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 A23187 can be reversibly blocked by the calmodulin antagonists TFP (30 \(\mu\)M) and W7 (100 \(\mu\)M), 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 (Ryzanov 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 requires 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 Ca\(^{2+}\) signal.

Abbreviations used in this paper: AM, acetoxymethyl ester; ASW, artificial seawater; ATP, adenosine 5-triphosphate; Ca\(^{2+}\)SW, calcium-free artificial seawater; CSF, cytosolic factor; D-600, enthoxyverapamil; DMSO, dimethylsulfoxide; eEF-2, eukaryotic elongation factor; EGTA, [ethylendiamine(tetraacetic acid)] tetraacetic acid; Fluo-3/AM, fluo-3 pentachloro methyl ester; GA, glutamine 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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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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} concentration. This is the case when oocytes are incubated in the presence of the Ca\textsuperscript{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 \textsuperscript{45}Ca\textsuperscript{2+} uptake, as observed in the bivalves Barnea (Dubre and Guerrier, 1982) and Spisula (Dubre 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\textsuperscript{2+} as observed for KCl and to produce a similar stimulation of the \textsuperscript{45}Ca\textsuperscript{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 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 (Dubre 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\textsuperscript{2+} concentration, such as fertilization, KCl or ionophore A 23187 stimulations. Moreover, we show that sperm, KCl- or

Fig. 1. Transient intracellular Ca\textsuperscript{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\textsuperscript{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.

Fig. 2. Pseudocolor scale imaging showing the relative changes in Ca\textsuperscript{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 (C), 50 mM excess KCl (D), or 1 mg emetine (E). 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.

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 mammillata* 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-
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 anticamandin 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 amino acid, 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
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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 p34cdc2 and could be precipitated with p13Src1 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,
which mimic fertilization in mobilizing Ca²⁺, 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 cyotstatic 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 Ca²⁺ transients that trigger activation.

### Intracellular Ca²⁺ surges and possible downstream involvement of a Ca²⁺/calmodulin-dependent kinase

Fertilization is the only event which has been unequivocally shown to trigger a transient and dramatic rise in the intracellular free Ca²⁺ concentration. Using Ca²⁺ sensitive microelectrodes or the photoprotein aequorin, this Ca²⁺ 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 Ca²⁺ 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 Ca²⁺. Oocytes of the mollusc 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 Ca²⁺ sensitive channels, has already been shown to trigger a ⁴⁵Ca²⁺ influx similar to those obtained following fertilization or serotonin stimulation of the bivalve oocyte (Dubé and Guerrier, 1982; Dubé et al., 1987; Dubé, 1988; Krantice et al., 1991). It appeared moreover that KCl- or ionophore-dependent activations did not occur in the absence of extracellular Ca²⁺ or when Ca²⁺ influx was cancelled using Ca²⁺ channel blockers such as Mn²⁺ 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 Ca²⁺ sensitive probe, that both fertilization and KCl actually induce a transient intracellular Ca²⁺ surge both in Ruditapes and Mytilus (Figs. 1 and 2). In these species, we also show that calmodulin antagonists such as trifluoperazine and W7 reversibly inhibit ionophore- of KCl-dependent activations. We have no tools to check if these drugs
Calcium and oocyte activation

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 echinoid 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\(^{mod}\) 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 Martasterias 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 \(\mu\)M), serotonin (10 \(\mu\)M) and...
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 Rudites 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 β-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.
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 35S-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
buffer (Laemmli, 1970). Samples were boiled for 5 min, loaded on 10% SDS-PAGE. Gels were stained with Coomassie blue and dried before exposition on X-ray film (Amersham & max).

**Assay for histone H1 kinase**

Oocytes were prepared as a 2% v/v suspension before fertilization or activation with epinephrine. At desired times, aliquots were withdrawn, centrifuged, washed once with CaFSSW 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, 2 mM EGTA, 5 mM β-glycerophosphate), adjusted to pH 7.4 with HCl 1 M 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 µl [32P]-ATP. 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 3-Max films (Amersham & max) at room temperature.

**Immunoblotting**

After polyacrylamide gel electrophoresis, using Blotrods minigel system, proteins were transferred onto nitrocellulose membranes (0.54 µm Blorad) in 0.3 M Tris, pH 10.4, 20% methanol at room temperature using a semi dry transfer apparatus operated at 1.06 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. 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 2h. After 3 washes in TBST (5-10 min per wash), blots were developed in 50 ml of Tris-HCl, pH 7.4, 150 mM NaCl, containing 30 µg α-chloronaphtol and 12 µl H2O2, 30%.

**Intracellular free Ca2+ 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 Fura-3/AM (Molecular probes, Eugene, Oregon, USA) prepared from a 1 mM stock solution in DMSO. Phosphorylase a (97.4 Kd), Bovine serum albumin (69 Kd), Ovalbumine (46 Kd), Carbonic anhydrase (30 Kd), Trpisin 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 & max).

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 (50 mM Hepes-KOH, pH 7.6; 10 mM MgOAc; 1 mM EGTA; 2 mM CaCl2; 5 mM Dithiothreitol; 50 µM [γ-32P]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 3-Max films (Amersham & max) at room temperature.

Fig. 11. Changes in the rate of uptake (A) and incorporation of 3H-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 CI, first and second cleavages.
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.

**3H-leucine incorporation**

Pulse labeling experiments were performed to measure the rate of protein synthesis before and after fertilization of metaphase 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 3H-leucine. 10 ml of ice-cold seawater 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 3H-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 glacial acetic acid buffer before staining them with Hoechst 33258 (Dubé, 1988).

**References**


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