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Protein Kinase C Activity in Mouse Eggs Regulates Gamete Membrane Interaction

Hiroto Akabane

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***PROTEIN KINASE C ACTIVITY IN MOUSE EGGS REGULATES GAMETE
MEMBRANE INTERACTION***

Thesis submitted to
the Graduate Collage of
Marshall University

In partial fulfillment of
the requirements for the degree of
Master of Science
in Biological Sciences

by

Hiroto Akabane

Dr. Simon Collier, Ph.D.
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Marshall University

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Symbols / Nomenclatures

AR	Acrosome Reaction
CGE	Cortical Granule Exocytosis
DAG	Diacylglycerol
FR	Fertilization Rate
FI	Fertilization Index
GVBD	Germinal Vesicle Breakdown
ICR	Institute of Cancer Research
IVF	In Vitro Fertilization
MPF	Maturation Promoting Factor
NCBI	National Centre for Biotechnology Information
NOS	Nitric Oxide Syhthase
PKC	Protein Kinase C
SEM	Scanning Electron Microscopy
SB	Sperm Binding Number

Drugs used in this study**Calphostin c****Chymotrypsin****DiC8**.....1,2-dioctanoyl-sn-glycerol**Formaldehyde****Glutaraldehyde****Gö 6976**.....12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-
carbazole**Gö 6983**.....

2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide

hCG.....human Chorionic Gonardotropin**Hyaluronidase****PMA**.....phorbol ester 12-tetradecanoylphorbol-13 acetate**PMSG**.....Pregnant Mare Serum Gonardotropin**Staurosporine****Y27632**.....(R)-(+)-*trans*-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide

Abstract

Every mammalian life develops from one cell after fertilization of an egg by the sperm. The molecular pathways governing this event are still poorly understood. Numerous reports indicate that mammalian eggs highly express various protein kinase C (PKC) isoforms. Accordingly, we hypothesize that PKC activity in the egg plays an important role during egg-sperm membrane binding and fusion. In this study, we tested our hypothesis in mouse gametes using two types of PKC inhibitors (calphostin c and staurosporine) and the typical PKC activator, phorbol ester 12-tetradecanoylphorbol-13 acetate (PMA). After treatment with the individual drug, eggs were inseminated with sperm. The sperm binding number (SB) and fusion rate (FR) were scored using light and fluorescence microscopy. By applying the Student's t-test, we confirmed that the FR and SB were significantly decreased after PKC activator or inhibitors treatments. All treatment groups, 1 μ M calphostin c, 1 μ M staurosporine, or 1 nM PMA showed the significant reduction of FR and SB. Further analysis indicated that the inhibition effects of calphostin c and PMA were dose- and time- dependent. Moreover, under scanning electron microscopy (SEM), we observed that the number and shape of microvilli on the PMA-treated eggs, but not on the calphostin c-treated eggs, was severely changed. Taken together, these results suggest that egg PKC activity plays an important role in the signaling regulation of sperm-egg membrane binding and fusion during fertilization.

Chapter One

Introduction

The inability to conceive is not abnormal in the United States. About 15 % of couples suffer from infertility (53). This problem stems from various factors such as low sperm count or motility, prevention of the egg from being fertilized and delivered to the uterus, sexually transmitted diseases, and so forth. Because more people tend to have children at older ages, the risk of the infertility is expected to be larger (53). *In vitro* fertilization is one of the common solutions for these infertile couples, however, it is anticipated, from new findings in this study area, that more simple solutions will be sought.

All mammalian life begins to develop after sperm-egg membrane fusion. The fertilized egg is implanted on the uterus of the maternal body, starts cell division, and develops into an embryo. The conception requires several steps in the maternal body including sperm-egg recognition and union, the fertilized egg's trip through the fallopian tube to the uterus, and the implantation.

Although these steps are shared by most mammals, the fertilization mechanism of the sperm and egg has barely been examined at a molecular level until now. The interaction of the sperm and an egg is the first event to start this series of processes. By understanding this initiation process at the molecular level, we can determine the important proteins and signals involved. Although both the sperm and the egg are responsible for this fertilization stage, this study was focused on the egg. The egg is one of the largest cells in the mammalian body, therefore, it is possible to observe the localization of specific molecules and treat with specific drugs on a single egg. For this study, many eggs had to

be isolated at one time. Thus, the mammal chosen for investigating fertilization must have certain characteristics. It should be small, easy to manage, and have enough eggs to be isolated. In this respect, CD-1 mice (common name ICR, known as Institute of Cancer Research) were purchased from Charles River labs (Wilmington, Massachusetts).

Identifying key molecules involved in sperm-egg membrane interaction is an intensive research area in the field of reproductive biology. Much progress has been made during the last decade. CD9, a member of tetraspanin membrane proteins, on the egg membrane (63) and Izumo, which is a novel immunoglobulin superfamily protein, on the sperm membrane (44) are two factors directly required for binding and fusion of the sperm and an egg. These two proteins are the only two proven requirements for these steps, even though an interaction of these two proteins is not reported thus far. CD9 is mainly expressed on the microvillus region of the egg plasma membrane (46). This microvillus region is the place where the sperm and an egg membrane interaction is mediated (25). Without CD9 and Izumo, the sperm and the egg cannot fuse with each other and conception will not be initiated. Despite these advancements, the signaling pathways inside the sperm and the egg are barely understood thus far. For this study we focused on one signaling pathway inside the egg, the protein kinase C (PKC) signaling. PKC is a superfamily of protein kinases which phosphorylate many other proteins and regulate their functions. The PKC superfamily consists of many isoforms divided into four subgroups, conventional PKC (cPKC), novel (nPKC), atypical PKC, and PKC related kinase (PRK) (27, 62). Several PKC isoforms have been reported in the egg (62). PKC activity is known to have an important role for egg meiosis resumption, egg activation, and further modification of eggs into zygotes (27, 44, 48). Many reports suggest involvement of

different PKC isoforms during egg activation, but only cPKC is proven to be involved in the step. Although it is known that cPKC activation leads to further development of the egg, any significant function of cPKC activity as well as other subgroups in the egg during fertilization and initiation of egg activation remains to be explored.

Calcium ion (Ca^{2+}) plays an important role during mammalian egg activation. After fertilization, we can observe repetitive intracellular Ca^{2+} increase in an egg (35, 45, 82). Calcium concentration in cytoplasm is repetitively and transiently raised after sperm fusion to an egg (16, 81). This calcium oscillation causes cortical granule exocytosis, which results in fertilization envelope formation (50). This protein layer makes a physical barrier that prevents further sperm fusion to the egg. Actin is involved in the modification of the egg after fertilization and interacts with PKC alpha, one isoform of cPKC (22). A recent study by Halet and his colleagues (36) also shows the close relationship between cPKC and calcium oscillation. From their research, it is expected that the long-term calcium level may be maintained by cPKC activity (36). Because the vigorous activity of PKC has been demonstrated inside the egg, we hypothesize that PKC is also involved in gamete membrane binding and fusion. To determine the importance of PKC activity during sperm-egg fertilization, various PKC activators and inhibitors were used to treat the mouse eggs. The ability of fusion and binding of the egg was then tested by in vitro fertilization (IVF). The collected results will determine the role, if any, of specific PKC isoforms in the sperm-egg binding and/or fusion. Moreover, PKC activity is expected to regulate actin-supported microvilli and CD9 which is localized on microvilli and physically attached by PKC on the egg (46, 55, 98). The morphological structure of the egg plasma membrane will also be viewed by scanning electron microscopy after drug

treatment.

This experiment aims specifically at the sperm-egg binding and fusion. So the eggs used in this experiment are zona-free eggs, which zona pellucida is chemically removed from the egg. Zona pellucida is the polymers of three glycoproteins forming the gel layer around the egg (24, 73). It induces acrosome reaction on the sperm and allows the sperm to go through this jelly layer (23, 64). It also protects the egg from multiple fertilizations by sperm (polyspermy) (92). To observe the pure interaction of sperm and egg membrane, we removed this outer layer. So the sperm binding and fusion number is not affected by the functions of zona pellucida.

Objectives

The objectives of this study are: 1) to determine the fertilization ability of zona-free mouse eggs after PKC activation or inhibition inside the eggs; 2) to determine whether there is an alteration of structure and localization of actin, CD9, chromosomal DNA, and microvilli after PKC activation or inhibition in the zona-free mouse egg. Regulation of PKC is thought to play a critical role in the egg's ability to bind and fuse with the sperm. Clarifying the effects of PKC activation and inhibition in the egg will further our understanding of the egg's signaling pathways during fertilization.

Specific Aims

PKC is involved in meiosis resumption and further egg activation. It is also the key regulator for biological activities of many target proteins in a cell. PKC is expected to have an important role during fertilization. Therefore, the goal of this study is to determine any alteration of fertilization ability of the mouse egg when PKC is activated or inhibited, and to determine any alteration of the structures of the important molecules (actin, CD9, chromosomal DNA, and microvillus) of the egg during fertilization.

Specific Aim 1

To determine the effects of PKC activation or inhibition in the mouse egg during gamete membrane interaction.

Hypothesis: PKC activation or inhibition blocks sperm binding and fusion to the egg.

Specific Aim 2

To determine the molecular and structural alternations in the mouse egg after PKC activation or inhibition.

Hypothesis: Both PKC activation and inhibition change the localization and the extent of some fertilization-related molecules and structures.

Chapter Two

Review of Literature

INTRODUCTION

This chapter briefly reviews reported studies about this area of research. The sections are consisted of three parts:

One: Important molecules participating in sperm-egg membrane interaction and further embryonic development

Two: Protein kinase C (PKC) as the important signaling molecules inside the egg during egg activation

Three: CD-1 (ICR) mice as an experimental model for the investigation of IVF

Important molecules participating in sperm-egg membrane interaction and further embryonic development

The molecular and cellular mechanisms underlying sperm-egg fertilization have begun to be unfolded in the last two decades and still are being extensively explored. Since this event consists the interaction of two kinds of cells, studies on both the sperm and the egg are necessary to understand this phenomenon. First of all, the acrosome reaction (AR) is the process by which the sperm acquires the fertilization competency. AR modifies the sperm membrane proteins and the sperm membrane, thus enables the sperm to fertilize an egg (72). The freshly ejaculated sperm is unable to fertilize without undergoing AR (72). This process is initiated when a sperm attaches to the zona-pellucida of the egg *in vivo*. It also can occur spontaneously after 2 hours capacitation in a suitable culture

medium *in vitro* (37, 84). This process is required for the sperm to penetrate through the zona pellucida of the egg by producing nitric oxide synthase (NOS) (11). NOS enhances the motility and AR of sperm which allow the sperm to fertilize with egg effectively (38, 74). On the other hand, oocytes undergo maturation steps inside the maternal body, but their meiosis is arrested at the second metaphase (MII). When oocytes reach this phase, they are defined as “egg” (59). The MII eggs do not experience further activation without sperm fusion. Several key cellular events follow sperm-egg fusion, activation of the egg, resumption of the egg meiosis, pronuclear formation, and initiation of the early embryonic development, among others.

However, little is known about fertilization at the molecular level. To date, only two proteins are known to be required factors during sperm-egg fusion, Izumo on the sperm membrane and CD9 on the egg membrane. A Japanese research group recently reported that sperm from Izumo-knockout male mice are infertile (44). Sperm from Izumo knockout mice cannot fuse with the egg *in vitro*, thus proving that the Izumo protein on the sperm membrane is essential for sperm fusion with the egg. The gene encoding Izumo was identified from crude sperm protein by gel electrophoresis and immunoblotting. A human homologue was an unverified gene in the National Centre for Biotechnology Information (NCBI) database. The predicted protein size was 56.4 kDa on mouse sperm and 37.2 kDa on human sperm. This protein was detectable only after the AR of the sperm.

CD9 knockout mice were also generated but only CD9 knockout females were infertile (18). The eggs from CD9 knockout female mice cannot be fused with sperm *in vitro* (34). CD9 is a member of the tetraspanin family. Proteins in this family have four transmembrane domains. Zhu and colleagues (99) reported the requirement of the large

extracellular loop of CD9 for fertilization. Using microinjection, injection of Izumo knockout sperm into a wild type egg or wild-type sperm into a CD9 knockout egg resulted in normal egg activation and further development of the resulting embryo to term. The derived offspring had no physical difference from wild-type mice (18, 44). Therefore, these two proteins are currently confirmed as required molecules (fusion factors) specifically involved in the process of sperm-egg fusion. These data indicate that each is necessary but not that the pair alone is sufficient for fertilization.

The discovery of these two important molecules involved in fertilization has led to further questions. What are the signaling pathways that regulate these molecules? What signaling pathways are activated or inhibited after fertilization? To explore these questions, we focused on PKC activity in mouse eggs. Because an egg is one of the biggest single cells in an organism, molecular localization and structural changes can be observed and analyzed with simple instruments. Zona pellucida is the final mechanical barrier to sperm (6). Because of this property, we need to remove zona pellucida for certain observation of sperm-egg membrane interaction.

Protein kinase C (PKC) as an important signaling pathway during egg activation

PKC is a family of serine/threonine kinases, which are enzymatic proteins that phosphorylate other target proteins to stimulate their cellular activity (47, 66). PKC comprises four subgroups based on differences in activation sites (86). The four subgroups are as follows: conventional PKCs (cPKC: PKC α , PKC β and PKC γ), atypical PKCs (aPKC: PKC δ , PKC ϵ , PKC η , and PKC θ), novel PKCs (nPKC: PKC ζ , PKC ι , and PKC λ), and PKC-related kinases (PRK). These subgroups are regulated by multiple activators

including calcium ion, diacylglycerol, and phosphatidylserine (Fig. 1-1). Many mechanisms and signaling pathways involving PKC activity have been described (76, 97).

One reason for studying PKC is that it is targeted by diacylglycerol and phosphatidylserine, which are components of the cellular membrane. Because fertilization is completed by gamete membrane interaction (gamete membrane binding and fusion) the involvement of PKC activity is a strong possibility. Moreover, one previous study (98) showed the association of PKC and CD9 after classical PKC activation by phorbol 12-myristate 13-acetate (PMA). Therefore, PKC activity may contribute to the signaling pathway(s) in the egg during sperm-egg membrane binding and fusion.

Many studies have investigated the significance of PKC activity in oocyte maturation and egg activation. Before oocyte is completely matured to a secondary oocyte, meiosis has to be resumed and prophase I to be finished (29). Germinal vesicle breakdown (GVBD) is one of the important steps during the final stage of oocyte maturation. PKC activation of mouse oocytes inhibit GVBD (26), but PKC activation by 12-myristate 13-acetate (PMA) stimulates further egg activation at the MII stage (58). Early events of oocyte activation, e.g., second polar body emission and pronuclear formation, are restrained after inhibition of PKC activity (27). Furthermore, most PKC isoforms are required for egg activation and the early stage of embryonic development (19, 27, 49). These findings indicate important and very complex activities of PKC during oocyte maturation and egg activation. Although this signaling pathway has been investigated in many studies, PKC activity in the regulation of sperm-egg membrane binding and fusion has not been explored.

There are many commercially available PKC activators and inhibitors. PMA and

1,2-Dioctanoyl-sn-glycerol (DiC8) are used as PKC activators, and calphostin c, staurosporine, (*R*)-(+)-*trans*-4-(1-Aminoethyl)-N-(4-Pyridyl) cyclohexane carboxamide dihydrochloride (Y-27632), 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a) pyrrolo (3,4-c)-carbazole (Gö 6976), and 2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide (Gö 6983) are used as PKC inhibitors. These chemicals target different PKC isoforms, so that the specific PKC isoform(s) playing an important role during fertilization of mouse gametes may be identified.

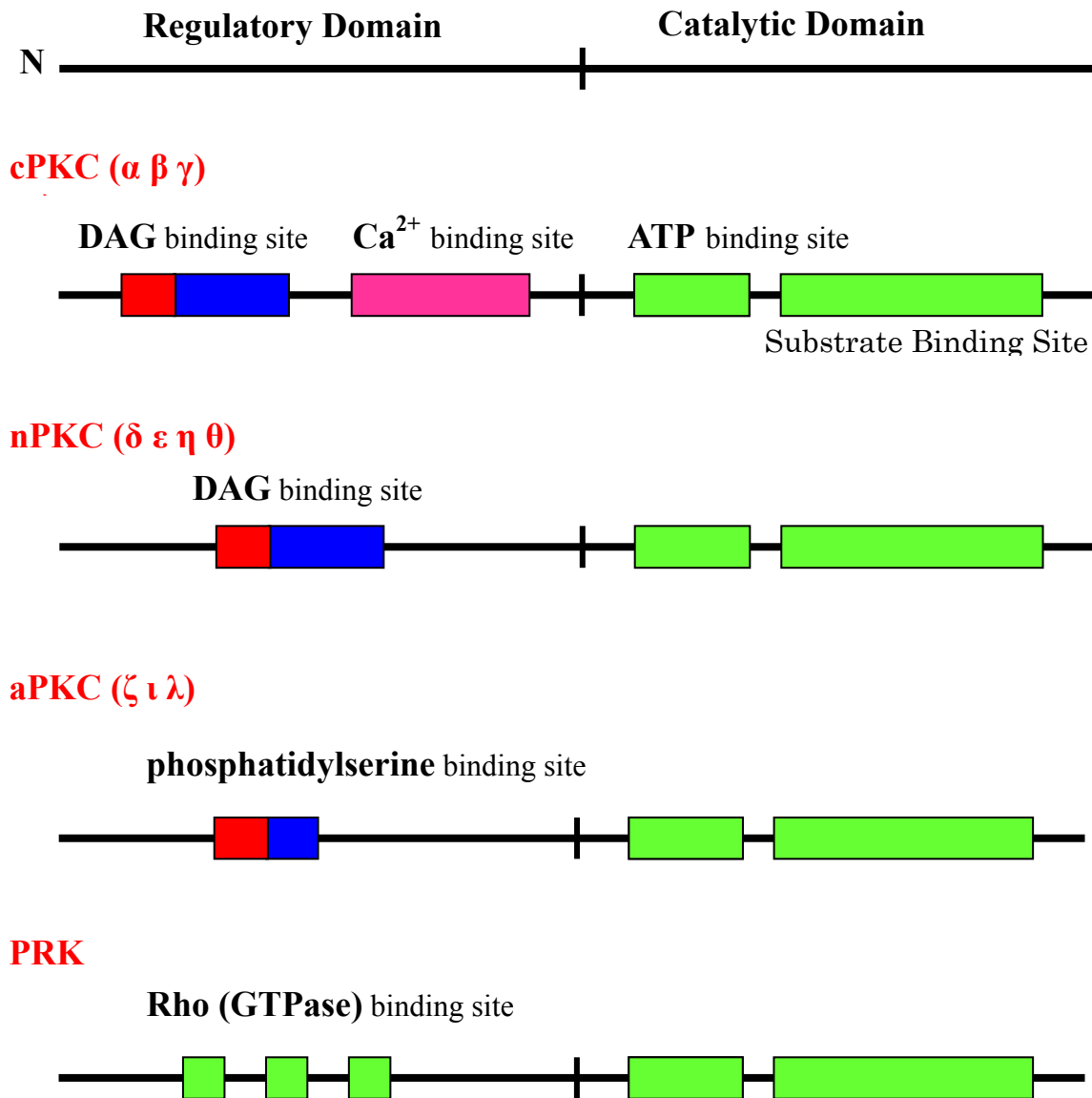


Figure1-1. Schematic structures of four PKC subgroups.

Each group has distinct regulatory and catalytic domains. To be activated, cPKC requires DAG and calcium ion; nPKC requires DAG; aPKC requires phosphatidylserine; and PRK requires Rho. ATP is an obligatory factor for all PKC isoforms to be stimulated.

(DAG: Diacylglycerol)

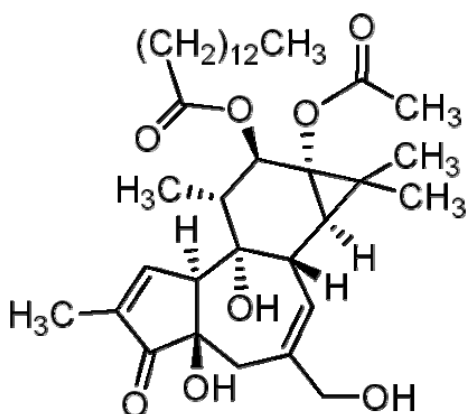


Figure 1-2. The structure of Phorbol 12-myristate 13-acetate (68).

It activates traditional PKC subgroups (cPKC and nPKC) by binding DAG binding site (80).

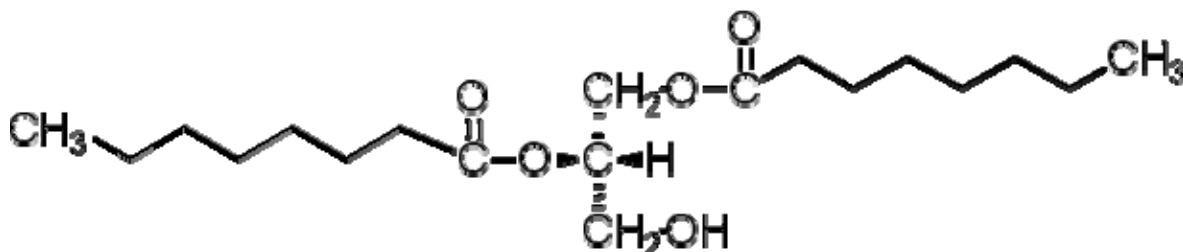


Figure 1-3. The structure of 1,2-Dioctanoyl-*sn*-glycerol (1).

It activates traditional PKC subgroups by binding DAG binding site (80)

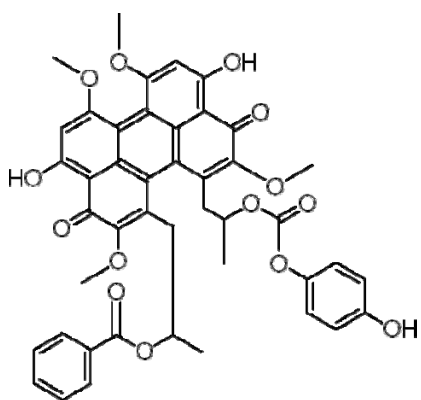


Figure 1-4. The structure of calphostin c (13).

