In vivo actions of protein phosphatase inhibitor-2 in *Xenopus* oocytes

J. Gordon Foulkes[+] and James L. Maller*

Department of Pharmacology, University of Colorado School of Medicine, 4200 East 9th Avenue, Box C236, Denver, CO 80262, USA

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Meiotic maturation of amphibian oocytes induced by progesterone is known to be regulated by protein phosphorylation. To investigate a possible role for protein phosphatase-1 in this process, the effect of phosphatase inhibitor-2 was determined on the in vivo rate of dephosphorylation of phosphorylase *a* and on the rate of oocyte maturation. Dephosphorylation of microinjected phosphorylase *a* was inhibited up to 40% in the presence of inhibitor-2, with half-maximal inhibition at an intracellular concentration of 0.6 μM. Inhibitor-2 also caused over a 3-fold increase in the half-time for maturation, suggesting a possible role for protein phosphatase-1 in the regulation of meiosis.

Microinjection *Protein phosphatase-1* *Inhibitor-2* Xenopus *oocyte* *Oocyte maturation* *Progesterone*

1. INTRODUCTION

As isolated from the ovary, ripe *Xenopus* oocytes in first meiotic prophase are stimulated to complete meiotic cell division by the addition of progesterone in vitro (review [1]). Hormone-stimulated oocytes progress to the 2nd meiotic metaphase, where they remain physiologically arrested until fertilization or activation. These oocytes are very large cells (1.4 mm diam.) and suitable for quantitative microinjection. The use of microinjection demonstrated that the signal releasing the oocyte from the 1st meiotic arrest is a decrease in activity of the cyclic AMP-dependent protein kinase. Thus, studies which altered the intracellular level of the free catalytic subunit [2] demonstrated that the observed decrease in cyclic AMP with progesterone was both necessary and sufficient to initiate the biological response [2–5]. As originally described in [6], progesterone inhibits the plasma membrane adenylate cyclase by a mechanism involving the guanine nucleotide regulatory protein [6–8]. Recent evidence from photoaffinity labelling studies indicates that this inhibition of adenylate cyclase is mediated by a steroid receptor on the plasma membrane of the oocyte [9].

All of these findings support the hypothesis that the prophase arrest of the ripe oocyte is maintained by maturation inhibiting phosphoprotein(s), whose phosphorylation state is controlled by the cyclic AMP-dependent protein kinase [2]. Hence, following the progesterone-induced inhibition of this enzyme, there would be a subsequent reduction in the degree of phosphorylation of the putative maturation-inhibiting protein(s) [2].

The phosphorylation state of any protein is a balance between the protein kinase and protein phosphatase activities. While it is clear that the cyclic AMP-dependent protein kinase activity is reduced by progesterone, almost nothing is known about the possible involvement of protein phosphatases in the maturation process [1]. An important question, for example, is whether progester-

* To whom reprint requests should be addressed

[+] Present address: The Center for Cancer Research, E17–517, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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one might also increase the rate of dephosphorylation of the maturation-inhibiting protein(s) due to an increase in protein phosphatase activity. This is of particular interest due to the recent findings that mammalian protein phosphatases appear to be controlled by a variety of hormones [10–17]. Enzymes of one class, termed protein phosphatase-1, have been partially characterized with respect to structure and substrate specificity, and are implicated in the regulation of a number of metabolic pathways. Protein phosphatase-1 is inhibited by nM levels of two heat-stable proteins termed inhibitor-1 and inhibitor-2 [10,20–24], while enzymes in the phosphatase-2 class are unaffected by the inhibitors [10,11,18,19]. Inhibitor-1 is inhibitory only when phosphorylated by the cyclic AMP-dependent protein kinase [20–24], while inhibitor-2 does not require phosphorylation [23,24]. Inhibitor-1 and inhibitor-2 have been shown to act as specific inhibitors of protein phosphatase-1 in a variety of mammalian tissue extracts [18–20], and the phosphorylation state of inhibitor-1 is controlled in vivo by both epinephrine [12–14] and insulin [13]. However, a conclusive demonstration that these proteins act as phosphatase inhibitors in vivo has not been made, nor has it been determined in a single cell what proportion of phosphorylase phosphatase is accounted for by the protein phosphatase-1 class. Furthermore, several reports [25–27] have led to the claim that protein phosphatase-1 is not present in 'fresh' tissue extracts, suggesting that the enzyme is artifactually generated by proteolysis during homogenization. In this paper, studies with inhibitor-2 are presented which suggest that protein phosphatase-1 is an active phosphorylase phosphatase in both oocyte extracts and in vivo in the intact living oocyte. Inhibitor-2 was also used as a probe to investigate a possible role for protein phosphatase-1 in the control of oocyte maturation. The results of these experiments are discussed in relation to the activity of protein phosphatase-1 in vivo.

