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Calmodulin, gametes and fertilisation

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Summary

The role of calmodulin in fertilisation events was examined in a zona-free mouse system by using a selective calmodulin inhibitor, calmidazolium (1 μM). The effects of this antagonist were studied either on the ooplasmic calcium oscillations induced by fertilisation by using the Ca^{2+} indicator, fluo-3/AM, or on pronucleus formation 4 h later by using the nucleic acid stain, Syto-15. When the calmidazolium treatment was applied to one or the other gamete before insemination, the fertilisation process was affected only when spermatozoa were treated: most of the oocytes were partially fertilised as demonstrated by the profile of Ca^{2+} oscillations and the presence of polar bodies with no typical male and female pronuclei. When the treatment was applied during insemination, more than half the oocytes were unfertilised and only a few were partially fertilised. These results demonstrate that: (1) the calmodulin-dependent events taking place in spermatozoa before insemination appear essential at least for regular Ca^{2+} oscillations and for pronucleus formation; (2) the inhibition of calmodulin by calmidazolium applied to metaphase II oocytes before insemination has no major impact on their fertilising ability; and (3) at the time of gamete fusion calmodulin, either from the oocyte or from the spermatozoon, is essential for fertilisation to occur.

Keywords: Calmidazolium, Confocal imaging, Fertilisation, Mouse, Spermatozoa

Introduction

Calmodulin, a 17 kDa highly conserved E–F hand protein (Smith & Dedman, 1986; Kretzinger *et al*., 1988) is implicated in calcium signalling in all eukaryotic cells that have been studied. Moreover, calmodulin is considered to be a potential target for the transient increase in Ca^{2+} that occurs following fertilisation in *Xenopus* (Lorca *et al.*, 1993) and mouse (Xu *et al.*, 1996). However, as calmodulin is present in male as well as in female gametes, the difficulty is to distinguish which gamete's calmodulin is acting at fertilisation. Sperm calmodulin, first described at the acrosomal level in spermatids, is also found in large quantities in the post-acrosomal region and tail of many mammalian spermatozoa (Jones *et al.*, 1980; Feinberg *et al.*, 1983; Weinmann *et al.*, 1986; Kann *et al.*, 1991). Its presence in compartments such as the acrosome or tail may account for physiological functions such as the acrosome reaction and motility (Camatini *et al.*, 1986; Pariset *et al.*, 1989). Furthermore, this calciprotein is stable at high concentration throughout the physiological processes in the post-acrosomal region (which is implicated in gametic fusion) and disappears in the ooplasm at the exact moment when sperm factor(s) initiate oocyte activation (Courtot *et al.*, 1994).

Our aim was thus to check whether sperm calmodulin plays a specific role in the first steps of fertilisation. As a calmodulin inhibitor we used calmidazolium, which is particularly characterised both by its ability to cross membranes and by its calmodulin affinity. We worked in a mouse homospecific system. To study the events occurring after the acrosome reaction we used zona-free oocytes and a calmidazolium concentration known to have no inhibitory effect on sperm motility (Aitken *et al.*, 1988). By using confocal imaging we analysed the effects of sperm treatment with calmidazolium on ooplasmic calcium changes in the minutes following insemination and on sperm attachment and subsequent fertilisation resulting in the formation of pronuclei 4 h later.

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Materials and methods

Media and reagents

Gametes were cultured in M16 and M2 media prepared as described by Fulton & Whittingham (1978) and supplemented with bovine serum albumin (BSA; Fraction V, Sigma, St Quentin Fallavier, France). A stock solution of 1 mM calmidazolium (Sigma) was prepared in absolute ethanol and stored at –20 °C. The Ca^{2+} indicator, fluo-3 acetoxymethyl ester (fluo-3/AM), pluronic F-127 and the live cell permeant nucleic acid stain, Syto-15, were obtained from Molecular Probes (Eugene, OR). Stock solutions of fluo-3/AM (0.5 mM), pluronic F-127 (0.16 mM) and Syto-15 (5 mM) were prepared in dimethyl sulphoxide (DMSO).

Gamete preparation and insemination

Gametes were recovered from mature F1 hybrids (C57 BL × CBA) (Iffa Credo, L'Arbresle, France). Female mice were superovulated by injecting 5 IU pregnant mare serum gonadotrophin (PMSG) followed 48 h later by 5 IU of human chorionic gonadotrophin (hCG). Cumulus–egg complexes were retrieved from the oviducts of the mice 14–15 h after the hCG injection. The eggs were freed from the surrounding cells by 0.1% hyaluronidase (Sigma) and the zona pellucida removed by brief treatment with α-chymotrypsin (0.01%). All these experiments were carried out in M2 medium supplemented with BSA (4 mg/ml) at 37 °C. After washing, the zona-free oocytes were incubated in M16 medium containing BSA (15 mg/ml) at 37 °C in a humidified atmosphere with 5% CO_2 for 1.5 h before insemination.

After being expelled from the cauda epididymis and the deferent ducts of mature male mice, the spermatozoa were dispersed in M16 medium with BSA (15 mg/ml) at 37 °C in a humidified atmosphere with 5% CO_2, to allow capacitation (1.5 h). The zona-free mouse oocytes were then inseminated with capacitated spermatozoa at 37 °C and incubated for 4 h.