It is from Fungi *Cladosporium cladosporioides*, activated by light and a very specific PKC activator (13).

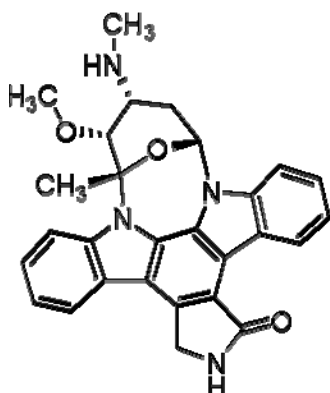


Figure 1-5. The structure of staurosporine (79).

It is from bacteria *Streptomyces* sp. It inhibits many protekin kinases inside a cell (39, 71).

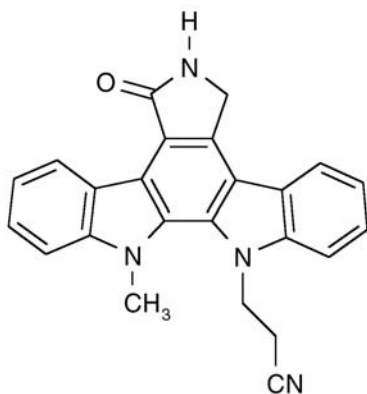


Figure 1-6. The structure of 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a) pyrrolo (3,4-c)-carbazole (Gö 6976) (30).

It is known to inhibit cPKC activity, but other subgroups are potentially inhibited (51, 77).

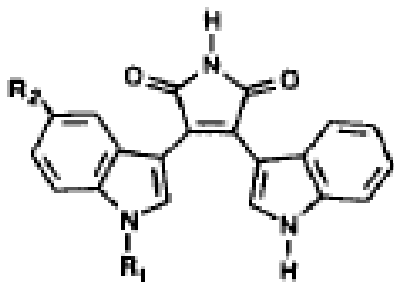


Figure 1-7. The structure of 2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide (Gö 6983) (34).

It is known to inhibit PKC activity except PRK (34).

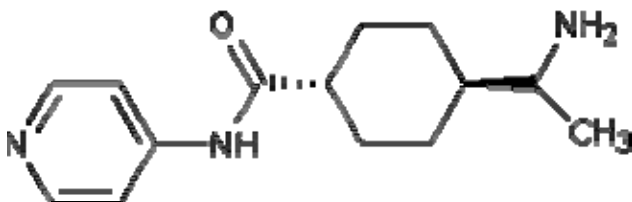


Figure 1-8. The structure of (*R*)-(+)-*trans*-4-(1-Aminoethyl)-*N*-(4-Pyridyl) cyclohexane carboxamide dihydrochloride (Y-27632) (96).

It is known to inhibit PRK activity by binding ATP binding site (96).

CD-1 (ICR) mice as an experimental model for the investigation of in vitro fertilization

Sperm-egg membrane binding and fusion are shared by all mammals as the part of reproduction event. To explore the underlying mechanisms, it is required that we can obtain enough numbers of eggs as needed from the animal. We also need to consider husbandry and fecundity of the animal. ICR (Institute of Cancer Research, where this mouse strain was developed) mice from Charles River Laboratories were used as the experimental model in this research. This strain was Swiss origin and established in 1926 from 9 albino mice by Dr. Clara Lynch (83). Mating and breeding of this strain results in more pups at a time comparing with normal non-albino mouse strain. Generally speaking, each ICR mouse can ovulate about 25 eggs after being injected with pregnant mares' serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) in our laboratory. Sacrificing 6 mice at each experiment, we were able to collect at least 60 eggs, which were enough to make more than 3 groups of experiments with more than 15 eggs per group. Thus, this experimental model is one of the best animal models to facilitate this fertilization research.

In our experiments, 8-week old ICR mice were purchased from the commercial vendor and normally spent 2 weeks in the animal facility of Marshall University to accommodate the new environment before being used for egg isolation. We injected two hormones PMSG and hCG into the mice to stimulate ovulation. The best egg numbers were obtained from the oviducts of the mice that were sacrificed 13.5 hours after hCG administration, which should be given at 47.5 after first PMSG injection. When the mice were sacrificed more than 14 hours after hCG injection, we had problems to remove zona pellucida from the eggs.

All animal care and use procedures described within were reviewed and approved by the Institutional Animal Care and Use Committee of Marshall University, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Chapter Three

Manuscript for submission

PROTEIN KINASE C ACTIVITY IN MOUSE EGGS REGULATES GAMETE
MEMBRANE INTERACTION

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Abstract

Every mammalian life develops from one cell after fertilization of an egg by the sperm. The molecular pathways governing this event are still poorly understood. Numerous reports indicate that mammalian eggs highly express various protein kinase C (PKC) isoforms. Accordingly, we hypothesize that PKC activity in the egg plays an important role during egg-sperm membrane binding and fusion. In this study, we tested our hypothesis in mouse gametes using two types of PKC inhibitors (calphostin c and staurosporine) and the typical PKC activator, phorbol ester 12-tetradecanoylphorbol-13 acetate (PMA). After treatment with the individual drug, eggs were inseminated with sperm. The sperm binding number (SB) and fusion rate (FR) were scored using light and fluorescence microscopy. By applying the Student's t-test, we confirmed that the FR and SB were significantly decreased after PKC activator or inhibitors treatments. All treatment groups, 1 μ M calphostin c, 1 μ M staurosporine, or 1 nM PMA showed the significant reduction of FR and SB. Further analysis indicated that the inhibition effects of calphostin c and PMA were dose- and time- dependent. Moreover, under scanning electron microscopy (SEM), we observed that the number and shape of microvilli on the PMA-treated eggs, but not on the calphostin c-treated eggs, was severely changed. Taken together, these results suggest that egg PKC activity plays an important role in the signaling regulation of sperm-egg membrane binding and fusion during fertilization.

Introduction

Sperm-egg membrane binding and fusion is a critical event, initiating the development of a new life, but the molecular signaling pathways governing this event are barely explored. The protein kinase C (PKC) signaling pathway has been shown to play an important role in diverse cellular processes, such as, cell differentiation, cell growth, and cell death (3, 43, 62, 89). In addition to these essential roles, oocyte maturation and egg activation are also regulated by PKC activity (27). There are four broad PKC subfamilies consisting of 13 isoforms; conventional PKC (cPKC; PKC α , PKC β , and PKC γ), novel PKC (nPKC; PKC δ , PKC ϵ , PKC η , and PKC θ), atypical PKC (aPKC; PKC ζ , PKC ι , and PKC λ), and PKC-related kinase (PRK) (62). These four subfamilies have different modes of regulation for their activity. Each activator binds to the regulatory domains of the target PKC isoforms, which allows the substrate to bind with catalytic domain of the PKC. cPKC requires 1,2-diacylglycerol (DAG) and calcium ion (Ca²⁺). nPKC requires DAG or phorbol esters. aPKC requires Phosphatidylserine. PRK requires Rho (3,62, 89). Rho is a family of GTP-binding proteins and modifies the actin cytoskeleton (9). A variety of PKC isoforms, have been confirmed in mouse eggs (28, 58).

Ca²⁺ inside the egg is also an important signaling molecule. Germinal vesicle stage mouse oocytes do not continue maturation to pronuclear formation after Ca²⁺ chelation (5). The Ca²⁺ level inside the egg is dramatically increased after fertilization with sperm (67), leading to cortical granule exocytosis (CGE) at the point of sperm fusion (49, 54, 67). When eggs are treated with Ca²⁺ chelator, CGE and second polar body formation are inhibited (49), and an increase in the number of polyspermy is observed (61). Both Ca²⁺ independent PKC, and Ca²⁺ dependent PKC are involved in early egg activation (85).

These data suggest that the increase in Ca^{2+} concentration is probably related to the PKC signaling inside the eggs, stimulating maturation and activation of mouse oocytes.

In the present study, we particularly focus on the egg PKC activity relevant to fertilization. PKC activity constitutes the necessary signaling pathway for the egg just before and immediately after fertilization. Moreover, PKC is recruited to the inner cell membrane in order to be activated inside the cell (3, 43, 62, 89). Accordingly, we hypothesize that PKC is a requirement for sperm-egg membrane binding and fusion. To test this, the effects of PKC activation and inhibition were examined. Eggs were treated with either a PKC activator or a PKC inhibitor before insemination, and then the egg's ability to fertilize was assessed. Staurosporine and calphostin c (PKC inhibitors), and PMA (a PKC activator), were applied. Staurosporine inhibits protein kinases, such as, protein kinase A (PKA), PKC, and protein kinase G (PKG) (39). Calphostin c competes and binds with DAG and phorbol ester binding sites (31), inhibiting the classic PKC isoforms (4, 12, 31, 56, 77). PMA is a phorbol ester required for activating classical PKC isoforms (cPKC, nPKC, and aPKC) (62). Both Ca^{2+} dependent and independent PKCs are activated by PMA (2, 95).

PKC is involved in and regulates many signaling pathways in a variety of cell types (3, 43, 62, 87, 89). We speculate that this drug-altered activity in the egg also leads to cellular responses other than fertilization events. Actin, CD9, and chromosomal DNA are known as important molecules involved in egg activation and maturation (61, 20, 46, 63, 69). Actin is a main component of the cytoskeleton and plays various functions in the cell. These functions include strengthening the cell, organizing the organelles, and controlling cell division (48, 94). In the egg, actin has been shown to function in the adhesion of

sperm to the egg and the formation of a polyspermy shield (61, 90). The actin polymer, F-actin is easily stained by phalloidin conjugated with a labeling dye, rhodamine.

CD9 is associated with microvilli, and is the only egg membrane protein which is confirmed as a required molecule for sperm-egg fusion (46, 63). Eggs from CD9 knockout female mice or those treated with the CD9 monoclonal antibody KMC8 are not able to fuse with sperm. In somatic cells, translocation of cPKC to the plasma membrane forms a physical association with CD9 (98). These data suggest that PKC may associate with and regulate CD9's function, which, in turn, regulates the egg's fertilization ability. It is our hypothesis that changes in the localizations and/or structures of actin, CD9, chromosomal DNA, and microvilli will be observed after PKC activation or inhibition. By analyzing the cellular dynamics of these molecules, we will determine the importance of PKC signaling in fertilization.

In this study, to test the effects of PKC signaling, the egg surface and some molecules involved in the egg fertilization and activation are examined after PKC activation/inhibition.

Methods and Materials

Collection of eggs and sperm cells

MII phase oocytes were isolated from 8 to 10 weeks old ICR (65) mice into 100 μ l of M199 Medium containing 0.3 % bovine serum albumin (BSA; Medium 1). Female mice were superovulated by intraperitoneal injection of 10 i.u. (100 μ l of 100 i.u./ml) of pregnant mares' serum gonadotropin (PMSG) into female mice. After 48 hours, 10 i.u. (100 μ l of 100 i.u./ml) of human chorionic gonadotropin (hCG) was injected

intraperitoneally. Approximately 13-14 hours post-hCG injection, mice were sacrificed by suffocation (CO₂ incubation in a plastic box at room temperature) for 2 minutes. The oocytes surrounded by cumulus cells were obtained from the oviducts. Hyaluronidase (300 µg / ml) treatment for 5 minutes was used to release the cumulus masses, followed by chymotrypsin (30 µg / ml) treatment for 3 minutes to remove the zona pellucida from the oocytes. Using a zona-pipette (appendix A), we applied physical pressure to the zona pellucida to obtain zona-free eggs. These zona-free eggs were stored in 100 µl of Medium 1 at CO₂ incubator (37 °C, 5% CO₂). A mouth pipette was used to transfer the eggs in the in vitro fertilization experiments. After each drug treatment, the eggs were washed three times with 100 µl of Medium 1 (appendix A). All drug treatments were completed in Medium 1.

After 8 to 12 weeks male mice were sacrificed by suffocation, caudal epididymis and vas deferens were obtained. Sperm were isolated from the caudal epididymis and vas deferens by placed and chopped in 500 µl of M199 Medium with 3 % BSA (Medium 2). The concentration of sperm in the medium was approximately 1×10^7 sperm / ml after sperm isolation from one male mouse. Because the sperm acrosome reaction is required before sperm fusion to the egg (75), sperm were capacitated (changes that enable sperm to undergo both the acrosome reaction and hyperactivation.) for two hours in 500 µl of Medium 2 in the CO₂ incubator. Sperm were diluted (1:10) from the original concentration to get the sperm concentration at 1×10^6 sperm / ml.

Drug Treatments of the eggs

All drugs used in this experiment are dissolved in dimethyl sulfoxide (DMSO).

Therefore DMSO treatment is used as the control group. After a 60 minutes resting interval, zona-free eggs were incubated with 1 μ M calphostin c or 1 % DMSO in 100 μ l of Medium 1 for 60 minutes. After three times washing with Medium 1, eggs were incubated with DAPI for 15 minutes and washed three times. The same procedure was applied to staurosporine and PMA treatments.

In Vitro Fertilization (IVF)

Ten μ l of sperm (1×10^6 sperm / ml) from the capacitated medium were transferred into 100 μ l of Medium 1 containing the drugtreated eggs (10:110 dilution of the sperm: approximately 1×10^5 sperm cells in the insemination medium). Fourty minutes after insemination, eggs were washed three times with Medium 1 to remove nonspecific sperm binding. Eggs were then fixed with 3.7% formaldehyde for 15 minutes and observed under $200 \times$ magnification. Sperm binding numbers and fusion numbers were counted. Under the phase contrast mode, the sperm tails attached to an egg were counted and defined as the sperm binding number (SB). Under the fluorescence mode, the number of chromosomal DNA stained with DAPI, excluding the egg chromosomal DNA, was defined as the sperm fusion number. The fertilization rate (FR) was defined as the percentage of the eggs fused with at least one sperm. The fertilization index (FI) was defined as the mean number of fused sperm per egg.

At least 15 eggs per sample group were prepared to normalize the each result. We repeated the IVF assays with calphostin c for five times, staurosporine for four times, and PMA for three times. Eggs and sperm were stored in a CO₂ incubator (37 °C and 5% CO₂) during all incubation steps.

Staining and immunocytochemistry of eggs by fluorescence microscopy (Triple staining)

After individual drug treatment, eggs were stabilized on a glass slide by incubation in M199 Medium without BSA for 30 minutes in a CO₂ incubator. Once the eggs were stacked on the glass, they were treated with 3.7 % formaldehyde in Medium 1 for 15 minutes. After fixation, they were incubated with rat anti-CD9 monoclonal antibody (KMC8; 50 ug/l final concentration) for 60 minutes and it is washed out though Medium 1 for three times, followed by the incubation with the goat anti-rat IgG antibody (20 ug/l) conjugated with FITC for 45 minutes. Then they were washed three times with Medium 1 and soaked in 1 % DAPI for 15 minutes to stain the chromosomal DNA and washed three times with Medium 1. Subsequently, the eggs were treated with 0.1 % triton-X 100 for 1 minute to permeabilize the membrane and washed three times with Medium 1. Finally, the eggs were incubated with rhodamine-phalloidin (2 ug/ml) for 15 minutes to stain the actin cytoskeleton and washed three times with Medium 1. The eggs were kept in Medium 1 at all times during these steps. Fluorescent signals of chromosome (blue), actin (red), and CD9 (green) were visualized under fluorescence microscopy.

Scanning Electron Microscopy (SEM)

Following each drug treatment, eggs were transferred on to a glass slide holding 50 µl of M199 Medium without BSA. After 30 minutes incubation in the CO₂ incubator, eggs were stabilized on the glass. Eggs were fixed with a 2.5 % glutaraldehyde solution (in 0.1 M sodium cacodylate, pH 7.3) overnight, and then treated with a 1 % osmium tetroxide solution (in 0.1 M sodium cacodylate, pH 7.3) for 1 hour. After washing with 0.1 M sodium cacodylate, the eggs were dehydrated by serial ethanol dehydrating steps (50 %, 70 %, 80 %, 90 %, 100 %).

70 %, 80 %, 95 %, and 100 %) in 70 μ l volume. The eggs were soaked in each concentration of ethanol for 15 minutes. The ethanol concentration was increased step by step. Dehydration with 100 % ethanol was repeated three times to completely dehydrate the sample. The slide was put into a plasma coater, and sputter coated with gold / palladium in argon gas. The coated eggs were examined under SEM with a magnification of $3,500 \times$ or $15,000 \times$ to observe the plasma membrane surface of the egg.

Results

IVF after PKC inhibition and activation

A. PKC inhibition

Because all drugs used in this study were dissolved in DMSO (table. 2-1), DMSO-treated eggs were always used as the control group to compare with the eggs treated with the drugs. From the previous studies, 1 % DMSO treatment on eggs has no effect on the egg's fertilization ability (16, our unpublished results). After PKC inhibition by 1 μ M of calphostin c, sperm binding and fusion numbers were significantly decreased (Fig. 2-1). We observed a clear difference between DMSO and calphostin c treated eggs under the fluorescence microscope (Fig. 2-5). On the other hand, even though there was a significant decrease of FR, FI, and SB after the staurosporine treatment (Fig. 2-2), this decrement was not as dramatic as that seen in the calphostin c treatment.

Drug Name	Activator / Inhibitor	Target PKC isoforms	Stock Concentration
Calphostin c	Inhibitor	cPKC, nPKC, aPKC	100 μ M
DiC8	Activator	cPKC, nPKC	5 mM
Gö 6976	Inhibitor	cPKC, PKC γ , PRK	100 μ M
Gö 6983	Inhibitor	cPKC, nPKC, aPKC	100 μ M
PMA	Activator	cPKC, nPKC	200 nM
Staurosporine	Inhibitor	cPKC, nPKC, aPKC	100 μ M
Y-27632	Inhibitor	PRK	200 μ M

Table 2-1. Characteristic and stock concentration of drugs used in this experiment.

Depending on the drug, its strength for the effective concentrations are different, so their stock concentrations were also different (71, 96).

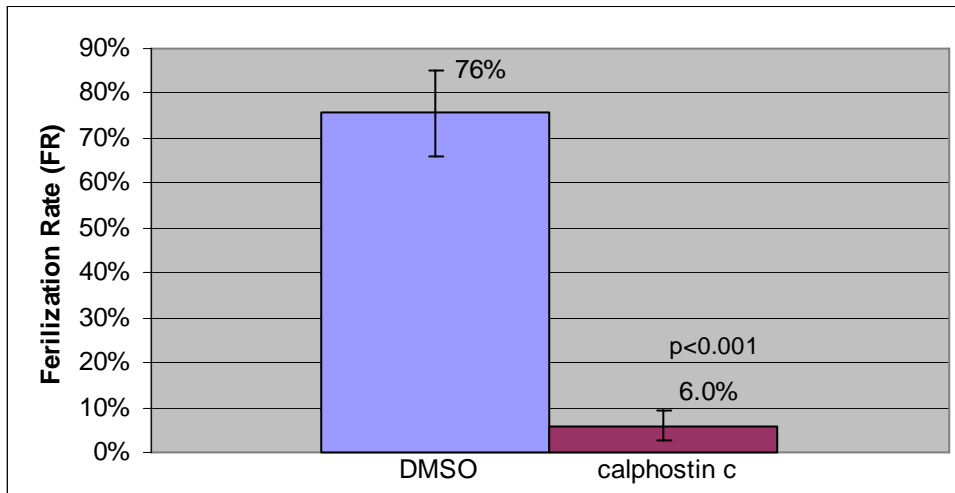
To determine the efficiency of PKC inhibition by calphostin c, treatments with different time increments (15, 30, and 60 minutes) were tested. The same inhibition effects were observed for all treatments at 15, 30, and 60 minutes (Fig. 2-6). Since 30 minutes treatment effectively inhibited both SB and FR (Fig. 2-6), eggs were treated with calphostin c for 30 minutes instead of 60 minutes hereafter. To determine whether the inhibition of calphostin c was dose-dependent, different concentrations of calphostin c (100 nM, 500 nM, and 1 μ M) were tested. Sperm binding numbers and fusion rates were both decreased as the inhibitor concentration was increased (Fig. 2-7), suggesting that the inhibition effect of calphostin c on the egg's fertilization ability is dose-dependent and specific.

Because there was significant blockage of sperm binding and fusion in the calphostin c treated eggs, we want to know which PKC isoform(s) have an important role in the regulation of these fertilization steps. For this purpose, we used three other PKC inhibitors to treat the eggs before IVF: (*R*)-(+)-*trans*-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632), 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Gö 6976), and 2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide (Gö 6983). Each drug has a unique target site, therefore, inhibiting different PKC isoforms (31, 77). Y-27632 effectively inhibits Rho-dependent protein kinases, ROCK-I, ROCK-II, and PRK2 (17, 89). Gö 6976 is competitive to the ATP binding site of Ca²⁺-dependent PKC (33), so it selectively inhibits cPKC (7, 17, 33, 77). Gö 6983 inhibits all PKC isoforms except PKC μ (34, 88); this drug is useful to differentiate PKC μ activity from other PKC activities. Gö 6976 (1 μ M; targets cPKC) and Gö 6983 (1 μ M; targets cPKC, PKC δ , and PKC ζ) were used, instead of calphostin c, to inhibit different PKC isoforms in the egg. Surprisingly, we did not see any inhibition in sperm binding and fusion (Fig. 2-4). Further experiments are needed to address this discrepancy. However, we did find an interesting result. After eggs were treated with 2 μ M of Y-27632, the polyspermy percentage (the percentage of the eggs with more than one fused sperm) was significantly reduced to 30.7 \pm 17.1 %, compared with the control's polyspermy percentage of 69.2 \pm 11.3 % (n=4, p < 0.02).