2. MATERIALS AND METHODS

2.1. *Isolation of oocytes and microinjection*

Xenopus laevis females were primed with 35 IU pregnant mare's serum gonadotropin 3 days prior to decapitation and removal of ovaries. Oocytes were manually dissected from the ovarian follicle with watchmaker's forceps. Detailed procedures for isolation of oocytes, preparation and calibration of micropipets are described in [28].

2.2. *Preparation of inhibitor-2 and phosphorylase a*

Inhibitor-2 was prepared from rabbit skeletal muscle and assayed as in [23]. Prior to microinjection, the inhibitor was diluted in buffer A (10 mM Tris, 0.01% Brij 35, pH 7.0). Units of inhibitor-2 and protein phosphatase-1 activity are as in [23]. The purified material had spec. act. 83000 units/mg, corresponding to an 87% homogenous preparation [23]. Assays confirmed the inhibitor-2 preparation was free of the heat-stable inhibitor of the cyclic AMP-dependent protein kinase. All concentrations of inhibitor-2 inside the oocyte are based on 1 μl cell water and assume homogenous distribution of material after injection. ^{32}P-labelled phosphorylase *a* was prepared as in [29], and stored at 0°C in 50 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, 20% ethanediol, 30 mM mercaptoacetic acid (pH 7.4). [γ-^{32}P]ATP was synthesized as in [30]. Protein was determined as in [31].

2.3. *Oocyte maturation*

Maturation was induced by exposure of oocytes to progesterone (10 μg/ml) in medium OR2 [32] followed by incubation at room temperature. A maturation response was assessed by the frequency of germinal vesicle breakdown as evidenced by a white spot in the animal pole and confirmed by manual dissection of oocytes fixed in 5% trichloroacetic acid.

2.4. *Determination of phosphorylase phosphatase activity in vitro*

Thirty oocytes were dropped into 0.4 ml 250 mM sucrose, 4 mM EDTA, 15 mM β-mercaptoethanol, 20 mM Tris–HCl (pH 8.0, 4°C) and homogenized in a Dounce hand homogenizer. The suspension was centrifuged for 40 min at 7000 × *g*, at 4°C. The supernatant was decanted and then centrifuged (1500 × *g*, 4 min) through a Sephadex G-50 (fine) column (6.5 × 0.7 cm), equilibrated in 50 mM Tris–HCl (pH 7.6, 4°C), 1 mM EDTA, 30 mM β-mercaptoethanol (buffer B). The gel-filtered extract was diluted 60-fold before assay into buffer B containing 6 mM $MnCl_2$ and 1 mg bovine serum albumin/ml. Assays were done at 30°C. Details of the phosphorylase phosphatase assay are in [23].

2.5. *Determination of phosphorylase phosphatase activity in vivo*

Oocytes (15–30) were individually injected with 50–60 nl [^{32}P]phosphorylase *a* (>2000 cpm) with or without inhibitor-2. The zero time of the reaction was taken immediately after all the injected material had entered the cell. The reaction was terminated by dropping oocytes into 1 ml ice cold 10% trichloroacetic acid; 0.1 ml 20 mg bovine serum albumin/ml was then added, followed by centrifugation in a microcentrifuge and liquid scintillation counting of the soluble and insoluble fraction. In some cases where longer incubation times were employed, several oocytes were injected over a 30 s interval and the zero time of the reaction taken as the half-time for completion of the injection. The time between the first and last oocyte of a group being injected never amounted to >10% of the total assay time. All experiments were carried out at least 3 times. All values shown are the mean of duplicate determinations on oocytes from the same female.

3. RESULTS AND DISCUSSION

In the presence of 1 mM Mn^{2+} to optimise total phosphatase activity, the specific activity of phosphorylase phosphatase in the oocyte extracts was 1.35 ± 0.16 (n = 6) units/mg protein, a level of activity comparable to that reported for a variety of mammalian extracts [18,19]. Phosphorylase phosphatase activity in such extracts is attributed to the action of both protein phosphatase classes 1 and 2 [11,18]. In the presence of 100 units inhibitor-2, 26 ± 4% (n = 6) inhibition was observed in the oocyte extract, indicating that ~25% of the activity was due to protein phosphatase-1, and 75% to protein phosphatase-2 (the inhibitor-2 insensitive activity). This is similar to the levels of these enzymes in rabbit liver extracts [18–20]. The dephosphorylation of phosphorylase *a* proceeds in a linear fashion with time after injection into the oocyte, and the rate is significantly reduced in the presence of inhibitor-2 (fig. 1). Buffer A alone had no effect. To determine whether the inhibition was saturable, dose–response curves were carried out with increasing concentrations of inhibitor-2 injected in a constant volume (60 nl), with a constant amount of phosphorylase *a*. Inhibition was saturable, with half-maximal inhibition at an internal