Kinetics of fertilisation events

To establish the kinetics of fertilisation events, control oocytes were examined every hour following insemination. The inseminated oocytes were incubated with Syto-15 (950 µM) in M2 medium for 10 min at 37 °C. After washing, they were put on a coverslip in a small drop of medium and fluorescence from eggs was monitored with a confocal laser scanning imaging system. A series of 25 images separated by a Z-step (3 µm) were collected (PlanApo ×20 objective) and then further projected so that they were all superimposed, allowing observation of the appearance of the chromatin in the whole oocyte.

Calmidazolium treatments

In one series of three experiments, gametes were treated before insemination with calmidazolium (1 µM) during either the whole preincubation period (1.5 h) or for the last 20 min. After this, gametes were washed in fresh medium before being inseminated with their untreated counterpart.

In another series of three experiments, untreated oocytes were inseminated with untreated spermatozoa in a calmidazolium-containing medium (1 µM) and were left in that medium either for 4 h or for short periods of 30 min (from 0 to 30 min or from 30 to 60 min).

Using a stock solution of calmidazolium prepared in absolute ethanol, control groups of eggs were inseminated with untreated spermatozoa diluted in M16 medium containing 0.2% ethanol.

Four hours after insemination, all the oocytes were loaded with Syto-15 and examined as described above. The oocytes were classified into three main categories: normally fertilised oocytes (with a second polar body, one female pronucleus and one or more male pronuclei); partly fertilised oocytes (with a second polar body, and male and female chromatin elements); and unfertilised oocytes (with a metaphase II plate).

Fluo-3/AM loading of oocytes and fluorescence measurements

Before insemination, untreated oocytes were loaded with the Ca^{2+} indicator fluo-3/AM (5 µM) dissolved simultaneously with pluronic F-127 (0.8 µM) into 0.5 ml M16 medium (15 mg/ml BSA) and put in the dark for 10 min in a humidified atmosphere with 5% CO_2 at 37 °C. After loading, the oocytes were washed twice in M2 medium, put into microdrops of M2 medium under paraffin oil (BDH, Poole, UK) in a plastic culture dish (35 × 10 mm; Costar, Cambridge, MA) and placed on the heating stage of the microscope at 33–34 °C. They were then inseminated with capacitated spermatozoa that had or had not been preincubated in a medium containing calmidazolium. Fluorescence measurements were performed with a Nikon Diaphot inverted microscope interfaced with a Bio-Rad MRC600 confocal laser scanning imaging system as previously described (Lefèvre *et al.*, 1995). Records were taken at 5 s intervals between two consecutive images.

Electron microscopic analysis

Oocytes recovered 4 h after insemination with 1 µM calmidazolium-treated spermatozoa were fixed in 0.2% glutaraldehyde, and 1% paraformaldehyde in 0.1 M cacodylate buffer, at pH 7.2, embedded in Lowicryl K4M and processed for ultrathin sections. After uranyl

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acetate staining the sections were observed under a Philips OMU3 electronic microscope. The acrosome reaction was assessed with reference to non-acrosome-reacted epididymal sperm and to ultrastructural criteria already published for mouse spermatozoa (Saling *et al.*, 1979).

Statistics

Pooling at least three experiments, percentages were compared using the χ^2 test with a 95% confidence interval.

Results

Kinetics of fertilisation events

The kinetics of fertilisation events in control oocytes (Fig. 1) showed that, in the first hour following insemination, the male chromatin appeared very swollen in the ooplasm and compared with the spermatozoa attached to the plasma membrane increased in volume 3- to 4-fold. Two hours after insemination the female gametes had resumed meiosis and extruded the second polar body. Within the third hour, male and female chromatin condensed to finally become round male or female pronuclei 4 h after insemination.

Four hours after insemination the oocytes configurations observed in the whole series of experiments were classified according to the main criteria shown in Fig. 2. Oocytes were considered as fertilised when they exhibited a polar body and two or more round pronuclei that were not abnormal in a zona-free system (Fig. 2*A*). Oocytes were considered as partially fertilised when they exhibited either a female pronucleus already formed or female chromatin at the telophase stage and swollen sperm nuclei (Fig. 2*B*, *C*). Unfertilised oocytes were characterised by the presence of the metaphase plate sometimes partially extruded in a polar body in formation (Fig. 2*D–F*). When spermatozoa were preincubated in the presence of calmidazolium most of the unfertilised oocytes exhibited numerous spermatozoa attached to the membrane (Fig. 2*E*).

Calmidazolium treatment of spermatozoa affects oocyte activation and pronucleus formation

Ethanol (0.2%) in the medium did not affect the fertilising ability of oocytes. A high fertilisation rate (80.6%) was observed 4 h after insemination of the control oocytes (Fig. 3). When the spermatozoa were capacitated in the presence of 1 µM calmidazolium the percentage of fertilised oocytes decreased dramatically (20.0%; $p < 0.05$); however, most oocytes were partially fertilised since they exhibited not only the second polar body but also male chromatin elements (64.0%) (Fig. 3). When the oocytes were incubated in medium containing 1 µM calmidazolium before insemination, the rate of fertilisation was not affected compared with that in the control group (95.2%; not significant). However, the fertilisation process was affected neither when the spermatozoa nor when the oocytes were incubated in the presence of 1 µM calmidazolium during the last 20 min preceding insemination (Fig. 4).

