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Original Article

Release from the metaphase I block in invertebrate oocytes: possible involvement of Ca^{2+}/calmodulin-dependent kinase III

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ABSTRACT Full grown mature oocytes of the prosobranch gastropod mollusc *Patella* or the bivalves *Mytilus* or *Ruditapes* provide an excellent model for studying the mechanisms which trigger cyclin degradation and exit from the M phase. They are naturally arrested in metaphase of the first maturation division and their fertilization or artificial activation rapidly results in destruction of the cyclins and completion of meiosis. In this paper, we establish the presence of Ca^{2+}/calmodulin-dependent kinase III or eEF-2 kinase in these oocytes and describe how the protein synthesis inhibitor emetine is able to release them from the metaphase block. Using the fluorescent Ca^{2+} indicator dye, fluo-3, we demonstrate moreover that both fertilization or KCl-dependent activation of *Ruditapes* and *Mytilus* oocytes actually trigger a measurable transient increase in cytosolic free Ca^{2+} concentration. We also show that the activations triggered by these signals as well as by the ionophore A 23187 can be reversibly blocked by the calmodulin antagonists TFP (30 μM) and W7 (100 μM), while these drugs have no effect upon emetine-dependent activations. Finally, we report that the rate of protein synthesis, measured in pulse experiments, decreases at each meiotic and mitotic cleavage following fertilization of metaphase I-arrested oocytes of *Mytilus*. On the basis of these experiments and as a working hypothesis, we thus propose that the Ca^{2+} surge which activates the oocyte may inhibit protein synthesis by triggering a transient phosphorylation of eEF-2. This would result in disappearance of the putative short-lived proteins which protect cyclins from degradation during the metaphase block.

KEY WORDS: *eEF-2 kinase, maturation promoting factor, metaphase block, oocyte activation, protein synthesis inhibition*

Introduction

Eukaryotic elongation factor-2 (eEF-2) is a 100 kDa protein which catalyzes the translocation of peptidyl t-RNA along the ribosome. In mammalian cells, it has been shown that eEF-2 is the target for a very specific Ca^{2+}/calmodulin-dependent eEF-2 kinase (Nairn *et al.*, 1985) and that phosphorylation of this factor results in a decrease in the rate of protein synthesis (Ryazanov and Spirin, 1990 for review). It has been already proposed by these authors that transient changes in intracellular Ca^{2+} concentration may thus inactivate short-lived molecules, by which new genes would be turned on. Here, we want to show that the same potential Ca^{2+}/calmodulin-dependent inhibition of translation may indirectly reduce the availability of important cell cycle control proteins such as the cyclins, which are the main regulatory subunit components of the M-phase promoting factor or MPF (Guerrier *et al.*, 1990a). Indeed, it has been recently shown, both *in vivo*, using pulse-chase experiments and *in vitro*, using acellular metaphase extracts, that cyclins are stable during the metaphase I stage which precedes fertilization in a number of marine invertebrate oocytes (Colas *et al.*, 1992). We also found that maintenance of that metaphase block required a continuous protein synthesis (Dubé and Dufresne, 1990; Guerrier *et al.*, 1990b; van Loon *et al.*, 1991). This clearly demonstrated that the stabilization of the MPF activity that we observed during metaphase I (Néant and Guerrier, 1988) actually depended on the

Abbreviations used in this paper: AM, acetoxymethyl ester; ASW, artificial seawater; ATP, adenosine 5'-triphosphate; CaFSW, calcium-free artificial sea water; CSF, cytostatic factor; D-600, emthoxyverapamil,; DMSO, dimethylsulfoxide; eEF-2, eukaryotic elongation factor; EGTA, [ethylenebis-(oxyethylenenitrilono)] tetraacetic acid; Fluo-3/AM, fluo-3 pentaacetomethyl ester; GA, glucamine acetate buffer; GVBD, germinal vesicle breakdown; MPF, M-phase promoting factor; SDS, sodium dodecyl sulfate; TFP, trifluoperazine; W7, N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide.

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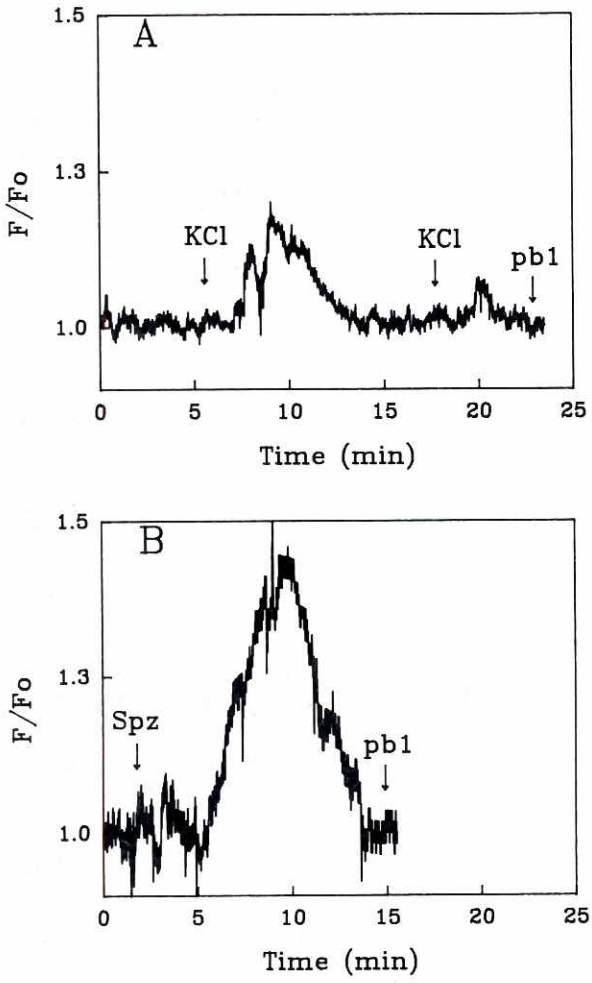

Fig. 1. Transient intracellular Ca^{2+} surges observed after KCl stimulation (A) or fertilization (B) of individual metaphase I-arrested oocytes of *Ruditapes philippinarum*. **(A)** *Two successive additions of 200 µl of an isotonic solution of 0.53 M KCl, which depolarize the oocyte plasma membrane, were performed in a total volume of 2 ml. The first addition was sufficient to trigger polar body emission (pb1) as also observed in the overall population of tested oocytes from the same batch. It produced a significant Ca^{2+} transient, higher than that triggered by the second addition.* **(B)** *A larger transient increase in fluorescence follows the addition of spermatozoa (spz). In both cases, first polar body (pb1) is emitted within 15 min.*

synthesis of short-lived proteins which did inhibit activation of the cyclin degradation pathway.

In this paper, we establish that eEF-2 kinase is present in oocytes of a number of marine invertebrates. We also extend our original observation that the protein synthesis inhibitor emetine, which induces a rapid disappearance of the cyclins (Dubé and Dufresne, 1990; Colas *et al.*, 1992), releases a number of invertebrate oocytes from their block in metaphase I. This inhibitor thus mimics various activating treatments which transiently increase cytosolic Ca^{2+} concentration, such as fertilization, KCl or ionophore A 23187 stimulations. Moreover, we show that sperm, KCl- or ionophore-dependent activations can be reversibly blocked by calmodulin antagonists such as trifluoperazine (TFP) or W7, whereas the same drugs do not affect emetine-dependent activations. Finally, we report that the rate of protein synthesis is also affected following fertilization of such metaphase-arrested oocytes.

Taken together, these data strongly suggest that the Ca^{2+} surge which releases invertebrate oocytes from their metaphase block may act by triggering a transient phosphorylation of eEF-2, which would stop protein synthesis and thus produce a rapid inactivation of MPF, via disappearance of its cyclin components.

