Inhibition of protein tyrosine phosphatases blocks calcium-induced activation of metaphase II-arrested oocytes of *Xenopus laevis*

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Abstract We have studied the effect of a protein tyrosine phosphatases (PTP) inhibitor on calcium-induced activation of *Xenopus laevis* oocytes arrested at metaphase II. Ammonium molybdate microinjection blocked pronucleus formation following A23187 treatment while cortical granules still underwent exocytosis. Pronuclei still occurred in ammonium molybdate-injected oocytes following 6-DMAP addition. Changes that usually occurred following A23187 exposure were inhibited in the presence of ammonium molybdate in the oocyte: MAPK dephosphorylation, $p34^{cdc2}$ rephosphorylation and cyclin B2 and $p39^{mos}$ proteolysis. These results suggest that a PTP is involved in the activation of the ubiquitin-dependent degradation machinery.

Key words: Oocyte; Calcium; Protein tyrosine phosphatase; Activation; *Xenopus*

1. Introduction

Entry into M phase is due to the activation of MPF, a kinase made up of two subunits: the catalytic subunit $p34^{cdc2}$ and the regulatory subunit cyclin [4,16]. Exit from M phase is dependent on MPF inactivation. Cyclin is proteolysed by the ubiquitin pathway [10,13] while $p34^{cdc2}$ is dephosphorylated on T161 [19]. Ubiquitination of proteins requires a 20S multisubunit complex that has been termed the anaphase-promoting complex (APC) which targets proteins for destruction by the 26S proteasome complex [34]. APC is believed to be the target of a cell cycle-dependent regulation. Degradation of cyclin B is stimulated in cell-free extracts prepared at interphase by addition of purified MPF [6,19]. The MPF has therefore been proposed to promote degradation of M phase cyclins by a mechanism that remains almost completely unknown [18,34].

In *Xenopus*, metaphase II arrest is due to a cytostatic factor (CSF) [21]. Although CSF has not yet been purified, the product of the protooncogene c-mos ($p39^{mos}$) [28] and mitogen-activated protein kinase (MAPK) [12] are thought to be involved in CSF activity. MAPK could mediate the CSF activity of $p39^{mos}$ by preventing the cyclin degradation pathway from being turned on and leading to the maintenance of a high level of MPF activity in the cell [1]. This might be due to the presence in the egg of an inhibitor acting either on APC [1] or on ubiquitin-dependent proteolysis [36].

The interaction of the sperm with the egg membrane at fertilization leads to egg activation. This is mediated by a transient increase in intracellular calcium which induces cortical granule exocytosis for fertilization membrane formation as well as release from metaphase II arrest. Following this increase in calcium, both CSF and MPF activities disappear but MPF is inactivated before CSF [28,38]. Calcium/calmodulin-dependent protein kinase II (CaMKII) has been shown to act downstream of intracellular calcium elevation [20,25]. Although $p39^{mos}$ could be cleaved by the protease calpain [30,37], its proteolysis is thought to occur by the ubiquitin pathway [26]. MAPK is inactivated but this event is not required for CaMKII to turn on the cyclin degradation machinery in CSF extracts [1]. This is consistent with the fact that MAPK is inactivated after cyclin B proteolysis. It was suggested that CaMKII could activate a phosphatase responsible for the dephosphorylation of an inhibitor of APC [1].

The role of some protein phosphatases has already been discussed as regulators of the APC. Cyclin B proteolysis has been found to be inhibited by the microinjection of okadaic acid (OA) into starfish oocytes [27]. It has recently been reported in *Xenopus* egg extracts that proteolysis of cyclin B is prevented by the use of OA, which induces an activity that inhibits cyclin B ubiquitination [36]. Nevertheless, this activity is still calcium-sensitive. Taken together, these results indicate a role for protein phosphatases in the metaphase-anaphase transition.