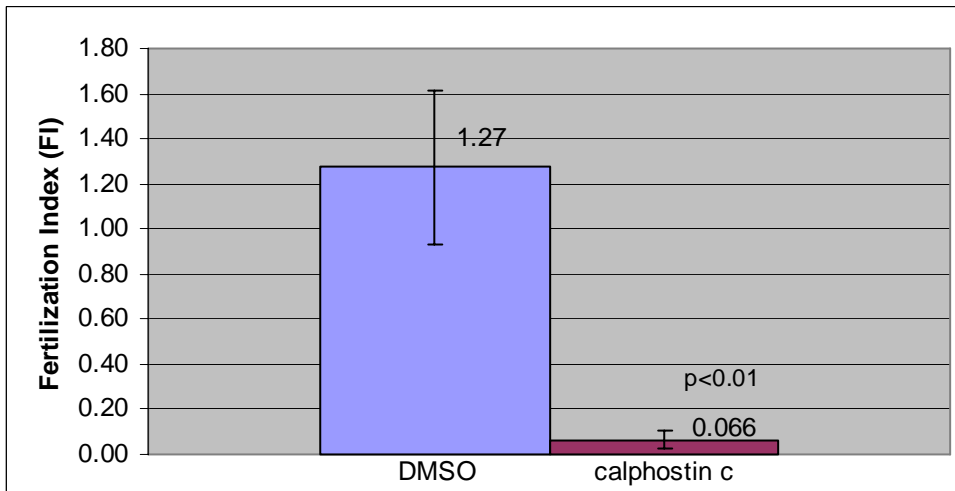
B. PKC activation

To determine the regulation of fertilization by PKC signaling, a PKC activator was

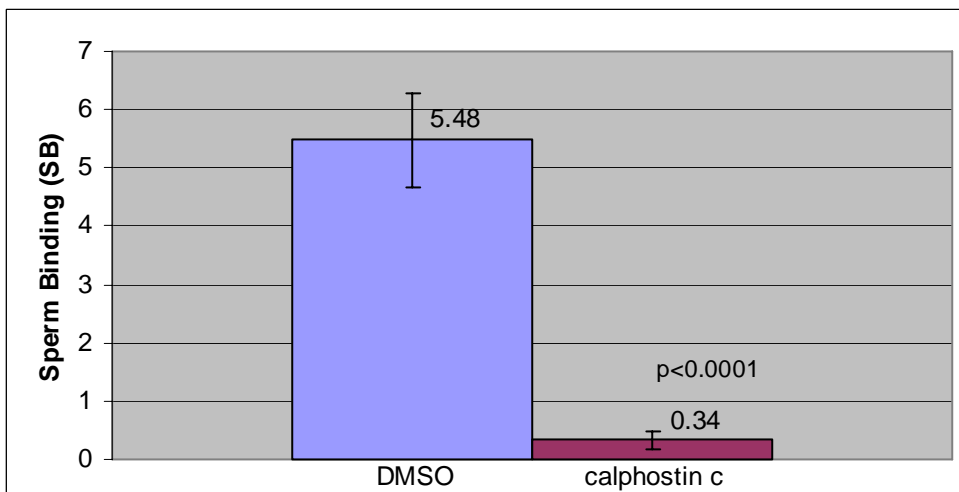
applied to the eggs and their fertilization ability was observed. Surprisingly, as we saw in the eggs treated with either calphostin c or staurosporine, the eggs treated with 2 nM of PMA showed significant blockage of sperm binding and fusion to an egg. FR, FI, and SB were all significantly reduced (Fig 2-3). Time-course experiments showed that the PMA treatment significantly inhibited sperm binding and fusion after 30 minutes treatment (Fig. 2-6). The dose-response curve clearly demonstrated that the treatment with 1 nM of PMA for 30 minutes effectively decreased the SB and FR (Fig. 2-7). Since PKC activation in eggs by PMA showed dramatically reduced sperm binding and fusion, another broad PKC activator, 1,2-Dioctanoyl-sn-glycerol (DiC8) was also tested. DiC8 mimics phorbol esters, which are required for activation of classical PKC isoforms (34). Eggs treated with 50 μ M of DiC8 for 30 minutes did not show any significant changes in all FR, FI, and SB (Fig. 2-3). Some eggs were lysed in the medium, and others became very fragile and lysed when transferred (table 2-2). Increasing the concentration of DiC8 to 100 μ M even caused higher percentage of egg death (103 out of 156 eggs died, 66 %). A much lower concentration of DiC8 will be tested in the future.



A



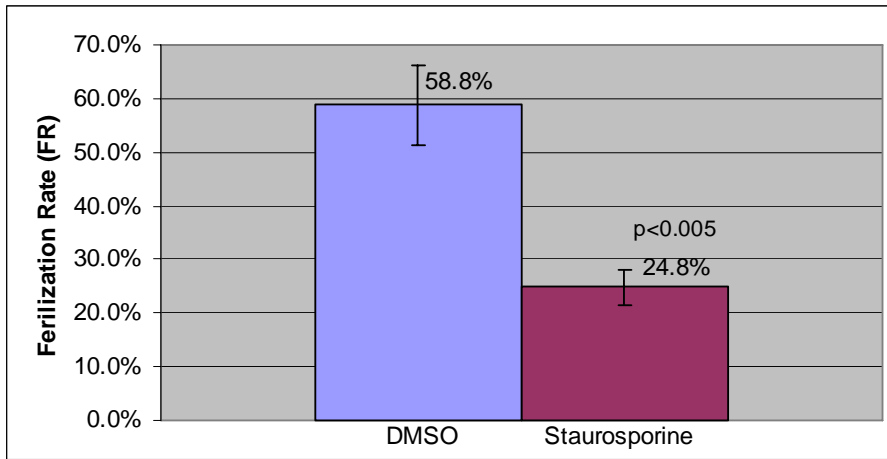
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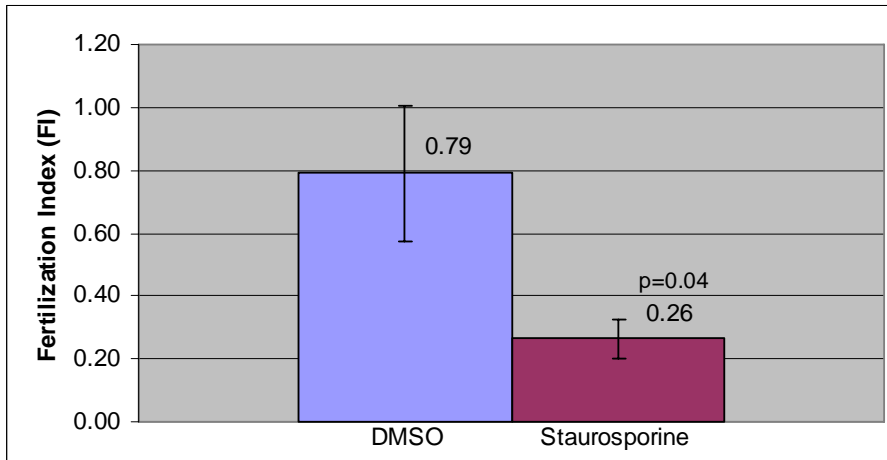
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Figure 2-1. Fertilization rate, fertilization index, and sperm bound of DMSO- and calphostin c-treated eggs. After 1 μ M calphostin c treatment, the eggs lost the ability to bind and fuse with sperm. FR, FI, and SB all were significantly decreased after either the calphostin c treatment or the PMA treatment. (A) FR of these two groups. The values were 75.6 ± 9.6 % (DMSO) and 6.0 ± 3.4 % (calphostin c) ($p < 0.001$). (B) FI of these two groups. The numbers were 1.27 ± 0.34 (DMSO) and 0.066 ± 0.036 (calphostin c) ($p < 0.01$). (C) SB of those two groups, 5.48 ± 0.81 (DMSO), 0.34 ± 0.15 (calphostin c) ($p < 0.001$).

A



B



C

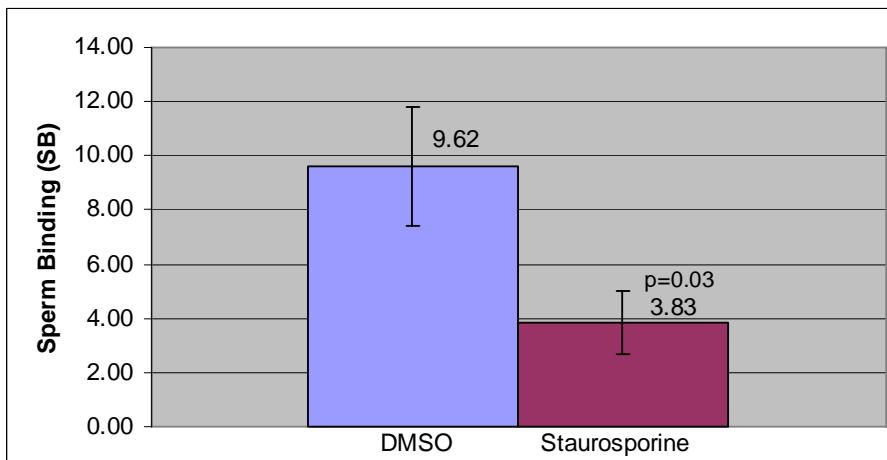
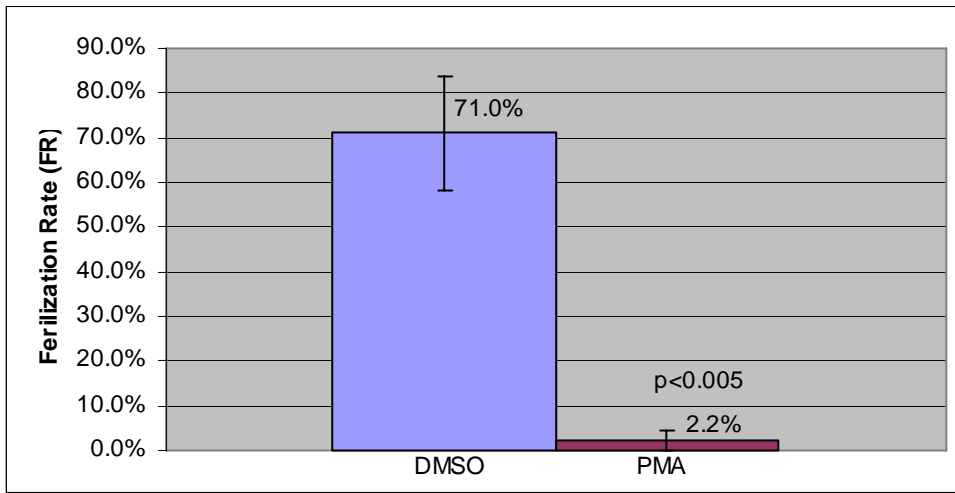
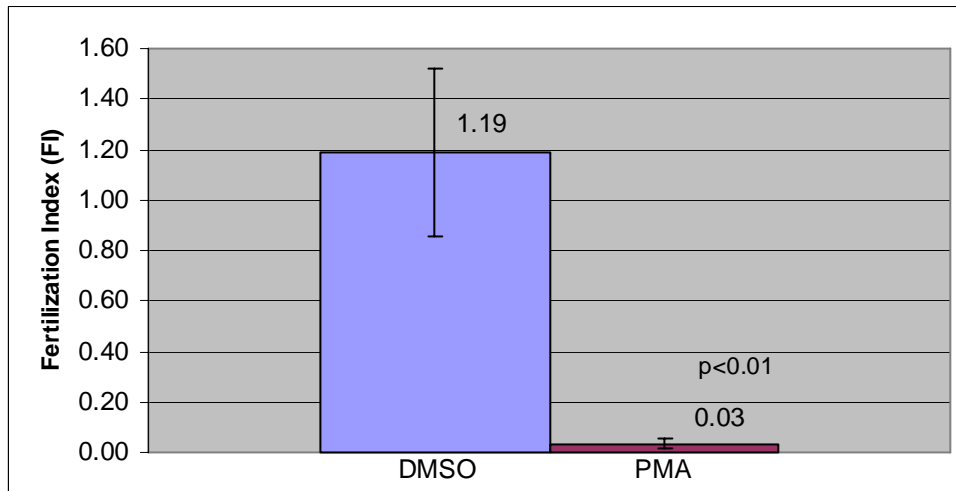


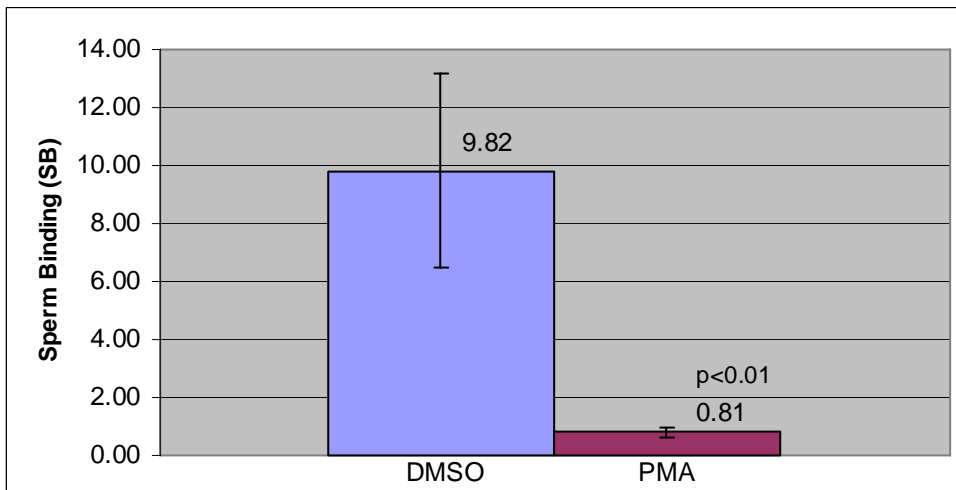
Figure 2-2. Fertilization rate, fertilization index, and sperm bound of DMSO- and staurosporine-treated eggs. After 1 μ M staurosporine treatment, the eggs' ability to bind and fuse with sperm was drastically decreased. There were significant differences between the DMSO and staurosporine group in all three values of FR, FI, and SB. (A) FR of these two groups. The values were 58.8 ± 7.4 % (DMSO) and 24.8 ± 3.3 % (staurosporine) ($p < 0.005$). (B) FI of these two groups. The numbers were 0.79 ± 0.22 (DMSO) and 0.26 ± 0.06 (staurosporine) ($p = 0.04$). (C) SB of those two groups, 9.62 ± 2.22 (DMSO) and 3.83 ± 1.15 (staurosporine) ($p = 0.03$).



A

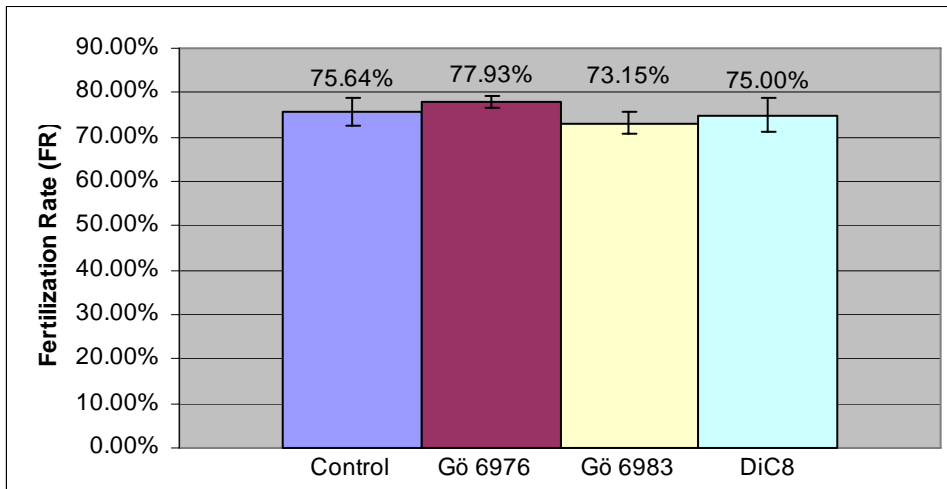


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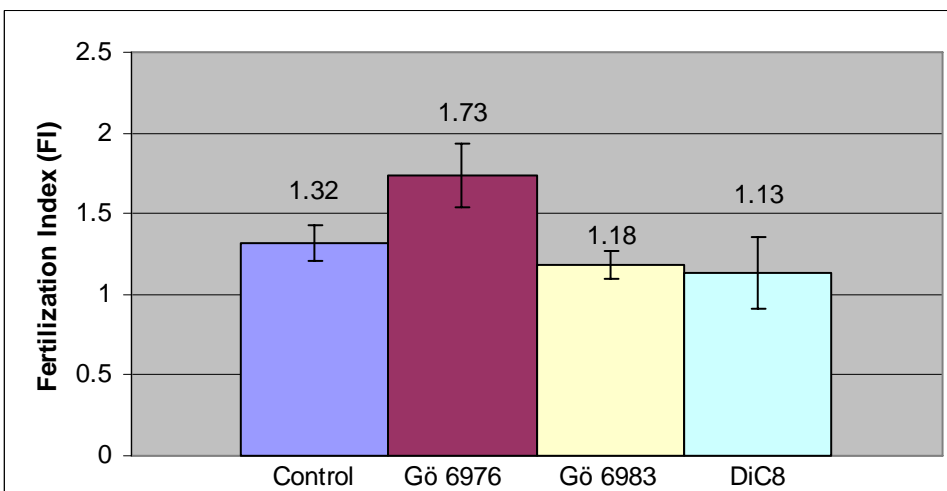


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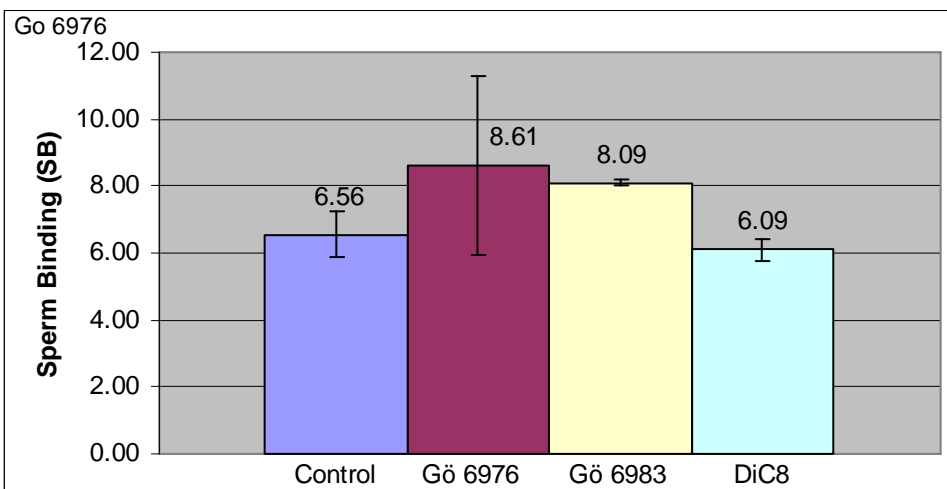
Figure 2-3. Fertilization rate, fertilization index, and sperm bound of DMSO- and PMA-treated eggs. After 2 nM PMA treatment, the eggs lost the ability to bind and fuse with sperm. FR, FI, and SB all were significantly decreased after the PMA treatment. (A) FR of these two groups. The values were 71.0 ± 12.9 % (DMSO) and 2.2 ± 2.2 % (PMA) ($p < 0.005$). (B) FI of these three groups. The numbers were 1.19 ± 0.33 (DMSO) and 0.03 ± 0.02 (PMA) ($p < 0.01$). (C) SB of those three groups, 9.82 ± 3.33 (DMSO) and 0.81 ± 0.17 (PMA) ($p < 0.01$).



A



B



C

Figure 2-4. Fertilization rate, sperm bound, and fertilization index to the eggs treated with DMSO, Gö 6976, Gö 6983, or DiC8. There were not significant differences between DMSO and drug treatment groups in all three values of FR, FI, and SB. (A) FR of these four groups. The values were 75.6 ± 3.2 % (DMSO), 77.9 ± 1.2 % (Gö 6976), 73.2 ± 2.5 % (Gö 6983), and 75.0 ± 3.8 % (DiC8). (B) FI of these four groups. The numbers were 1.32 ± 0.11 (DMSO), 1.73 ± 0.20 (Gö 6976), 1.18 ± 0.08 (Gö 6983), and 1.13 ± 0.32 (DiC8). (C) SB of those four groups, 6.56 ± 0.67 (DMSO), 8.64 ± 2.68 (Gö 6976), 8.09 ± 0.76 (Gö 6983), and 6.09 ± 0.22 (DiC8).