Results

Evidence for the existence of an intracellular Ca^{2+} surge following fertilization or activation of metaphase I-arrested oocytes

Metaphase I-arrested oocytes of various invertebrates are usually activated following fertilization or the application of diverse treatments known to produce a transient increase in cytosolic Ca^{2+} concentration. This is the case when oocytes are incubated in the presence of the Ca^{2+} ionophore A 23187 or after increasing external potassium concentration, a treatment which is known to depolarize the plasma membrane and to trigger a measurable $^{45}Ca^{2+}$ uptake, as observed in the bivalves *Barnea* (Dubé and Guerrier, 1982) and *Spisula* (Dubé *et al.*, 1987; Dubé, 1988). The neurohormone serotonin or 5-hydroxytryptamine (5-HT), which also triggers meiosis reinitiation in *Spisula* oocytes (Hirai *et al.*, 1988) was found to present the same dependency towards external Ca^{2+} as observed for KCl and to produce a similar stimulation of the $^{45}Ca^{2+}$ uptake (Krantic *et al.*, 1991). It is worth noting that, in these two species, i.e., *Barnea candida* and *Spisula solidissima*, fertilization occurs at the germinal vesicle stage and is directly responsible for reinitiation of

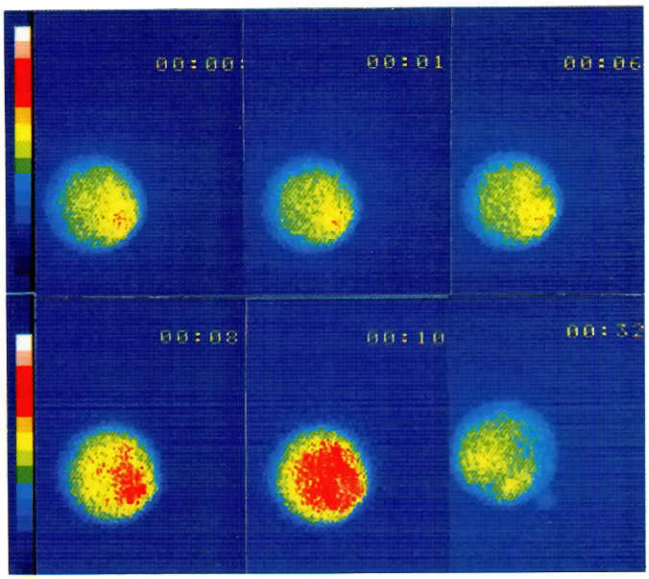

Fig. 2. Pseudocolor scale imaging showing the relative changes in Ca^{2+} concentration during fertilization of a metaphase I-arrested oocyte of *Mytilus galloprovincialis*. *The cloud of swimming spermatozoa approached the oocyte at 00:06 minutes. Similar records were obtained following KCl activation.*

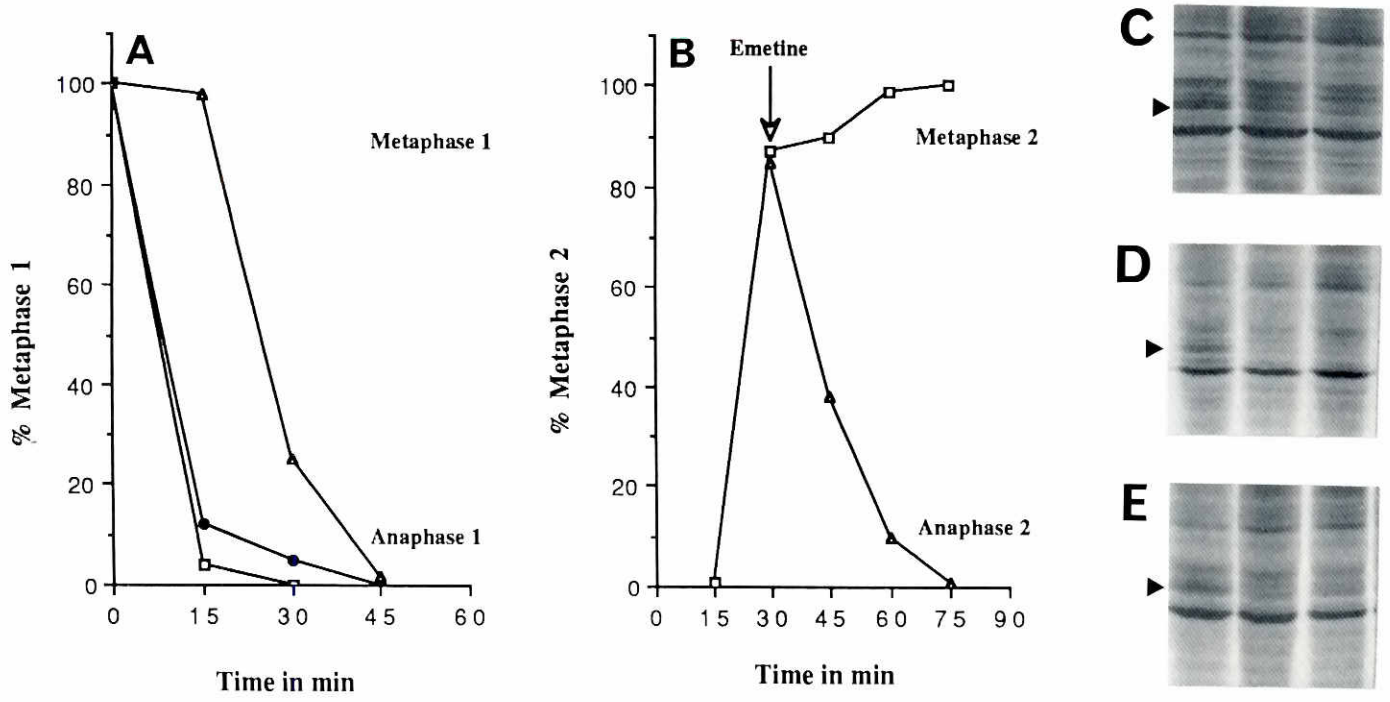

Fig. 3. Kinetics of the transition from metaphase I to anaphase I (A) and from metaphase II to anaphase II (B) in oocytes of *Mytilus edulis*. *Oocytes were treated with 10 μM ionophore A 23187 (□), 50 mM excess KCl (●) or 1 mg emetine (Δ). Companion photographs illustrate moreover the disappearance of a cyclin-like band of about 50 kDa following fertilization* **(C)** *or activation by KCl* **(D)** *or ionophore A 23187* **(E)**. *Samples were prepared at 0, 7.5 and 15 min after stimulation.*

the meiotic divisions. This is not the case for two other bivalves, *Mytilus* and *Ruditapes*, which must first reach metaphase of the first maturation division before they can be fertilized (Osanai and Kuraishi, 1988; Dubé and Dufresne, 1990). In these species, no objective data existed so far demonstrating the potential involvement of Ca^{2+} in triggering the first step in their activation, i.e., the metaphase-anaphase transition.

Here, we report that fertilization, ionophore A 23187 (EC_{50}=1.5 μM) and excess KCl (+50 mM to +100 mM) actually trigger first polar body extrusion in both species, provided that Ca^{2+} is present in the external medium. This biological response is preceded by a measurable transient intracellular Ca^{2+} surge, as monitored using Fluo-3 as a probe (Figs. 1A,B and 2). In the absence of external Ca^{2+} (CaFSW+2mM EGTA), KCl produces no Ca^{2+} transient and does not trigger activation.

Evidence that inhibition of protein synthesis mimics calcium signals in releasing invertebrate oocytes from the metaphase I block

Fig. 3 illustrates kinetics of the effects of KCl and the Ca^{2+} ionophore A 23187 in triggering metaphase-anaphase transition, when added to an oocyte suspension of metaphase-arrested oocytes of *Mytilus edulis*. In this species, as in *Mytilus galloprovincialis* and *Ruditapes philippinarum*, both treatments trigger extrusion of the first polar body within 10-20 min (Fig. 3A), as also observed after fertilization (Figs. 1 and 11). This evolution, which included disappearance of the cyclin B component of MPF (Fig. 3C,D,E), could be reproduced following a treatment of the oocyte with the protein synthesis inhibitor emetine. This treatment not only triggers cyclin disappearance (Dubé and Dufresne, 1990), but also mimics fertilization in inactivating histone H1 kinase (Fig. 4). Under these conditions, the oocytes of *Ruditapes*, like those of *Mytilus* (Guerrier *et al.*, 1990b) remain blocked in interphase, exhibiting a large diploid nucleus (Fig. 4). No second meiotic division can occur since emetine actually blocks de novo cyclin synthesis, which obviously precludes MPF reactivation.