However, less is known about the role of protein tyrosine phosphatases (PTP) in the metaphase-anaphase transition. We have addressed the question of involvement of these PTP in the activation of *Xenopus* metaphase II-arrested oocytes, by using ammonium molybdate, a PTP inhibitor [15,22]. We report that inhibition of PTP blocks A23187-induced pronucleus formation. At the molecular level, ammonium molybdate inhibited not only the dephosphorylation of MAPK but also the proteolysis of cyclin B and $p39^{mos}$. This inhibitory effect may be due to a PTP implicated in the regulation of the APC.

2. Materials and methods

2.1. Handling of oocytes and gametes

Adult *Xenopus laevis* females were purchased from C.R.B.M. (C.N.R.S., Montpellier France). Full-grown stage VI oocytes [5] were obtained as previously described [9] and used in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5/NaOH). Progesterone was used overnight at 10 μM to obtain metaphase II-arrested oocytes. A23187 (Boehringer Mannheim) was used at 10 μM from a stock solution (100 mM) prepared in DMSO. 6-DMAP (Sigma) was diluted in ND96, and used at 12 mM.

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This concentration was chosen since it was reported to induce egg activation within 30 min [39]. All experiments were performed at 20°C and at least in triplicate.

2.2. Microinjections

The PTP inhibitor ammonium molybdate [15,22] (Sigma) was microinjected into in vitro matured oocytes, by the use of a positive displacement digital micropipette (Nichiryo) in a calcium-limited medium (CaLM: 120 mM NaCl, 7.5 mM KCl, 22.5 mM HEPES, 400 μM EDTA, 500 μM $MgSO_4$, 150 μM $CaCl_2$, pH 7.4), in order to prevent activation due to pricking [39]. After injection of ammonium molybdate (1.6 mM final), the oocytes were allowed to heal for 15 min in this medium, and then incubated for 45 min in ND96 for the ammonium molybdate to diffuse throughout the oocytes, before treatment with A23187 or 6-DMAP.

2.3. Cytological analysis

Oocytes were fixed overnight in Smith's fixative, sectioned and stained with nuclear red to detect nuclei and chromosomes, and with picroindigo carmine which reveals cytoplasmic structures [9].

In order to detect cortical granule exocytosis, oocytes were fixed in ND96 medium pH 7.5 containing 2.5% glutaraldehyde, after cutting into two parts to improve fixation. They were then dehydrated in a graded series of acetone, embedded in EMbed 812 and sectioned with a Reichert OmU2 ultramicrotome (Reichert, Vienna, Austria). Sections (1 μm thick) were stained with a 0.1% toluidine blue solution.

2.4. Electrophoresis and Western blotting

Oocytes (20 per batch) were homogenized in homogenization buffer [2] and centrifuged for 10 min at $10\,000 \times g$ (4°C) to eliminate yolk platelets. Supernatants were then incubated at 4°C for 30 min, under constant rotation with 10 μl $p9^{CKShs1}$-Sepharose beads. After a brief centrifugation, one volume of 2×Laemmli sample buffer was added to one volume of supernatant for $p39^{mos}$ and MAPK analysis. Beads were washed three times with ice-cold bead buffer [2] and treated with 2×Laemmli sample buffer for detection of $p34^{cdc2}$ and cyclin B2. Oocyte proteins were denatured by heating the mixture (100°C, 5 min) and then separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In the particular case of MAPK immunodetection, proteins were resolved by 15% SDS-PAGE (prepared from a stock solution containing 29.82% acrylamide/0.18% bisacrylamide). Such gels allowed a good discrimination between active and inactive MAPK [3]. Separated proteins were subjected to Western blot analysis as previously reported [9]. $p34^{cdc2}$ was detected using the monoclonal antibody A17 [11] (1/1000 in TBS). MAPK was detected using the anti-Erk2 monoclonal antibody D-2 (Santa Cruz Biotechnology, Heidelberg, Germany) (1/2500 in TBS). Cyclin B2 was detected using the monoclonal antibody X121 diluted at 1/1000 in TBS. $p39^{mos}$ was detected using the rabbit polyclonal antibody C-237 (Santa Cruz Biotechnology, Heidelberg, Germany), 1/1000 in TBS.