If the 1 µM calmidazolium treatment continued throughout the culture period following insemination (Fig. 5), more than half the inseminated oocytes were unfertilised (57.5% vs 8.3%; $p < 0.05$) and few of them were partially fertilised (6.1%). It was interesting to note that this inhibitory effect of calmidazolium took place essentially during the first 30 min following insemination (49.0% of fertilised oocytes; $p < 0.05$) since the percentage of fertilised oocytes observed when calmidazolium was added 30 min after insemination was similar to that in the control group (79.0%).

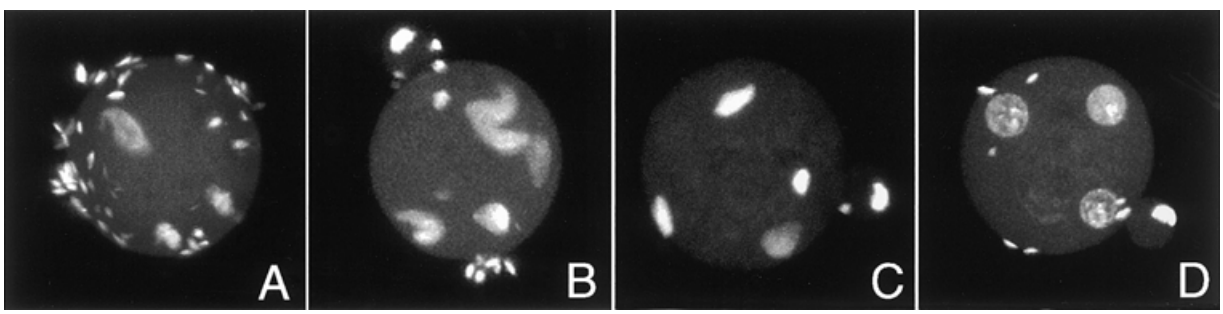


Figure 1 Kinetics of pronucleus formation in control gametes. Zona-free mouse oocytes inseminated with spermatozoa were examined every hour after insemination under a confocal microscope following Syto-15 chromatin staining (see Materials and Methods). (*A*) One hour post-insemination: the chromatin of the fertilising spermatozoa swelled in the ooplasm and, compared with the spermatozoa attached to the plasma membrane, increased in volume 3- or 4-fold. (*B*) Two hours post-insemination: oocytes resumed meiosis and extruded the second polar body. (*C*) Three hours post-insemination: condensation of male and female chromatin. (*D*) Four hours post-insemination: the male and female round pronuclei were formed.

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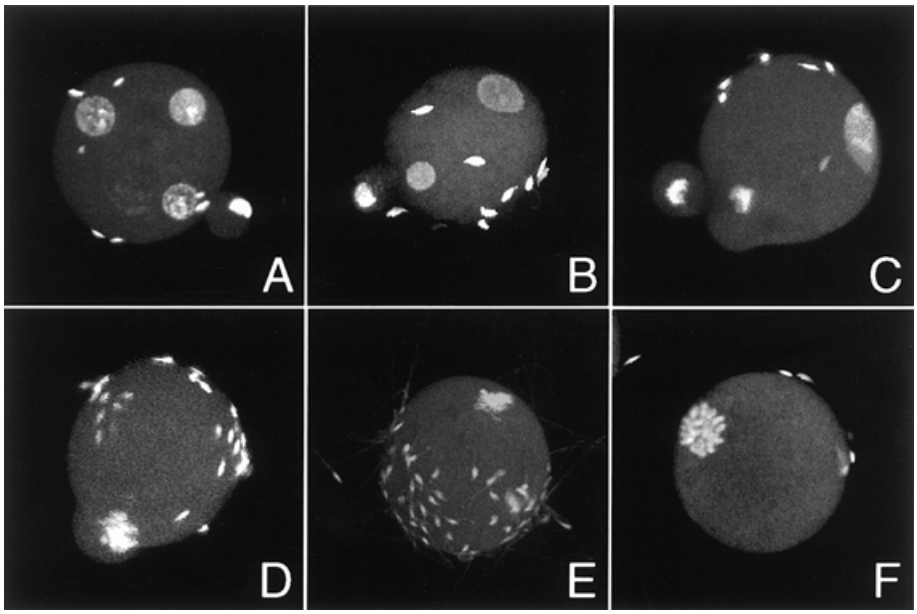


Figure 2 Classification of oocytes observed 4 h post-insemination in our experimental conditions (control and treated). All the zona-free mouse oocytes were examined following Syto-15 chromatin staining 4 h after insemination. The zona-free oocytes were classified into three groups: (*A*) Fertilised oocytes: oocytes with three pronuclei and the second polar body. (*B*), (*C*) Partly fertilised oocytes: (*B*) oocyte with a female pronucleus already formed and a swollen sperm nucleus: (*C*) oocytes exhibiting a significant delay or blockage of formation of both pronuclei so that the sperm nucleus was still at the swollen stage and the female chromatin at the telophase stage. (*D*)–(*F*) Unfertilised oocytes: (*D*) atypical activation of the oocyte; (*E*) oocyte with numerous sperm attached to its membrane, this situation often being observed when spermatozoa have been preincubated with calmidazolium; (*F*) oocyte with a classical metaphase plate.