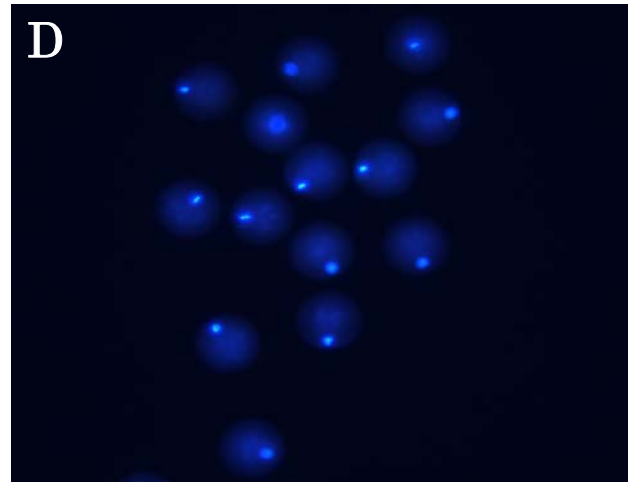
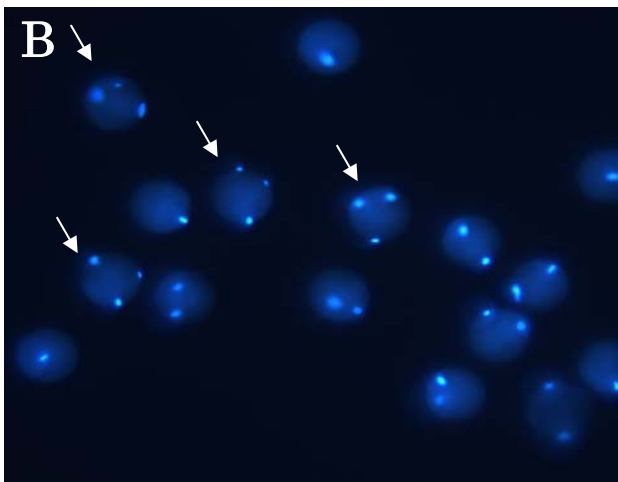
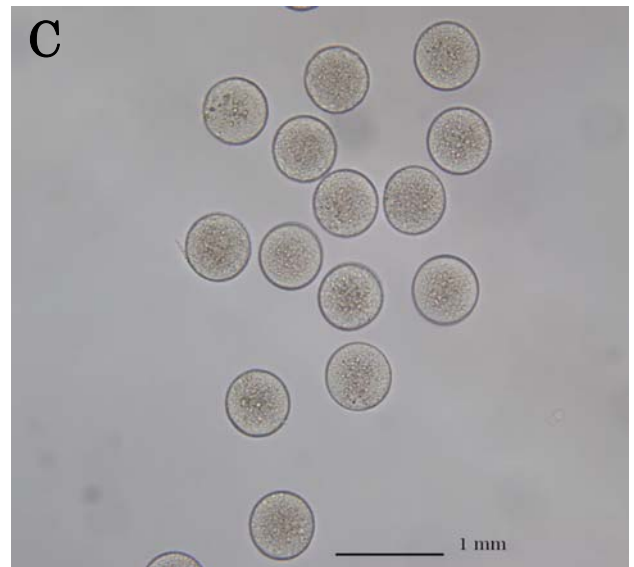
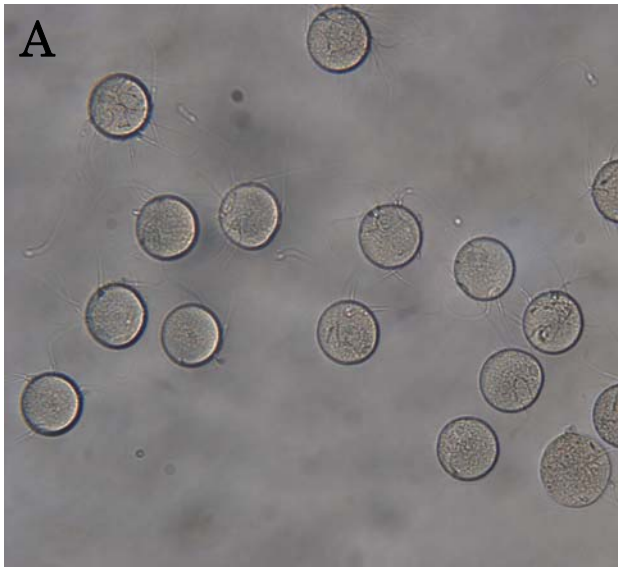


Figure 2-5. Microscopic images of the DMSO-treated (A and B) and calphostin c-treated (C and D) eggs. The phase contrast image of DMSO-treated eggs (A) shows many sperm binding (SB) to the eggs. In contrast, no SB is seen in the phase contrast image of calphostin c-treated eggs (C). Blue dots show chromosomal DNA stained by DAPI in the fluorescence image of the DMSO-treated eggs (B) and the calphostin c-treated eggs (D). More than two dots are observed in each egg in picture B, but all eggs have only one chromosomal DNA dot in picture D. This means that there is no sperm fusion to the eggs treated with calphostin c. Polyspermy eggs with three or more blue dots (indicated by arrows) are also observed in picture B.

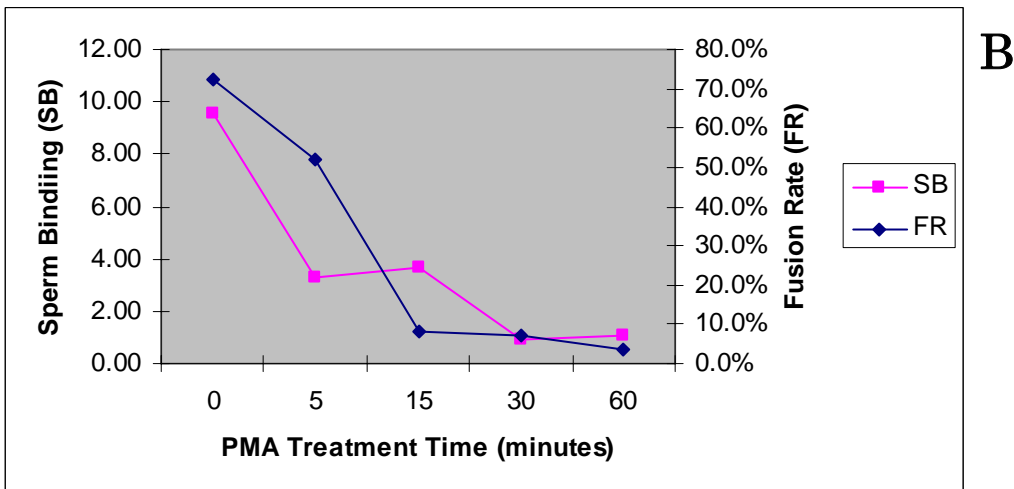
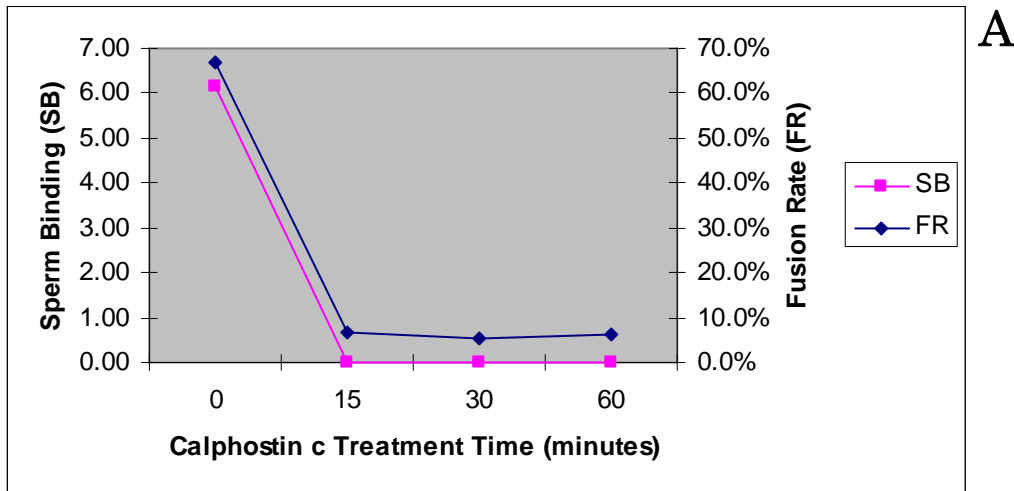


Figure 2-6. Changes in sperm binding and fusion rate over the time course of 1 μ M calphostin c (A) or 2 nM PMA (B) treatment. As the treatment time increases the SB and FR decreases. Each tested group contained at least 20 eggs and was repeated three times.

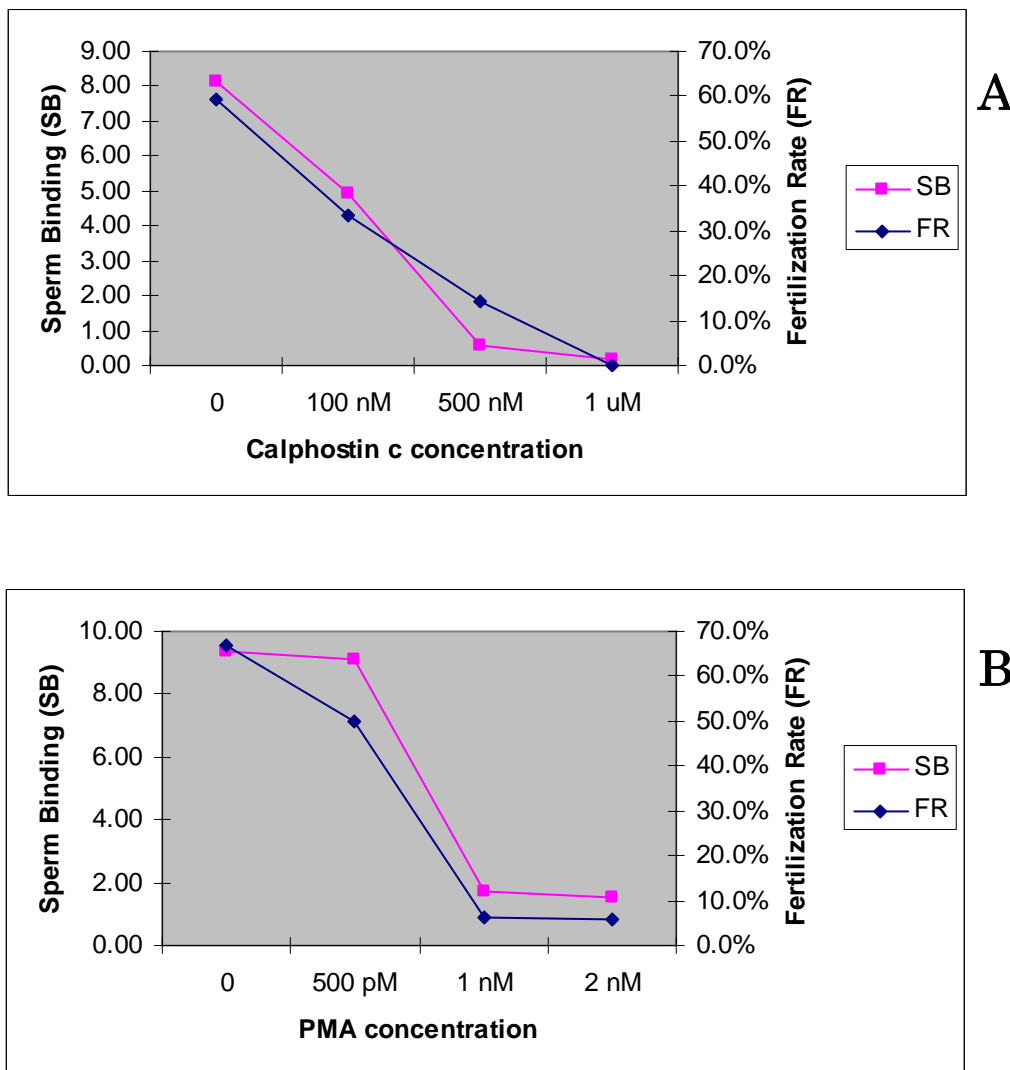


Figure 2-7. The dose-response curve after 30 minutes exposure to calphostin c (A) or PMA (B) treatment. SB and FR of the calphostin c-treated eggs gradually decreased as concentration increased. In contrast, both SB and FR of PMA-treated eggs were suddenly blocked at 1 nM of PMA. 30 minutes treatments were applied to all experiments. Each tested group contained at least 20 eggs and was repeated three times.

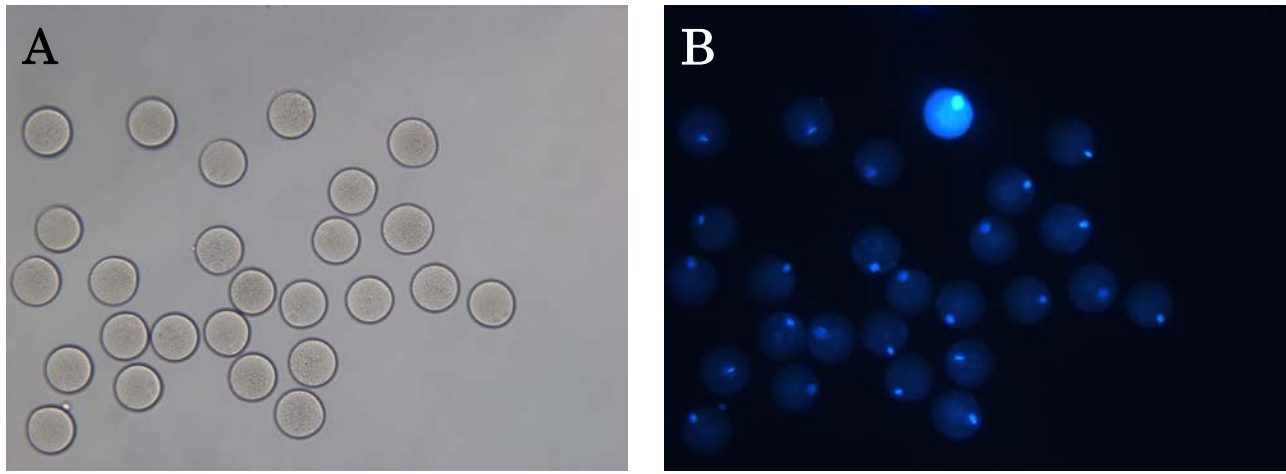


Figure 2-8. Microscopic images of the eggs after insemination with sperm then followed by 2 nM PMA treatment for 60 minutes. Egg pictures are of phase contrast (A) and DAPI fluorescence (B). No sperm tails were observed in picture A, indicating no sperm binding to the eggs. All eggs showed only egg chromosomal DNA in picture B, indicating no fusion of sperm to the eggs.

Treatment (40 minutes)	fertilized eggs # (FR)	fused sperm # (FI)	sperm bind # (SB)	Total egg #
DMSO	138 (72.6 %)	219 (1.15)	1468 (7.73)	190
50 μ M DiC8	120 (65.2 %)	210 (1.14)	1477 (8.03)	184
100 μ M DiC8	103/156 (66.0 % of eggs lysed)			156

Table 2-2. Fertilization ability of the eggs treated with DiC8. There was no statistical difference between the DMSO- and DiC8-treated group ($P=0.21$). The eggs treated with 100 μ M DiC8 intent to die in the medium. The egg membrane became delicate to physical stress. The transferring procedure broke some cells and movement of the medium provoked cell lysis.

Localization of chromosomal DNA, actin, and CD9 after PKC inhibition or activation

It is possible that PKC activity regulates the molecules known to have a critical role in the fertilization process. These molecules include actin, CD9, and chromosomal DNA (18, 61, 63). Actin is required for further activation of the egg after sperm fusion (61). CD9 is a tetraspanin protein, and is expressed on the egg's microvilli. It is required for sperm fusion (46, 63, 99). Association of egg and sperm chromosomal DNA influences further steps of egg activation and zygote development. If PKC is involved in the regulation of these molecules, both the structure and localization of these molecules could be changed after PKC inhibition (by calphostin c) or activation (by PMA). Actin, CD9, and chromosomal DNA were visualized by rhodamine-phalloidin, FITC-conjugated

secondary antibody, and DAPI respectively. Under fluorescence microscopy, rhodamine, FITC, and DAPI appear red, green and blue, respectively. A 0.1 % triton X-100 solution was used to permeabilize the egg membrane so that rhodamine-phalloidin could stain the actin inside (20, 69). This permeabilization also allowed for the detection of any CD9 in the cytoplasm, although CD9 is usually found on the egg plasma membrane. Our results indicated that PKC inhibition by calphostin c did not elicit any changes in the localization and structure of actin, CD9, and chromosomal DNA (Fig 2-9). The actin meshwork in the cell cortex (just beneath the cell plasma membrane) and the actin cap (just overlying the egg chromosomal DNA) were not significantly changed. CD9 was still restricted to the microvillus region of the egg plasma membrane. And the egg chromosomal DNA appeared normally. Similar results were obtained when eggs were treated with 2 nM PMA. No significant change was seen for actin, CD9 or egg chromosomal DNA.

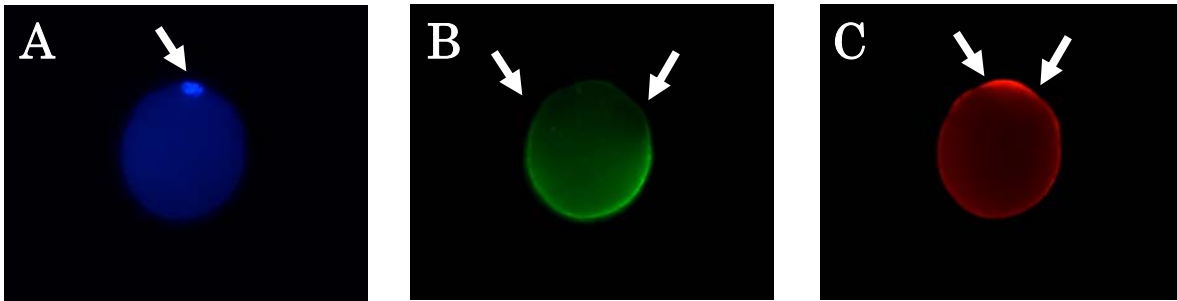


Figure 2-9. DAPI (A), CD9 (B), and actin (C) staining of an egg after calphostin c treatment. The arrow in A indicates the chromosome of the egg. The two arrows in B show the boundaries of the microvilli (major region) and the amicrovilli (minor region) of the egg surface. CD9 is restricted to the microvillus region. The two arrows in C show the actin cap of the egg. Similar staining patterns of DAPI, CD9 and actin were observed in a control egg.

SEM imaging of the egg

After calphostin c or PMA treatment, eggs lost their ability to be fertilized with sperm. This result may come from the regulation of the microvillus structure on the egg surface by PKC activity. Microvilli physically permit sperm to bind with the egg (25). PKC may negatively modify this structure so that the egg is not able to bind with sperm. To approach this question, we used electron microscopy to visualize this structure on the egg membrane. Because we used toxic chemicals to fix the egg, i. e. paraformaldehyde and osmium, mouth pipetting was not used for transferring eggs. Therefore, the eggs were attached to a glass slide so that we could change the solution, instead of transferring the eggs. We made sure that the eggs had stuck on to the glass before fixation, otherwise eggs would not attach to the glass after fixation. BSA in the routine culture medium does not

allow the living eggs to attach to the Petri dish or the slide glass. Thus, we established a protocol that 30-minutes incubation of the eggs in M199 without BSA successfully attached the eggs on the slide glass. In this way, the eggs would not be drained off the slide glass when the solution was changed with a pipette. After fixation, dehydration, and coating with gold, the eggs were observed under SEM. Normal microvillus structure and an amicrovillus region were clearly seen on the surface of the egg (Fig 2-9). There was no difference between the calphostin c-treated egg and the control egg in both $3,500 \times$ and $15,000 \times$ magnifications. On the other hand, there was a dramatic change on the microvillus structure of the PMA treated egg. The microvillus number was obviously reduced and the microvilli shape became distinctly longer and bigger.

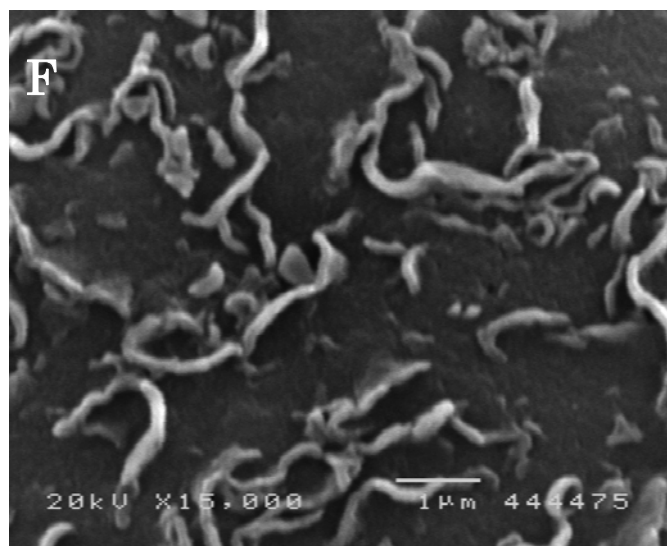
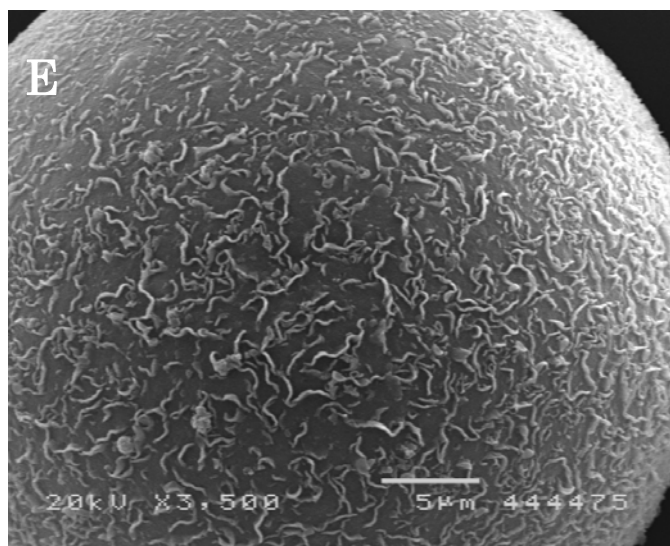
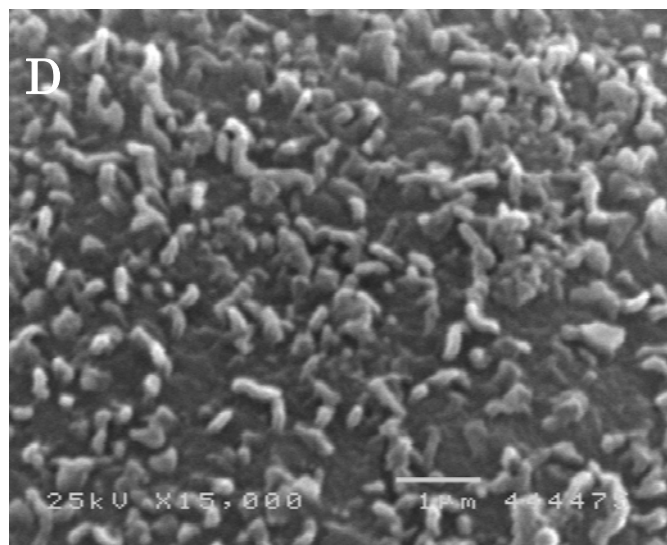
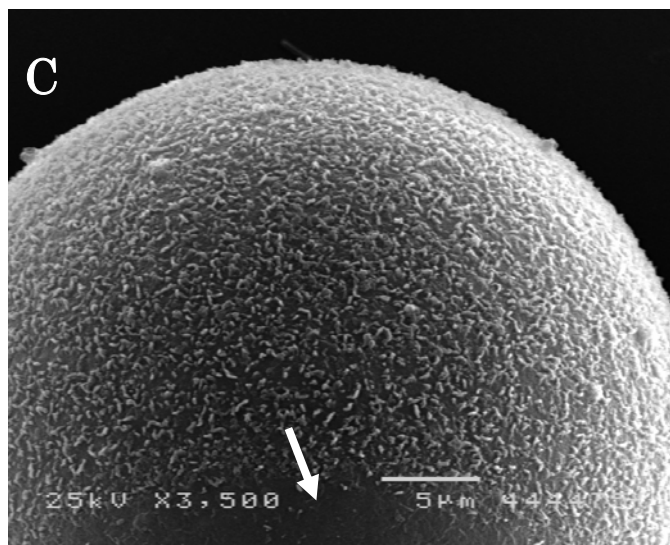
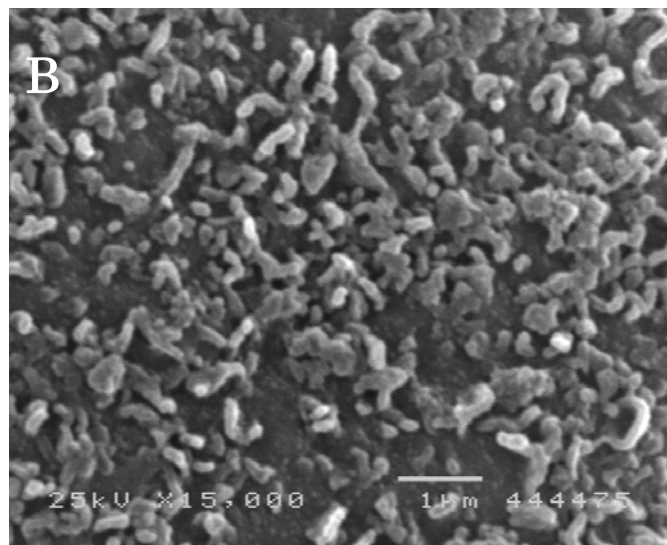
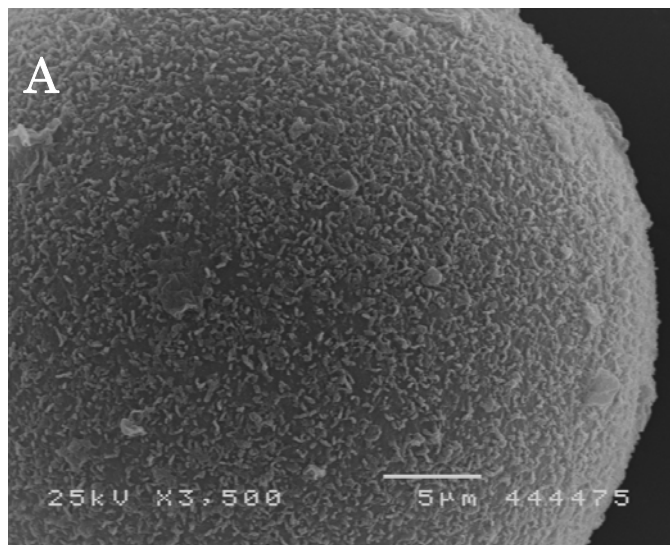