On the contrary, excess KCl was found to be capable of driving the oocytes across the first and second meiotic cleavages (data not shown), while ionophore stopped them in metaphase II (Fig. 3B). However, when added to such ionophore-activated, metaphase II-arrested oocytes, emetine could also trigger the second metaphase-anaphase transition.

Fig. 5 illustrates the cytological effect of emetine on two other invertebrate oocytes arrested in first metaphase, i.e., the oocytes of the ascidian *Phallusia mammilata* and of the prosobranch gastropod *Patella vulgata*. In both cases, after emission of the first polar body, internal chromosomes decondense and reconstitute an interphase nucleus.

Evidence for the existence of eEF-2 kinase in invertebrate oocytes

Although eEF-2 kinase had been shown to be present in *Xenopus* in a form similar to that found in mammalian cells (Severinov *et al.*, 1990), no information existed so far concerning the presence of this enzyme in invertebrates. Here, we show that incubating different oocyte extracts with γ ^{32}P ATP reveals a high molecular weight protein of about 100 kDa as one of the major proteins which can be phosphorylated *in vitro*, no matter whether oocytes are arrested in prophase or in metaphase (Fig. 6). This protein has been unequivo-

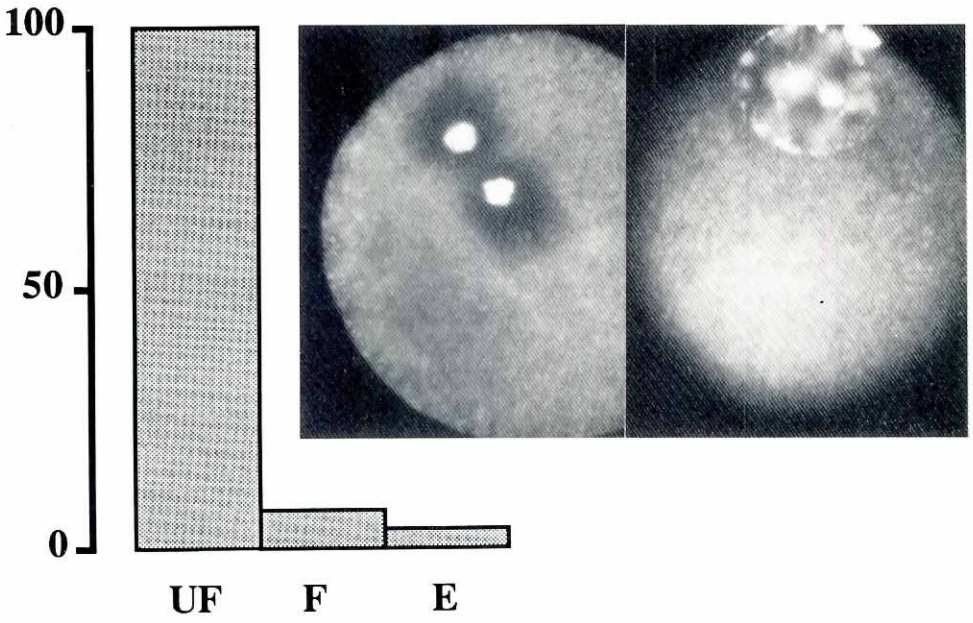

Fig. 4. Biochemical and cytological effect of emetine. *The blocks refer to percent relative values of histone H1 kinase activity measured before (UF) and after activation of metaphase I-arrested unfertilized oocytes of Mytilus by sperm (F) or 100 µg/ml emetine (E). Samples were taken at 0 min (UF) or 15 min (F) and 30 min (E) after activation. The two adjacent photographs present oocytes of Ruditapes philippinarum vitally stained with Hoechst 33342, 20 min (anaphase) and 70 min after treatment with 100 mg/ml emetine. At 70 min a bright first polar body and a very big diploid nucleus is observed, which results from decondensation of the potential internal second metaphase chromosomes.*

cally identified as eEF-2 by comigration and cophosphorylation with purified eEF-2 (Fig. 6) and by Western blot analysis (Fig. 7). Fig. 8 illustrates moreover the Ca^{2+} dependency of eEF-2 phosphorylation, as observed in extracts prepared from oocytes of the mollusc *Patella vulgata* and the starfish *Asterias rubens:* eEF-2 is the only protein to disappear from the picture when Ca^{2+} is omitted from the reaction mixture. We verified moreover that addition of 100 µM TFP to the assay did also preclude eEF-2 phosphorylation.

Evidence for the involvement of a Ca^{2+}/calmodulin-dependent step in releasing invertebrate oocytes from the metaphase block

In *Mytilus* and *Ruditapes*, the anticalmodulin drugs TFP (30 µM) and W7 (100 µM) were found to block both KCl- and ionophore-induced activations but not emetine-dependent activations (Fig. 9A-F). These inhibitions could be released by washing the oocytes in FSW after 30 min incubation, returning them to the presence of the effective signals. In *Ruditapes*, we also observed that the same drugs did not inhibit the effect of the natural hormone serotonin, which releases oocytes from the prophase block and drives them to the metaphase I stage within 30 min (Fig. 10A,B,C). We also checked the effect of these drugs upon physiological activation. Since TFP and W7 were found to preclude sperm penetration, they were added to the oocyte suspensions some 5 to 10 min after insemination, when sperm heads had already entered the oocyte. Under these conditions, these drugs again proved able to inhibit release of the oocytes from the metaphase block. This effect is quite striking, since first polar body is normally extruded very soon in control fertilized eggs, e.g., within 15 min after insemination (Fig. 10D,E,F). Oocytes incubated for 30 min with the inhibitors could also be fertilized after intensive washing.

Evidence that fertilization induces a transient decrease in the rate of protein synthesis

Results of a typical experiment in which 14 min pulses of ^{3}H-leucine were given to aliquot suspensions of metaphase-arrested oocytes or fertilized eggs of *Mytilus galloprovincialis* are presented in Fig. 11. Three important features emerge from that picture. First, the uptake of the aminoacid, which remains rather low during the metaphase block, increases dramatically and continuously after fertilization (Fig. 11A). Second, the rate of protein synthesis, as expressed in percent incorporation of total uptake, slightly decreases just after fertilization to burst dramatically after second polar body extrusion (Fig. 11B). Third, this rate decreases again during first and second cleavage, beginning after the metaphase stage has been reached.

Discussion

Generally, meiosis reinitiation appears as a two-step process since release from the prophase block, which leads to germinal vesicle breakdown (GVBD), is followed by a second block which takes place in metaphase. This is the case for the molluscs *Patella* (Guerrier *et al.*, 1986), *Mytilus* (Dufresne-Dubé *et al.*, 1983), *Ruditapes* (Osanai and Kuraishi, 1988), the polychaete annelid *Arenicola* (Meijer, 1980) and the ascidians. This constitutes a very useful situation since such oocytes stay for an unusually long time in M-phase before they become fertilized or activated. This would afford a better understanding of the mechanisms leading to the opposite processes of stabilization and exit from the M-phase.

Protein synthesis and the metaphase block

Release from the metaphase block normally depends upon fertilization and results in MPF inactivation. Thus, in *Xenopus*, cytoplasmic transfers performed some eight min only after fertilization had already failed to reinitiate meiosis in recipient GV-arrested oocytes (Gerhart *et al.*, 1984). The same MPF inactivation, which leads to chromosome decondensation and nuclear reformation, has been found to occur when metaphase acellular extracts were treated with Ca^{2+} (Lohka and Masui, 1984; Newport and Kirschner, 1984; Lohka and Maller, 1985; Shibuya and Masui, 1988; Lorca *et al.*, 1991).