Calmidazolium treatment of spermatozoa induces modifications in the transient calcium oscillations at fertilisation

Calcium oscillations following insemination occurred in 80.0% of the control oocytes and in 54.4% of the oocytes inseminated with 1 µM calmidazolium-treated spermatozoa. Moreover, the calmidazolium treatment induced oscillations with a larger interspike interval (Fig. 6*C*) and irregular baselines and peaks (Fig. 6*D*) than those seen in the control group (Fig. 6*A*, *B*).

Acrosome status following calmidazolium treatment

Compared with non-acrosome-reacted epididymal sperm (Fig. 7*A*), 90% of the treated spermatozoa in close contact with zona-free oocytes were reacted or had a reacting acrosome (Fig. 7*B*). Moreover, some spermatozoa were involved in the fusiogenic step of fertilisation (Fig. 7*C*).

Discussion

In this study the role of calmodulin in fertilisation events was examined in a zona-free mouse system by using a selective calmodulin inhibitor, calmidazolium, to block its action either in one or the other gamete before insemination or in both gametes during insemination and fertilisation. According to the timing and the duration of the calmidazolium treatment, different inhibitory effects were observed.

Before insemination, the fertilising ability of spermatozoa was affected by treatment when calmidazolium was applied throughout the incubation period. In these conditions, acrosome-reacted spermatozoa were able to fuse with the oocyte plasma membrane and sperm heads were able to swell in the ooplasm. However, most of the oocytes inseminated with calmidazolium-treated spermatozoa were only partially fertilised: the oocytes were indeed activated, as demonstrated by the exhibition of Ca^{2+} oscillations in more than half of them and the presence of polar bodies; however, these oscillations were slow and irregular and most of the oocytes never exhibited typical male and female pronuclei 4 h post-insemination. Therefore, the calmodulin-dependent events that take place in spermatozoa before insemination appear essential, at least for regular Ca^{2+} oscillations and for pronucleus formation. However, the inhibition of calmodulin by calmidazolium applied to metaphase II oocytes before insemination has no major impact on their fertilising ability.

Recent papers show contradictory actions of calmodulin on the fertilising ability of spermatozoa. The inhibition of calmodulin by trifluoroperazine accelerated sperm capacitation and stimulated the fertilisation *in*

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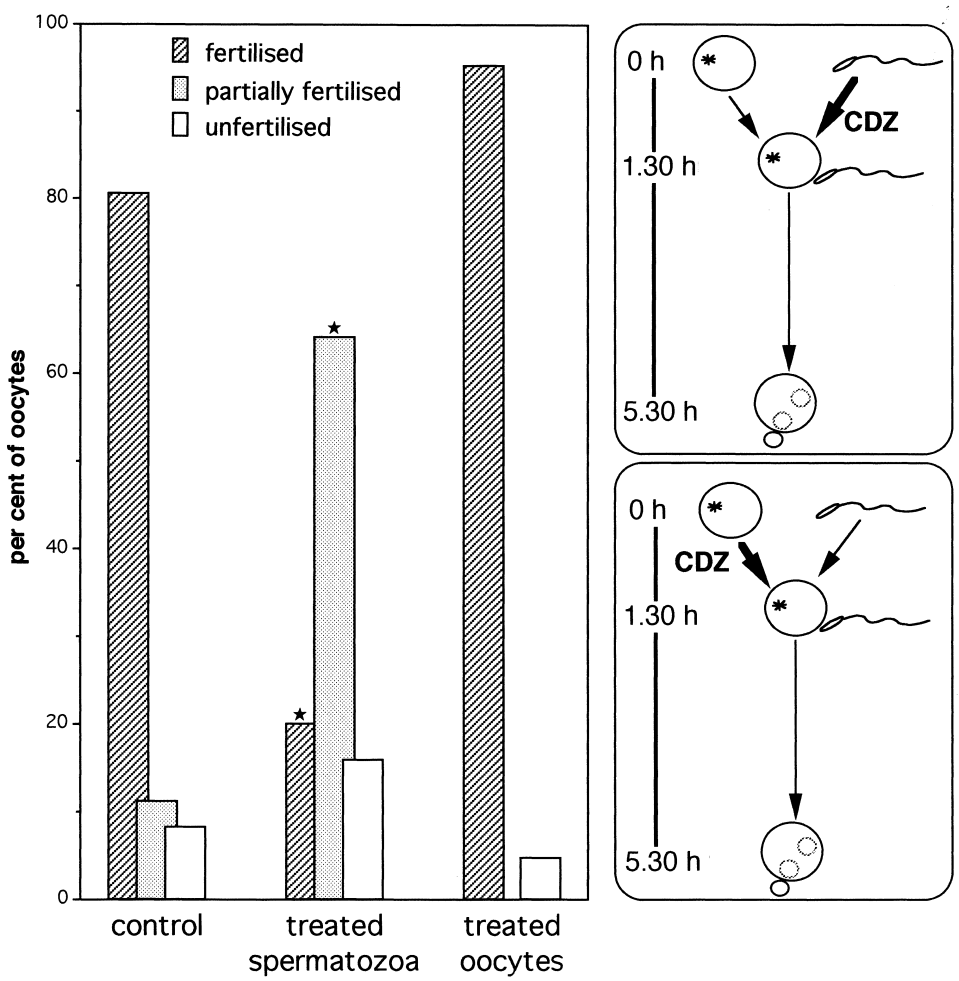