Figure 2-10. Scanning electron microscopy (SEM) images of the drug-treated eggs. The egg samples were observed with two magnifications, 3500 X (A, C, E) and 15,000 X (B, D, F). Eggs treated with DMSO showed normal microvillus structure (A, B). Calphostin c-treated eggs also showed normal morphology of the microvillus and amicrovillus (arrow in C) regions (C, D). An unusual microvillus structure was found on the egg surface after PMA treatment (E, F). The microvillus number was reduced and its shape became longer.

Discussion

Several findings are revealed from the series of experiments in this study. As was hypothesized, PKC activity in the mouse egg is involved in the signaling regulation of the egg-sperm membrane binding and fusion. Surprisingly, we find that both inhibition and activation of PKC in mouse eggs results in the blockage of sperm binding and fusion, suggesting that PKC activity in eggs may be precisely balanced to regulate gamete membrane interaction in a biphasic mode. Under fluorescence microscopy, PKC inhibition by calphostin c or activation by PMA did not affect the structure and localization of actin, CD9, and chromosomal DNA in the egg. These molecules play an important role during fertilization and development of the egg (20, 46, 48, 61, 63, 69,90, 94). Interestingly, under scanning electron microscopy, the structural morphology of egg surface microvilli was severely altered in the PMA-treated eggs, but not in the calphostin c-treated eggs.

Both PKC inhibitors, calphostin c and staurosporine, significantly disrupted the egg's ability to bind and fuse with sperm. Staurosporine is the non-specific PKC inhibitor and targets many other protein kinases (15). Therefore, compared with calphostin c, it is expected that 1 μ M staurosporine treatment was not as effective as 1 μ M calphostin c

treatment. Indeed, the reduced level of FR, FI and SB in the staurosporine-treated eggs was less than those in the calphostin c-treated eggs. Moreover, calphostin c induces irreversible PKC inhibition (31), whereas staurosporine induces a competitive and reversible PKC inhibition (78). Different extent of decrease in SB and FR after these two drug treatment could be explained by these characteristics. It is anticipated that there is still some PKC available for the eggs when they are treated with staurosporine. When there is PKC available in the egg cytoplasm, egg would still hold or recover the ability for fertilization.

By examining the time-course and dose-response of the calphostin c treatments, we found out that 1 μ M calphostin c treatment for 30 minutes was required to cause significant inhibition of sperm binding and fusion to the egg. A shorter treatment time or a lower concentration was not able to significantly block fusion and binding of sperm. Although some structural or localization alterations of actin, CD9, chromosomal DNA, or microvilli were expected, no changes were observed under fluorescence microscopy and SEM. Signaling pathways that block sperm binding or fusion to the egg might be turned on or signaling pathways that prompt SB and FR might be turned off after PKC inhibition by calphostin c. Consequently, PKC inhibition by calphostin c in mouse eggs specifically leads to the shut-down of sperm binding or fusion without altering other known molecules involved in sperm fusion and binding to the egg. Strangely enough, other kinds of specific PKC inhibitors, Gö 6976 and Gö 6983, had no effect on the egg's ability for fertilization with sperm. Concentrations and treatment times applied for both drugs in this study were considered to effectively inhibit target PKC isoforms in mouse eggs. This was also indicated in other studies (7, 17, 33, 34, 77, 88), in which many cell lines from several

species were tested. The reasons for this inconsistency are not clear at this moment and will be further investigated.

In our hands, fertilized eggs normally show more than 60 % polyspermy rate after IVF. Interestingly, we found that only 30 % of the eggs treated with 2 μ M Y-27632 showed polyspermy. Y-27632 is a very specific inhibitor of Rho-dependent protein kinases (17), and shows low suppression of other kinases. Accordingly, it is possible that Rho-dependent protein kinases inhibited by this treatment are responsible for the reduced polyspermy rate. Because SB and FR were not changed, it seems that the Y-27632 treatment specifically blocked the second sperm from fusing. There is a time lag between the first sperm fusion and the egg mechanism that refuses the second sperm. Harmonious sperm fusion after sperm binding may be assisted by Rho-dependent protein kinases. Therefore, it would be intriguing to see whether Rho-dependent protein kinases play a role in facilitating the transition of sperm binding to sperm fusion.

One remarkable observation about two PKC inhibitors (Gö 6976 and Gö 6983) was that the treated eggs seemly underwent immediate egg activation to form the second polar body after 40 minutes fertilization with sperm (Fig. 2-10). 40-50 % of fertilized eggs experienced this abnormal activation. Unfertilized eggs did not display this abnormal activation. This instant activation was not seen in the other IVF experiments, such as in the treatments of calphostin c, PMA, and DiC8. In the previous studies, this phenomenon was observed 1-2 hours after insemination and pronucleus formation was confirmed 8 hours after PKC inhibition (2 μ M staurosporine) and activation (PMA and DiC8) (54, 57). In this study, both zona-free and zona-intact eggs treated with either Gö 6976 or Gö 6983 did not experience pronucleus formation even after 8 hours without sperm fertilization.

Since both 1 μM Gö 6976 and 1 μM Gö 6983 effectively inhibit Ca^{2+} dependent PKC isoforms, cPKC, there are two possibilities considered. The rapid activation of MII phase oocytes is either induced by cPKC inhibition or suppressed by cPKC activity. Nevertheless, it is difficult to conclude that only the cPKC isoforms, which are also inhibited by calphostin c, control the second polar body emission. Moreover, it is stated that spindle stability and high maturation promoting factor (MPF) activity are required for the MII arrest of mouse oocytes (10). Thus these drugs may affect and change microtubule stability or MPF activity, because they have potent actions other than PKC inhibition (17, 77). Not only the PKC family, but other kinases are also inhibited or activated by these drugs. The significance of the observation that Gö 6976- and Gö 6983-treated eggs immediately emitted the second polar body after fusion with sperm remains to be addressed.

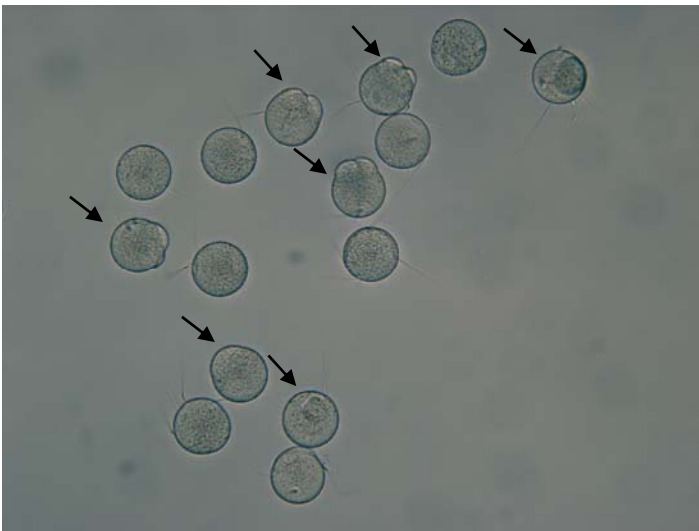


Figure 2-11. Phase contrast image of inseminated eggs treated with 1 μM Gö 6976. The arrows show the egg's abnormal shape, which indicates an extrusion of the second polar body from the egg. This event was induced only by Gö 6976 and Gö 6983 in this study.

The eggs treated with PMA showed similar reduction in sperm binding and fusion as the eggs treated with calphostin c. One remarkable difference between these two drugs is that the structure of microvilli on the egg surface was drastically changed after the PMA but not calphostin c treatment. This alteration of microvilli on the egg is considered to be the reason for the decreased sperm binding and fusion. From the previous data, PMA treatment induced pronuclear formation in both GV oocytes and MII phase eggs. It is anticipated that PKC activity controls the pronuclear formation, or egg activation into an embryo, in mouse eggs (58). We speculate that this promotion of eggs' maturation and activation, eventually, might establish a sperm fertilization blocking system inside the egg, such as the alteration of the microvillus structure as seen in this study.

Even though DiC₈ treatment induced pronuclear formation in the previous report (58), normal sperm binding and fusion was observed in this study. PMA induces the structural change of egg microvilli, which blocks sperm binding and fusion rapidly. However, DiC₈ may not quickly enough induce the microvillus change on the egg surface. Different from DiC₈, PMA activates ATPase and promotes cAMP formation (2, 95). This ATP regulation could also modulate the egg's fertilization ability.

Calphostin c treatment gradually reduced sperm binding and fusion to the egg. However, PMA treatment suddenly destroyed the egg's ability to bind and fuse with sperm. This result suggests that there might be a threshold of PKC activity. Increase of PKC activity to a certain level may switch on a blocking system, which in turn totally inhibits fertilization of the egg. With this respect, we speculate that the level of PKC activity activated by 1 nM PMA is equivalent to that activated after sperm fusion to the egg.

In summary, our results show that the eggs treated with both PKC activation and

inhibition significantly decreased their fertilization ability to bind and fuse with sperm. PKC inhibition by calphostin c significantly reduces sperm binding and fusion to the egg. However, this treatment does not affect the egg's viability and activation (this study and 57), nor does it shift the distribution of actin, CD9, chromosomal DNA, and microvilli. On the other hand, PKC activation by PMA not only remarkably reduces sperm binding and fusion to the egg, but also markedly alters the number and shape of microvilli on the egg membrane, even though no distinct changes were observed for the localization and structure of actin, CD9 and chromosomal DNA. Taken together, these results suggest that PKC activity in eggs may be precisely balanced to regulate gamete membrane interaction in a biphasic mode, and this biphasic regulation is executed through two different mechanisms.

Chapter Four

Conclusions

1. PKC inhibition by calphostin c or staurosporine in the mouse egg significantly diminished sperm fusion and binding. Calphostin c did not change the microvillus structure on the egg surface which is considered as a critical structure for sperm-egg membrane binding. The data from dose-dependent curve showed that the higher concentration of calphostin c treatment resulted in the less sperm binding and fusion to the egg. Collectively, these results sustain that PKC activity in the egg is necessary for sperm-egg membrane binding and fusion, highlighting that the PKC signaling plays a critical role during fertilization of the mouse egg and sperm.
2. PKC activation by PMA in the mouse egg significantly decreased sperm fusion and binding. Moreover, this treatment also markedly changed the structure of the microvilli on the egg surface. PKC activation in the mouse egg is known to induce egg activation. In addition, the dose-response curve from this study shows sudden decrease in sperm binding and fertilization rate between 500 pM to 1 nM PMA treatment, suggesting a threshold of PKC activity exists in the regulation of the egg's ability to bind and fuse with sperm. Sperm fusion to the egg may create a polyspermy blocking mechanism involving the microvillus structure. Taken together, these data support the important role of PKC signaling in the regulation of

sperm-egg membrane binding and fusion, a view that has not been appreciated before.

3. The localization of CD9, actin, and chromosomal DNA in the mouse egg were not changed by PMA or calphostin c treatment under the protocols described in this study. It is possible that the regulation of these molecules may require some time after PKC activation or inhibition. If so, we may miss any changes since these molecules were detected shortly after each drug treatment. Alternatively, the regulation of these molecules may be mediated through other signaling pathways.

Future Directions

For future studies based on this research results, we can explore more pathways regulated by PKC signaling inside the mouse oocyte (egg).

Several previous studies report the automatic resumption of meiosis after PKC activation (57, 58). We found that the PKC- activated egg already compromised the ability to bind and fuse with sperm. Therefore, it is reasonable to hypothesize that PKC activation mimics the fertilization of an egg with sperm. However, blockage of sperm binding and fusion to the egg by calphostin c are not expected. For further understanding of PKC activity during fertilization, our research focus will be on PKC inhibition, which is still shrouded in mystery, rather than PKC activation.

PKC signaling activates many other signaling modules, such as extracellular signal-regulated kinase (ERK), epidermal growth factor (EGF), and mitogen-activated protein kinase (MAPK) (41, 42, 52, 70). Because this complex signaling network is either regulating PKC or regulated by PKC, we can explore the signaling pathways downstream or upstream of PKC. One previous study showed the involvement of Raf (a serine/threonine kinase) in oocyte development (14). Raf is also regulated by PKC activity (40). Accordingly, contribution of the Raf pathway in gamete membrane interaction is possible and worthy of further investigation. Designing in vitro fertilization (IVF) experiments with the activation or inactivation of the Raf pathway inside the egg, we may clarify the participation of Raf signaling module during fertilization.

Calcium ion is another candidate to examine its function inside the egg during sperm-egg membrane binding and fusion. One subfamily of the PKC isoforms, conventional PKC, requires calcium to be activated, and calcium oscillation is observed

inside the egg after sperm fusion with the egg. In the previous study, it has been shown that calcium chelator treated eggs showed polyspermy after IVF (61). Treating eggs with both a PKC activator and a calcium chelator, we may identify the specific role of calcium ion during sperm and egg fertilization. Furthermore, the combination of PKC activators/inhibitors and calcium ionophore or chelators in the treatment of mouse eggs, we will be able to investigate the fundamental relationship of PKC and calcium ion during sperm-egg binding and fusion.

Although this study clearly demonstrates the importance of PKC pathway in the egg, PKC signaling may also play a role in sperm during sperm-egg membrane binding and fusion. One paper suggests that the PKC signaling and calcium ion pathway are required for capacitation of sperm (74). Instead of treating eggs with PKC activators/inhibitors, our next research experiments may be designed to investigate these drugs' effects on sperm during fertilization. To do so, capacitated sperm will be treated with a PKC activator or a PKC inhibitor, and then be used in the IVF assays. Sperm may also be treated with a calcium ionophore/chelator to determine the involvement of calcium ion during sperm-egg binding and fusion. Crosstalk of PKC and calcium ion will be also explored. It is our hypothesis that PKC signaling and calcium ion inside the sperm also play an important role during sperm-egg membrane binding and fusion.

References

1. 1,2-Dioctanoyl-*sn*-glycerol. Sigma-Aldrich. Available at: <http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/D5156>. Accessed April 26 , 2006.
2. Allard B, Long E, Block E, Zhao X. Dependence of superoxide anion production on protein kinase C in bovine neutrophils. *Can J Vet Res.* 1999;63:13-17.
3. Almholt K, Arkhammar PG, Thastrup O, Tullin S. Simultaneous visualization of the translocation of protein kinase C α -green fluorescent protein hybrids and intracellular calcium concentrations. *Biochem J.* 1999;337:211-218.
4. Areces LB, Kazanietz MG, Blumberg PM. Close similarity of baculovirus-expressed n-chimaerin and protein kinase C α as phorbol ester receptors. *J Biol Chem.* 1994;269:19553-19558.
5. Azuma T, Kondo T, Ikeda S, Imai H, Yamada M. Effects of EDTA saturated with Ca²⁺ (Ca-EDTA) on pig, bovine and mouse oocytes at the germinal vesicle stage during maturation culture and the involvement of chelation of Zn²⁺ in pronuclear formation induction by Ca-EDTA. *Reproduction.* 2002;124:235-240.
6. Baltz MJ, Katz FD, Cone AR. Mechanics of sperm-egg interaction at the zona pellucida. *Biophys J.* 1988;54:643-654.
7. Baron GM, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J Biol Chem.* 1993;268:9194-9197.

8. Becker WM, Deamer DM. The World of the Cell. Second Edition. Redwood City CA: Benjamin/Cummings;1991.
9. Brandt D, Gimona M, Hillmann M, Haller H, Mischak H. Protein kinase c induces actin reorganization via a Src- and Rho-dependent pathway. J of Biol. Chem. 2002;277:20903-20910.
10. Brunet S, Maro B. Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space. Reprod. 2005;130:801-811.
11. Burgess M. BioScience 2004 Report. Biochem Soci. 2004;35-57.
12. Caloca MJ, Fernandez N, Lewin NE, Ching D, Modali R, Blumberg PM, Kazanietz MG. β 2-chimaerin is a high affinity receptor for the phorbol ester tumor promoters. J Biol Chem. 1997;272:26488-26496.
13. Calphostin C from *Cladosporium cladosporioides*. Sigma-Aldrich. Available at: <http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/C6303>. Accessed April 26 , 2006.
14. Chen HD, Chen TC, Zhang Y, Liu AM, Campos-Gonzalez R, Pan TB. Characterization of p96^{h2bk}: immunoreaction with an anti-Erk (extracellular-signal-regulated kinase) peptide antibody and activity in *Xenopus* oocytes and eggs. Biochem J. 1998;335:43-50.
15. Cogolludo A, Moreno L, Bosca L, Tamargo J, Perez-Vizcaino F. Thromboxane A2-induced inhibition of voltage-gated K⁺ channels and pulmonary vasoconstriction, role of protein kinase C ζ . Circulation Research. 2003;93:656-663.

16. Cuthbertson KS, Cobbold PH. Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+} . *Nature*. 1985;316:541-542.
17. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. 2000;351:95-105.
18. Davis RJ, Ganong BR, Bell RM, Czech MP. Sn-1,2-Dioctanoylglycerol: A cell-permeable diacylglycerol that mimics phorbol diester action on the epidermal growth factor receptor and mitogenesis. *J Biol Chem*. 1985;260:1592-1566.
19. Dehghani H, Hahnel AC. Expression profile of protein kinase C isozymes in preimplantation mouse development. *Reproduction*. 2005;130:441-451.
20. Discher DE, Winardi R, Schischmanoff PO, Parra M, Conboy JG, Mohandas N. Mechanochemistry of protein 4.1's spectrin-actin-binding domain: ternary complex interactions, membrane binding, network integration, structural strengthening. *J cell Biol*. 1995;897-907.
21. Eckert JJ, McCallum A, Mears A, Rumsby MG, Cameron IT, Fleming TP. PKC signaling regulates tight junction membrane assembly in the pre-implantation mouse embryo. *Reproduction*. 2004;127:653-667.
22. Eliyahu E, Tsaadon A, Shtraizent N, Shalgi R. The involvement of protein kinase C and actin filaments in cortical granule exocytosis in the rat. *Reprod*. 2005;129:161-170.
23. Esterhuizen AD, Franken DR, Lourens JGH, Rooyen LH. Clinical importance of zona pellucida-induced acrosome reaction and its predictive value for IVF. *Human Reprod*. 2001;16:138-144.