Oocyte activation could also result from an inhibition of protein synthesis which would first affect short-lived proteins. This has been shown to occur in mouse oocytes which are arrested in second

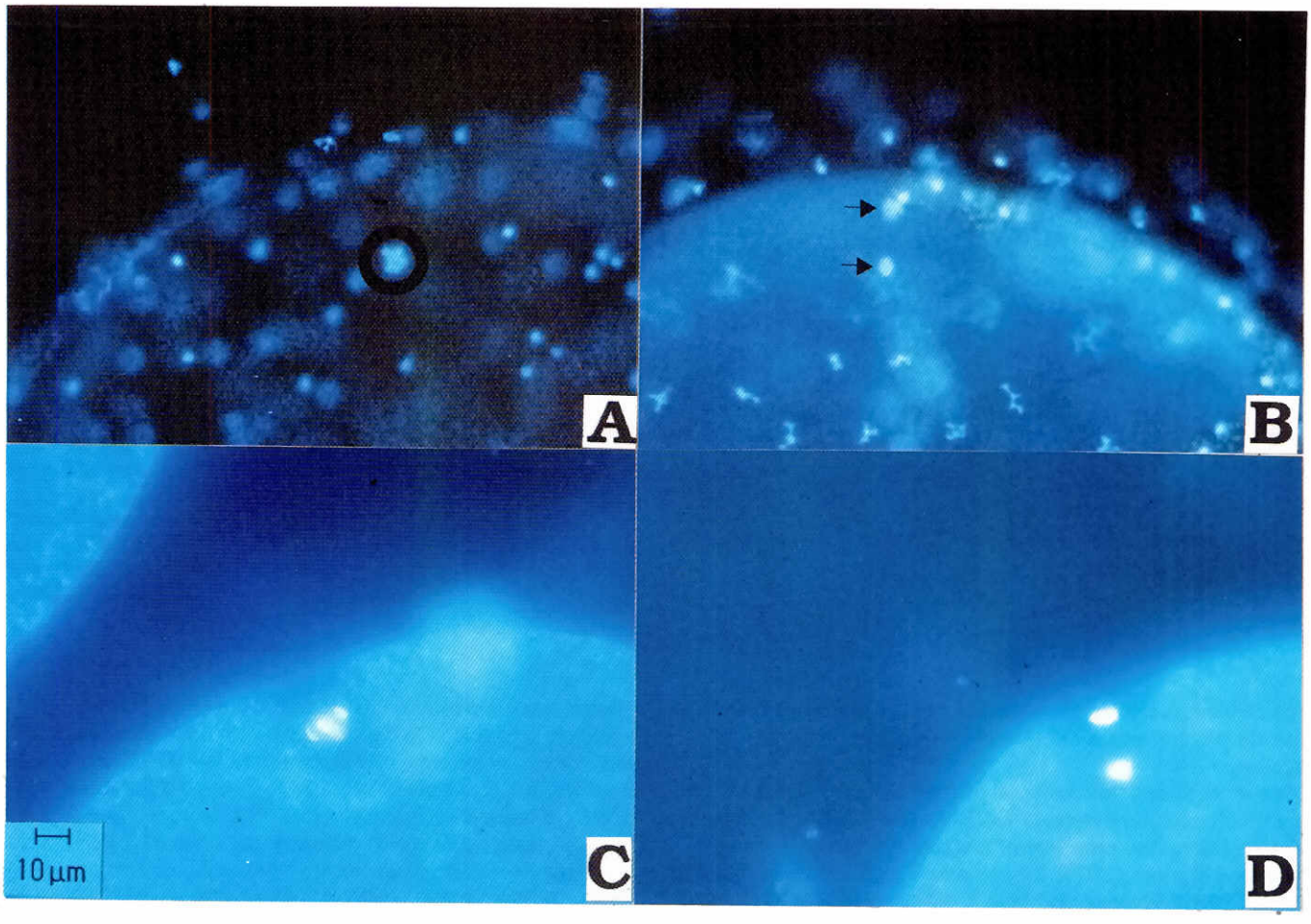

Fig. 5. Effects of the addition of 100 µg/ml emetine to metaphase-arrested oocytes of *Phallusia mammilata* (A-B) and *Patella vulgata* (C-D). *In A and C, the metaphasic chromosome plate is visible. In B and D, the two sets of chromosomes have reached the poles of the first meiotic spindle (pictures taken 20-25 min after emetine addition).*

metaphase (Clarke and Masui, 1983) and in oocytes of the annelid *Chaetopterus* (Zampetti-Bosseler *et al.*, 1973), the molluscs *Patella* (Néant and Guerrier, 1988) and *Mytilus* (Dubé and Dufresne, 1990), which are blocked in metaphase 1. In this paper, we also report that emetine triggers the same metaphase-anaphase transition in oocytes of the mollusc *Ruditapes philippinarum* and the ascidian *Phallusia mammilata*. This effect of protein synthesis inhibitors strongly suggested that maintenance of the metaphase condition required a continuous supply of newly synthesized short-lived proteins which might be either the cyclins themselves or other regulatory proteins which would block the cyclin degradation pathway. In the first case, one must assume that emetine might have disrupted an equilibrium existing between the processes of cyclin synthesis and degradation which are both working simultaneously. In the second case, cyclins would be stable in metaphase, while conditions for their degradation will only appear following activation or emetine treatment.

Pulse-chase experiments, performed on metaphase-arrested oocytes of *Patella vulgata*, coupled to *in vitro* incubation of labeled cyclins A and B in acellular metaphase extracts, favoured this second possibility (Colas *et al.*, 1992). These experiments demonstrated indeed that both cyclin A and cyclin B were stable in metaphase, while they disappeared following emetine addition. In this species, we had previously shown that emetine effect could be reproduced *in vivo* following the intracellular microinjection of antisense oligonucleotides directed against both cyclins A and B (Van Loon *et al.*, 1991). The specificity of this effect was attested by the facts that these oligonucleotides only suppressed the synthesis of their respective cyclins in a rabbit reticulocyte lysate system containing RNAase H and that the injection of only one of them did not release oocytes from the metaphase block. This last observation indicated that cyclins A or B were equivalent in their capacity to maintain the metaphase arrest. We also reported that cyclins, which were coupled to $p34^{cdc2}$ and could be precipitated with $p13^{suc1}$ beads, disappeared quite rapidly following treatment of the metaphase I-arrested oocytes with emetine.

In *Mytilus*, it has been also reported that both fertilization and emetine treatment triggered the selective and rapid disappearance of a 50 Kd protein, which periodically appeared and disappeared during the meiotic and mitotic cell cycles (Dubé and Dufresne, 1990).

Here, we extended these observations, showing first that histone H1 kinase was simultaneously inactivated after fertilization and emetine treatment and, secondly, that ionophore A 23187 and KCl,

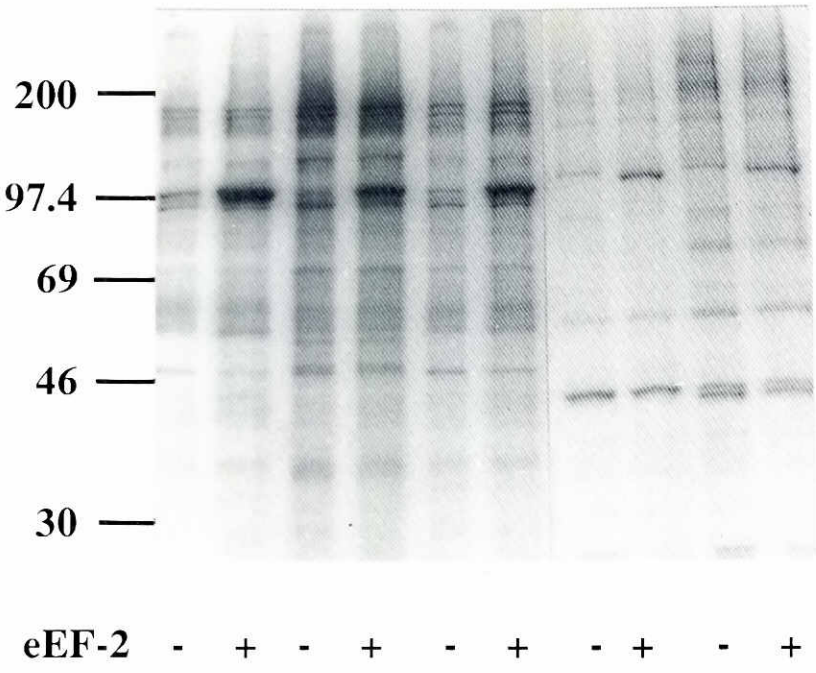

Fig. 6. *In vitro* phosphorylation of eEF-2 as observed in the 26000 g supernatant fractions prepared from oocytes of *Mytilus galloprovincialis* (1), *Spisula ovalis* (2), *Ruditapes philippinarum* (3), *Perinereis cultrifera* (4) and *Sabellaria alveolata* (5). *The signs + and - indicate the presence or absence of exogenous purified eEF-2 from rabbit reticulocytes. No exogenous calmodulin needed to be added to the incubation medium. All extracts were prepared from unfertilized oocytes, either arrested in metaphase I (Mytilus) or in prophase (all others). The mobility (molecular weight) of eEF2 seems to be slightly different in mollusc and annelid oocytes (two different gels).*

which mimic fertilization in mobilizing Ca^{2+}, also led to the disappearance of the same 50 Kd protein before first polar body extrusion.