Figure 3 Effect on fertilisation of incubation (1.5 h) of either spermatozoa or oocytes with 1 μM calmidazolium before insemination. The percentages of fertilised oocytes were similar in control and calmidazolium (CDZ)-treated oocytes. When spermatozoa were incubated in the presence of calmidazolium the percentage of fertilised oocytes was significantly lower (*$P < 0.05$) than in the controls and significantly more partly fertilised oocytes (cf. Fig. 2) were observed (*$P < 0.05$). The percentages were estimated after pooling three experiments with a total number of 118 oocytes.

vitro in a mouse zona-intact system (Adeoya-Osiguwa & Fraser, 1996). These authors have demonstrated that the inhibitory effect of calmodulin was essentially due to the activation of a calmodulin-dependent Ca^{2+}-ATPase present in the post-acrosomal region. We can hardly compare these experiments to ours since we worked in a zona-free system and examined the oocytes 4 h after insemination, while Adeoya-Osiguwa & Fraser estimated fertilisation 65 min after insemination according to the presence of a second polar body and decondensed sperm heads. No one knows whether the decondensed sperm heads would have been transformed in pronuclei some hours later or would have been blocked at this stage. On the contrary, a positive role for this sperm calciprotein, in the fertilisation events occurring downstream of the acrosome reaction, has been shown in the human–hamster heterospecific system where sperm–oocyte fusion was inhibited by calmidazolium (Aitken *et al.*, 1988).

Immunohistochemical studies with mammalian spermatozoa have revealed that calmodulin is localised in the acrosomal region of the cell, then in the equatorial and post-acrosomal segments (Moore & Dedman, 1984; Jones *et al.*, 1980; Noland *et al.*, 1985; Olson *et al.*, 1985; Courtot *et al.*, 1994). The presence of these proteins in this region of the sperm head is intriguing since it is this area of the cell that initiates fusion with the oocyte. The acquisition of fusogenic properties occurs at the time of the acrosome reaction and involves the creation of particle-free zones in the overlying plasma membrane (Friend *et al.*, 1977). The influx of calcium associated with the acrosome reaction may help to create such fusogenic zones by inducing changes in the underlying cytoskeleton which, if mediated by calmodulin, would be sensitive to antagonists such as trifluoperazine or calmidazolium (Aitken *et al.*, 1988). Moreover, the fact that calmodulin of the post-acrosomal region of the human sperm disappeared abruptly at

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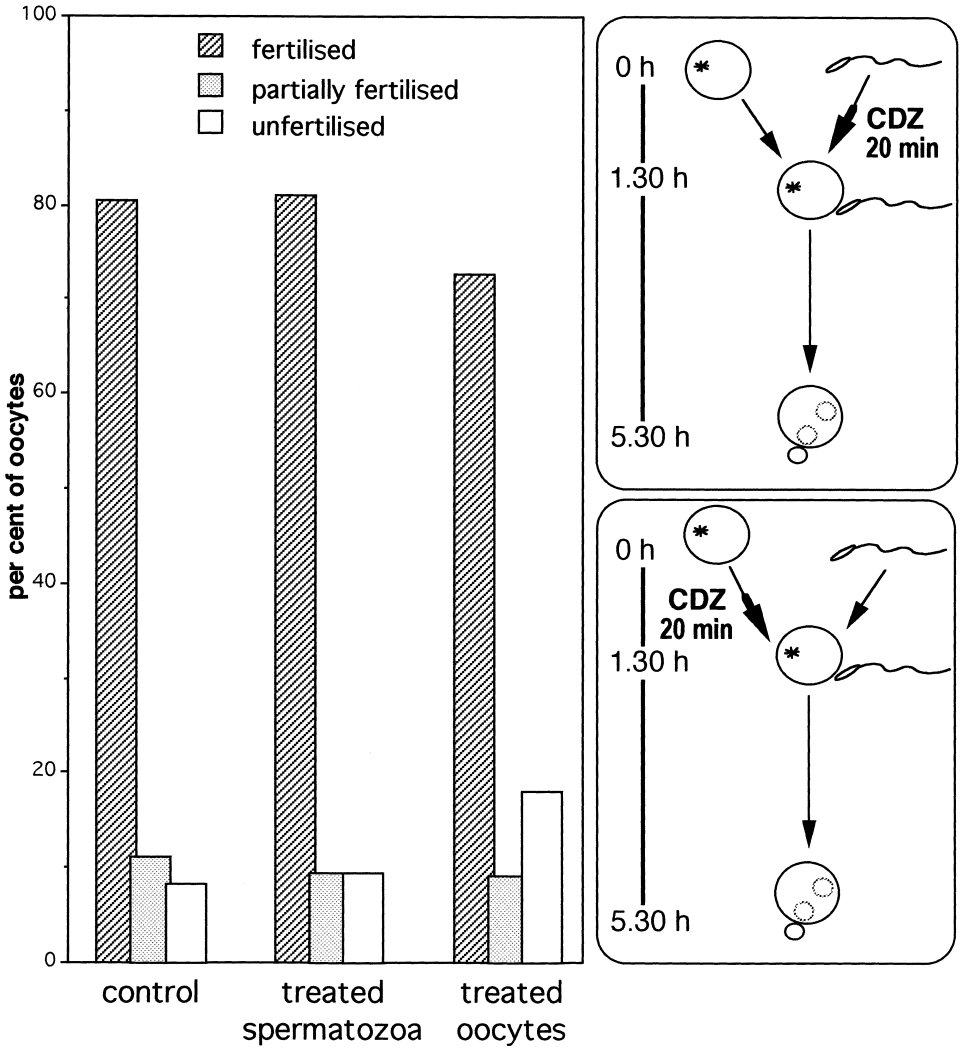