24. Evans PJ. Getting sperm and egg together: things conserved and things diverged. *Biol Reprod.* 2000;63:355-360.
25. Evans JP, Fooster JA, McAvey BA, Gerton GL, Kopf GS, Schultz RM. Effects of perturbation of cell polarity on molecular markers of sperm-egg binding sites on mouse eggs. *Biol Reprod.* 2000;62:76-84.
26. Fan HY, Huo LJ, Chen DY, Schatten H, Sun QY. Protein kinase C and mitogen-activated protein kinase cascade in mouse cumulus cells: cross talk and effect on meiotic resumption of oocyte. *Biol Reprod.* 2004;70:1178-1187.
27. Gallicano GI, McGaughey RW, Capco DG. Activation of protein kinase C after fertilization is required for remodeling the mouse egg into the zygote. *Mol Reprod Dev.* 1997;46:587-601.
28. Gangeswaran R, Jones KT. Unique protein kinase C profile in mouse oocytes: lack of calcium-dependent conventional isoforms suggested by rtPCR and Western blotting. *FEBS Lett.* 1997;412:309-312.
29. Gavin AC, Cavadore JC, Schorderet-Slatkine S. Histone H1 kinase activity, germinal vesicle breakdown and M phase entry in mouse oocytes. *J of Cell Sci.* 1994;107:275-283.
30. Gö 6976, >99%. LC Laboratories, division of PKC pharmaceuticals Inc. Available at: <http://www.lclabs.com/PRODFILE/G-K/G-6203.php4>. Accessed April 26, 2006
31. Gopalakrishna R, Chen ZH, Gundimeda U. Irreversible oxidative inactivation of protein kinase C by photosensitive inhibitor calphostin C. *FEBS.* 1992;314:149-154.

32. Gordon SE, Park JD, Tam B, Zimmerman AL. Diacylglycerol analogs inhibit the rod cGMP-gated channels by a phosphorylation-independent mechanism. *Biophys J.* 1995;69:409-417.
33. Greco A, Muscella A, Elia MG, Romano S, Storelli C, Marsigliante S. Mitogenetic signaling by B2 Bradykinin receptor in epithelial breast cells. *J Cell Physiol.* 2004;201:84-96.
34. Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, Johannes FJ. Inhibition of protein kinase C μ by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS.* 1996;392:77-80.
35. Halet G, Tunwell R, Balla T, Swann K, Carroll J. The dynamics of plasma membrane PtdIns(4,5)P₂ at fertilization of mouse eggs. *J Cell Sci.* 2002;115:2139-2149.
36. Halet G, Tunwell R, Parkinson JS, Carroll J. Conventional PKCs regulate the temporal pattern of Ca²⁺ oscillations at fertilization in mouse eggs. *J Cell Biol.* 2004;164:1033-1044.
37. Harrison PAR. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. *Reprod Fertil Dev.* 1996;8:581-594.
38. Herrero MB, Lamirande E, Gagnon C. Nitric oxide regulates human sperm capacitation and protein-tyrosine phosphorylation in vitro. *Biol Reprod.* 1999;61:575-581.
39. Hirsh KJ, Silinsky EM. Inhibition of spontaneous acetylcholine secretion by 2-chloroadenosine as revealed by a protein kinase inhibitor at the mouse neuromuscular junction. *British J Pharm.* 2002;135:1897-1902.

40. Ho DP, Zechner KD, He H, Dillmann HW, Glembotski CC, McDonough MP. The Raf-MEK-ERK cascade represents a common pathway for alternation of intracellular calcium by Ras and protein kinase C in cardiac myocytes. *J Biol Chem.* 1998;273:21730-21735.
41. Hornberg JJ, Tijssen RM, Lankelma J. Synergistic activation of signaling to extracellular signal-regulated kinases 1 and 2 by epidermal growth factor and 4 β -phorbol 12-myristate 13-acetate. *Eur J Biochem.* 2004;271:3905-3913.
42. Hornia A, Lu Z, Sukezane T, Zhong M, Joseph T, Frankel P, Foster A. Antagonistic effects of protein kinase C α and δ on both transformation and phospholipase D activity mediated by the epidermal growth factor receptor. *Mol and Cell Biol.* 1999;19:7672-7680.
43. Hug H, Sarre TF. Protein kinase c isozymes: divergence in signal transduction?. *Biochem J.* 1993;291:329-343.
44. Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature.* 2005; 434: 234-238.
45. Jones TK, Soeller C, Cannell BM. The passage of Ca²⁺ and fluorescent markers between the sperm and egg after fusion in the mouse. *Development.* 1998;125:4627-4635.
46. Kaji K, Oda S, Shikano T, Ohnuki T, Uematsu Y, Sakagami J, Tada N, Miyazaki S, Kudo A. The gamete fusion process is defective in eggs of CD9-deficient mice. *Nature gene.* 2000;24:279-282.

47. Kawakami Y, Kitaura J, Yao L, McHenry WR, Kawakami Y, Newton CA, Kang S, Kato MR, Leitges M, Rawlings JD, Kawakami T. A Ras activation pathway dependent on Syk phosphorylation of protein kinase C. *PNAS*. 2003;100:9470-9475.
48. Kim NH, Chung KS, Day BN. The distribution and requirements of microtubules and microfilaments during fertilization and parthenogenesis in pig oocytes. *J Reprod Fertil*. 1997;111:143-149.
49. Kline D, Kline JT. Reproductive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev Biol*. 1992;149:80-89.
50. Knott GJ, Poothapillai K, Wu H, He LC, Fissore AR, Robl MJ. Porcine sperm factor supports activation and development of bovine nuclear transfer embryos. *Biol Reprod*. 2002;66:1095-1103.
51. Koivunen J, Aaltonen V, Koskela S, Lehenkari P, Laato M, Peltonen J. Protein kinase c α/β inhibitor Gö6976 promotes formation of cell junctions and inhibits invasion of urinary bladder carcinoma cells. *Cancer Research*. 2004;64:5693-5701.
52. Koyama N, Kashimata M, Sakashita H, Sakagami H, Gresik WE. EGF-stimulated signaling by means of PI3K, PLC γ 1, and PKC isozymes regulates branching morphogenesis of the fetal mouse submandibular gland. *Dev Dynamics*. 2003;227:216-226.
53. Kreider MR. Number, timing, and duration of marriages and divorces: Household Econ Stud. 2001;2005.70-97.

54. Lawrence Y, Whitaker M, Swann K. Sperm-egg fusion is the prelude to the initial Ca^{2+} increase at fertilization in the mouse. *Development*. 1997;124:233-241.
55. Levy S, Shoham T. The tetraspanin web modulates immune-signalling complexes. *Nature*. 2005;5:136-148.
56. Lorenzo PS, Beheshti M, Pettit GR, Stone JC, Blumberg PM. The guanine nucleotide exchange factor RasGRP is a high – affinity target for diacylglycerol and phorbol esters. *Mol Pharmacol*. 2000;57:840-846.
57. Lu Q, Smith GD, Chen DY, Han ZM, Sun QY. Activation of protein kinase C induces mitogen-activated protein kinase dephosphorylation and pronucleus formation in rat oocytes. *Biol Reprod*. 2002;67:64-69
58. Luria A, Tennenbaum T, Sun QY, Rubinstein S, Breitbart H. Differential localization of conventional protein kinase c isoforms during mouse oocyte development. *Biol Reprod*. 2000;62:1564-1570.
59. Malcov M, Ben-Yosef D, Glaser T, Shalgi R. Changes in calpain during meiosis in the rat egg. *Mol Reprod Dev*. 1997;48:119-126.
60. Maro B, Johnson MH, Pickering SJ, Flach G. Changes in actin distribution during fertilization of the mouse egg. *J Embryol Exp Morphol*. 1984;81:211-237.
61. McAvey BA, Wortzman GB, Williams CJ, Evans JP. Involvement of calcium signaling and the actin cytoskeleton in the membrane block to polyspermy in mouse eggs. *Biol Reprod*. 2002;67:1342-1352.
62. Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem J*. 1998;332:281-292.

63. Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, Suzuki K, Kosai K, Inoue K, Ogura A, Okabe M, Mekada E. Requirement of CD9 on the egg plasma membrane for fertilization. *Science*. 2000;287:321-324.
64. Mollova M, Nedkova R, Ivanova M, Djarkova T, Peknicova J, Kyurkchiev S. Role of a capacitation-related protein on some sperm functional parameters. *Folia Biol*. 2002;48:232-236.
65. Neuber E, Powers RD. Is the mouse a clinically relevant model for human fertilization failures?. *Human Reprpd*. 2000;15:171-174.
66. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J*. 1995;9:484-496.
67. Parrington J, Jones KT, Lai FA, Swann K. The soluble sperm factor that causes Ca^{2+} release from sea-urichin (*Lytechinus pictus*) egg homogenated also triggers Ca^{2+} oscillations after injection into mouse eggs. *Biochem J*. 1999;341:1-4.
68. Phorbol 12-myristate 13-acetate. Sigma-Aldrich. 2006. Available at: <http://www.sigmaaldrich.com/catalog/search/ProductDetail/FLUKA/79346>. Accessed April 26, 2006.
69. Picart C, Discher DE. Actin protofilament orientation at the erythrocyte membrane. *Biophys J*. 1999;77:869-878.
70. Plessis SS, Page C, Franken RD. The zona pellucida-induced acrosome reaction of human spermatozoa involves extracellular signal-regulated kinase activation. *Andrologia*. 2001;33:337-342.

71. Protein Kinase C Isozymes. Sigma-Aldrich. Available at: http://www.sigmaaldrich.com/Area_of_Interest/Biochemicals/Enzyme_Explorer/Key_Resources/Protein_Kinase_Explorer/Protein_Kinase_C_Isozymes_Overview/Protein_Kinase_C_Isozymes.html. Accessed April 26 , 2006.
72. Punkazhenthil SB, Wildt ED, Ottinger AM, Howard J. Inhibition of domestic cat spermatozoa acrosome reaction and zona pellucida penetration by tyrosine kinase inhibitors. *Mol Reprod Dev.* 1998;49:48-57.
73. Read A, Strachan T. *Human Molecular Genetics, Third Edition.* England: Garland Science; 2003
74. Revelli A, Costamagna C, Moffa F, Aldieri E, Ochetti S, Bosia A, Massobrio M, Lindblom B, Ghigo D. Signaling pathway of nitric oxide-induced acrosome reaction in human spermatozoa. *Biol Reprod.* 2001;64:1708-1712.
75. Sathananthan AH, Szell A, Ng SC, Kausche A, Lacham-Kaplan O, Trounson A. Is the acrosome reaction a prerequisite for sperm incorporation after intracytoplasmic sperm injection (ICSI)? *Reprod Fertil Dev.* 1997;9:703-709.
76. Scott TM, Ingram A, Ball LK. PKD1-dependent activation of atypical PKC leads to degradation of the p21 tumour modifier protein. *EMBO J.* 2002;21:6771-6780.
77. Shah BH, Olivares-Reyes A, Catt KJ. The protein kinase C inhibitor Gö6976 [12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] potentiated agonist-induced mitogen-activated protein kinase activation through tyrosin phosphorylation of the epidermal growth factor receptor. *Mol Pharmacol.* 2004;67:184-194.

78. Staurosporine. Stressgen Bioreagents. Available at: <http://antibody.stressgen.com/pdf/HPK-112.pdf?-Token.Z=>. Accessed April 26, 2006.
79. Staurosporine from *Streptomyces* sp. Sigma-Aldrich. Available at: <http://www.sigmaaldrich.com/catalog/search/ProductDetail/FLUKA/85660>. Accessed April 26, 2006.
80. Steinberg SF. Distinctive activation mechanisms and functions for protein kinase C δ . *Biochem J.* 2004;384:449-459.
81. Swann K. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development.* 1990;110:1295-1302.
82. Swann K, Parrington J. Mechanism of Ca²⁺ release at fertilization in mammals. *J Exp Zool.* 1999;285:267-275.
83. Taddesse-Heath L, Chattopadhyay KS, Dillehay LD, Lander RM, Nagashfar Z, Morse III CH, and Hartley WJ. Lymphomas and high-level expression of murine leukemia viruses in CFW mice. *J Virol.* 2000;74:6832-6837.
84. Tardif S, Dube C, Bailey LJ. Porcine sperm capacitation and tyrosine kinase activity are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only associated with calcium. *Biol Reprod.* 2003;68:207-213.
85. Tatone C, Monache SD, Francione A, Gioia L, Barboni B, Colonna R. Ca²⁺-independent protein kinase C signaling in mouse eggs during the early phases of fertilization. *Int J Dev Biol.* 2003;47:327-333.
86. Toker A. Signaling through protein kinase C. *Frontiers Biosci* 1998;3:1134-1147.

87. Toker A, Cantley LC. Signalling through the lipid products of phosphoinositide-3-PH kinase. *Nature*. 1997;387:673-676.
88. Tsuru M, Katagiri H, Asano T, Yamada T, Ohno S, Ogihara T, Oka Y. Role of PKC isoforms in glucose transport in 3T3-L1 adipocytes: insignificance of atypical PKC. *Am J Physiol Endocrinol Metab*. 2002;283:338-345.
89. Uehara M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature*. 1997;389:990-994.
90. Valentine MT, Perlman ZE, Mitchison TJ, Weitz DA. Mechanical properties of *Xenopus* egg cytoplasmic extracts. *Biophys J*. 2005;88:680-689.
91. Violin JD, Zhang J, Tsien RY, Newton AC. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J Cell Biol*. 2003;161:899-909.
92. Wassarman P, Chen J, Cohen N, Litscher E, Liu C, Qi H, Williams Z. Structure and function of the mammalian egg zona pellucida. *J Experimental Zool*. 1999;285:251-258.
93. Welling A, Hofmann F, Wegener JW. Inhibition of L-type $\text{Ca}_v1.2.\text{Ca}^{2+}$ channels by LY294002 and Gö6983. *Mol. Pharm*. 2005;67:541-544.
94. Wu J, Carrell DT, Wilcox AL. Development of in vitro-matured oocytes from porcine preantral follicles following intracytoplasmic sperm injection. *Biol Reprod*. 2001;65:1579-1585.

95. Xiao GQ, Qu Y, Sun ZQ, Mochly-Rosen D, Boutjdir M. Evidence for functional role of ϵ PKC isozyme in the regulation of cardiac Na^+ channels. *Am J Physiol Cell Physiol*. 2001;281:1477-1486.
96. Y-27632 dihydrochloride. Sigma-Aldrich. Available at: <http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/Y0503>. Accessed April 26 , 2006.
97. Zang Q, Lu Z, Curto M, Barile N, Shalloway D, Foster AD. Association between v-Src and protein kinase C δ in v-Src-transformed fibroblasts. *J Biol Chem*. 1997;272:13275-13280.
98. Zhang XA, Bontrager AL, Hemler ME. Transmembrane-4 superfamily proteins associate with activated protein kinase C (PKC) and link PKC to specific β_1 integrins. *J Bio Chem*. 2001;276:25005-25013.
99. Zhu GZ, Miller BJ, Boucheix C, Rubinsten E, Liu CC, Hynes RO, Myles DG, Primakoff P. Residues SFQ (173-175) in the large extracellular loop of CD9 are required for gamete fusion. *Development*. 2002;129:1995-2002.

Appendix A

Detail of Methods

Preparing transfer pipette and zona pipette

Eggs are small ($\sim 100 \mu\text{m}$), delicate, and low number (80-200 eggs from 6 female mice) when isolated from a mouse; therefore, we have to use mouth pipettes for fine adjustment of the pressure to the pipettes. “Transfer-pipette” is used to transfer eggs and “zona-pipette” is used to remove the zona pellucida from around the eggs. These pipettes are made by hand in the lab. A 9” disposable Pasteur pipette tip is held and heated in an alcohol lamp flame until it is red in color. When the pipette becomes red and softens, take it out of the flame and pull both sides of the pipette. The diameter of a transfer-pipette is 120 - 200 μm , the diameter of a zona-pipette is 50 - 100 μm (Fig. 1). Since they are very thin and easily broken, extra pipettes are also created.

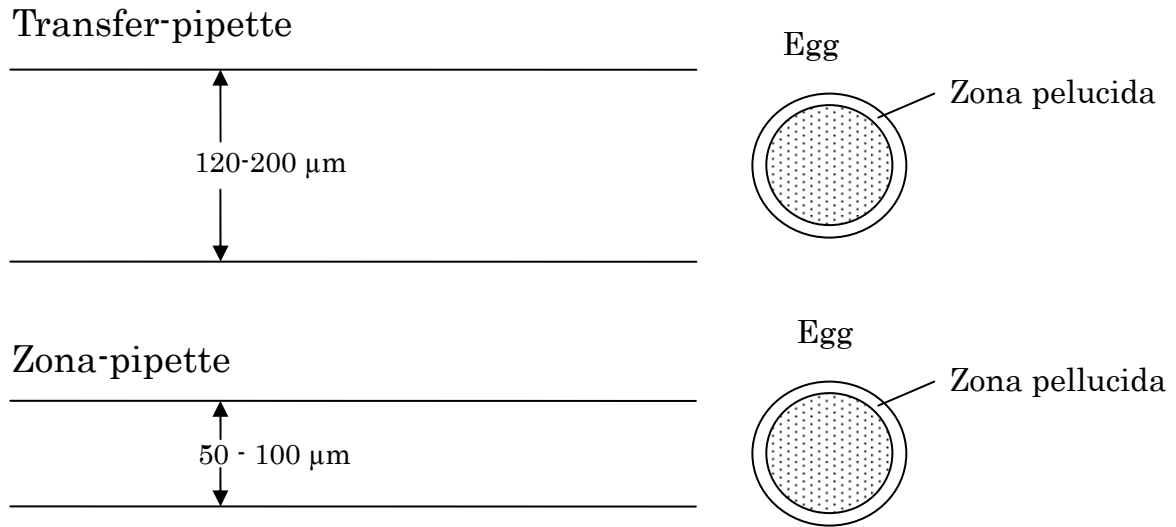


Figure Ap-1.

Simple figures of the transfer and zona pipette used for this study. Depending on the person, the diameter of each pipette will be different.

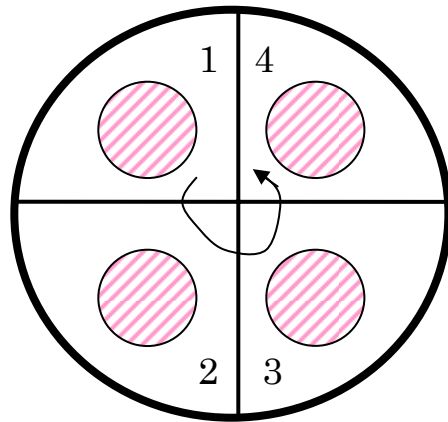


Figure Ap-2.

When a drug is put into drop 1, eggs are washed through 2, 3, and 4 (35 X 10 mm Petri dishes are used). 100 μl of Medium 1 is used to make each drop.

Data figures

Subsection A: Egg images after IVF in phase contrast and fluorescence

Figure A-1. phase contrast image of DMSO-treated eggs after IVF.

(November 17, 2004)



Figure A-2. fluorescence image of DMSO-treated eggs after IVF.

(November 17, 2004)

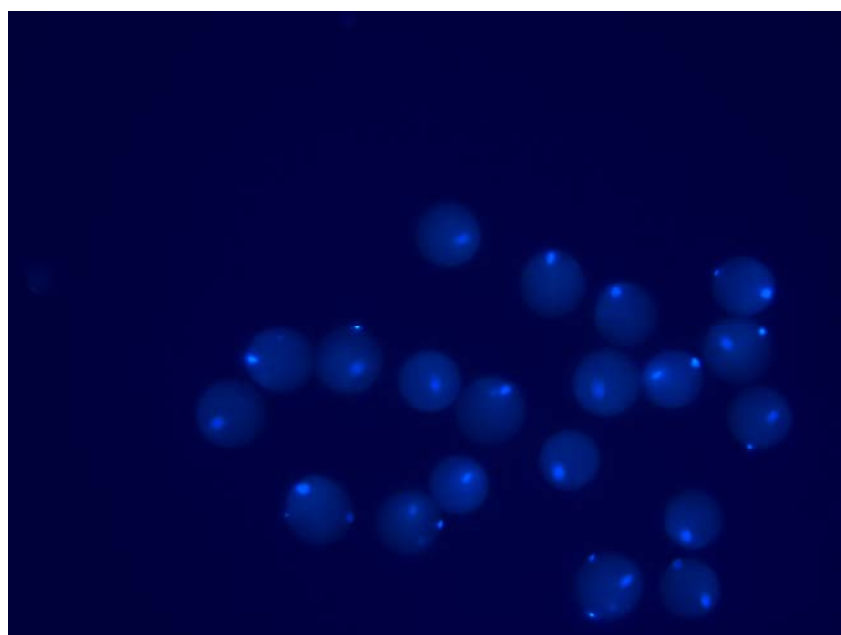


Figure A-3. phase contrast image of calphostin c-treated eggs after IVF.

(November 17, 2004)

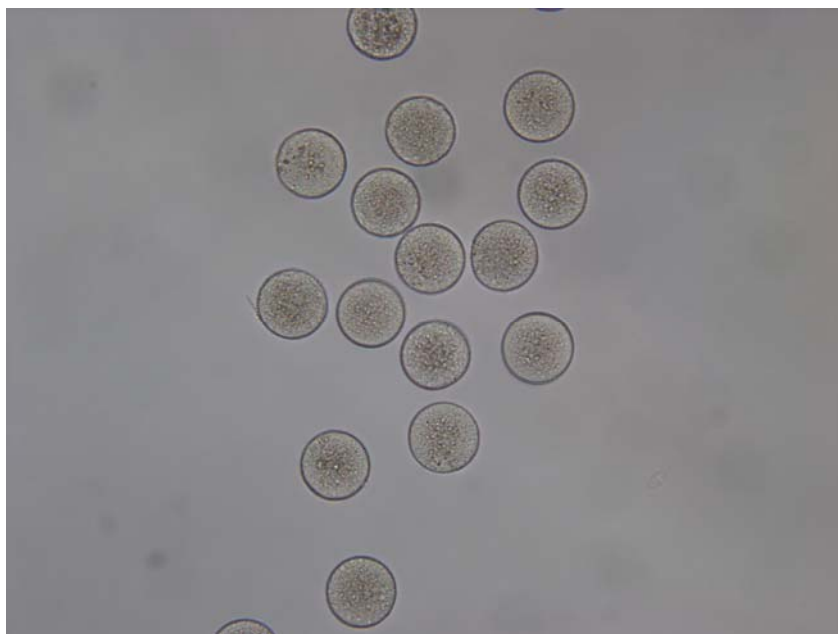


Figure A-4. fluorescence image of calphostin c-treated eggs after IVF.