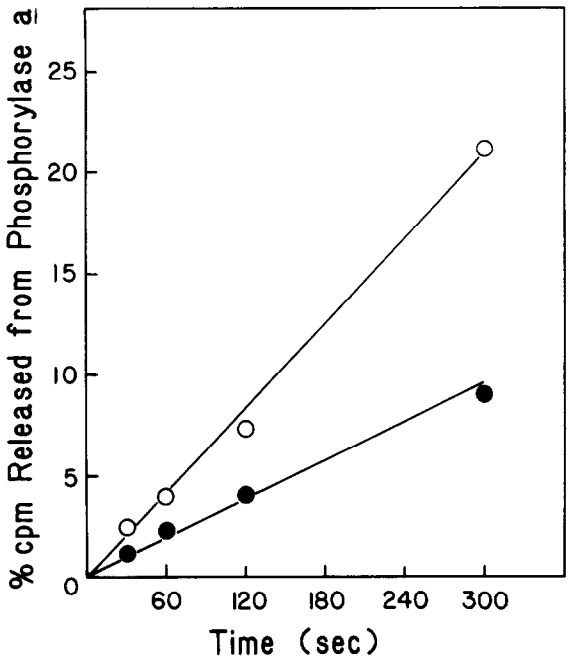

Fig. 1. Effect of inhibitor-2 on the dephosphorylation of phosphorylase *a* in vivo. [^{32}P]Phosphorylase *a* was mixed with an equal volume of buffer A; (10 mM Tris, 0.01% Brij-35, pH 7.0) (○—○) or inhibitor-2 (●—●) and the rate of dephosphorylation after microinjection determined as in section 2. The final concentration of inhibitor-2 in the cytoplasm was ~4 μM.

concentration of 0.6 μM inhibitor-2 (fig. 2). This is similar to the concentration of inhibitor-2 in rabbit skeletal muscle, 0.35 μM [23]. Saturating concentrations of inhibitor-2 resulted in a 40% decrease in the rate of phosphorylase *a* dephosphorylation, demonstrating unambiguously that inhibitor-2 can act as a phosphatase inhibitor in vivo.

The fact that inhibitor-2 acts as a saturable inhibitor of phosphorylase phosphatase in the intact oocyte suggests that only one class of phosphatase in the oocyte is subject to inhibition by inhibitor-2. We suggest that the inhibited activity corresponds to protein phosphatase-1. The possibility that the non-inhibited activity represents proteolysis has been ruled out by demonstrating all radioactivity released comigrates with P_i on thin-layer chromatography.

To determine whether protein phosphatase-1 acts on the putative maturation-inhibiting phos-

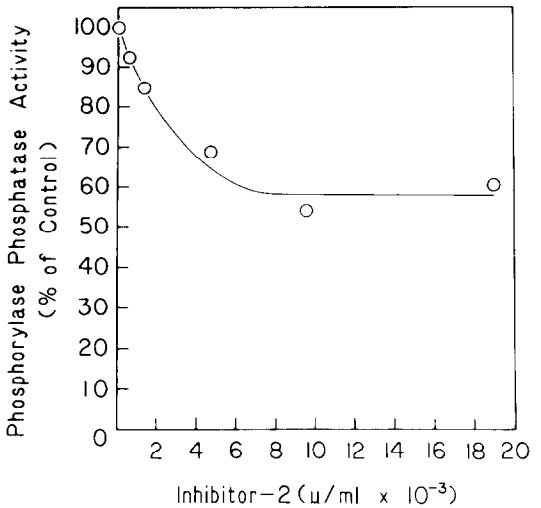

Fig. 2. Dose–response curve for inhibition of dephosphorylation of phosphorylase *a* in vivo. Oocytes (10–30) were injected with 60 nl [^{32}P]phosphorylase *a* (>2000 cpm) containing increasing concentrations of inhibitor-2. After 3 min, the reaction was terminated and the extent of dephosporylation determined as in section 2. The abscissa represents the intracellular concentration of inhibitor-2 in units/ml; 1500 units/ml corresponds to a concentration of ~0.6 μM [23].

