Effects of protein kinase C activation and inhibition on sperm-, thimerosal-, and ryanodine-induced calcium responses of human oocytes

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Previous data have shown that protein kinase C (PKC) participates in the mechanism of sperm-induced calcium oscillations in mammalian oocytes, but the actual role of this enzyme in the oscillation mechanism is still unknown. In this study we show that drugs modulating PKC activity disturb the oscillations induced by spermatozoa, thimerosal and ryanodine, but in a different way for each of the three oscillogenic agents. Moreover, PKC inhibition interferes with the return of the intracellular free calcium concentration to basal values during the sperm- and ryanodine-induced calcium oscillations, but not during the thimerosal-induced calcium oscillations. When the PKC-modulating drugs were applied before any of the three oscillogens, the subsequent calcium oscillations were also disturbed. However, the first calcium spike induced by spermatozoa and thimerosal was little influenced by PKC activation or inhibition. On the other hand, ryanodine failed to produce any calcium response when the PKC activity was clamped to a high level. These data suggest that sustained high PKC activities impede calcium oscillations by interfering with the opening of the ryanodine-sensitive calcium release channel, whereas sustained low activities of the enzyme paralyse the channel in the open state.

Key words: calcium oscillations/oocyte activation/protein kinase C/ryanodine/thimerosal

Introduction

Calcium is a second messenger employed by a variety of cells to convey signals generated by hormones and other stimuli, and involved in many biological and pathological processes (reviewed in Berridge and Dupont, 1994). In mammalian cells, calcium typically carries out its signalling role in very short bursts (spikes) of increased free intracytoplasmic calcium concentration ([Ca2+]i) that repeat periodically. This phenomenon is called calcium oscillations. In mammalian fertilization, calcium oscillations represent an early response of the oocyte to the fertilizing spermatozoon and play an essential role in the transduction of the sperm-generated signal to downstream links of the oocyte activation cascade (reviewed in Swann and Ozil, 1994). A highly conserved soluble sperm protein (oscillin), capable of promoting calcium oscillations in mammalian oocytes and probably also in somatic cells, has been identified (Parrington et al., 1996). However, many questions about the mechanism of calcium oscillations in cells and about the biological significance of this special form of second messenger signal in the fertilized oocyte still remain unresolved.

Theories explaining the mechanism of calcium oscillations are all based on the ability of calcium channels localized in the plasma membrane (controlling calcium influx) or in the endoplasmic reticulum membrane (controlling calcium release from intracellular stores) to switch between the open and closed state in a temporally- and spatially-ordered fashion (Berridge and Dupont, 1994; Clapham, 1995). The understanding of the biological significance of calcium oscillations is dependent on the unravelling of the mechanism by which the cell modulates the frequency and amplitude of the oscillations.

Protein kinase C (PKC) is a molecule of special interest with regard to this question although experimental data relative to the role of PKC in the regulation of calcium oscillations are not consistent. Based on studies with PKC activators and inhibitors using the hamster oocyte as a model, PKC has been suggested to operate as part of a negative feedback loop responsible for desensitization in the agonist-induced calcium oscillations (Swann et al., 1989; Miyazaki et al., 1990; Bird et al., 1993). Possible mechanisms of the PKC action, including the removal of free calcium ions from the cytosol, either across the plasma membrane or into an internal store, and down regulation of calcium release from internal stores sensitive to inositol 1,4,5-trisphosphate (InsP3), either by increasing InsP3 degradation or decreasing its production, have been discussed (Swann et al., 1989). On the other hand, an opposite effect of PKC has been reported in smooth muscle (Xuan et al., 1994) and exocrine pancreatic cells (Bode and Göke, 1994) in which PKC activated capacitative calcium entry through voltage-dependent calcium channels of the plasma membrane. In another study, PKC has been shown to be able to both activate and inhibit the capacitative calcium entry depending on the activity level (Petersen and Berridge, 1994). Activation of PKC with phorbol esters and diacylglycerols has also been shown to cause cortical granule exocytosis, second polar body emission and pronuclear formation (Cuthbertson and Cobbold,
9 uM Fluo-3-AM [prepared from a stock solution of 890 uM Fluo-
described (Tesarik and Kopecny, 1989a,b). Zona-free oocytes were
pronase (Sigma, St. Louis, MO, USA) in Tyrode’s salt solution as
of Fluo-3 does not permit a reliable calibration of the emitted
because of its ability to give a better spatial resolution in confocal
fluorescence in terms of absolute \([\text{Ca}^{2+}]\); values. Thus, changes in
visible wavelengths (Minta 1989). On the other hand, the use
Probes, Eugene, OR, USA). Fluo-3 was chosen as calcium probe
the use of the acetoxymethyl ester of Fluo-3 (Fluo-3-AM) (Molecular

