase in intracellular Ca^{2+}. The presence of a functional ryanodine-sensitive Ca^{2+} store in mammalian eggs is controversial. Microinjection of mouse eggs with ryanodine (final concentration of 200-400 μM) has been reported to result in an increase in the intracellular Ca^{2+} concentration (Swann, 1992). Nevertheless, it is not apparent from that study if the increase in intracellular Ca^{2+} is similar to that which occurs following fertilization or if any events of egg activation are induced. Results of other studies, however, suggest that mammalian eggs do not possess this Ca^{2+} store. For example, microinjection of mouse eggs with ryanodine (final concentration of 160-875 μM) does not induce an increase in intracellular Ca^{2+} (Kline and Kline, 1994). Moreover, immunoblot analysis of hamster egg extracts does not detect the ryanodine receptor, whereas the IP_3 receptor is detected (Miyazaki et al., 1992). Also consistent with the absence of a ryanodine-sensitive Ca^{2+} store is that mouse eggs microinjected with cyclic ADP ribose, which is believed to release Ca^{2+} from ryanodine-sensitive Ca^{2+} stores, neither brings about an increase in intracellular Ca^{2+} (Kline and Kline, 1994) nor induces any events of egg activation (Kurasawa, Kopf and Schultz, unpublished observations). Regardless of whether mouse eggs possess a functional ryanodine-sensitive Ca^{2+} store that is used during the course of egg activation, the results described here indicate that Ca^{2+} released from IP_3-sensitive stores is essential for mouse egg activation.

Although IP_3-induced Ca^{2+} release appears necessary for sperm-induced mouse egg activation, it is not apparent if it is sufficient for total egg activation, i.e., induction of both early and late events. The basis for this statement is that microinjection of IP_3 results in CG exocytosis (Cran et al., 1988; Ducibella et al., 1993) and the full complement of ZP modifications but does not initiate the resumption of the cell cycle or recruitment of maternal mRNAs (Kurasawa et al., 1989). Likewise, microinjection of either sea urchin eggs (Whitaker and Irvine, 1984) or *Xenopus* eggs (Picard et al., 1985) with IP_3 also does not induce complete egg activation. A possible explanation for the inability of microinjected IP_3 to induce both early and late events of egg activation is that fertilization activates additional pathways that are necessary for complete egg activation. For example, G proteins are implicated in egg activation (Miyazaki, 1988; Williams et al., 1992; Moore et al., 1993) and activation through this pathway could stimulate various effectors such as phospholipase C (Birnbaumer, 1992; Clapham and Neer, 1993). Activation of this enzyme would result in the production of both IP_3 and diacylglycerol, which can activate protein kinase C (Nishizuka, 1988). In fact, treatment of eggs with biologically active phorbol diesters results in CG exocytosis (Ducibella et al., 1993) and ZP modifications, but not in cell cycle resumption and mobilization of maternal mRNAs (Endo et al., 1987).* Thus, complete egg activation may require the input of several signaling pathways that must act either in concert in a synergistic manner or in a parallel and independent fashion.

Another explanation for the inability of microinjected IP_3 to induce both early and late events of egg activation is that the sustained production of IP_3 is required. Fertilization results in multiple intracellular Ca^{2+} transients (Miyazaki et al., 1993) that have different characteristics. Whereas the initial Ca^{2+} transient occurs independently of extracellular Ca^{2+}, subsequent transients are dependent on extracellular Ca^{2+} and this dependency is postulated to be required to fill the depleted intracellular stores (Miyazaki et al., 1993). Microinjection of a single bolus of IP_3 induces a single Ca^{2+} transient that is similar in amplitude and duration to that induced by the sperm, whereas continuous microinjection of IP_3 results in multiple Ca^{2+} transients (Swann, 1992). It was not shown, however, in that study that these multiple Ca^{2+} transients result in either CG exocytosis or resumption of the cell cycle. Although these results suggest that multiple Ca^{2+} transients are involved in egg activation, this interpretation is confounded by the observation that treatment of eggs with calcium ionophore results in complete egg activation but elicits only a single Ca^{2+} transient whose amplitude is similar to the initial calcium transient that occurs following fertilization (Kline and Kline, 1992). Thus, the multiple Ca^{2+} transients that occur following the initial transient may not lead to any biological sequelae, but may simply manifest inherent properties of the Ca^{2+} release and uptake systems that operate in the fertilized egg.

