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Microinjection of acylphosphatase blocks *Xenopus laevis* oocytes maturation induced by *ras*-p21

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ras proteins induce germinal vesicle breakdown (GVBD) when microinjected into *Xenopus laevis* oocytes. The mechanism of action is still unresolved, although several hypotheses have been proposed. Acylphosphatase is a cytosolic enzyme that specifically catalyses the hydrolysis of the carboxylphosphate bond of acylphosphate for the removal of acylphosphate residues of various membrane pumps. A direct effect of acylphosphatase on the regulation of ionic balance of a cell by interaction with ionic membrane pumps has been proposed. We have analyzed the effect of microinjecting acylphosphatase, by itself or along with *ras*-p21 proteins or progesterone, into oocytes. The enzyme alone is unable to induce GVBD, but increases oocyte maturation induced by progesterone. By contrast, acylphosphatase blocked GVBD induced by microinjection of oncogenic *ras*-p21. These data suggest that acylphosphatase acts synergistically or antagonistically with factors involved in proliferating signals by altering the intracellular ionic conditions of the cell, conforming the hypothesis that the intracellular ionic condition of the cell is important in the induction of proliferating signals, and that its perturbation may have a serious effect on signal transduction.

ras; Acylphosphatase; Progesterone; *Xenopus laevis* oocyte

1. INTRODUCTION

Acylphosphatase (EC 3.6.1.7) is a cytosolic enzyme catalysing the hydrolysis of organic acylphosphates [1]. It is present in mammalian tissues in two isoforms, muscular and erythrocytic [2]. The structural and functional properties of acylphosphatases purified from skeletal muscle of various vertebrate species have been investigated [3–5]; they all share similar kinetic properties and a highly conserved primary structure [5].

The biological function of acylphosphatase is still unresolved. Several studies have shown that it is able to hydrolyse the phosphoenzyme intermediate of various membrane pumps, such as Na^+/K^+-ATPase, Ca^{2+}/Mg^{2+}-ATPase and Ca^{2+}-ATPase [6–9]. This effect is mediated by dephosphorylation of an aspartate residue [10] that belongs to the highly conserved sequence, CSDK, found in several ATPases of the family of the E_1E_2-type of transport ATPases. This sequence seems to be the target for the phosphorylation–dephosphorylation mechanism of such membrane proteins resulting in the modulation of the ionic homeostasis of the cell.

Xenopus laevis oocytes represent a population of cells blocked in the G_2/M phase of the first meiotic division [11]. Various effectors, either hormonal, such as progesterone and insulin, or not, such as TPA or activated *ras*-p21 [12], can release this block by different mechanisms, inducing germinal vesicle breakdown (GVBD), a process similar to mitogenic induction of mammalian cells. In keeping with the analogy between oocytes and mammalian cells, microinjection of oncogenic *ras*-p21 proteins into fibroblasts induce synthesis of DNA [13]. This process seems to be related to the activation of tyrosine kinase receptors by growth factors, such as platelet-derived growth factor (PDGF) and insulin [14].

ras-p21 proteins belong to the superfamily of small GTPases that play a critical role in signal transduction of multiple cellular functions [15], among them the regulation of proliferation and differentiation. *ras* protein also play an important role in the generation of a large variety of tumors in animals and humans [16]. In spite of the fact that *ras* proteins have been largely investigated, elucidation of their mechanism of action still remains elusive. The possible regulatory effect of *ras* proteins on K^+ channels [17] and intracellular pH [18] has been described in mammalian cells. Since acylphosphatase may regulate some of the structural elements of ionic fluxes, we have investigated the effect of microinjection of acylphosphatase into the *X. laevis* oocyte system. Specifically we have injected purified active rat skeletal muscle acylphosphatase into oocytes stimulated by either *ras*-p21 or progesterone. Our results indicate that acylphosphatase specifically antagonizes the biological function of *ras* proteins, but increases progesterone-induced GVBD, a recognized independent mitogenic signalling pathway in oocytes.

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2. MATERIALS AND METHODS

2.1. *Oocytes preparation and microinjection*

Stage VI oocytes were obtained and microinjected as previously described [19]. Oocytes were maintained in Ringer's buffer (NaCl 100 mM, KCl 1.8 mM, $MgCl_2$ 2 mM, $CaCl_2$ 1 mM, $NaHCO_3$ 4 mM, pH 7.8) [19]. Groups of 20–30 oocytes were microinjected with indicated amounts of BSA, acylphosphatase or *ras*-p21 proteins in a final volume of 50 nl 20 mM MES, pH 7.0.

2.2. *Protein purification*

Rat skeletal muscle acylphosphatase was isolated according to Ramponi et al. [20]. Recombinant activated *ras*-p21 was isolated as previously described [19]. Both proteins were resuspended in 20 mM MES, pH 7.0.

