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Okadaic acid, a specific protein phosphatase inhibitor, induces maturation and MPF formation in *Xenopus laevis* oocytes

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Micro-injection of, or incubation with okadaic acid (OA), a specific phosphatase inhibitor, can induce formation of maturation-promoting factor (MPF) and germinal vesicle breakdown (GVBD) in *Xenopus laevis* oocytes. Comparison of the dose-response curves of OA on maturation, isolated enzymes and phosphatase activities in crude oocyte preparations suggests that inhibition of both polycation-stimulated (PCS) and ATP,Mg-dependent (AMD) phosphatases is sufficient but requires that a critical phosphorylation level is attained of one or several of their substrates, resulting in the formation of active MPF and meiotic maturation.

Okadaic acid; Oocyte maturation; Protein phosphatase

1. INTRODUCTION

The concept that phosphorylation processes are implicated in the regulation of the cell cycle gained new impetus with the discovery that highly purified preparations of MPF from *Xenopus* oocytes contain a protein kinase activity which appears to be the *Xenopus* homologue of the *cdc*2 gene product of the fission yeast *Schizosaccharomyces pombe* [1,2]. The human *cdc*2 gene, as well as yeast *cdc*2, encodes a 34 kDa protein kinase [3]. The cyclic rise and fall of MPF activity during the cell cycle is apparently due to posttranslational modification rather than de novo synthesis [4,5]. The transition between inactive pre-MPF and active MPF could be due to inactivation of a protein phosphatase [5] and it has been shown by genetic analysis and sequencing, that genes coding for protein kinases [6,7] can act as positive and negative effectors of $p34^{cdc2}$, implicating protein phosphorylation in MPF regulation. In addition, subunit rearrangements appear to be involved in activation of the $p34^{cdc2}$ kinase in human cells [8]. The appearance of MPF activity leads ultimately to a major burst in protein phosphorylation, approximately coincident with the GVBD [9]. Several distinct protein kinase activities are enhanced at the time of the appearance of MPF [10], e.g. ribosomal protein S_6 kinase II [11] and independent histone kinase [12]. The major 34 kDa band in highly purified independent histone kinase from starfish oocytes was identified as the $p34^{cdc2}$ kinase [13].

The concept of protein kinase cascades certainly is familiar in the field of protein phosphorylation, but protein kinases and phosphatases represent an intertwining network requiring efficient coordination of both activities. Only a small number of multifunctional phosphatases appear to control the major regulatory proteins. Four different types of Ser/Thr protein phosphatases can be distinguished [14-16], classified according to enzyme-directed regulation [16] as PCS phosphatases, AMD

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Abbreviations: OA, okadaic acid; MPF, maturation-promoting factor; GVBD, germinal vesicle breakdown; PCS, polycation-stimulated; AMD, ATP,Mg-dependent; *cdc*, cell division cycle; *p*NPP: *p*-nitrophenyl phosphate

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phosphatases, calcineurin and Mg^{2+}-dependent protein phosphatase. The PCS and AMD phosphatases appear to be highly regulated enzymes and the former could play a central role in cellular regulation [17], since they are involved in the regulation of AMD phosphatase, phosphorylase kinase, casein kinase 2, protein kinase C and ribosomal protein S_6 kinase, etc. In addition, they can be converted from a Ser/Thr phosphatase into a Tyr protein phosphatase [18–20]. The lack of a specific inhibitor was a hindrance in the study of the in vivo role of PCS phosphatase. Okadaic acid [21], a strong inhibitor of AMD and PCS phosphatases [22], is also a strong non-TPA-like tumor promoter [23]. Its acute effect on intestine smooth muscle contraction was ascribed to specific inhibition of myosin light chain phosphatase activity [24,25]. Phosphorylation/dephosphorylation processes are believed to be the driving force in regulation of the cell cycle. We further substantiate this concept, showing that microinjection of, or incubation with OA, a specific phosphatase inhibitor, can induce MPF formation and germinal vesicle breakdown (GVBD) in *X. laevis* oocytes. Comparison of dose-response curves of OA on maturation, isolated enzymes and phosphatase activities in crude oocyte preparations suggests that inhibition of both PCS and AMD phosphatases is required to reach a critical phosphorylation level of one or several of their substrates. Since OA is also recognized as a tumor promoter [23], it is tempting to speculate as to the role of phosphatases in cell proliferation in general.