Figure 4 Effect on fertilisation of incubation (20 min) of either spermatozoa or oocytes with 1 μM calmidazolium (CDZ) before insemination. In these conditions no differences in the percentages of fertilised oocytes were observed (three experiments, 126 oocytes).

gamete fusion (Courtot *et al.*, 1994) leads to the hypothesis of calmodulin being one of the biochemical elements brought by the sperm into the oocyte at fertilisation. Thus, its inhibition before insemination may impair one of these important transformations of the sperm taking place downstream of the acrosome reaction, leading to a further incomplete fertilisation.

When calmidazolium was added to the insemination medium during the whole experiment or during the first 30 min of contact between the two gametes, we observed a deleterious effect on the subsequent events: more than half the oocytes were unfertilised and only a few of them were partially fertilised. However, when the gametes were treated during the 20 min preceding insemination or the 30 min after insemination, no deleterious effect was observed. These results demonstrate that, at the time of gamete fusion, calmodulin, either from the oocyte or from the spermatozoon, is essential for fertilisation to occur. It is interesting to note that the inhibition of sperm calmodulin before insemination does not affect the fertilisation process in the same way as when calmodulin was inhibited at the time of insemination. Although in the first case we observed only a blockage of the events after egg activation and sperm head swelling, in the second case fertilisation was totally inhibited.

Some questions remain to be answered: At which essential steps is calmodulin acting and which gamete generates this active calmodulin? It is known that in most of the animal species studied spermatozoa activate oocytes by inducing transient Ca^{2+} oscillations (Swann & Ozil, 1994). Two hypotheses have been elaborated to explain how a spermatozoon could induce such an event in the oocyte. The first one is that, in fusing with the oocyte membrane, the spermatazoon induces the synthesis of inositol triphosphate ($InsP_3$)

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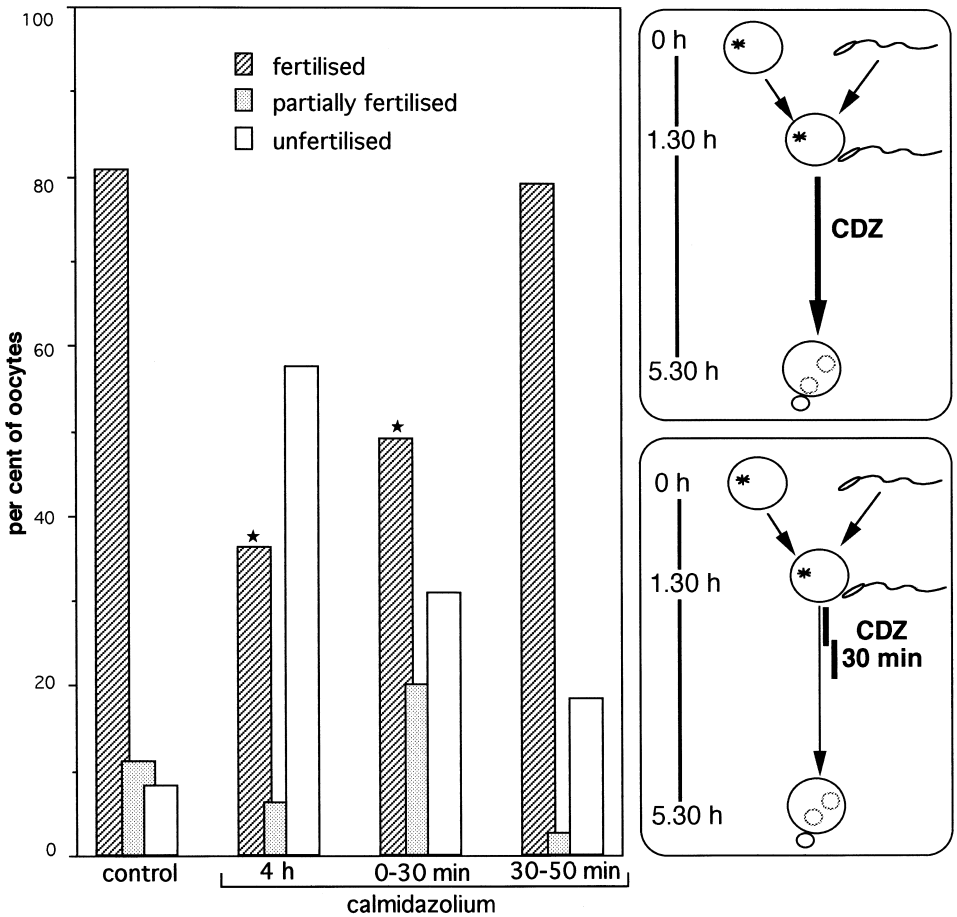