One must stress that *Xenopus* oocytes behave quite differently since their second metaphase block does not depend on protein synthesis but on the presence of a stable cytostatic factor or CSF (Masui and Markert, 1971; Ziegler and Masui, 1976; Masui and Shibuya, 1987; Shibuya and Masui, 1989; Masui, 1991), which has been tentatively identified as the product of the *mos* protooncogene (Sagata *et al.*, 1989).

At present, we know nothing about the exact nature of those short-lived proteins which maintain cyclin stability in invertebrate metaphase-arrested oocytes and must be inactivated following the Ca^{2+} transients that trigger activation.

Intracellular Ca^{2+} surges and possible downstream involvement of a Ca^{2+}/calmodulin-dependent kinase

Fertilization is the only event which has been unequivocally shown to trigger a transient and dramatic rise in the intracellular free Ca^{2+} concentration. Using Ca^{2+} sensitive microelectrodes or the photoprotein aequorin, this Ca^{2+} surge has been demonstrated to occur following fertilization of the sea urchin (Steinhardt *et al.*, 1977), the fish medaka (Gilkey *et al.*, 1978), the amphibian *Xenopus* (Busa and Nuccitelli, 1985), the hamster (Igusa and Miyazaki, 1986; Miyazaki *et al.*, 1986; Miyazaki, 1991) and the mouse (Cuthbertson *et al.*, 1981; Cuthbertson and Cobbold, 1985; Kline and Kline, 1992). Indirect observations also argue for a positive role of Ca^{2+} in releasing oocytes from the metaphase block. Thus, artificial activation of metaphase 1-arrested oocytes of *Sabellaria* (Peaucellier, 1977, 1978) or *Arenicola* (Meijer, 1980) was obtained under the influence of various agents supposed to result in the release of intracellular Ca^{2+}. Oocytes of the molluscs *Patella* (Guerrier *et al.*, 1986) and *Mytilus* (Dufresne-Dubé *et al.*, 1983) were also activated upon treatment with the ionophore A 23187 or following a KCl-induced depolarization. Such a depolarization, which is thought to open voltage-dependent Ca^{2+} sensitive channels, has already been shown to trigger a $^{45}Ca^{2+}$ influx similar to those obtained following fertilization or serotonin stimulation of the bivalve oocyte (Dubé and Guerrier, 1982; Dubé *et al.*, 1987; Dubé, 1988; Krantic *et al.*, 1991). It appeared moreover that KCl- or ionophore-dependent activations did not occur in the absence of extracellular Ca^{2+} or when Ca^{2+} influx was cancelled using Ca^{2+} channel blockers such as Mn^{2+} ions or D-600 (Schuetz, 1975; Dubé and Guerrier, 1982; Guerrier *et al.*, 1981).

In this paper, we directly demonstrate, using Fluo 3 as a Ca^{2+} sensitive probe, that both fertilization and KCl actually induce a transient intracellular Ca^{2+} surge both in *Ruditapes* and *Mytilus* (Figs. 1 and 2). In these species, we also show that calmodulin antagonists such as trifluoperazin and W7 reversibly inhibit ionophore- or KCl-dependent activations. We have no tools to check if these drugs

Fig. 7 Characterization of eEF-2 by western blot using a purified anti-eEF-2 antibody. (Lane 1) *Mytilus oocytes in metaphase I;* **(lanes 2 and 3)** *Patella oocytes in metaphase and in prophase;* **(lane 4)** *Asterias rubens oocytes in prophase;* **(lanes 5 and 6)** *Spisula oocytes in metaphase and in prophase;* **(lanes 7 and 8)** *Ruditapes oocytes in metaphase and in prophase. Specificity of the antibody leaves no doubt since only one protein of 98 kDa was recognized over the entire range of molecular weight, which did not appear when incubation was performed in the presence of exogenously added purified eEF-2.*

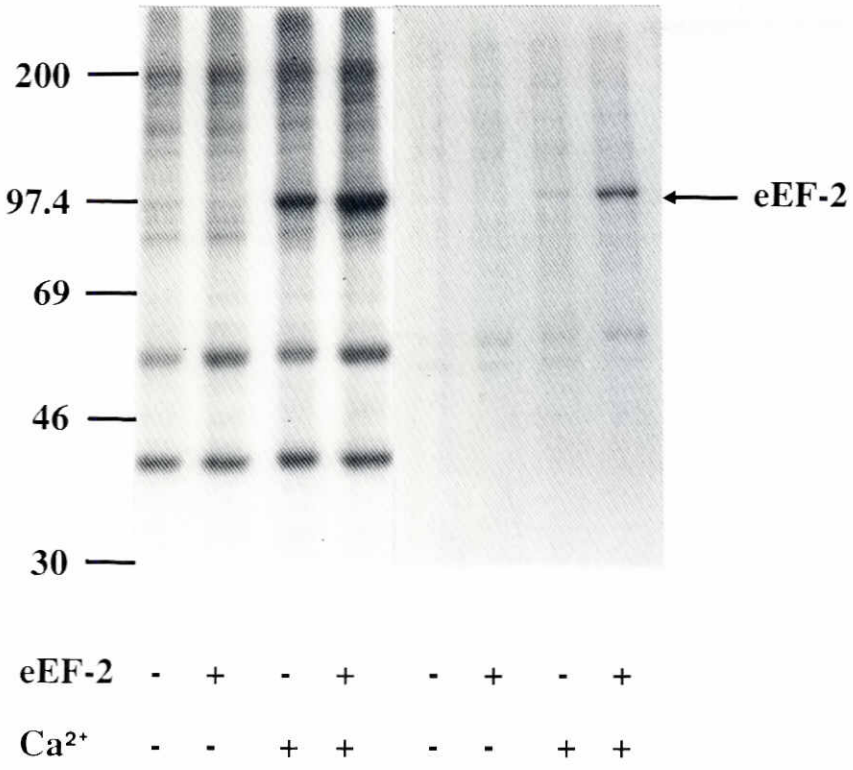

Fig. 8. Ca^{2+} dependency of eEF-2 phosphorylation as observed in the 26000 g supernatant fractions prepared from oocytes from *Patella vulgata* (Lanes 1 to 4) and *Asterias rubens* (lanes 5 to 8) collected in metaphase I. *The signs + and – point to the presence or absence of Ca^{2+} or exogenous eEF-2 in the assay (see Materials and Methods).*

act *in vivo* by inhibiting a Ca^{2+}/calmodulin-dependent kinase such as eEF-2 kinase. It remains possible that they act upstream to this event, by directly precluding the Ca^{2+} influx step, as previously shown for TFP during the artificial activation of prophase-arrested, GV-bearing oocytes of the echiuroid *Urechis* or the bivalve *Barnea* (Meijer *et al.*, 1983). However, the fact that both drugs remain effective when added up to 10 min after insemination, i.e., during the height of the Ca^{2+} surge process, indicates that they must hit a target situated downstream to that event. These *in vivo* collected data agree with some recent *in vitro* experiments performed by Lorca *et al.* (1991) on acellular extracts prepared from metaphase II-arrested *Xenopus* oocytes. These authors demonstrated that both cyclin proteolysis and exit from the M-phase could occur independently of $p39^{mos}$ destruction. Moreover, these processes, which occurred in the presence of micromolar physiological Ca^{2+} concentrations, could not be inhibited by calpastatin and seemed to involve a Ca^{2+}/calmodulin-dependent enzyme.