3. Results and discussion

3.1. Cytological analysis of ammonium molybdate-injected oocytes

The results of this analysis are summarized in Fig. 1. A pronucleus was observed in 100% of the in vitro matured oocytes that had been exposed to calcium ionophore A23187 (10 μM) or 6-DMAP (12 mM) for 60 min. Ammonium molybdate microinjection itself did not trigger activation of the oocytes. When treated by calcium ionophore, 14% of oocytes microinjected ($n = 14$) showed pronucleus formation. Ammonium molybdate did not block pronucleus formation induced by 6-DMAP, since pronuclei were found in 70% of the oocytes ($n = 10$).

This result showing that ammonium molybdate inhibits calcium-induced pronucleus formation suggests that PTP play an important role in the cascade of events leading to pronucleus formation. This confirms a recent report indicating that inhibition of PTP with sodium orthovanadate leads to disruption of sperm-induced *Xenopus* egg activation [31], even if these authors observed only cortical reaction without pronucleus analysis.

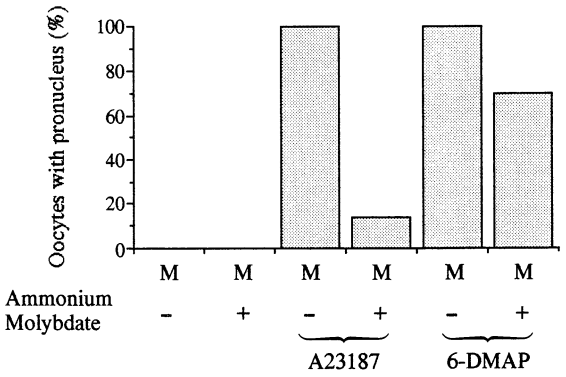

Fig. 1. Pronucleus formation after treatment with A23187 or 6-DMAP of metaphase II-arrested oocytes previously injected with ammonium molybdate. Oocytes were injected or not with ammonium molybdate and 1 h later they were incubated for 1 h in control or test solutions. Results are expressed as percentage of oocytes showing a pronucleus on the histological sections. No pronuclei were found in matured oocytes, even 2 h after the injection of ammonium molybdate. Matured oocytes showed a pronucleus in all cases when analyzed after 1 h of A23187 or 6-DMAP exposure. In the presence of the PTP inhibitor, only 14% ($n = 14$) of the A23187-treated oocytes showed a pronucleus whereas such a structure was found in 70% ($n = 10$) of the 6-DMAP-treated oocytes.

One might argue that the lack of pronucleus might be due to a toxic effect of the drug. However, this does not seem to be the case since 6-DMAP, a kinase inhibitor able to activate *Xenopus* eggs in the absence of calcium changes [39], induced pronucleus formation in these injected oocytes.

Another explanation might be that the calcium increase could not occur in these injected oocytes. Since cortical granule exocytosis is a reliable event associated with calcium changes in the egg, we analyzed the cortical granule content of in vitro matured oocytes. In control oocytes, cortical granules were located under the plasma membrane (Fig. 2A), while these granules underwent exocytosis after A23187 treatment (Fig. 2B). Ammonium molybdate injection itself did not trigger cortical granule exocytosis (Fig. 2C). The presence of the PTP inhibitor did not block exocytosis induced by the calcium ionophore (Fig. 2D). The vitelline envelope was not present on the sections because oocytes were cut into two halves to aid fixation. The fact that cortical granule exocytosis still occurred in oocytes injected with ammonium molybdate following A23187 addition demonstrated that the PTP inhibitor did not prevent the increase in intracellular calcium.

3.2. Analysis of MPF and CSF in ammonium molybdate-injected oocytes

First, phosphorylation states of $p34^{cdc2}$ and MAPK were analyzed. $p34^{cdc2}$ may appear as two forms on Western blots: the slow migrating band is inactive and phosphorylated on three residues (Thr-14, Tyr-15 and Thr-161), the fast migrating band is either unphosphorylated and inactive or monophosphorylated (Thr-161) and active [32]. Two forms of MAPK may also be detected: an active tyrosine phosphorylated form and an unphosphorylated faster migrating inactive form [24].