2.3. *Maturation assay*

Oocytes were incubated after microinjection for different times at room temperature in Ringer's buffer supplemented with indicated amounts of progesterone. Oocytes were fixed in 10% trichloroacetic acid (TCA) and monitored by dissection for the disappearance of the germinal vesicle (GVBD) under a Zeiss stereomicroscope GSZ.

2.4. *Acylphosphatase activity assay*

Oocytes were either injected with 10 ng of acylphosphatase or with 10 ng of BSA, both being dissolved in 20 mM MES, pH 7.0. At indicated times after microinjection, different sets of oocytes were treated by the acetone powder method [21]. The powder obtained was finally dissolved in ice-cold 0.1 M sodium acetate, pH 5.3, and centrifuged. Acylphosphatase activity was analyzed in the supernatants with 5 mM benzoylphosphate as substrate [22].

3. RESULTS AND DISCUSSION

3.1. *Stability of injected acylphosphatase after microinjection*

The soluble fraction of *X. laevis* oocytes was obtained by the acetone powder method (see section 2). As previously reported [1], due to its structural properties, the acylphosphatase enzyme conserves its activity after purification by this method providing a rapid method for detection and analysis of acylphosphatase activity in *X. laevis* oocytes using the benzoylphosphate assay [22]. Both acylphosphatase and control, BSA-injected oocytes were tested. As shown in Fig. 1, the oocytes injected with active enzyme showed about 250-times more activity than control, BSA-injected oocytes at 10 min or 18 h after injection. The homogenates assayed at longer times after microinjection showed a slight decrease in acylphosphatase activity compared to the oocytes injected and processed at 10 min. The decrease in activity was less than 20% of the total, demonstrating that injected acylphosphatase remains stable and enzymatically active for several hours after injection, sufficient to ascertain its biological activity in the *X. laevis* oocyte system.

3.2. *Biological effect of acylphosphatase injection*

We then investigated whether acylphosphatase by itself was able to participate in signal transduction processes by inducing GVBD when microinjected into the oocytes. Different amounts of either acylphosphatase or BSA were injected, and the disappearance of the germinal vesicle analyzed by split-open, TCA-fixed oocytes after 20 h of incubation in Ringer's buffer. As shown in Table I, no increase in the GVBD rates was detected in acylphosphatase-injected oocytes compared to the control, BSA-injected oocytes. These results suggest that acylphosphatase does not play a significant role in the induction of GVBD by itself; however, the putative implication of acylphosphatase in the regulation of ionic cellular balance, particularly on Ca^{2+} homeostasis, has been investigated, indicating that acylphosphatase affects Ca^{2+} entry into the oocytes by inhibiting the function of the ionic machinery [23]. Thus, acylphosphatase could either enhance or interfere with the signalling process of other well-known initiators of oocyte maturation.

Table I

Effect of microinjection of acylphosphatase into *X. laevis* oocytes

	Microinjection (ng/oocyte)	% GVBD
Control*	–	10 ± 2
BSA	10	14 ± 3
	170	14 ± 3
Acylphosphatase	10	16 ± 1
	170	14 ± 3
ras-p21	30	95 ± 5

Oocytes were selected and microinjected with the indicated proteins in 50 nl of 20 mM Mes, pH 7.0, as described in section 2. GVBD was analyzed 20 h after injections as described.
*Not microinjected.

3.3. *Effects of acylphosphatase injection on* ras-*p21 and progesterone-induced GVBD*

It has been reported that injection of *ras*-p21 protein into *X. laevis* oocytes is able to induce GVBD [12]. The

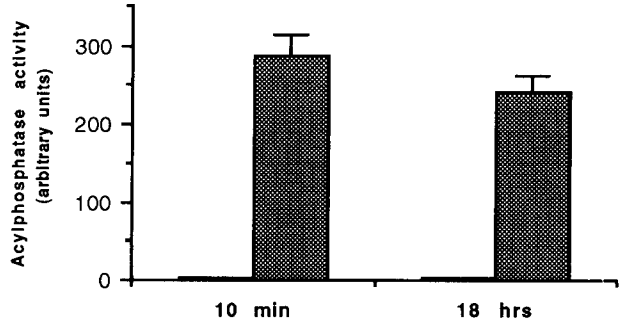

Fig. 1. Acylphosphatase activity assay. Activity of homogenates from oocytes previously injected with either 10 ng/oocyte BSA or 10 ng/oocyte acylphosphatase. The assays were carried out by incubating the oocyte homogenates for 30 min in 5 mM benzoylphosphate as substrate. Each point, consisting of sets of 5 oocytes, was normalized to 10 μg of protein. The results are expressed as times of activity of homogenates above the spontaneous hydrolysis of benzoylphosphatate as control (▨) BSA-injected oocytes. (■) Acylphosphatase-injected oocytes.