Materials and methods

Source and preparation of the gametes
Spermatozoa came from semen samples obtained from healthy donors showing normal values of sperm count, concentration, motility and morphology. After liquefaction at room temperature (30 min), semen samples were diluted in sperm preparation medium (SPM; Medi-
Cult, Copenhagen, Denmark) and centrifuged for 10 min at 600 g to
wash the spermatozoa from the seminal plasma. The resulting sperm
pellets were then resuspended in SPM equilibrated with 5% CO\(_2\) in
air and incubated at 37°C for an additional 3 h.

Oocytes were recovered from large ovarian follicles by ultrasonically-guided follicular aspiration in cycles stimulated with human menopausal gonadotrophin (HMG) and human chorionic gonado-
triph (HCG) after pituitary desensitization with a gonadotrophin-
leaving hormone (GnRH) agonist and used in a therapeutic in-vitro
fertilization (IVF) attempt. Most of the oocytes used in this study were those that did not show signs of fertilization (formation of
pronuclei, emission of the second polar body) by 46 h after in-vitro
insemination and that were still at the second metaphase with no
signs of cytoplasmic degeneration or fragmentation. Such oocytes are
referred to as aged oocytes throughout this study. Oocytes obtained
under identical conditions have been shown to be usable as a model
for the study of early developmental processes in human zygotes
when reinseminated in the zona-free state (Tesarik, 1989; Tesarik and
Kopecn, 1989a,b,c), when injected subzonaly with freshly prepared
human spermatozoon (Tesarik, 1993) or when fertilized by intracyto-
plasmic sperm injection (ICSI; Tesarik and Sousa, 1994; Tesarik and
Testart, 1994; Tesarik et al., 1994, 1995).

Supernumerary fresh mature oocytes, not used in clinical IVF
attempts, were also included in these experiments. All of these oocytes
were donated by consenting patients for research purposes.

Evaluation of stimulus-induced changes in \([\text{Ca}^{2+}]\); 
Sperm- and drug-induced changes in \([\text{Ca}^{2+}]\); were visualized with the use of the acetoxymethyl ester of Fluo-3 (Fluo-3-AM) (Molecular
Probes, Eugene, OR, USA). Fluo-3 was chosen as calcium probe because of its ability to give a better spatial resolution in confocal
studies than ratiometric probes and because it can be excited with visible wavelengths (Minta et al., 1989). On the other hand, the use
of Fluo-3 does not permit a reliable calibration of the emitted fluorescence in terms of absolute \([\text{Ca}^{2+}]\); values. Thus, changes in
\([\text{Ca}^{2+}]\); occurring with time are expressed as changes in relative fluorescence intensity throughout this study.

Oocytes were freed from the zona pellucida with the use of 1%
pronase (Sigma, St. Louis, MO, USA) in Tyrode’s salt solution as
described (Tesarik and Kopecn, 1989a,b). Zona-free oocytes were
then loaded with Fluo-3 by incubating them for 30 min at 37°C with
9 µM Fluo-3-AM [prepared from a stock solution of 890 µM Fluo-
3-AM in dimethylsulphoxide (DMSO)] in SPM equilibrated with 5%
CO\(_2\) in air. After brief washing, oocytes were placed in SPM and left
to adhere to a Falcon plastic culture dish coated with poly-L-lysine
(Sigma). Oocytes were then examined in a Biorad MRC 600 confocal
laser scanning microscopy unit with the use of an Argon laser and a
Nikon BHS filter. The fluorescence emitted from the equatorial plane
of each oocyte was monitored at 2-5 s intervals depending on
experiment. Images were saved in a Panasonic 1-Gb rewritable
magneto-optical disk and later analysed using the Biorad Time Course
Ratiometric Software Module.