Immature oocytes contain two forms of $p34^{cdc2}$ and the dephosphorylated form of MAPK whereas matured oocytes contain only the fast migrating band for $p34^{cdc2}$ and the slow

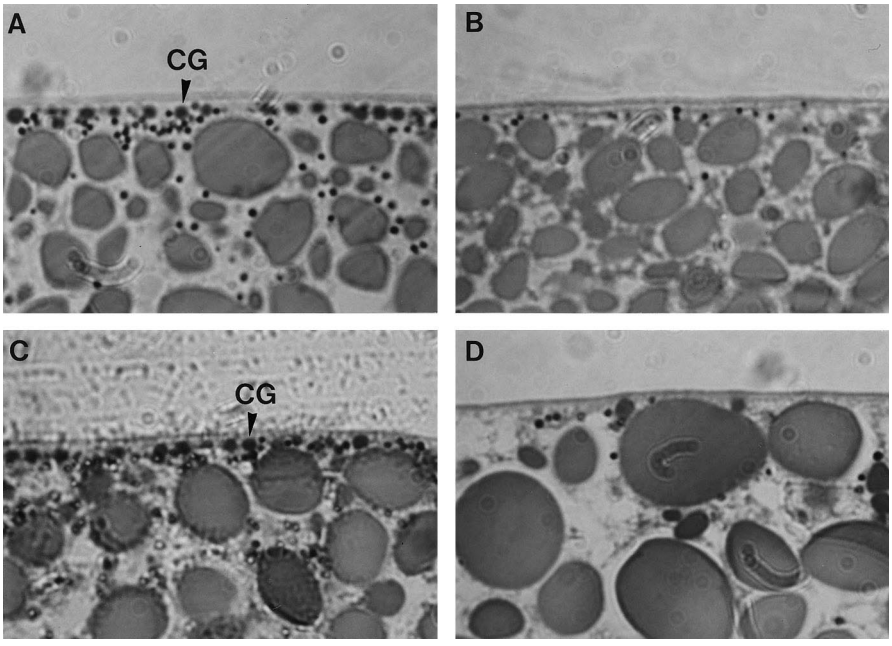

Fig. 2. Sections of matured oocytes showing cortical granule content following ammonium molybdate microinjection and treatment with A23187. In vitro matured oocytes were injected or not with ammonium molybdate and 1 h later, they were treated or not with A23187 for 20 min. Cortical granules (CG) appeared located under the plasma membrane in control metaphase II-arrested oocytes (A) as well as in oocytes injected with ammonium molybdate (C). A23187 induced CG exocytosis in matured oocytes (B) even in the presence of ammonium molybdate (D) (×1000). The vitelline envelope is not observed on the sections due to its loss following the cutting of the oocytes into two halves to improve fixation.

migrating band for MAPK (Fig. 3). In metaphase II-arrested oocytes previously injected with ammonium molybdate, calcium ionophore failed to induce changes in the immunodetection profiles of $p34^{cdc2}$ and MAPK (Fig. 3).

Ammonium molybdate inhibited calcium-induced dephosphorylation of MAPK, providing efficiency of its inhibitory action. Indeed, it is well-known that tyrosine dephosphorylation of MAPK is a key event for its inactivation [7]. MAPK phosphatases have been found in *Xenopus* egg extracts: an uncharacterized 47 kDa PTP was purified from the cytosolic fraction of *Xenopus* eggs [29], and XCL100, close to the mouse homologue CL100, has been cloned in *Xenopus* embryos and shown to inactivate MAPK [17]. In metaphase II-arrested extracts of *Xenopus* eggs, ectopic overexpression of a C-terminally truncated form of CL100 results in premature inactivation of MAPK and exit from metaphase arrest [23].

The role of MAPK in the metaphase II arrest has recently been discussed. MAPK has been shown to prevent the cyclin B degradation rather than to inhibit it [1]. Indeed, in metaphase II-arrested oocytes extracts, CaMKII is able to release metaphase block, and to trigger cyclin degradation without inactivation of MAPK. This is consistent with the fact that MAPK inactivation does not occur earlier than 20–30 min after fertilization, which is well after cyclin degradation [1].