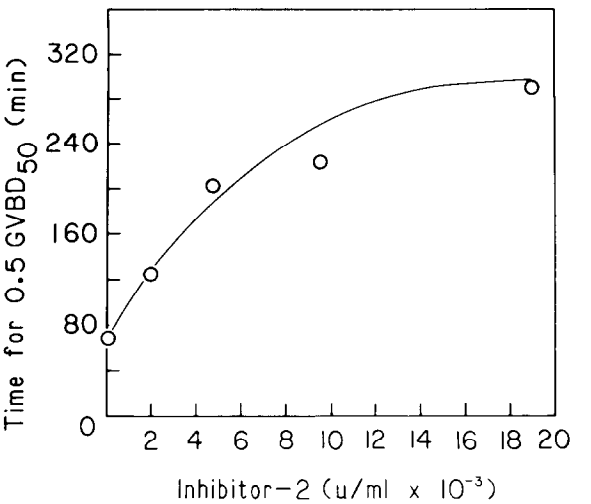

Fig. 3. Effect of inhibitor-2 on oocyte maturation. Groups of 25–30 oocytes were injected with increasing concentrations of inhibitor-2 prior to progesterone stimulation, and the time course for germinal vesicle breakdown determined. Controls were injected with buffer A. Progesterone (10 μM) was administered 15–30 min after injection of inhibitor-2, 0.5 GVBD 50 represents 50% of the time required for 50% of the responding oocytes to undergo germinal vesicle breakdown. The abscissa represents the final intracellular concentration of inhibitor-2 in units/ml.

phoprotein(s), single doses of increasing concentrations of inhibitor-2 were injected into oocytes, followed by progesterone administration. It was anticipated that if protein phosphatase-1 played an obligatory role during initial progesterone action, then injection of inhibitor-2 should block maturation. Inhibitor-2 was capable of delaying, in a dose-dependent fashion, the time required for germinal vesicle breakdown (fig. 3). Apparently saturating concentrations of inhibitor-2 delayed the half-time for 50% of the oocytes to undergo germinal vesicle breakdown by >3-fold. Half-maximal effects were seen at an internal concentration of 2.0 μM inhibitor-2. If the steady state level of a maturation-inhibiting phosphoprotein can be rapidly affected by changes in phosphatase activity and protein phosphatase-1 was the only phosphatase acting at this step, one might have predicted, a priori, that inhibitor-2 would have completely inhibited maturation, as seen with microinjection of catalytic subunit of cyclic AMP-dependent protein kinase [1,2], Complete inhibition is not observed with inhibitor-2 which suggests, but does not prove, that the hormonal control of the steady state level of the maturation-inhibiting phosphoprotein(s) is not controlled primarily by changes in phosphatase-1 activity. However, several other explanations for incomplete inhibition of maturation may also be proposed:

(1) More than one protein phosphatase could be involved, with the second enzyme being insensitive to inhibitor-2. The existence of such an enzyme(s) is indicated in fig. 2 and, in addition, Ca^{2+} also regulate oocyte maturation [1], and we have found significant levels of a calcium/calmodulin-dependent protein phosphatase-2 [33] in oocytes (unpublished).
(2) The kinetics of inhibition in vitro indicate that inhibitor-2 acts in a hyperbolic manner, suggesting that complete inhibition would be difficult to achieve;
(3) Inhibitor-2 may be inactivated with time, either by proteolysis, or perhaps in a more physiologically relevant manner [34,35].

While this work was in progress, it was reported that microinjection of the phosphorylated form of inhibitor-1 could also delay but not prevent maturation [36], similar to the results presented here for inhibitor-2. Failure to totally block maturation was interpreted as being due to a dephosphorylation of inhibitor-1. Clearly this cannot provide an explanation for the similar effects observed here with inhibitor-2. The ability of inhibitor-1 and inhibitor-2 to delay maturation supports the concept that phosphatase-1 could be involved in oocyte maturation. The resting activity of phosphatase-1 might become regulatory only in the event of reduced protein kinase activity with progesterone, or there could be a net activation of the phosphatase. Conclusive evidence for this latter possibility will require the demonstration that this enzyme is activated in response to progesterone, possibly after inhibitor-1 dephosphorylation. These considerations also lead to the prediction that microinjection of purified protein phosphatase-1 alone would be sufficient to initiate the maturation response. These predictions are currently under investigation in order to evaluate the possibility that inhibitor-1 is in fact one of the maturation inhibiting phosphoproteins.

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