Pharmacological experiments
Ryanodine, thimerosal, ionophore A23187, 4β-phorbol 12-myristate
13-acetate (PMA), 4α-phorbol 12,13-didecanoate (PDD), and cheleryth-
rine were purchased from Sigma. Stock solutions of ryanodine, ionophore A23187, PMA, PDD, and chelerythrine were made in
DMSO at respective concentrations of 160 mM (in 40% DMSO),
4 mM, 3.24 mM, 2.97 mM, and 5.21 mM. Sphingosine was purchased
from Calbiochem (La Jolla, CA, USA); stock solutions of this drug
were made at a concentration of 20 mM in ethanol. Shortly before
use, the drugs were diluted in SPM to the double of the final
concentration desired. Additions of drugs to oocytes were made by
mixing equal volumes of these latter solutions with medium containing
oocytes. Consequently, the final concentrations of DMSO and ethanol
were <1%. Preliminary experiments showed that at these concentra-
tions the solvents did not induce oocyte activation nor did they
produce degeneration of oocytes or spermatozoa. Thimerosal was
prepared in SPM shortly before use. Final concentrations of the drugs
used were 4 mM ryanodine, 0.67 mM thimerosal, 100-200 nM PMA or
PDD, 10-100 µM sphingosine, 0.5-1.0 µM chelerythrine and
10 µM ionophore A23187. These concentrations corresponded to
those previously shown to be effective and non-toxic in mammalian
oocytes (Miyazaki et al., 1990; Swann, 1992; Fissore and Robl, 1993;
Gallicano et al., 1993; Homa and Swann, 1994; Sousa et al.,
1996) and human vascular smooth muscle cells (Schuhmann and
Groschner, 1994).

In-vitro insemination
For in-vitro insemination studies, spermatozoa were incubated for
3 h in capacitating medium (Tesarik et al., 1990) and then given an
decosme reaction-inducing stimulus in order to shorten the time
between sperm addition to oocytes and sperm-oocyte fusion. Briefly,
spermatozoa were incubated with 10 µM ionophore A23187 in SPM
for 15 min and then washed by four cycles of centrifugation (600 g;
5 min) and resuspension in fresh SPM. To make sure that no
biologically relevant traces of the ionophore remained in the final
sperm suspension, the supernatant was tested for its ability to
induce oocyte activation; no activity has been detected in any case.
Insemination was carried out by adding 1×10^5/ml motile spermatozoa
to zona-free oocytes.

Results
Sperm-induced calcium oscillations
In the absence of any PKC modulating agent, the fusion of
oocytes with spermatozoa produced a long-lasting (~2 h) series of
calcium oscillations as described previously (Taylor et al.,
1993; Tesarik and Sousa, 1994; Sousa et al., 1996). The same
form of calcium response was obtained when oocytes were
preincubated with PDD, a PKC-non-activating phorbol ester,
before the exposure to spermatozoa (Figure 1A; representative
data from an experiment performed with five aged and four
Figure 1. Effects of sustained protein kinase C (PKC) stimulation and inhibition on calcium oscillations induced in oocytes by spermatozoa. Living oocytes were loaded with Fluo-3 (see Materials and methods section) and exposed to spermatozoa (Sp). 4β-phorbol 12-myristate 13-acetate (PMA) or chelerythrine (Che) were added at the times indicated. Fluo-3 fluorescence emitted from the equatorial plane of the oocytes was recorded at intervals of 2 s. (A) Control oocyte exposed to spermatozoa in the presence of 100 nM 4α-phorbol 12,13-didecanoate (PDD) (data from a single oocyte representative of five aged and four fresh oocytes). (B) Oocyte exposed to 100 nM PMA before the addition of spermatozoa (data from a single oocyte representative of nine aged and four fresh oocytes). (C) Oocyte exposed to 100 nM PMA in the course of the sperm-induced calcium oscillations (data from a single oocyte representative of 10 aged and four fresh oocytes). (D) Oocyte exposed to 1 μM chelerythrine before the addition of sperm (data from a single oocyte representative of 12 aged and four fresh oocytes). Similar data were obtained with 100 μM sphingosine (experiment performed with 12 aged and four fresh oocytes). (E) Oocyte exposed to 1 μM chelerythrine in the course of the sperm-induced calcium oscillations (data from a single oocyte representative of 17 aged and four fresh oocytes). Similar data were obtained with 100 μM sphingosine (experiment performed with 17 aged and four fresh oocytes).
fresh oocytes). In contrast, when PKC activity was clamped to a high level by pretreatment of oocytes with PMA, a PKC-activating phorbol ester, a marked inhibitory effect on the sperm-induced calcium oscillations was observed although the PMA treatment had relatively little effect on the primary sperm-induced \([Ca^{2+}]_j\) increase (Figure 1B; representative data from an experiment performed with nine aged and four fresh oocytes). Similarly, ongoing calcium oscillations, provoked by previous sperm addition, were inhibited by subsequent addition of PMA although the arrest of the oscillation was not instantaneous (Figure 1C; representative data from an experiment performed with 10 aged and four fresh oocytes). Unlike PMA, PDD addition did not inhibit the pre-existing sperm-induced calcium oscillations (data not shown).