(November 17, 2004)

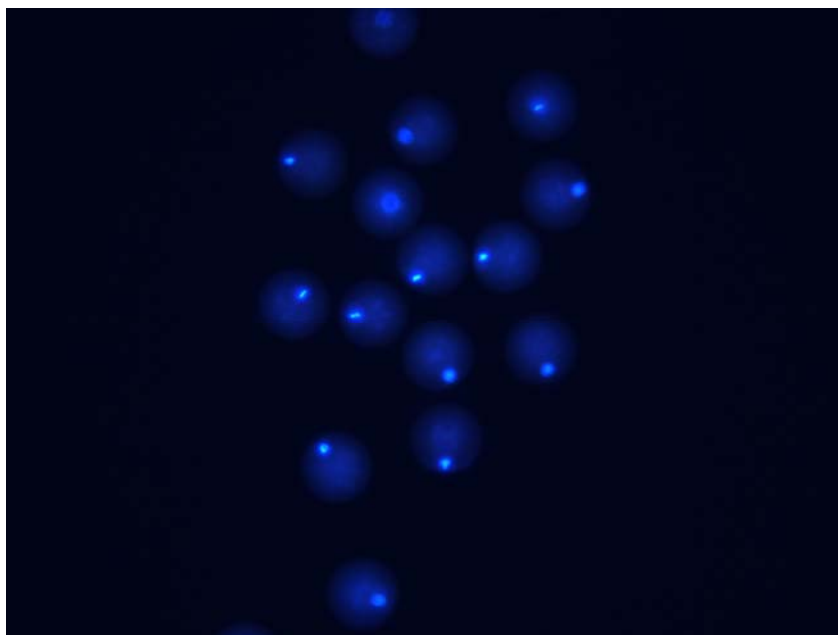


Figure A-5. phase contrast image of PMA-treated eggs after IVF.

(November 17, 2004)

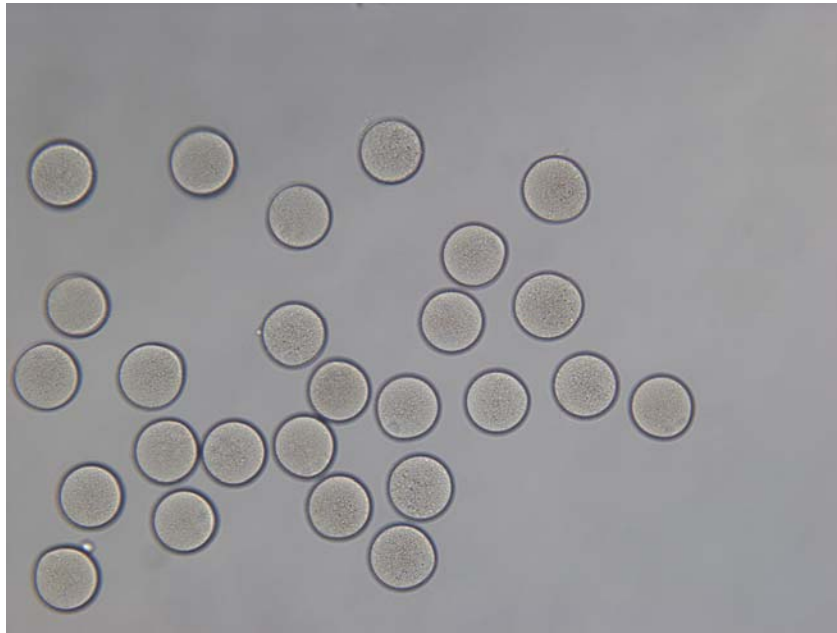


Figure A-6. fluorescence image of PMA-treated eggs after IVF.

(November 17, 2004)

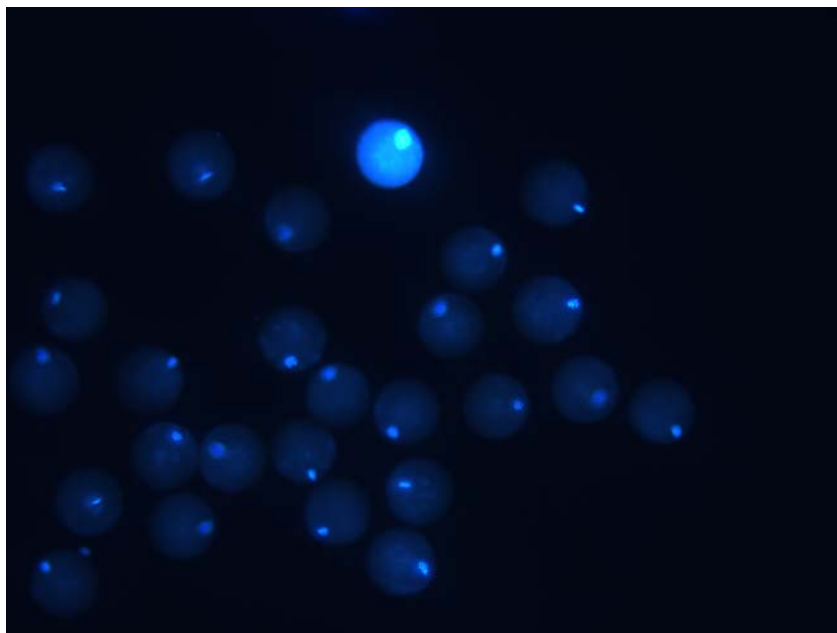


Figure A-7. phase contrast image of Gö 6976-treated eggs after IVF

(February 25, 2005)



Figure A-8. fluorescence image of Gö 6976-treated eggs after IVF

(February 25, 2005)

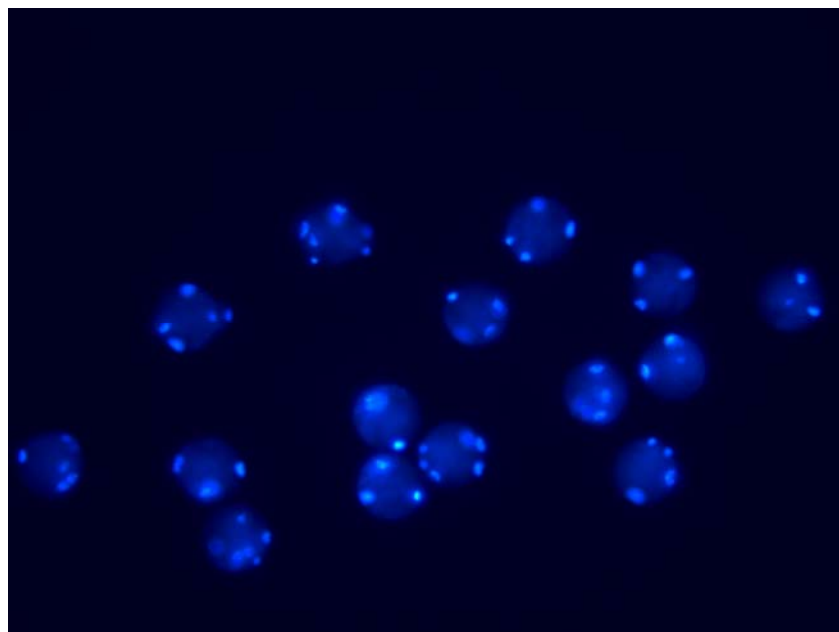


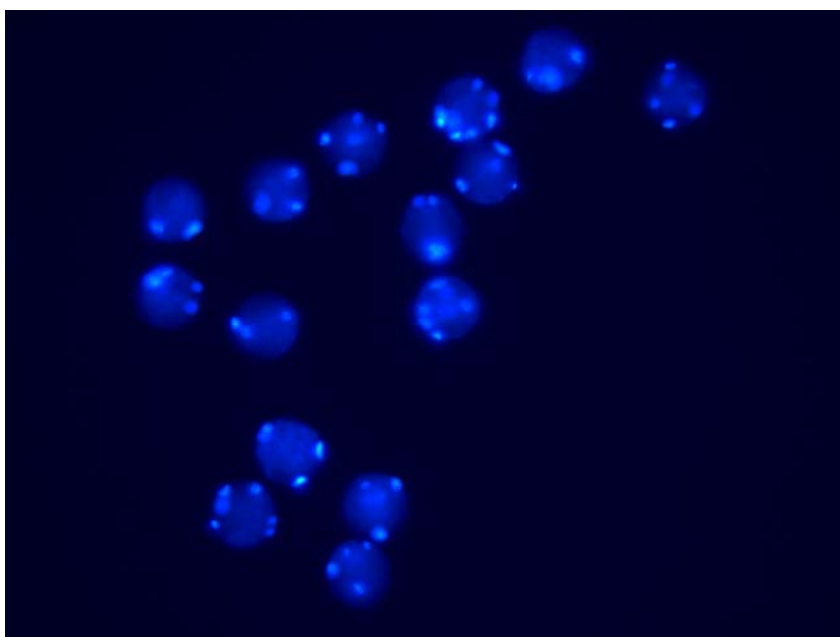
Figure A-9. phase contrast image of Gö 6983-treated eggs after IVF

(February 25, 2005)



Figure A-10. fluorescence image of Gö 6983-treated eggs after IVF

(February 25, 2005)



Subsection B: Localization of actin, CD9, and chromosomal DNA inside the mouse egg

(June 24, 2004)

Figure B-1. actin

Actin localization after calphostin c treatment. Rhodamine-phalloidin was used to visualize actin inside the egg.

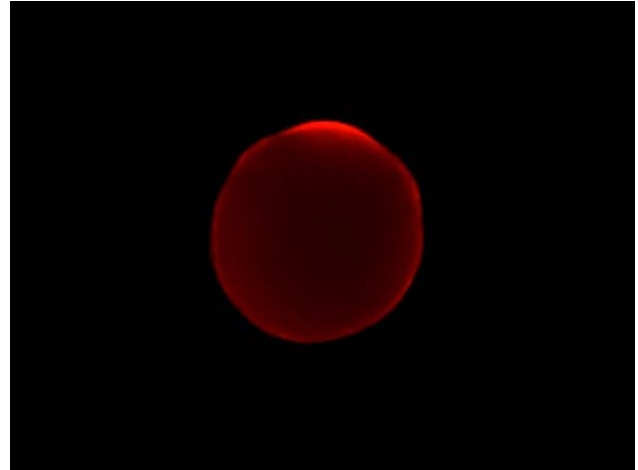


Figure B-2. CD9

CD9 localization after calphostin c treatment. After rat anti-CD9 antibody treatment, FITC-conjugated goat anti-rat antibody was used for immunohistochemistry.

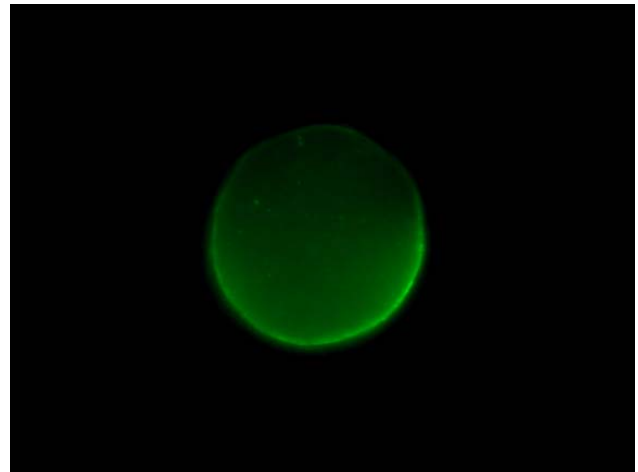


Figure B-3. chromosomal DNA

Chromosomal DNA localization after calphostin c treatment. DAPI is used to stain the egg chromosomal DNA.



Subsection C: Scanning Electron Microscopy images of eggs**(March 25, 2004)**

Figure C-1. control (DMSO-treated)
egg surface (X 3500)

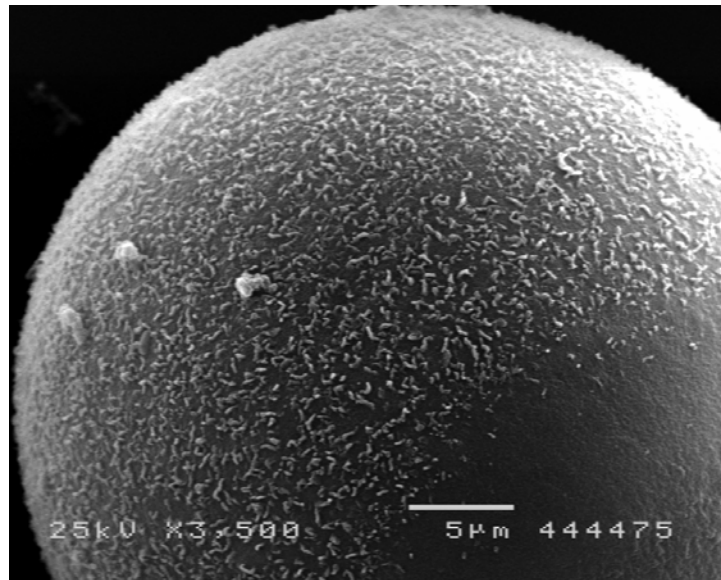


Figure C-2. control (DMSO-treated)
egg surface (X 15000)

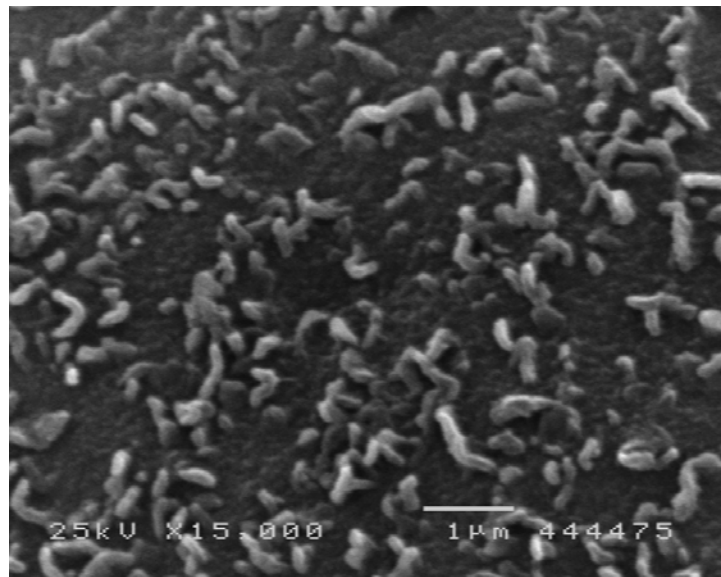


Figure C-3. calphostin c-treated egg surface (X 3500)

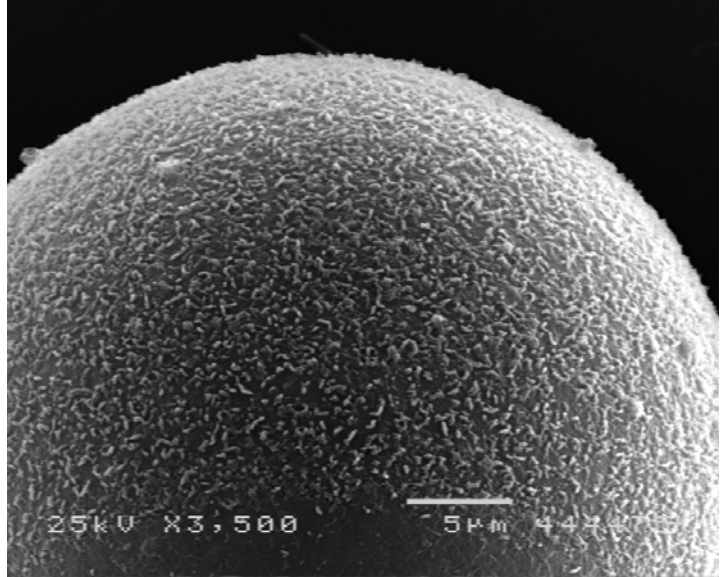


Figure C-4. calphostin c-treated egg surface (X 15000)

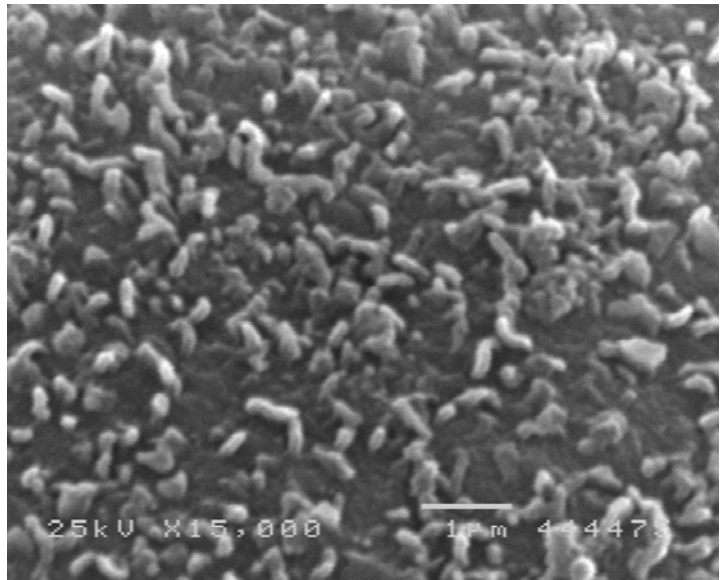


Figure C-5. PMA-treated egg
surface
(X 3500)

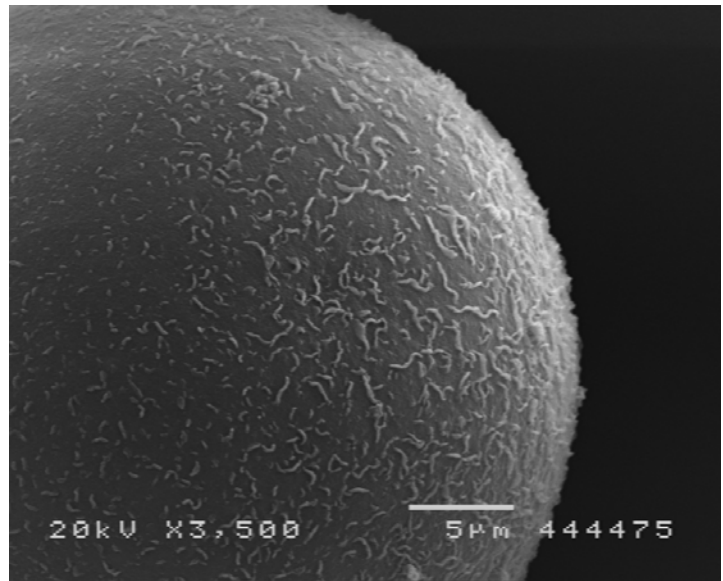
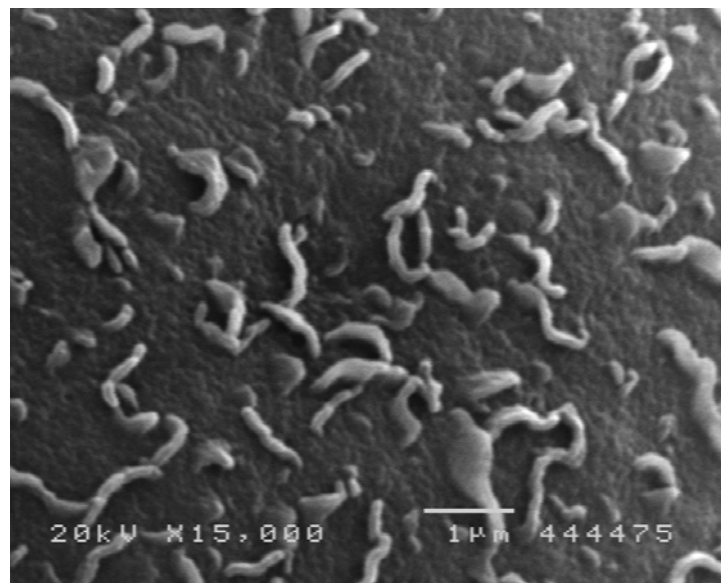


Figure C-6. PMA-treated egg
surface
(X 15000)



Subsection D: Change in fertilization ability after staurosporine, calphostin c, and PMA treatment of mouse eggs