What is common to the fertilization-induced Ca^{2+} transients and the single Ca^{2+} transient induced by ionophore is that following the initial Ca^{2+} transient the basal intracellular Ca^{2+} concentration is reset at a higher value (Miyazaki et al., 1993; Kline and Kline, 1992). This resetting is not observed following the microinjection of a bolus of IP_3 (Miyazaki et al., 1992). This increase may be due to the continuous entry of Ca^{2+} (Miyazaki et al., 1992) that is necessary for the refilling of the intracellular Ca^{2+} stores. More intriguing, however, is the possibility that establishing a higher basal concentration of intracellular Ca^{2+} is required for resumption of the cell cycle and recruitment of maternal mRNAs. This hypothesis may be tested by elevating in a stepwise manner the basal Ca^{2+} concentration of unfertilized eggs with Ca^{2+} buffers and assaying for early and late events of egg activation.

This research was supported by grants from the NIH (HD 22732 to G. S. K. and R. M. S.; HD 06274 to G. S. K. and HD 22681 to R. M. S.). The authors would like to thank Stuart Moss for constructive criticisms regarding the manuscript.

*It should be noted that a brief treatment of hamster eggs with biologically active phorbol diesters results in emission of the second polar body and possibly pronucleus formation (Gallicano et al., 1993). The experiments reported by Endo et al. (1989) exposed mouse eggs to phorbol diesters for 1 hour. This difference in experimental protocols is unlikely to account for the differences in egg activation in hamster and mouse eggs. We have confirmed the report of Gallicano et al. (1993) and have shown that a brief treatment of hamster eggs results in emission of the second polar body. In contrast, a similar treatment of mouse eggs does not (Moore, Kopf and Schultz, unpublished observations). Thus, species differences may in fact account for the reported differences of these eggs in response to activators of protein kinase C.

REFERENCES

Birnbaumer, L. (1992). Receptor-to-effector signaling through G proteins: Roles of βγ dimers as well as α subunits. *Cell* **71**, 1069-1072.

Bleil, J. D. and Wassarman, P. M. (1980a). Structure and function of the zona pellucida: Identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev. Biol.* **76**, 185-202.

Bleil, J. D. and Wassarman, P. M. (1980b). Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* **20**, 873-882.

Bleil, J. D. and Wassarman, P. M. (1981). Mammalian sperm-egg interaction: Fertilization of mouse eggs triggers modifications of the major zona pellucida glycoprotein, ZP2. *Dev. Biol.* **86**, 189-197.

Bleil, J. D. and Wassarman, P. M. (1983). Sperm-egg interactions in the mouse: Sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* **95**, 317-324.

Bleil, J. D. and Wassarman, P. M. (1986). Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *J. Cell Biol*. **102**, 1363-1371.

Bleil, J. D., Greve, J. M. and Wassarman, P. M. (1988). Identification of a secondary sperm receptor in the mouse egg zona pellucida: Role in maintenance of binding of acrosome-reaction sperm to eggs. *Dev. Biol.* **128**, 376-385.

Bonner, W. M. and Laskey, R. A. (1974). A film detection method for tritium-labeled protein and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**, 83-88.

Bornslaeger, E. A. and Schultz, R. M. (1985) Zona-free mouse oocytes contain adenylate cyclase. *Exp. Cell Res.* **156**, 277-281.

Cascio, S. M. and Wassarman, P. M. (1982). Program of early development in the mammal: Post-transcriptional control of a class of proteins synthesized by mouse oocytes and early embryos. *Dev. Biol.* **89**, 397-408.

Chatot, C. L., Ziomek, C. A., Bavister, B. D., Lewis, J. L. and Torres, L. (1989). An improved culture medium supports development of random-bred 1-cell embryos in vitro. *J. Reprod. Fertil.* **86**, 679-688.

Choi, T., Aoki, F., Mori, M., Yamashita, M., Nagahama, Y. and Kohmoto, K. (1991). Activation of $p34^{cdc2}$ protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. *Development* **113**, 789-795.