When PKC activity was clamped to a low level by oocyte pretreatment with chelerythrine or sphingosine, the development of calcium oscillations after subsequent addition of spermatozoa was also disturbed (Figure 1D; representative data from experiments performed with 12 aged and four fresh oocytes for chelerythrine and with 12 aged and four fresh oocytes for sphingosine). Typically, the first \([Ca^{2+}]_j\) increase was little affected, but there always was a considerable increase in the frequency and a diminution of the amplitude of the secondary \([Ca^{2+}]_j\) rises until a complete inhibition occurred. The addition of chelerythrine and sphingosine to oocytes during ongoing sperm-induced calcium oscillations produced a rapid and complete block of the calcium oscillations by preventing \([Ca^{2+}]_j\), from returning to the basal value (Figure 1E; representative data from experiments performed with 17 aged and four fresh oocytes for chelerythrine and with 17 aged and four fresh oocytes for sphingosine).

Sequential confocal images of human ooocytes taken at 2 s intervals during the first sperm-induced \([Ca^{2+}]_j\) increase showed that calcium release began at a focus localized in the oocyte cortex (the putative sperm fusion site) and propagated as a calcium wave throughout the oocyte cytoplasm (Figure 2A). When the PKC activity was clamped at a high level, by pretreatment of oocytes with PMA, or at a low level, by pretreatment of oocytes with chelerythrine or sphingosine, the sperm-induced calcium discharge was inhibited both in the oocyte cortex and in the rest of ooplasm (Figures 2B and C). A similar effect was observed when PMA was added during ongoing sperm-induced calcium oscillations (data not shown).

On the other hand, the addition of chelerythrine or sphingosine in the course of sperm-induced calcium oscillations prevented \([Ca^{2+}]_j\), from returning to the basal value mainly in the central ooplasm with relatively less effect in the cortical region (Figure 2D).

**Thimerosal-induced calcium oscillations**

The calcium oscillations induced by thimerosal had amplitude and frequency very similar to those induced by spermatozoa; the preincubation of oocytes with PDD did not modify this calcium response (Figure 3A; representative data from experiments performed with eight aged and four fresh oocytes). Similarly to the sperm-induced calcium oscillations, the thimerosal-induced calcium oscillations were inhibited by PMA added before thimerosal (Figure 3B; representative data from an experiment performed with 21 aged and four fresh oocytes) or after thimerosal (Figure 3C; representative data from an experiment performed with nine aged and four fresh oocytes). Chelerythrine or sphingosine added before thimerosal (Figure 3D; representative data from an experiment performed with 21 aged and eight fresh oocytes) or after thimerosal (Figure 3E; representative data from an experiment performed with 25 aged and eight fresh oocytes) also blocked the thimerosal-induced calcium oscillations. However, unlike the sperm-induced calcium oscillations, chelerythrine and sphingosine did not tend to paralyse \([Ca^{2+}]_j\), at elevated values even at the highest concentration used (1 \(\mu\)M chelerythrine and 100 \(\mu\)M sphingosine).

The analysis of sequential confocal images of oocytes taken at 2 s intervals during the first thimerosal-induced calcium release showed that the main \([Ca^{2+}]_j\) increase was localized at the oocyte periphery (Figure 4A). When the PKC activity was clamped to a high level by pretreatment of oocytes with PMA, this peripheral thimerosal-induced calcium discharge was inhibited so that more calcium was released in the central ooplasm in this condition (Figure 4B). The same phenomenon was observed when the PKC activity was clamped to a low level by oocyte pretreatment with chelerythrine or sphingosine although the central calcium discharge was lower than for the oocytes treated with PMA (Figure 4C).