Is eEF-2 the final target responsible for exit from the metaphase stage?

In transformed human amnion cells, it has been found that eEF-2 becomes significantly phosphorylated during mitosis and that protein synthesis significantly declines at this stage (Celis *et al.*, 1990). Here, we have shown that both eEF-2 and eEF-2 kinase are present in a number of invertebrate oocytes. We also observed that the rate of protein synthesis decreased both after fertilization and during the first cleavages of *Mytilus*. If cyclins are really stabilized by a limited threshold level of short-lived proteins, then such an eEF-2-dependent and transient arrest of the protein synthesis machinery would account for the fact that cyclins actually disappear during oocyte activation. One may thus conceive that the effect of the fertilization or activation-dependent Ca^{2+} surges would be to activate eEF-2 kinase. This will transiently reduce the rate of protein synthesis, make the cyclins disappear and finally trigger metaphase-anaphase transition. To strengthen this working hypothesis, it remains to be shown that eEF-2 kinase transiently phosphorylates eEF-2 *in vivo*, after the Ca^{2+} surge.

Alternatively, one may still conceive that the Ca^{2+}/calmodulin-dependent step required to initiate metaphase-anaphase transition, both in our model systems and in *Xenopus* (Lorca *et al.*, 1991), would directly activate the cyclin degradation pathway, without significantly interfering with protein synthesis. However, this last unifying hypothesis, which also applies to *Xenopus* oocytes, would not explain why emetine activates invertebrate metaphase-arrested oocytes, unless it is also assumed that our postulated stabilizing short-lived proteins may negatively control activation of this enzyme and are Ca^{2+} labile. Work is now in progress to test the validity of these two alternative hypotheses.

Materials and Methods

Handling of oocytes

Most living animals were collected in the vicinity of Roscoff or Concarneau. They were kept in running sea water tanks until used. Oocytes of the starfish *Asterias rubens* and *Marthasterias glacialis*, the polychaete annelid *Perinereis cultrifera*, the gastropod mollusc *Patella vulgata* and the bivalves *Spisula ovalis* and *Ruditapes philippinarum* were extracted directly from the gonad. Oocytes of the bivalves *Mytilus edulis* (collected near Rimouski, Québec) or *Mytilus galloprovincialis* (Roscoff, France), which lyse when extracted at the germinal vesicle stage, were respectively obtained following electrical stimulation (Dubé and Dufresne, 1990) or by raising the temperature of the sea water up to 30°C (Dufresne-Dubé *et al.*, 1983). Under these conditions, they undergo germinal vesicle breakdown (GVBD) and are blocked in first meiotic metaphase. 1-methyladenine (1 μM), serotonin (10 μM) and

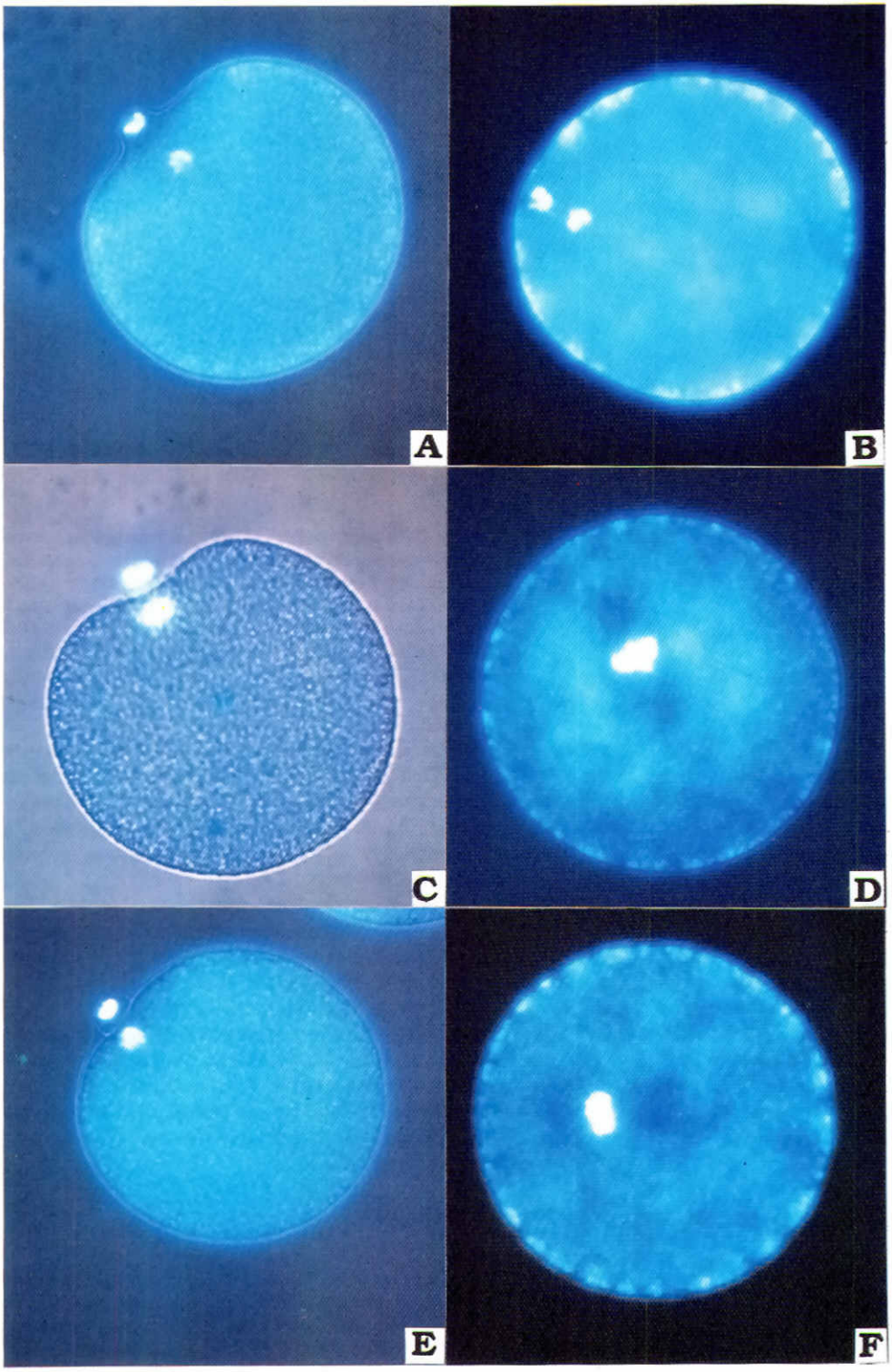

Fig. 9. Effects of the calmodulin antagonist W 7 on artificial activation of metaphase I-arrested oocytes of the Japanese clam *Ruditapes philippinarum*. **(A)** *First polar body extrusion following addition of 90 μM emetine.* **(B)** *Anaphase of the first maturation division occurring normally after emetine addition to an oocyte pretreated with 100 μM W 7.* **(C)** *First polar body extrusion following addition of 50 mM KCl.* **(D)** *Inhibition of KCl-induced activation in an oocyte pretreated with 100 μM W 7.* **(E)** *First polar body extrusion following the addition of 36 μM of the calcium ionophore A 23187.* **(F)** *Inhibition of ionophore-induced activation in an oocyte pretreated with 100 μM W 7. Identical results were obtained in oocytes pretreated with 30 μM TFP.*

ammonia (10 mM) were used respectively to trigger GVBD in starfish, bivalve and *Patella* oocytes. While meiosis proceeds to completion in *Spisula* and the starfish, oocytes of *Patella* and *Ruditapes* exhibit a second block in metaphase I, which is only released upon further fertilization or activation.