Figure 5 Effect on fertilisation of the addition of 1 μM calmidazolium (CDZ; 4 h or 20 min) during co-incubation of the gametes. When calmidazolium was applied during the 4 h following insemination the percentage of fertilised oocytes was significantly affected (*$P < 0.05$). When calmidazolium was added for 20 min only, its effect depended on the time elapsed from the insemination time: when calmidazolium was added during the first 30 min after insemination a deleterious effect was observed on the percentages of fertilised oocytes (*$P < 0.05$). After that period and until 50 min post-insemination, the presence of calmidazolium did not impede fertilisation (three experiments; 200 oocytes).

that in turn induces a calcium release participating in the generation of fertilisation-associated $[Ca^{2+}]_i$ increases. This hypothesis has been demonstrated in several mammalian oocytes (Miyazaki *et al.*, 1992; Peres, 1990; Fukiwara *et al.*, 1993; Fissore & Robl, 1994; Mehlmann & Kline, 1994). No effect of calmodulin on $InsP_3$-sensitive stores has been reported (Lee *et al.*, 1994). However, a calmodulin binding domain in the mouse $InsP_3$ receptor type 1 has been determined (Yamada *et al.*, 1995). Thus, this point remains to be further investigated. The second hypothesis is the existence of a sperm factor responsible for calcium oscillations at fertilisation, as first described by Swann (1990). This sperm factor, named oscillin, has recently been isolated and purified (Parrington *et al.*, 1996; Swann & Lai, 1997). However, questions remain concerning its means of action and no one can currently assert that it is acting alone. Our results show that sperm calmodulin is also implicated in the generation of the events leading to oocyte activation, in particular in the generation of Ca^{2+} oscillations. Although these oscillations were not inhibited by calmidazolium, they were slowed down and distorted, leading to the conclusion that calmodulin could be necessary for oscillin to act. The Ca^{2+} transients induced by oscillin could activate a calmodulin-dependent kinase whose inhibition by the absence of calmodulin could be deleterious to the fertilisation process. Indeed, this hypothesis is relevant since calmodulin is known to be important during activation (Lorca *et al.*, 1993; Xu *et al.*, 1996). Its action during the exit of the oocyte from metaphase II is known to be mediated mainly by the calmodulin-dependent kinase II in *Xenopus* (Lorca *et al.*, 1993) and mouse (Inagaki *et al.*, 1997).

In conclusion, by using calmidazolium as a calmodulin inhibitor we have demonstrated that the calmodulin-dependent events taking place in the sperm before insemination and in the egg at the time of fusion are

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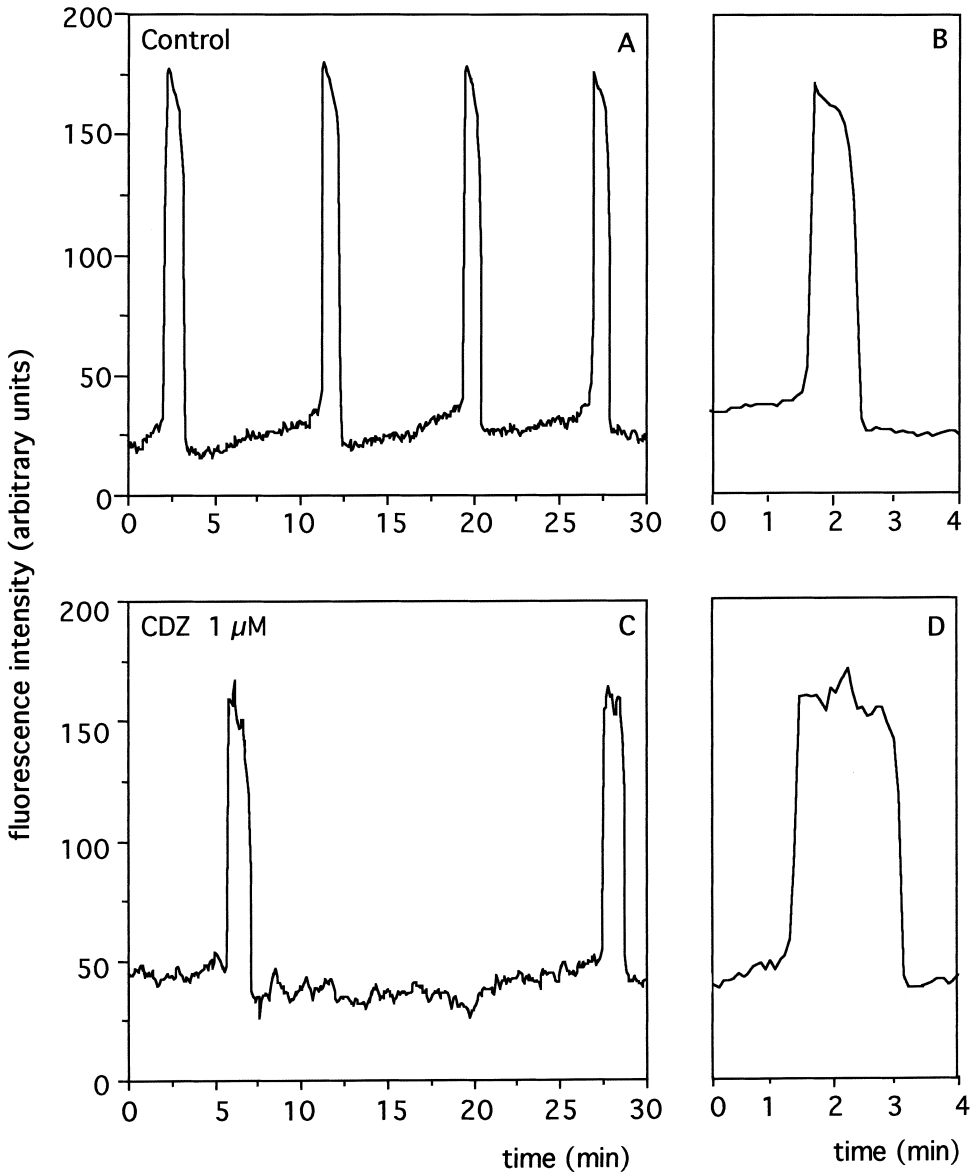