**Ryanodine-induced calcium oscillations**

Ryanodine induced a rapid \([Ca^{2+}]_j\), increase followed by calcium oscillations of variable amplitude and frequency which were not disturbed by PDD (Figure 5A; representative data from an experiment performed with eight aged and four fresh oocytes). PMA blocked efficiently the ryanodine-induced calcium oscillations both when added before ryanodine (Figure 5B; representative data from an experiment performed with six aged and four fresh oocytes) or after ryanodine (Figure 5C; representative data from an experiment performed with six aged and four fresh oocytes). Moreover, unlike the calcium oscillations induced by spermatozoa (cf. Figure 1B) or thimerosal (cf. Figure 3B), PMA also strongly affected the first ryanodine-induced \([Ca^{2+}]_j\), increase, which was completely abolished in PMA-pretreated oocytes (Figure 5B), and stopped rapidly the ongoing ryanodine-induced calcium oscillations (Figure 5C). The effects of chelerythrine and sphingosine on the ryanodine-induced calcium oscillations were similar to those on the sperm-induced calcium oscillations in that both produced a complete block of the oscillations when added before ryanodine (Figure 5D; representative data from an experiment performed with nine aged and four fresh oocytes for chelerythrine and with seven aged and four fresh oocytes for sphingosine) and an immediate arrest of the existing oscillations when added after ryanodine (Figure 5E; representative data from an experiment performed with 12 aged and four fresh oocytes for chelerythrine and with 12 aged and four fresh oocytes for sphingosine). As for the sperm-induced calcium oscillations (cf. Figure 1E), there also was a paralysis of \([Ca^{2+}]_j\), at elevated values after the addition of chelerythrine to oocytes in the course of the ryanodine-induced calcium oscillations (Figure 5E).
PKC and calcium oscillations

Figure 2. Confocal images of the first intracellular calcium concentration \([\text{Ca}^{2+}]_i\) increase induced by spermatozoa in fresh oocytes loaded with Fluo-3. (A) Oocyte pretreated with 100 nM 4α-phorbol 12,13-didecanoate (PDD). (B) Oocyte pretreated with 100 nM 4β-phorbol 12-myristate 13-acetate (PMA). (C) Oocyte pretreated with 1 μM chelerythrine. (D) Oocyte exposed to 1 μM chelerythrine during ongoing sperm-induced calcium oscillations. Images of Fluo-3-loaded oocytes were taken from the oocytes' equatorial plane at 2 s intervals. For each image presented in the individual series, the time (s) elapsed from the first image (time 0) is indicated in the upper left corner. Fluorescence intensity is expressed in pseudocolour according to the scale bar where the lowest values are coded black. Similar results were obtained with aged oocytes.

The analysis of sequential confocal images of oocytes taken at 2 s intervals during the first \([\text{Ca}^{2+}]_i\) increase after the addition of ryanodine showed that the calcium discharge began nearly simultaneously throughout the oocyte cytoplasm except for a thin cortical region; maximal values were attained in the central ooplasm (Figure 6A). When the PKC activity was clamped to a high level by PMA, no \([\text{Ca}^{2+}]_i\) increases could be detected irrespective of whether PMA was added before or after ryanodine (data not shown). When the PKC activity was clamped to a low level by the pretreatment of oocytes with chelerythrine or sphingosine, only one relatively small calcium discharge was observed after the subsequent ryanodine addition (data not shown). When these drugs were added in the course of ongoing ryanodine-induced \([\text{Ca}^{2+}]_i\) oscillations, \([\text{Ca}^{2+}]_i\) remained elevated throughout the oocyte cytoplasm (Figure 6B).

Discussion

Various models have been proposed to explain the mechanism of calcium oscillations in cells. Some of them postulate cyclic elevations of the intracellular concentration of InsP₃, resulting from cyclic stimulation of phospholipase C by the released calcium, to act as a positive feedback element releasing more calcium from the InsP₃-sensitive intracellular stores (Bezprozvanny et al., 1991; Meyer and Stryer, 1991; De Young and Keizer, 1992). Other models presume sustained InsP₃ production with alternating calcium release and uptake by separate calcium stores with different sensitivity to calcium-induced calcium release (CICR). These models are based on calcium exchanges between the InsP₃-sensitive and the ryanodine-sensitive stores (Goldbeter et al., 1990; Galione et al., 1993; Kasai et al., 1993; Lee et al., 1993) or between two types of the InsP₃-sensitive store with different sensitivities to the agonist (Bootman et al., 1992). Both InsP₃-sensitive and ryanodine-sensitive calcium stores have been detected in mammalian oocytes (Swann, 1992; Fissore and Robl, 1993; Miyazaki et al., 1993a,b; Ayabe et al., 1995; Yue et al., 1995). Human oocytes appear to use a two-store mechanism for the sperm-induced calcium oscillations (Tesarik et al., 1995) in which one of the stores is ryanodine-sensitive and the other ryanodine-insensitive and presumably InsP₃-sensitive (Sousa et al., 1996).