Living oocytes were washed 3 times with calcium-free sea water (CaFSW) prepared according to Shapiro (1941). Then, they were crushed in a glass homogenizer at 4°C using lysis buffer A (0.1 M Pipes, pH 6.6; 5 mM EGTA; 1mM MgSO4; 0.9 M glycerol; 1mM Dithiothreitol; 10 μg/ml soybean trypsin inhibitor; 0.1M benzamidine; 1mM ß-glycerophosphate and 1mM leupeptin). Homogenates were centrifuged at 26000 g for 30 min at 4°C and supernatants were preserved for assaying eEF-2 kinase. Protein concentration was determined according to Esen (1978) and the fractions adjusted to 1mg/ml.

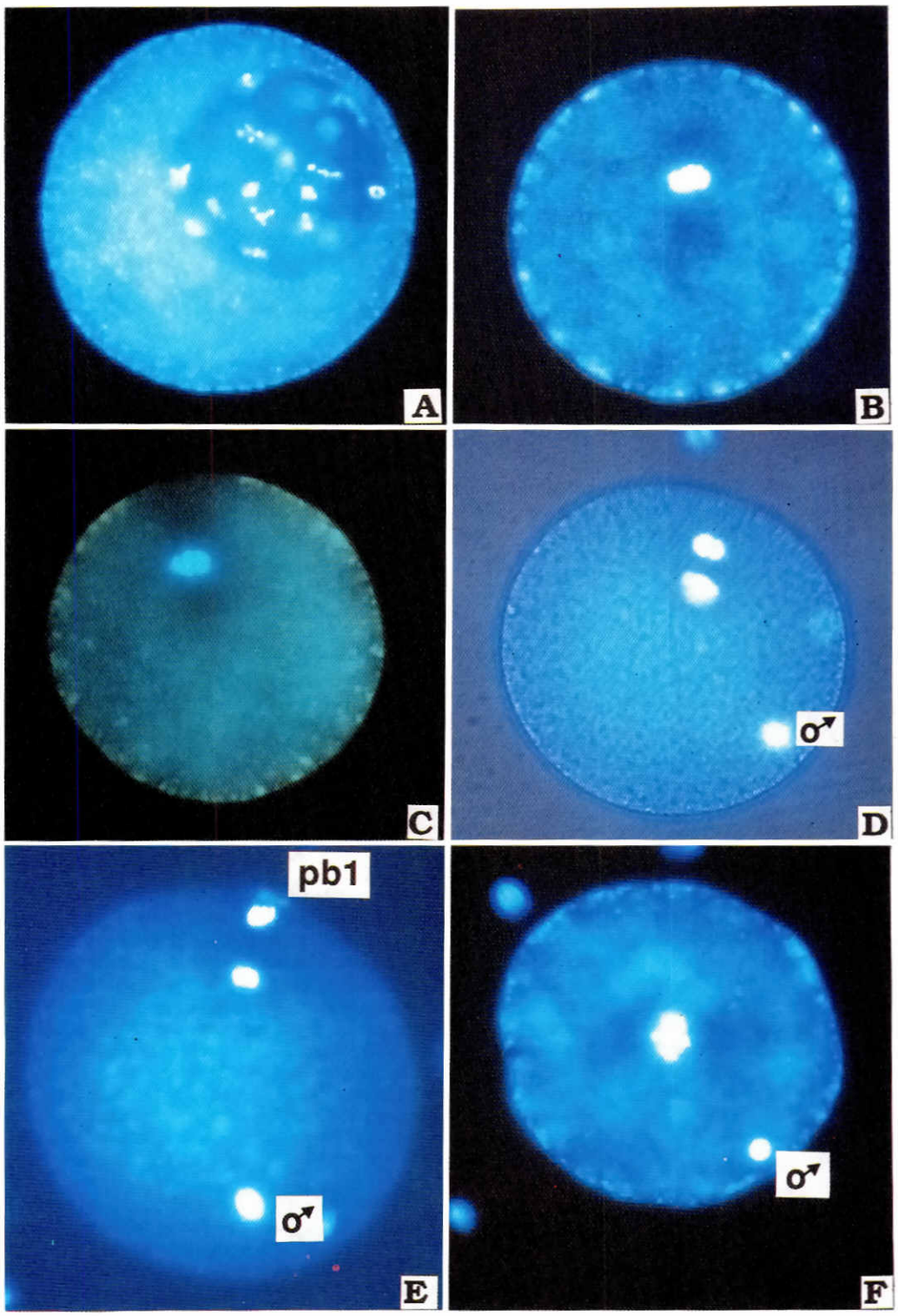

Fig. 10. Effects of the calmodulin antagonists W 7 and Trifluoperazine on oocyte maturation and fertilization in the Japanese clam *Ruditapes philippinarum*. **(A)** *A prophase-arrested oocyte with its huge germinal vesicle, pretreated with 100 μM W 7.* **(B)** *A metaphase 1-arrested oocyte obtained from the same batch, 30 min after the addition of 10 μM 5-HT.* **(C)** *A metaphase 1-arrested oocyte obtained after the addition of 10 μM 5-HT to an oocyte suspension pretreated with 30 μM TFP.* **(D)** *Anaphase of the first maturation division, 15 min after fertilization of a control oocyte.* **(E)** *Anaphase of the second maturation division, 45 min after fertilization of a control oocyte.* **(F)** *Inhibition of activation in a fertilized oocyte which received 100 μM W 7 at 5 min after insemination. The egg is still blocked in Metaphase I, 90 min after fertilization, when controls are at the 2 cell stage. Identical results were obtained with 30 μM TFP. Both drugs preclude sperm penetration. pb1, first polar body; ♂, male pronucleus.*

Fertilization was performed using a 0.5% suspension of *Mytilus* or *Ruditapes* oocytes. Sperm dilution varied from 2500 to 20000.

Oocyte labeling and SDS-PAGE analysis of synthesized proteins

200 μCi/ml of ^{35}S- methionine was added to a 10% v/v oocyte suspension and the incubation was run for two hours. Oocytes were then washed 3 times with ASW and adjusted to 2% (v/v). For autoradiogram analysis of synthesized proteins, 300 μl samples were recovered, centrifuged and the pellet dissolved in 2-fold concentrated Laemmli sample buffer. Sodium dodecyl sulfate (SDS)-polyacrylamide gels (12.5%, 1.0 mm thick) were prepared according to Laemmli (1970) and the lanes were loaded with an equal volume of sample (25 μl). Molecular weight standards

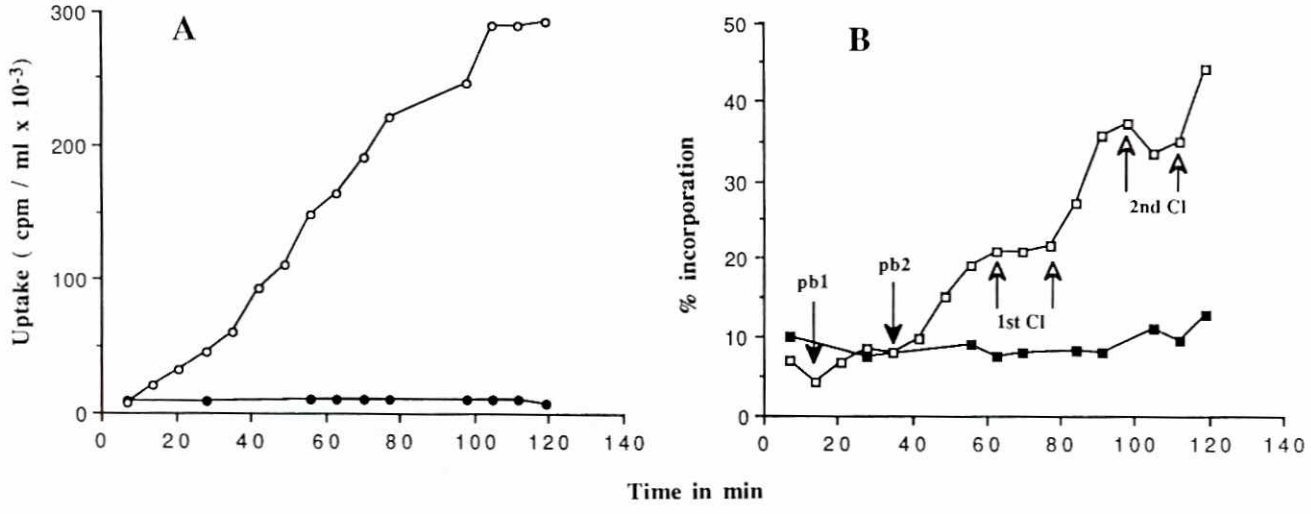

Fig. 11. Changes in the rate of uptake (A) and incorporation of ^{3}H-leucine (B) following fertilization of metaphase I-arrested oocytes of *Mytilus galloprovincialis*. *15 min pulse incorporations were started each 7 min. ●, ■, uptake and incorporation by unfertilized oocytes; ○, □, uptake and incorporation by fertilized eggs. pb1 and pb2, first and second polar bodies; 1st and 2nd Cl, first and second cleavages.*

(Amersham) included myosin (200 Kd), Phosphorylase a (97.4 Kd), Bovine serum albumin (69 Kd), Ovalbumine (46 Kd), Carbonic anhydrase (30 Kd), Trypsin inhibitor (21.5 Kd) and Lysozyme (14.3 Kd). The gels were stained with Coomassie blue and dried before exposition on X ray film (Amersham ß max).