Figure 6 Effect on ooplasmic calcium signals of insemination with calmidazolium-preincubated spermatozoa. The oocytes were loaded with the calcium indicator, fluo-3/AM, for 10 min before insemination. Fluorescence measurements were recorded with a Bio-Rad MRC600 confocal inverted microscope as soon as the spermatozoa were added to the oocytes. The left-hand panel shows the noticeable differences in the frequencies of the calcium oscillations recorded for 30 min according to the sperm treatment. The right-hand panel shows the alterations of the peak profile. (*A*), (*B*) Control; (*C*), (*D*) calmidazolium (CDZ)-treated group.

essential for fertilisation. Calmodulin appears to be one of the biochemical elements necessary for the cascade of events leading to fertilisation. Further investigations are necessary to determine the precise role of calmodulin in this cascade.

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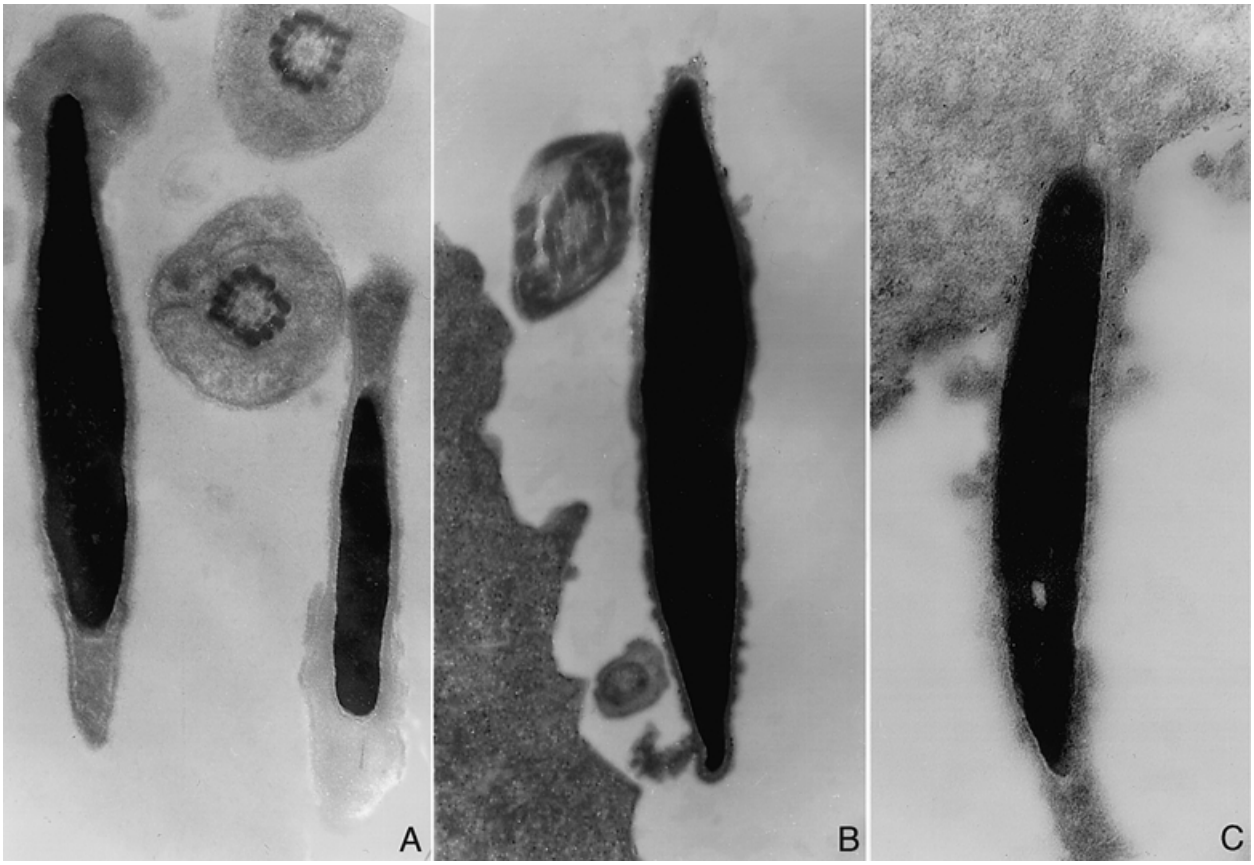


Figure 7. Effects of calmidazolium treatment on mouse spermatozoa at the ultrastructural level. (*A*) Control epididymal mouse spermatozoa with their acrosome. (*B*), (*C*) Incubation of spermatozoa with 1 μM calmidazolium before insemination: (*B*) the spermatozoa observed near the oocyte were acrosome-reacted and (*C*) some of them were fusing with the oocyte membrane.

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