2. MATERIALS AND METHODS

Different dilutions of OA were prepared from a 2.5 mM stock solution in dimethyl sulfoxide. Oocytes were prepared as described in [18], and the different oligomeric forms of PCS phosphatases (PCS_H, PCS_M, PCS_L) were purified to homogeneity from rabbit skeletal muscle [26], and the PCS_L phosphatase also from *Xenopus* oocytes [18]. The catalytic subunit of the PCS phosphatases (PCS_C phosphatase) and the AMD phosphatase was purified from rabbit skeletal muscle [27] and that of the AMD phosphatase also from dog liver [28]. The inactive form of AMD phosphatase was purified from rabbit skeletal muscle and activated as described [29], the deinhibitor-stimulated form of the AMD phosphatase being from dog liver as described previously [30]. Myosin light chains were prepared as in [31]. Tyrosine kinase was purified to apparent homogeneity from the particulate fraction of pig spleen based on a previous procedure as modified; the method is detailed in [32]. The purification includes detergent extraction, DEAE-Sephacel, tyrosine agarose, heparin-Sepharose and Mono Q-FPLC chromatography. This procedure was designed with particular care being taken to eliminate endogenous aryl phosphatases. Kinase activity was followed using the synthetic peptide poly[Glu:Tyr] (4:1) (Sigma) as substrate. The final preparation could incorporate 109 nmol P_i/min per mg in this substrate and showed an autophosphorylating protein doublet of 55 kDa in SDS-PAGE. Phosphatase assays were performed with phosphorylase as substrate according to [33] unless stated otherwise, and *p*NPP phosphatase as in [18]. Phosphotyrosyl phosphatase activity was assayed with 0.3 μM phosphomyosin light chains, phosphorylated exclusively on phosphotyrosyl residues to a level of 0.05 mol/mol by the porcine spleen tyrosine kinase (see above), as monitored by thin-layer electrophoresis at pH 3.5 after acid hydrolysis as in [34].

3. RESULTS AND DISCUSSION

In the absence of any other physiological stimulus, microinjection of OA can induce GVBD and formation of active MPF within 30–60 min. The dose-response curve of OA-induced GVBD (fig.1) shows that maturation of 50% of the oocytes is obtained by injection of 50 nl of 2.5 μM OA. Assuming rapid distribution of OA, and a volume of 500 nl for the cytosolic compartment, this represents an intracellular concentration of about 0.25 μM. Initiation of GVBD was very rapid (30–60 min) compared with progesterone-induced (4–7 h) and MPF-induced (90–120 min) maturation. Oocytes not matured within 60 min did not mature afterwards. The reaction of the oocyte to OA was rather violent, resulting in an irregular white spot on the animal pole followed by rapid cytolysis (within 15 min). The germinal vesicle disappeared, within 60 min, as confirmed on dissection of oocytes after fixation in 10% trichloroacetic acid. As soon as the white spot appeared, cytoplasm ($\pm$ 50 nl) was transferred to 2–4 recipient oocytes, which showed MPF-induced maturation in 100% of cases, through the appearance of the typical white spot within 90–120 min and GVBD as confirmed upon dissection after trichloroacetic acid fixation. This secondary maturation was not caused by OA still present after the transfer, without formation of MPF. Indeed, the steep dose-response curve of the OA-induced maturation (fig.1) implies that OA, at the expected low concentrations, cannot induce maturation. In addition, the difference in maturation time and the appearance of normal secondary maturation, in contrast with the atypical maturation induced by OA, indicate the involvement of a different

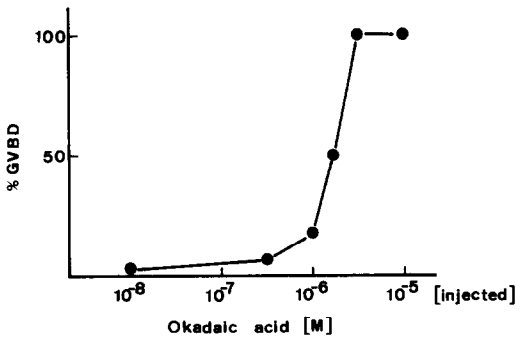

Fig.1. Dose-response curve of okadaic acid-induced maturation. Batches of 10 oocytes prepared as in [18] were microinjected with 50-nl dilutions of OA dissolved in 10 mM Hepes, 5 mM dithiothreitol, 1 mg/ml bovine serum albumin (pH 7.4), prepared from a stock solution of 2.5 mM OA in dimethyl sulfoxide. Maturation was scored between 30 and 60 min after microinjection and confirmed by dissection after trichloroacetic acid fixation.