Assay for histone H1 kinase

Oocytes were prepared as a 2% v/v suspension before fertilization or activation with emetine. At desired times, aliquots were withdrawn, centrifuged, washed once with CaFSW and frozen in liquid nitrogen. The pellets (100 µl packed eggs) were homogenized with 1 ml of PK buffer (20 mM Tris, 1 mM Dithiothreitol, 10 mM MgCl2, 2mM EGTA, 5 mM ß-glycerophosphate), adjusted to pH 7.4 with HCl 1M at 4°C. The homogenates were centrifuged for 15 minutes at 14 000 g in a cold chamber and supernatant frozen in liquid nitrogen. After determining protein concentration according to Bradford (1976), 1 µg/µl of proteins were mixed with 0.1 mM cold ATP with or without 1 µg/ml added histone in a final volume of 200 µl. The reaction started by addition of 0.7 µCi/µl γ-^{32}P-ATP, was carried out at 30°C for 15 min and stopped by adding 1 ml of 20% ice-cold TCA. Samples were then run on SDS-PAGE as already described, silver stained (Bio-Rad kit) to visualize the histone bands. These were carefully excised from the gels, transferred to scintillation vials containing 5ml of scintiverse BD (Fisher scientific) for CPM determination. Triplicate determinations were performed and results expressed as the difference between CPM in samples with added histones minus those without histones.

Assay for eEF-2 kinase

eEF-2 kinase activity was measured as described previously (Ryazanov *et al.*, 1988). Assays were performed for 10 min at 25°C in a total volume of 40 µl containing 10 µl of the supernatant fractions in phosphorylation buffer B (50mM Hepes-KOH, pH 7.6; 10 mM MgOAc; 1mM EGTA; 2mM CaCl2; 5 mM dithiothreitol; 50 µM (γ-^{32}P)ATP, 110 T Bq/m mol, Amersham) with or without 0.4 µg calmodulin and 3 µg of purified eEF-2 from rabbit reticulocytes. The reaction was stopped by addition of Laemmli sample buffer (Laemmli, 1970). Samples were boiled for 5 min, loaded on 10% SDS-PAGE. Gels were stained with Coomassie blue, dried and exposed overnight on ß-max films (Amersham) at room temperature.

Immunoblotting

After polyacrylamide gel electrophoresis, using Biorad minigel system, proteins were transferred onto nitrocellulose membranes (0.54 µm Biorad) in 0.3 M Tris, pH 10.4, 20% methanol at room temperature using a semi dry transfer apparatus operated at 106 mA for 1 h. Blots were blocked with Tris-buffered saline (TBST: 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20, containing 5% BSA (Sigma, fraction V) for 2 h at room temperature. Membranes were washed 3 times, 5-10 min with TBST, before incubation with the first antibody, anti eEF-2, diluted 1:500 in TBS containing 1.5% BSA, 0.1% Tween 20 for 2 h at room temperature. Blots were washed again before incubation with the second antibody (Goat-anti-rabbit IgG) coupled to peroxidase at dilutions of 1:2000 in antibody solution for 2 h. After 3 washes in TBST (5-10 min per wash), blots were developed in 50 ml of Tris-HCl, pH7.4, 150 mM NaCl, containing 30 mg α-chloronaphtol and 12 µl H2O2, 30%.

Intracellular free Ca^{2+} measurements

Oocytes, suspended in filtered seawater (FSW) or artificial seawater (ASW) prepared according to Shapiro (1941), were incubated for 30 min in the presence of 5 µM Fluo-3/AM (Molecular probes, Eugene, Oregon, USA) prepared from a 1mM stock solution in DMSO. 5 µl pluronic acid 25% was added to that medium in order to facilitate penetration of the Ca^{2+} indicator. Excess dye was removed by washing the oocytes in ASW or FSW. Loaded oocytes were then stuck to a coverslip at bottom of the observation chamber, using a 1% solution of polylysine in ASW.

Measurements were performed via an inverted Nikon Diaphot microscope equipped with a Hamamatsu R 268 photomultiplier operated at 900 volts. Epifluorescence illumination of individual oocytes was obtained through a 100 W Xenon lamp, the intensity of which being controlled by addition of neutral filters.

Excitation was performed at 490 nm and the emitted light was recorded on a Nikon dichroic mirror at 510 nm with an additional barrier filter at 515nm. Observations and measurements were done through a Nikon CF 40x Fluor objective with a numeric aperture of 0.85. Calibration of the Ca^{2+} response was performed with 10 µM ionomycine and 20 mM $MnCl_2$, as described by Kao *et al.* (1989).

Under these conditions, the concentration of cytosolic free Ca^{2+} is given by the formula:

$(Ca^{2+})_i = Kd\ (F\text{-}F_{min})/\ (F_{max}\text{-}F)$, with Kd= 400nM.

Since free Ca^{2+} resting levels varied from batch to batch, relative changes in fluorescence (F/F0), which are linearly proportional to the actual Ca^{2+} concentration (Cornell-Bell *et al.*, 1990), were adopted to present our kinetic recordings.

The ARGUS 10 equipment from Hamamatsu, coupled to a videoprinter Mitsubishi, which allowed pseudocolor image processing was also used occasionally to monitor Ca^{2+} transients.

^{3}H-leucine incorporation

Pulse labeling experiments were performed to measure the rate of protein synthesis before and after fertilization of metaphase I-arrested oocytes of *Mytilus galloprovincialis*. Each 7 min, two ml duplicate aliquot samples of a 0.5% oocyte or egg suspension were pulse labeled for 14 min with 185 kBq ^{3}H-leucine. 10 ml of ice-cold sea water containing 0.1 mg/ml cold leucine was added to end the pulse. The eggs were then rapidly centrifuged and the pellets further washed 3 times with 10 ml of the same medium. 1 ml of 10% TCA plus 1 mg/ml leucine was added to the pellets which were left overnight at 4°C. 200 µl of the TCA soluble fraction were mixed with 5ml ACS Amersham scintillation fluid to determine total uptake. The pellets were then washed 3 times with 5 ml TCA 5% plus 1 mg/ml leucine, dissolved in 1 ml 0.5 N NaOH, mixed with 5 ml ACS and counted in a Packard 1500 scintillation counter. The results are expressed as mean percentage incorporation of ^{3}H-leucine in the TCA insoluble fraction over total uptake of duplicate samples.

Cytological observations

Most of the observations were performed after *in vivo* labeling of the oocytes with the fluorescent dye Hoechst 33342. However, for kinetic studies, oocytes were first fixed in glucamine acetate buffer before staining them with Hoechst 33258 (Dubé, 1988).

Solutions

Chemicals and drugs were obtained from Sigma. Stock solutions of KCl (0.52 M, pH 8) and NH4Cl (2M, pH 8.5) were prepared in 50 mM Tris-HCl. Ionophore A 23187 was prepared as a 2mM stock solution in DMSO-Ethanol (1:3). TFP (5mM) was dissolved in CaFSW and W7 (20 mM) in Methanol. Controls were systematically performed in presence of the vehicle. They behaved normally.

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