Figure D-1. change in FR after PMA treatment of eggs

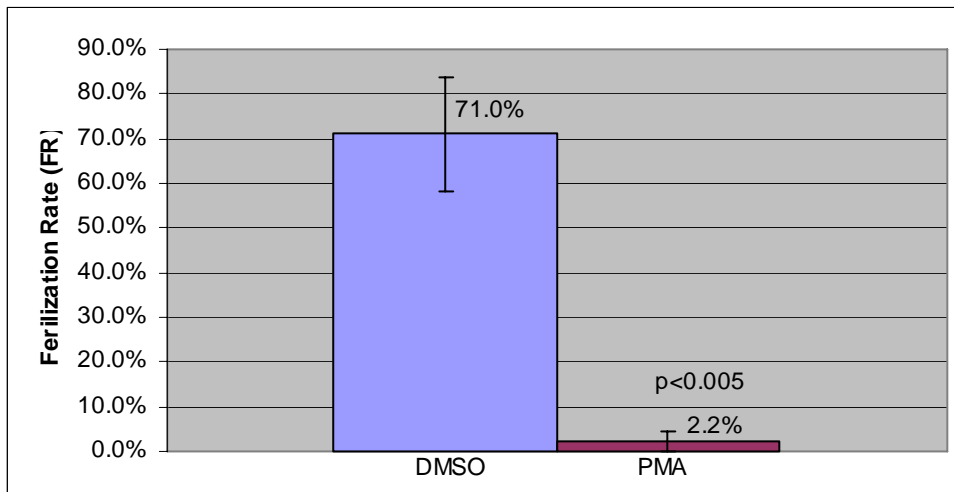


Figure D-2. change in FI after PMA treatment of eggs

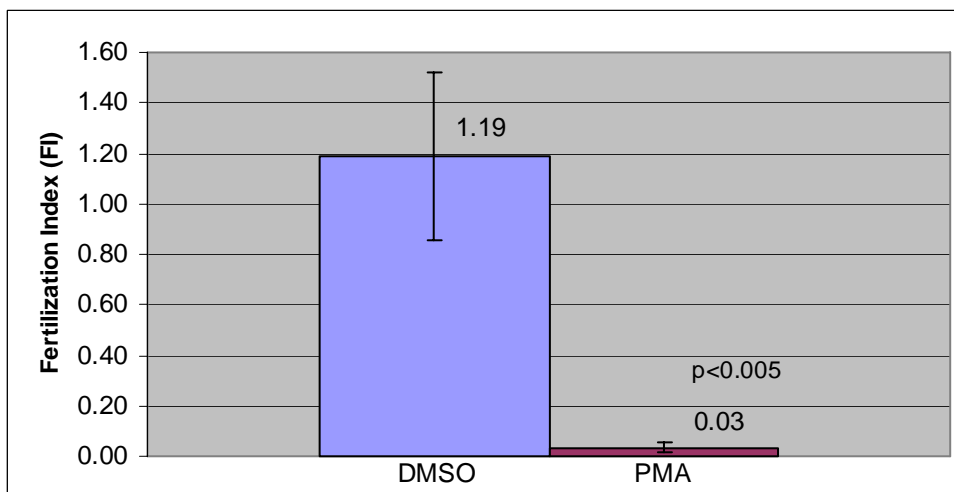


Figure D-3. change in SB after PMA treatment of eggs

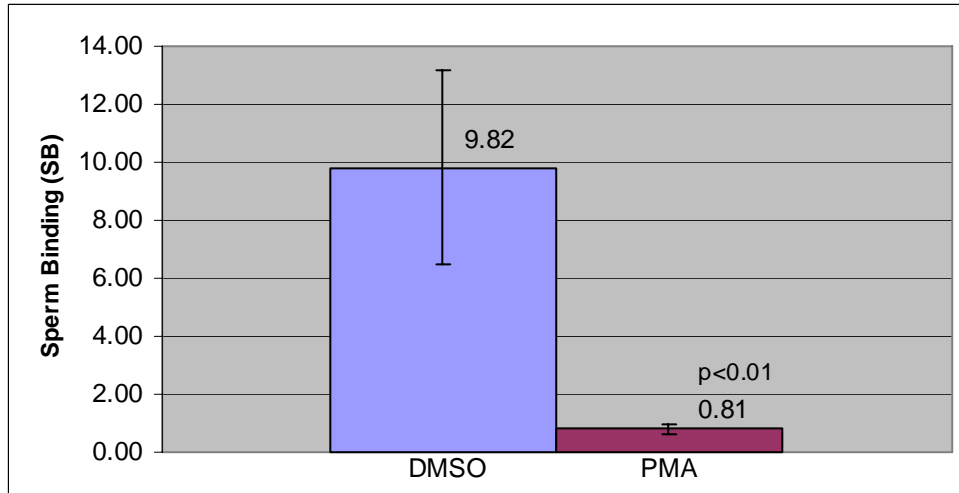


Figure D-4. change in FR after calphostin c treatment of eggs

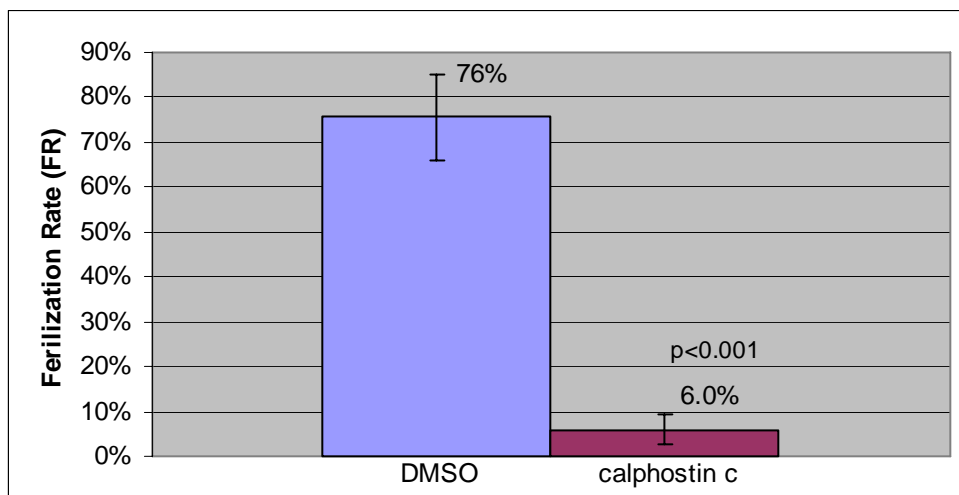


Figure D-5. change in FI after calphostin c treatment of eggs

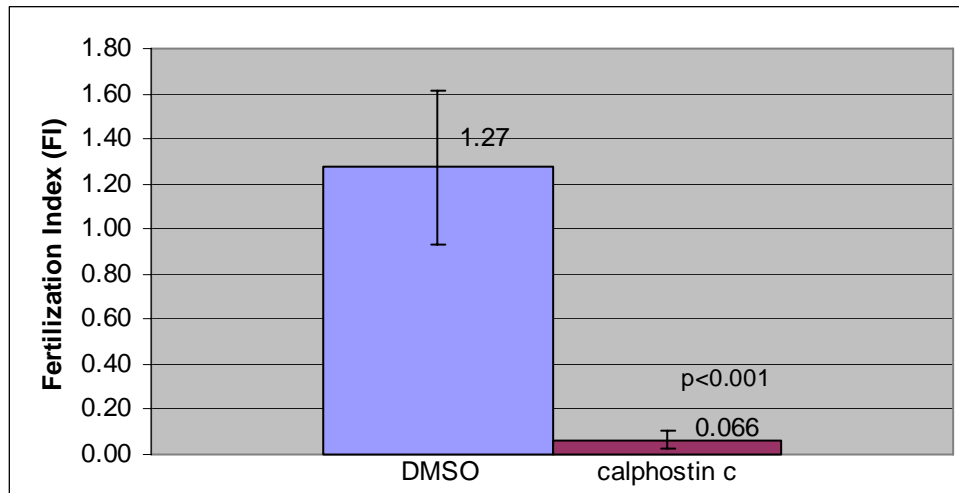


Figure D-6. change in SB after calphostin c treatment of eggs

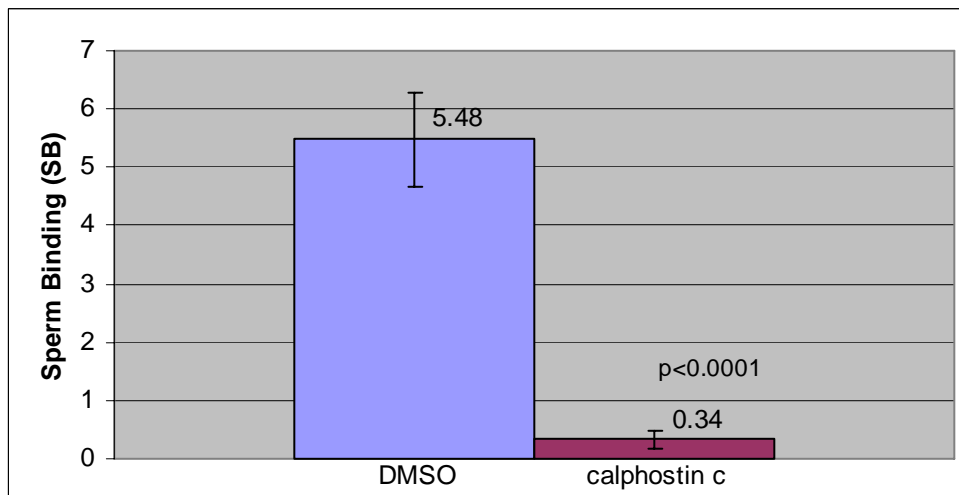


Figure D-7. change in FR after staurosporine treatment of eggs

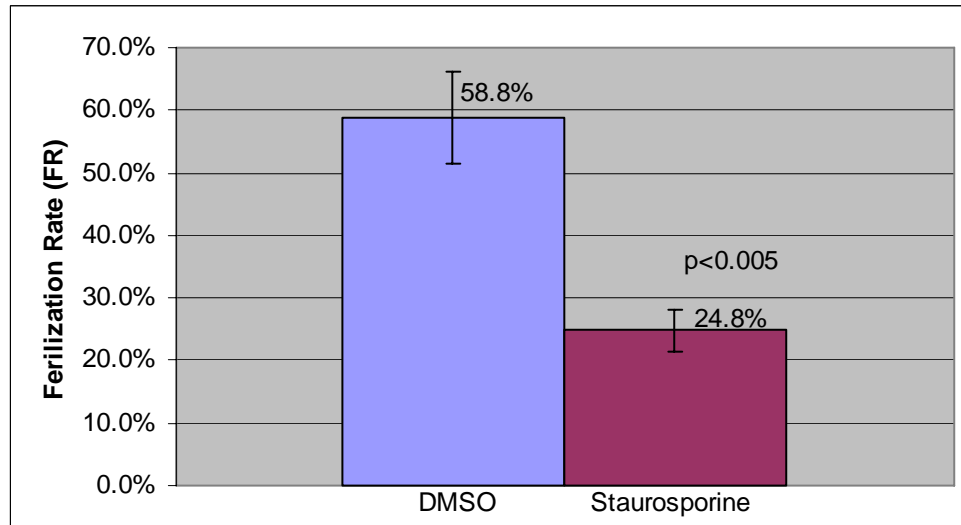


Figure D-8. change in FI after staurosporine treatment of eggs

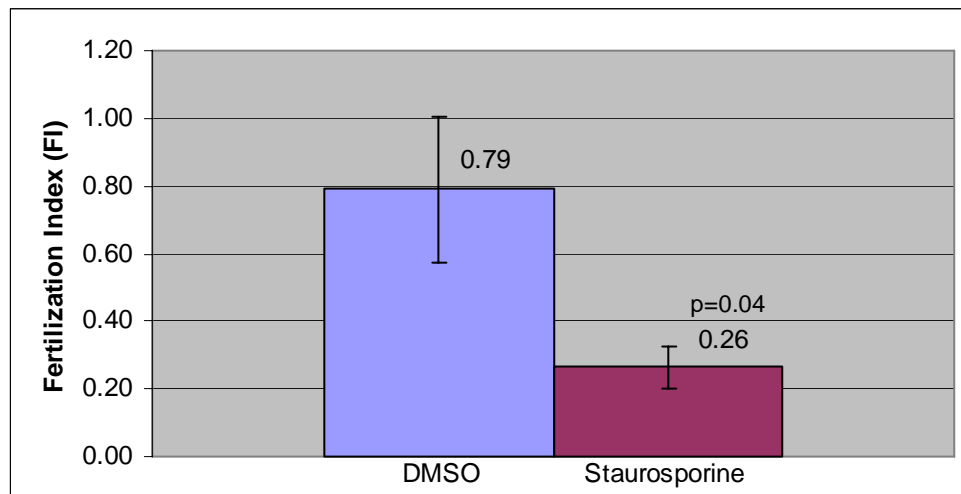


Figure D-9. change in SB after staurosporine treatment of eggs

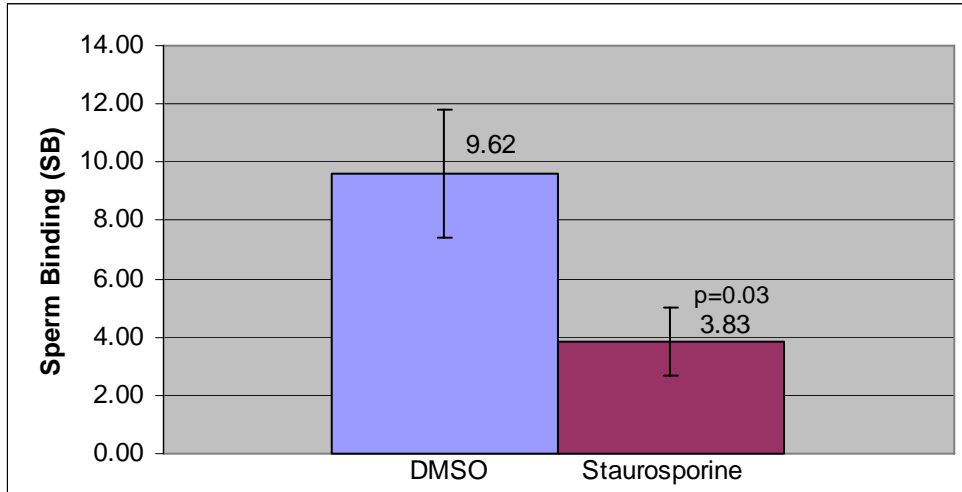


Figure D-10. dose-dependent effects of calphostin c to the sperm binding and fusion rate

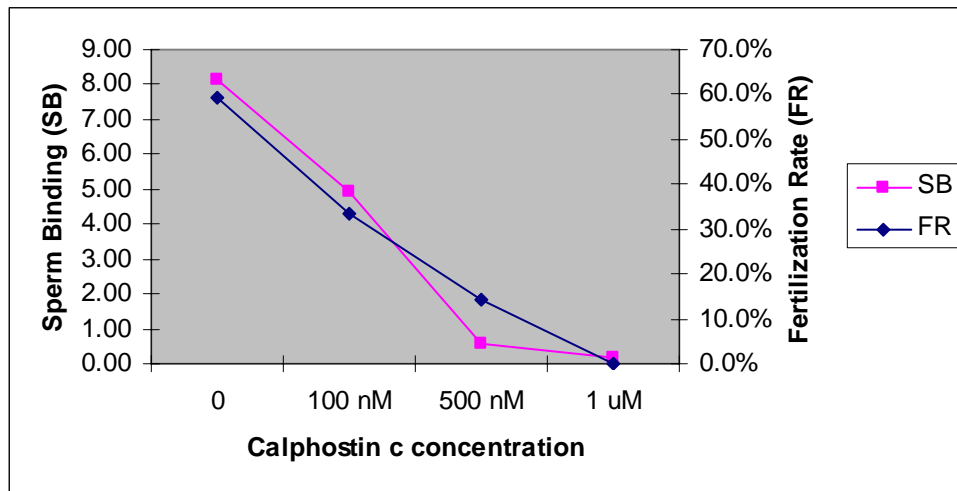


Figure D-11. dose-dependent effects of PMA to the sperm binding and fusion rate

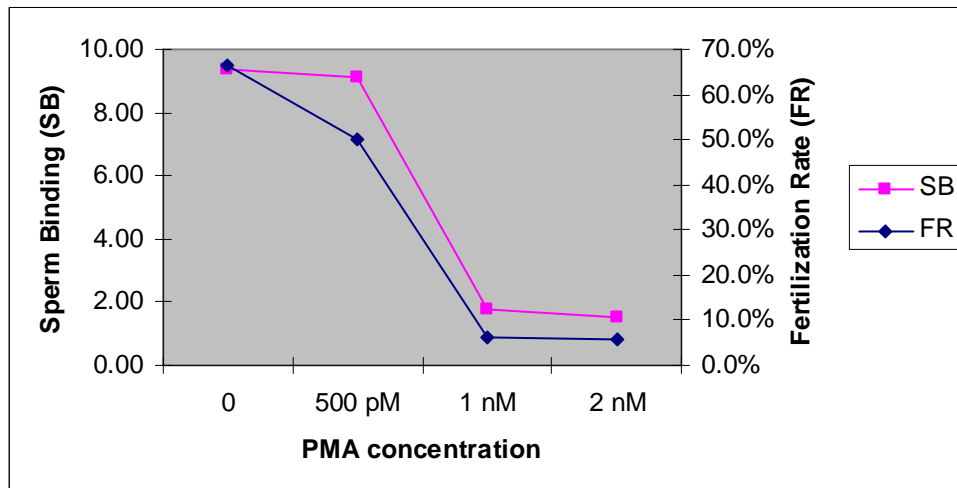


Figure D-12. time-dependent effects of calphostin c to the sperm binding and fusion rate

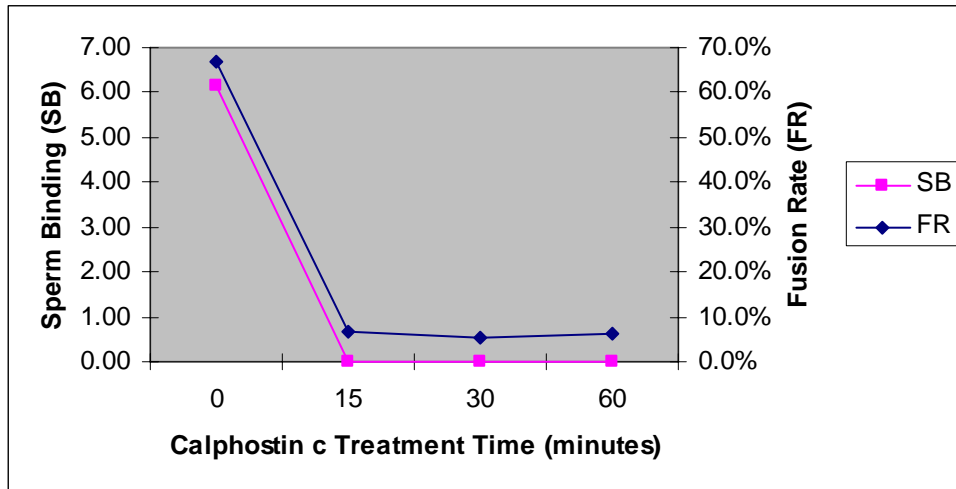


Figure D-13. time-dependent effects of PMA to the sperm binding and fusion rate

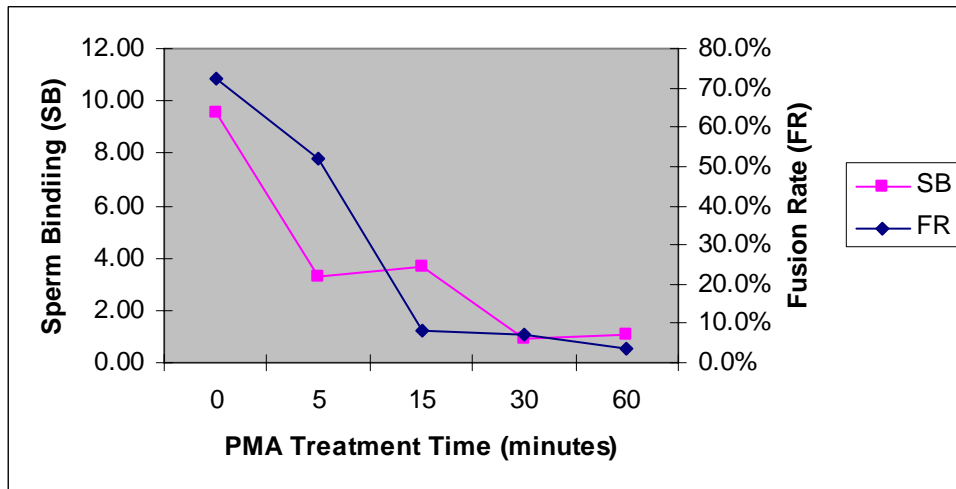


Figure D-14. change in FR after Gö 6976, Gö 6983, and DiC8 treatment of eggs

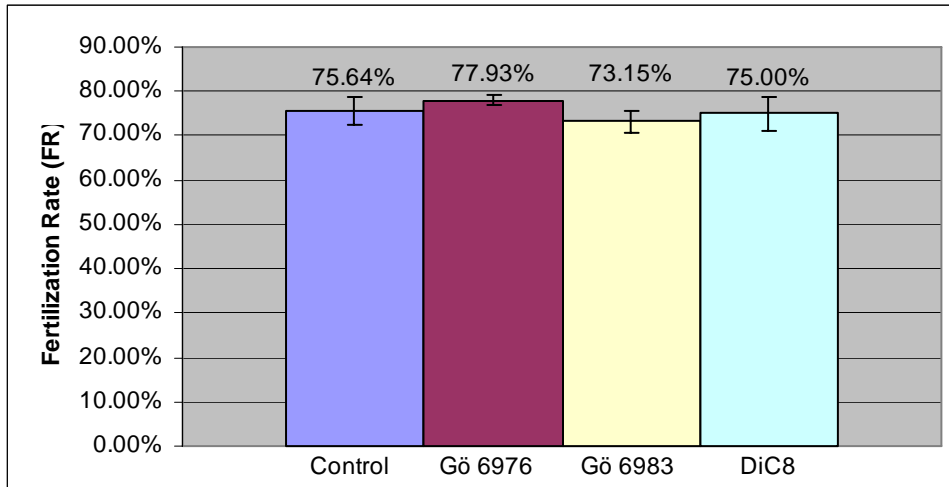


Figure D-15. change in FI after Gö 6976, Gö 6983, and DiC8 treatment of eggs

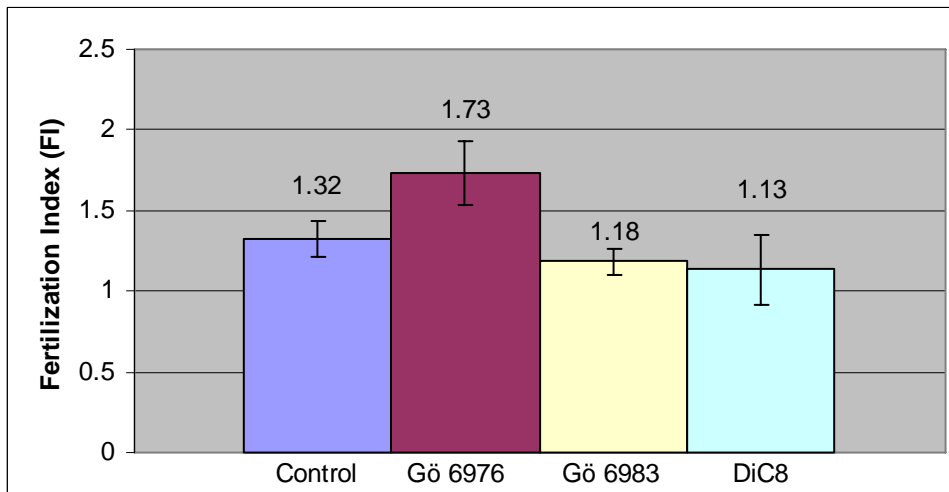


Figure D-16. change in SB after Gö 6976, Gö 6983, and DiC8 treatment of eggs