mechanism. The transfer from matured recipient oocytes into third generation recipient oocytes further substantiates the formation of authentic MPF. When oocytes were incubated in different concentrations of OA (75 μM–2.5 nM), maturation was observed between 25 and 50 μM. Maturation in these cases was much less violent and slower than after microinjection of OA. The time course of maturation was descreased compared to progesterone-induced maturation. $GVBD_{50}$ was attained within 4 h with progesterone and within 3 h with 50 μM OA. With slowly maturing oocytes ($GVBD_{50}$ 6 h with progesterone), this time was reduced to 3 h in the presence of 50 μM OA.

We investigated the inhibitory effect of OA on the oligomeric forms of homogeneously purified PCS [26] and AMD [27,28] phosphatases, as well as on their respective catalytic subunits [29,30]. The values for 50% inhibitory concentration obtained with PCS phosphatases were between 10 and 500 pM ($PCS_M < PCS_C = PCS_L < PCS_H$), also depending on the enzyme concentrations in the assay, and between 80 and 200 nM for AMD phosphatases. The pronounced inhibition by OA observed with PCS phosphatases is clearly at variance with observations for PCM phosphatase in [22]. However, the PCM phosphatase was shown to be a mixture of PCS and AMD phosphatases [26].

The microenvironment, aspecific binding, effector metabolism, etc. could largely influence the sensitivity of the different phosphatases in vivo. Therefore, the effect of OA on phosphorylase phosphatase activity was also ascertained in crude oocyte fractions under conditions allowing selective expression of PCS and AMD phosphatase activities [33]. Basically the same picture emerges as with the purified enzymes: under these conditions the AMD phosphatase (particulate fraction incubated in the presence of deinhibitor protein and *p*NPP), displayed an I_{50} of 80 nM, that of the PCS phosphatase (high-speed supernatant incubated with protamine) being 2 nM.

Because of the importance of Tyr phosphorylation in signal transduction and since the Tyr-phosphatase activity associated with PCS phosphatases can be regulated [17–20] and represents a significant fraction of soluble Tyr-phosphatase activity in oocytes [19], we tested the effect of OA on this activity using *p*NPP or myosin light chains phosphorylated on Tyr residues. An I_{50} of 1 nM was determined for both ATP-stimulated PTPase and *p*NPPase activity of the PCS_L phosphatase and I_{50} values of 2 and 5 nM were noted for the basal PTPase and *p*NPPase activity, respectively, somewhat greater than those observed with phosphorylase *a* as substrate.

OA at concentrations up to 10 μM was without effect on commercially available *E. coli* alkaline phosphatase (type III-R, Sigma). Since the I_{50} for calcineurin is 10 μM [22], whereas the Mg^{2+}-stimulated phosphatase is unaffected by OA at the same concentration [22], the inhibitory effect of OA on isolated phosphatases in the low concentration range (< 1 μM) seems to be limited to the PCS and AMD phosphatases.

4. CONCLUSIONS

Our results suggest that a critical phosphorylation level of one (or several) protein(s) is sufficient but required to initiate meiotic maturation and convert pre-MPF into active MPF in *Xenopus* oocytes. The dose-response curves indicate that, at least in one link of the chain of events leading to meiosis induced by OA, inhibition of both PCS and AMD phosphatases is required. Our experiments do not exclude the possible involvement of the PCS and/or AMD phosphatases in signal transduction of other mitotic agents such as progesterone. In the presence of OA, the kinase/phos-

phatase equilibrium at the substrate level might be completely in favor of the kinase so that kinase activation (possibly by dephosphorylation) is no longer required to obtain a critical level of substrate phosphorylation. The very short maturation time indeed suggests that other processes could be bypassed. The high affinity of OA for PCS phosphatases implies that these enzymes could function as the receptor for OA in vivo. The fact that a tumor promoter is an inhibitor of protein phosphatases constitutes a challenge in further investigations of the importance of these enzymes in cellular control.

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