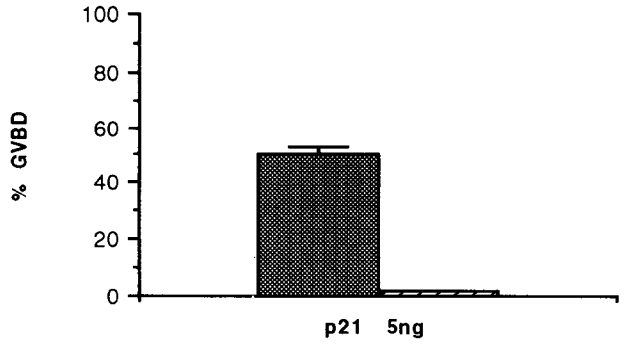

Fig. 2. Effect of acylphosphatase on GVBD induced by *ras*-p21. Oocytes were microinjected with the indicated amounts of *ras*-p21 plus BSA (■) or plus acylphosphatase (▨), and analyzed after 20 h of incubation in Ringer's buffer at room temperature.

mechanism of action of *ras* oncogene in *X. laevis* oocytes is still unclear, however *ras* oncogene pathways have been widely investigated in eukaryotic cell systems. Thus, it has been suggested that ionic fluxes may somehow be regulated by *ras* protein through its GTPase-activating protein, GAP-*ras* [24]. Particularly a rise of intracellular pH can be observed after microinjection of activated *ras* protein [18], and a decrease of K^+ fluxes through the plasma membrane can be observed in cells injected with *ras*-p21 [17].

A dose–response curve was performed using a *ras*-p21 protein constitutively activated by two point mutations at Val^{12} and Thr^{59} [25] to calculate the concentration of protein injected required to obtain semi-maximal rates of GVBD ($GVBD_{50}$) at 20 h of incubation. 5 ng/oocyte of the *ras* oncoprotein was found to give a $GVBD_{50}$ at the indicated time point (data not shown).

Thus, we microinjected 5 ng/oocyte of *ras*-p21 along with 85 ng/oocyte of purified rat skeletal muscle acylphosphatase in a final volume of 50 nl/oocyte. As shown in Fig. 2, *ras*-induced GVBD was totally abolished by acylphosphatase. To investigate further if the blocking action of acylphosphatase was due to a specific or non-specific effect of the enzyme in the *X. laevis* oocyte system, we performed a control experiment using progesterone to induce GVBD.

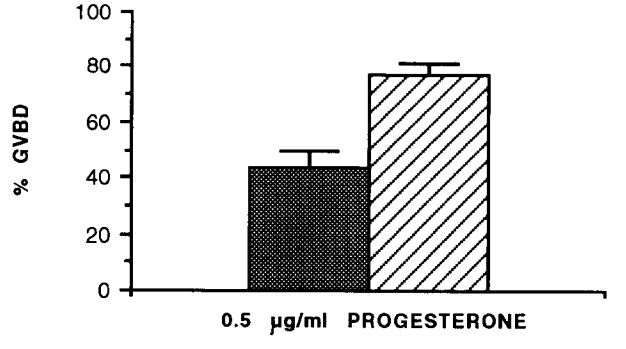

Fig. 3. Effect of acylphosphatase on progesterone-induced GVBD. Oocytes were microinjected with the indicated amounts of BSA (■) or acylphosphatase (▨), and then treated with 0.5 µg/ml progesterone. The rate of GVBD was observed after 20 h incubation in Ringer's buffer.

For comparison with the results obtained by co-injection of *ras*-p21 and acylphosphatase, a dose–response curve was carried out with progesterone to obtain the $GVBD_{50}$. The optimal dose for a $GVBD_{50}$ at 20 h of incubation was found to be 0.5 µg/ml (data not shown). When the same amount of acylphosphatase as used with the *ras*-p21 protein was injected in 50 nl/oocyte, and the oocytes were treated with 0.5 µg/ml of progesterone, an increase in GVBD by acylphosphatase microinjection was observed (Fig. 3). The effect of progesterone treatment on *X. laevis* oocytes has been widely investigated [11,26,27], but nevertheless the mechanism of action of this hormone in *X. laevis* oocytes is still not fully understood. It is accepted, however, that progesterone-induced GVBD follows a different pattern from that induced by *ras*-p21 [28]. These results suggest that acylphosphatase action on *ras*-induced GVBD is not due to non-specific blockage of cellular metabolism.

In this study we have carried out microinjection experiments in *X. laevis* oocytes to study the effects of acylphosphatase on *ras*-induced GVBD. Previous reports have suggested that acylphosphatase may affect the regulation of ionic homeostasis of the cell by controlling membrane ATPases [6–9]. We show that this enzyme may act on intracellular signalling pathways with different results, since acylphosphatase blocked *ras*-induced GVBD but increased that induced by progesterone. Thus, our results suggest an important role for acylphosphatase on the regulation of cell signalling. As previously described, ionic homeostasis of the cell could cooperate in the biological function of *ras* proteins, particularly the regulation of intracellular pH [18] and K^+ fluxes [17]. Our results strongly support this hypothesis by using an enzyme involved in the regulation of ionic fluxes. Further research using this system will be useful for a better understanding of the involvement of membrane pumps in the biological function of *ras* proteins both in the oocyte system and in mammalian cells.

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