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 oocytes, calcium typically carries out its signalling role in very short bursts (spikes) of increased free intracytoplasmic calcium concentration (\([Ca^{2+}]_i\)) 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 possibly also in somatic cells, has been identified (Parrington et al., 1994). 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 activated 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 activitiy 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,
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1985; Colonna et al., 1989), presumably by a calcium-independent mechanism (Gallicano et al., 1993).

This study was undertaken to determine whether and how drugs modulating PKC activity interfere with sperm-induced calcium oscillations in human oocytes. To address the question of how PKC is integrated in the sperm-induced calcium oscillation mechanism, we also compared these data with those obtained by similar experiments using two artificial Oscillogenic agents, thimerosal and ryanodine. Data suggest that PKC essentially regulates the ryanodine-sensitive calcium release channel.

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; MediCult, 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% CO2 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 gonadotrophin (hCG) after pituitary desensitization with a gonadotrophin-releasing 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 Kopecny, 1989,a,b,c), when injected subvazally with freshly prepared human spermatozoa (Tesarik, 1993) or when fertilized by intracytoplasmic 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 [Ca2+]i

Sperm- and drug-induced changes in [Ca2+]i were visualized with the use of the acetoxyethyl 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 [Ca2+]i values. Thus, changes in [Ca2+]i 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 Kopecny, 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% CO2 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 Argen 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 rewrtable magneto-optical disk and later analysed using the Biorad Time Course Ratiometric Software Module.

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). 4α-phorbol 12,13-didecanoate (PDD) 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$^{2+}$]i 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 thimerosal 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$^{2+}$]i increase was little affected, but there always was a considerable increase in the frequency and a diminution of the amplitude of the secondary [Ca$^{2+}$]i increase, 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$^{2+}$]i 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$^{2+}$]i 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$^{2+}$]i 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$^{2+}$]i at elevated values even at the highest concentration used (1 µM chelerythrine and 100 µM 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$^{2+}$]i 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$^{2+}$]i 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$^{2+}$]i 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$^{2+}$]i 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ oscillations, $[Ca^{2+}]_i$ 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 InsP$_3$, resulting from cyclic stimulation of phospholipase C by the released calcium, to act as a positive feedback element releasing more calcium from the InsP$_3$-sensitive intracellular stores (Bezprozvanny et al., 1991; Meyer and Stryer, 1991; De Young and Keizer, 1992). Other models presume sustained InsP$_3$ 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 InsP$_3$-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 InsP$_3$-sensitive store with different sensitivities to the agonist (Bootman et al., 1992). Both InsP$_3$-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 store is ryanodine-sensitive and the other ryanodine-insensitive and presumably InsP$_3$-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 InsP$_3$ 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 Gorschner, 1994), to stimulate calcium pump activity (Balasubramanyam and Gardner, 1995) and to participate in the release of calcium from the InsP$_3$-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-sensitive 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). 4ß-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 4ß-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 |ÌM 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 |ÌM sphingosine (experiment performed with seven aged and four fresh oocytes). (E) Oocyte exposed to 1 |ÌM chelerythrine in the course of the ryanodine-induced calcium oscillations. Similar data were obtained with 100 |ÌM sphingosine (experiment performed with 12 aged and four fresh oocytes).
PKC and calcium oscillations

falling phase of hormone-induced [Ca^{2+}], oscillations in rat hepatocytes (Sanchez-Bueno et al., 1990; Berrie and Cobbold, 1995) which are hypothesized mainly as relying on the InsP^3-sensitive calcium stores. The mechanism of this effect may thus be different from that underlying the action of PKC inhibitors in human oocytes in which they appear to affect preferentially the ryanodine-sensitive calcium stores.

According to a recently proposed mechanism of calcium oscillations in human oocytes (Tesarik et al., 1995), calcium ions are drained continuously by one type of intracellular calcium store during the period between individual spikes, and each new spike is launched as soon as the draining capacity of this store is saturated so that the excess calcium triggers CICR from another store of a different type. The former store is proposed to be InsP^3-sensitive and the latter ryanodine-sensitive (Sousa et al., 1996). Because the data presented in this study suggest that PKC inhibition compromises the resorption of calcium by the ryanodine-sensitive stores, this situation can be expected to interfere with the calcium-draining function of the ryanodine-sensitive stores. Hence, the resulting faster accumulation of free calcium ions in the cytosol would lead to a shortening of the interval between sequential calcium spikes, whereas the lower calcium loading of the ryanodine-sensitive store would account for the lower spike amplitude. This would explain the modification (inhibition and paralysis of [Ca^{2+}], at high levels) of calcium oscillations by chelerythrine observed in this study. A similar modification of the sperm-induced calcium oscillations has also been described to occur in hamster oocytes in which PKC was inhibited by sphingosine (Swann et al., 1989; Miyazaki et al., 1990). In view of the results of this study, this effect appears to be at least partly attributable to PKC action at the ryanodine-sensitive calcium stores, although other mechanisms, implying modulation of the function of the InsP^3-sensitive calcium stores (Berrie and 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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