Clapham, D. E. and Neer, E. J. (1993). New roles for G-protein βγ-dimers in transmembrane signalling. *Nature (London)* **365**, 403-406.

Cran, D. G., Moor, R. M. and Irvine, R. F. (1988). Initiation of the cortical reaction in hamster and sheep oocytes in response to inositol trisphosphate. *J. Cell Sci.* **91**, 139-144.

Cuthbertson, K. S. R. and Cobbold, P. H. (1985). Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. *Nature (London*) **316**, 541-542.

Cuthbertson, K. S. R., Whittingham, D. G. and Cobbold, P. H. (1981). Free Ca^{2+} increases in exponential phases during mouse oocyte activation. *Nature (London)* **294**, 754-757.

Ducibella, T., Anderson, E., Albertini, D. F., Aalberg, J. and Rangarajan, S. (1988). Quantitative studies of changes in cortical granule number and distribution in the mouse oocyte during meiotic maturation. *Dev. Biol*. **130**, 184-197.

Ducibella, T., Kurasawa, S., Duffy, P., Kopf, G. S. and Schultz, R. M. (1993). Regulation of the polyspermy block in the mouse egg: Maturation-dependent differences in cortical granule exocytosis and zona pellucida modifications induced by inositol 1,4,5-trisphosphate and an activator of protein kinase C. *Biol. Reprod.* **48**, 1251-1257.

Endo, Y., Kopf, G. S. and Schultz, R. M. (1986). Stage-specific changes in protein phosphorylation accompanying meiotic maturation of mouse oocytes and fertilization of mouse eggs. *J. Exp. Zool.* **239**, 401-409.

Endo, Y., Schultz, R. M and Kopf, G. S. (1987). Effects of phorbol esters and a diacylglycerol on mouse eggs: Inhibition of fertilization and modification of the zona pellucida. *Dev. Biol.* **119**, 199-209.

Fissore, R. A., Dobrinsky, J. R., Balise, J. J., Duby, R. T. and Robl, J. M. (1992). Patterns of intracellular calcium concentrations in fertilized bovine eggs. *Biol. Reprod.* **47**, 960-969.

Florman, H. M. and Wassarman, P. M. (1985). O-linked oligosaccharides of mouse egg ZP3 accounts for its sperm receptor activity. *Cell* **41**, 313-324.

Fujiwara, T., Nakada, K., Shirakawa, H. and Miyazaki, S. (1993). Development of inositol trisphosphate-induced calcium release mechanism during maturation of hamster oocytes. *Dev. Biol.* **156**, 69-79.

Fulton, B. P. and Whittingham, D. G. (1978). Activation of mammalian oocytes by intracellular injection of calcium. *Nature (London)* **273**, 149-151.

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989). Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. *Nature (London)* **342**, 32-38.

Galione, A., McDougall, A., Busa, W. B., Willmott, N., Gillot, I. and Whitaker, M. (1993). Redundant mechanisms of calcium-induced calcium release underlying calcium waves during fertilization in sea urchin eggs. *Science* **261**, 348-352.

Gallicano, G. I., Schwarz, S. M., McGaughey, R. W. and Capco, D. G. (1993). Protein kinase C, a pivotal regulator of hamster egg activation, functions after elevation of intracellular free calcium. *Dev. Biol.* **156**, 94-106.

Howlett, S. K. (1986). A set of proteins showing cell cycle dependent modification in the early mouse embryo. *Cell* **45**, 387-396.

Igusa, Y. and Miyazaki, S. (1983). Effects of altered extracellular and intracellular calcium concentration on hyperpolarizing responses of the hamster egg. *J. Physiol.* **340**, 611-632.

Kline, D. and Kline, J. T. (1992). Repetitive calcium transients and the role of calcium exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80-89.

Kline, J. T. and Kline, D. (1994). Regulation of intracellular calcium in the mouse egg: Evidence for inositol trisphosphate-induced calcium release, but not for calcium,-induced calcium release. *Biol. Reprod.* **50**, 193-203.

Kurasawa, S., Schultz, R. M. and Kopf, G. S. (1989). Egg-induced modifications of the zona pellucida of the mouse egg: Effects of microinjected inositol 1,4,5-trisphosphate. *Dev. Biol.* **133**, 295-304.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **277**, 680-685.