There are several possible ways in which PKC modulating agents may influence calcium oscillations. PKC has been suggested to be partly responsible for desensitization of the calcium-release mechanism by acting on the pathway leading from receptor activation to InsP₃ production (Llano and Marty, 1987; Woods et al., 1987; Maruyama, 1989), possibly by modulating the function of a putative G protein (Swann et al., 1989; Miyazaki et al., 1990). PKC has also been suggested as being implicated in the control of calcium entry into the cell.
Figure 3. Effects of sustained protein kinase C (PKC) stimulation and inhibition on calcium oscillations induced in Fluo-3-loaded oocytes by the addition of thimerosal (Thi). 4β-phorbol 12-myristate 13-acetate (PMA) or chelerythrine (Che) were added at the times indicated. Fluo-3 fluorescence emitted from the equatorial plane of the oocytes was recorded at intervals of 2 s. PMA or chelerythrine (Che) were added at the times indicated. Fluo-3 fluorescence emitted from the equatorial plane of the oocytes was recorded at intervals of 2 s. (A) Control oocyte exposed to thimerosal in the presence of 100 nM 4β-phorbol 12,13-didecanoate (PDD) (data from a single oocyte representative of eight aged and four fresh oocytes). (B) Oocyte exposed to 100 nM PMA before the addition of thimerosal (data from a single oocyte representative of 21 aged and four fresh oocytes). (C) Oocyte exposed to 100 nM PMA in the course of the thimerosal-induced calcium oscillations (data from a single oocyte representative of nine aged and four fresh oocytes). (D) Oocyte exposed to 1 μM chelerythrine before the addition of thimerosal (data from a single oocyte representative of 11 aged and four fresh oocytes). Similar data were obtained with 100 μM sphingosine (experiment performed with 10 aged and four fresh oocytes). (E) Oocyte exposed to 1 μM chelerythrine in the course of the thimerosal-induced calcium oscillations (data from a single oocyte representative of 15 aged and four fresh oocytes). Similar data were obtained with 100 μM sphingosine (experiment performed with 10 aged and four fresh oocytes).
PKC and calcium oscillations

Figure 4. Confocal images of the first thimerosal-induced intracellular calcium concentration \([Ca^{2+}]_i\) increase in fresh oocytes loaded with Fluo-3. (A) Oocyte pretreated with 100 nM 4α-phorbol 12,13-didecanoate (PDD). (B) Oocyte pretreated with 100 nM 4β-phorbol 12-myristate 13-acetate (PMA). (C) Oocyte pretreated with 1 μM chelerythrine. Images of Fluo-3-loaded oocytes were taken from the oocytes' equatorial plane at 2 s intervals. For each image presented in the individual series, the time (s) elapsed from the first image (time 0) is indicated in the upper left corner. Fluorescence intensity is expressed in pseudocolour according to the scale bar where the lowest values are coded black. Similar results were obtained with aged oocytes.

by regulating plasma membrane calcium channels (Di Virgilio et al., 1986; Bode and Göke, 1994; Petersen and Berridge, 1994; Schuhmann and Groschner, 1994), to stimulate calcium pump activity (Balasubramanyam and Gardner, 1995) and to participate in the release of calcium from the InsP₃-sensitive intracellular stores (Fasolato et al., 1988). The possibility that the function of the other major intracellular calcium store, the ryanodine-sensitive one, can be influenced by PKC has been evoked only indirectly by in-vitro studies demonstrating a relationship between the PKC-dependent phosphorylation of the ryanodine receptor and its ligand binding activity (Takasago et al., 1991).