So we focused on the proteolysis of the components of MPF and CSF. When calcium ionophore A23187 was applied on matured oocytes, cyclin B2 was degraded in the first minutes and was not detected on the Western blots performed after 20 min. The kinase $p39^{mos}$ was not detected on the Western blots performed after a 60 min A23187 exposure (Fig. 3). PTP inhibition by itself did not affect the stability of the two proteins (Fig. 3). In homogenates of oocytes exposed for 20 min to 10 μM A23187 after ammonium molybdate injection, cyclin B2 and $p39^{mos}$ were still detected (Fig. 3).

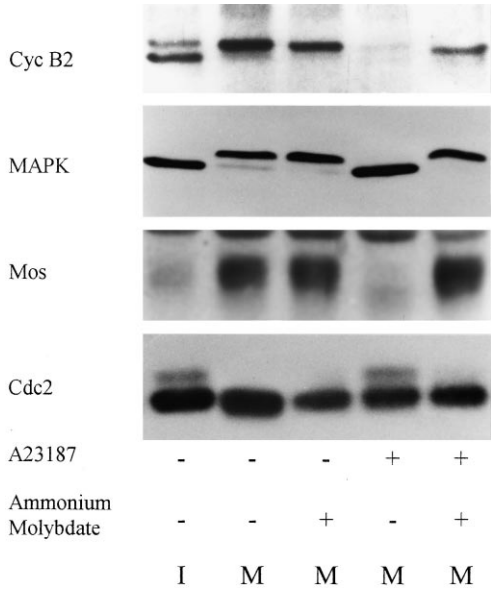

Fig. 3. Western blot analysis of $p34^{cdc2}$, $p39^{mos}$, MAPK and cyclin B2 in metaphase II-arrested oocytes microinjected with ammonium molybdate and treated with A23187. Metaphase II-arrested oocytes were injected or not with ammonium molybdate and 1 h later they were treated or not with A23187. Analyses were performed after 20 min for cyclin B2 and after 60 min for the other proteins. In homogenates from immature oocytes (I), we observed two forms of $p34^{cdc2}$ the absence of $p39^{mos}$, the dephosphorylated form of MAPK and two cyclin B2 isoforms. In matured oocytes (M), we found the fast migrating form of $p34^{cdc2}$, $p39^{mos}$ which was synthesized in response to progesterone, the phosphorylated active form of MAPK and the phosphorylated form of cyclin B2. Microinjection of the PTP inhibitor alone did not modify these profiles. Treatment with 10 μM A23187 induced rephosphorylation of $p34^{cdc2}$, degradation of $p39^{mos}$, dephosphorylation of MAPK and degradation of cyclin B2. These changes failed to occur when A23187 was applied on oocytes previously microinjected with the PTP inhibitor.

Why are these oocytes unable to carry out the proteolysis of cyclin B2 and P39^{mos} when PTP are inhibited? In the budding yeast *Saccharomyces cerevisiae*, Cdc14p, a dual specificity PTP, is required for cell cycle progression [33]. Cdc14p mutants, arrested in late mitosis, expressed a phenotype similar to that of cells expressing a non-degradable form of mitotic cyclins [8]. It has been proposed that Cdc14p induces degradation of mitotic cyclins through dephosphorylation of Cdh1/Hct1, the function of which is to activate APC [14,35]. Cdh1/Hct1 belongs to a family of proteins containing seven WD-40 repeats in their C-termini, involved in the regulation of the ubiquitin-proteasome pathway in both yeast and eukaryotes. A similar explanation might be proposed in *Xenopus* oocytes in which a PTP might be located downstream of calcium and CaMKII and upstream of APC.

In conclusion, our study shows that the calcium ionophore A23187 cannot induce pronucleus formation when PTP are inhibited. Since this effect is mediated not only by the inhibition of MAPK dephosphorylation but also by the inhibition of cyclin B2 and p39^{mos} proteolysis, we suggest that a PTP sensitive to ammonium molybdate is involved in APC regulation. The identification of this enzyme could be of great interest for the understanding of cell cycle regulation.

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