Lee, H. C., Aarhus, R. and Walseth, T. F. (1993). Calcium mobilization by dual receptors during fertilization of sea urchin eggs. *Science* **261**, 352-355.

Maeda, N., Niinobe, M., Nakahira, K. and Mikoshiba, K. (1988). Purification and characterization of P400 protein, a glycoprotein characteristic of Purkinje cell, from mouse cerebellum. *J. Neurochem.* **51**, 1724-1730.

Miller, D. J., Gong, X., Decker, G. and Shur, B. D. (1993). Egg cortical granule N-acetylglucosaminidase is required for the mouse zona block to polyspermy. *J. Cell. Biol.* **123**, 1431-1440.

Miyazaki, S. (1988). Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. *J. Cell Biol.* **106**, 345-353.

Miyazaki, S., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y. and Hiramoto, Y. (1986). Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. *Dev. Biol*. **118**, 259-267.

Miyazaki, S., Shirakawa, H., Nakada, K. and Honda, Y. (1993). Essential role of inositol 1,4,5-trisphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. *Dev. Biol.* **158**, 62-78.

Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakade, S., Mikoshiba, K. (1992). Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science* **257**, 251-255.

Moller, C. C. and Wassarman, P. M. (1989). Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse oocytes. *Dev. Biol.* **132**, 103-112.

Moore, G. D., Kopf, G. S. and Schultz, R. M. (1993). Complete mouse egg activation in the absence of sperm by stimulation of an exogenous G protein-coupled receptor. *Dev. Biol.* **159**, 669-678.

Moos, J., Kalab, P., Kopf, G. S. and Schultz, R. M. (1994). A rapid, nonradioactive, and quantitative method to analyze zona pellucida modifications in single mouse eggs. *Mol. Reprod. Dev*. **38**, 91-93.

Nakade, S., Maeda, N. and Mikoshiba, K. (1991). Involvement of the C-terminus of the inositol 1,4,5-trisphosphate receptor in Ca2+ release analyzed using region-specific monoclonal antibodies. *Biochem. J*. **277**, 125-131.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature (London)* **334**, 661-665

Picard, A., Giraud, F., Le Bouffant, F., Sladeczek, F., Le Peuch, C. and Dorée, M. (1985). Inositol 1,4,5-trisphosphate microinjection triggers activation, but not meiotic maturation in amphibian and starfish oocytes. *FEBS Lett.* **182**, 446-450.

Poueymirou, W. T. and Schultz, R. M. (1989). Regulation of the mouse preimplantation development: Inhibition of synthesis of proteins in the two-cell embryo that require transcription by inhibitors of cAMP-dependent protein kinase. *Dev. Biol*. **133**, 588-599.

Sun, F. Z., Hoyland, J., Haung, X., Maxson, W. and Moor, R. M. (1992). A

comparison of intracellular changes in porcine eggs after fertilization and electroactivation. *Development* **115**, 947-956.

Swann, K. (1992). Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store. *Biochem. J.* **287**, 79-84.

Tombes, R. M., Simerly, C., Borisy, G. G. and Schatten, G. (1992). Meiosis, egg activation, and nuclear envelope breakdown are differentially reliant on Ca^{2+}, whereas germinal vesicle breakdown is Ca^{2+} independent in the mouse oocyte. *J. Cell Biol.* **117**, 799-811.

van Blerkom, J. (1981). Structural relationship and post-translational modification of stage-specific proteins synthesized during preimplantation development in the mouse. *Proc. Natl. Acad. Sci. USA* **78**, 7629-7633.

Wassarman, P. M. (1988). Zona pellucida glycoproteins. *Ann. Rev. Biochem.* **57**, 414-442.

Whitaker, M. J. and Irvine, R. F. (1984). Inositol 1,4,5-trisphosphate microinjection activates sea urchin eggs. *Nature (London)* **312**, 636-639.

Williams, C. J., Schultz, R. M. and Kopf, G. S. (1992). Role of G proteins in mouse egg activation: Stimulatory effects of acetylcholine on the ZP2 to $ZP2_f$ conversion and pronuclear formation in eggs expressing a functional m1 muscarinic receptor. *Dev. Biol.* **151**, 288-296.

(Accepted 5 April 1994)