This study is the first to show that PKC modulators can affect the function of the ryanodine-sensitive calcium store in living cells. In fact, the ryanodine-sensitive store appears to be the main target of PKC during calcium oscillations, at least in our system. This conclusion is based on the comparison of the effects of PKC activation on \([Ca^{2+}]_i\), changes induced by each of the three oscillogens (spermatozoa, thimerosal, ryanodine) used in this study. The sustained activation of PKC inhibited the calcium oscillations induced in oocytes by spermatozoa, thimerosal and ryanodine, but it had relatively little effect on the first \([Ca^{2+}]_i\) increase induced by spermatozoa and thimerosal. In contrast, ryanodine failed to produce any \([Ca^{2+}]_i\) increase in these conditions.

The sperm-induced calcium oscillations in human oocytes have been hypothesized to be maintained by calcium exchanges between one type of calcium store prevailing in the oocyte periphery and a different type of store prevailing in the rest of the ooplasm (Tesarik et al., 1995). The latter store is now known to be sensitive to ryanodine while the former is not (Sousa et al., 1996). Spermatozoa and thimerosal might thus generate the initial calcium spike by acting on the ryanodine-insensitive store even under conditions in which the ryanodine-sensitive store is blocked by sustained PKC activation. However, further calcium spiking is disabled because of the inability of the ryanodine-sensitive store to participate correctly in the calcium exchanges that are needed for ongoing oscillations.

In fact, the present spatial analysis of the initial sperm-induced \([Ca^{2+}]_i\) increase has shown that PMA disturbs the propagation of the calcium wave across the central ooplasm (cf. Figures 2A,B) where the ryanodine-sensitive stores are well-developed (Sousa et al., 1996). The reason why PMA inverted the typical spatial propagation of the thimerosal-induced \([Ca^{2+}]_i\) increases, leading to a major calcium release in the central ooplasm instead of the oocyte periphery (cf. Figures 4A,B), is not known. It is possible that a blockage by PMA of the capacitative calcium entry from the extracellular space, similar to that reported in frog oocytes (Petersen and Berridge, 1994), is involved in this phenomenon.

On the other hand, the maintenance of low PKC activity by adding chelerythrine or sphingosine to oocytes in the course of sperm- or ryanodine-induced calcium oscillations tended to paralyse \([Ca^{2+}]_i\), at elevated values. The reason for the failure of chelerythrine to produce the same phenomenon with the thimerosal-induced calcium oscillations is not known. The confocal imaging has confirmed that the inhibition of calcium resorption due to the action of the PKC inhibitors concerns mainly the central ooplasm. This is in agreement with the spatial distribution of the ryanodine-sensitive calcium stores in human oocytes (Sousa et al., 1996). Interestingly, inhibitors of PKC have also been shown to provoke an elongation of the
Figure 5. Effects of sustained protein kinase C (PKC) stimulation and inhibition on calcium oscillations induced in Fluo-3-loaded oocytes by the addition of ryanodine (Ry). 4β-phorbol 12-myristate 13-acetate (PMA) or chelerythrine (Che) were added at the times indicated. Fluo-3 fluorescence emitted from the equatorial plane of the oocytes was recorded at intervals of 2 s. (A) Control oocyte exposed to ryanodine in the presence of 4α-phorbol 12,13-didecanoate (PDD) (data from a single oocyte representative of eight aged and four fresh oocytes). (B) Oocyte exposed to 100 nM PMA before the addition of ryanodine (data from a single oocyte representative of six aged and four fresh oocytes). (C) Oocyte exposed to 100 nM PMA in the course of the ryanodine-induced calcium oscillations (data from a single oocyte representative of six aged and four fresh oocytes). (D) Oocyte exposed to 1 μM chelerythrine before the addition of ryanodine (data from a single oocyte representative of nine aged and four fresh oocytes). Similar data were obtained with 100 μM sphingosine (experiment performed with seven aged and four fresh oocytes). (E) Oocyte exposed to 1 μM chelerythrine in the course of the ryanodine-induced calcium oscillations. Similar data were obtained with 100 μM sphingosine (experiment performed with 12 aged and four fresh oocytes).
Cobbold, 1995) or regulation of the capacitative calcium entry (Petersen and Berridge, 1994) by PKC may also be involved.

In conclusion, the present data show that PKC is intrinsically implicated in the mechanism of calcium oscillations of human oocytes. The ryanodine receptor/calcium release channel of the endoplasmic reticulum may be one of the main targets of this PKC action. Because PKC can be regulated by different cell signalling pathways, it may play an important role in the physiological regulation of the frequency and amplitude of the sperm-induced calcium oscillations of human oocytes.

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