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Acylphosphatase synergizes with progesterone during maturation of *Xenopus laevis* oocytes

Fabrizio Dolfi[a], Amancio Carnero[a], Antonio Cuadrado[a], Giampietro Ramponi[b] and Juan Carlos Lacal[a]

[a]*Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid, Spain* and [b]*Universitá degli Studi di Firenze, Dipartimento di Scienze Biochimiche, Viale Morgagni 50, 50134 Firenze, Italy*

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Xenopus laevis oocytes are physiologically arrested in the G2/M phase border of the first meiotic division. A number of different stimuli can trigger off the re-entry into the cell cycle as a consequence of activation of either membrane-dependent or -independent intracellular signals. This system has been widely used to study signal transduction mechanisms induced by hormones. Among those more intensively researched, special attention has been devoted to elucidate the mechanism of activation induced by progesterone. However, despite intense efforts to understand the intracellular signalling mechanism of progesterone, a clear notion of the most relevant events involved in this process has not yet been elucidated. We provide evidence that acylphosphatase, an enzyme responsible for the regulation of membrane pumps in eukaryotic cells, synergizes with progesterone for induction of oocyte maturation. We deduced that this synergism may be related to the regulation of intracellular Ca^{2+} levels for several reasons: (1) maturation of oocytes by extracellular Ca^{2+} is blocked by acylphosphatase; (2) both progesterone and acylphosphatase drastically reduced Ca^{2+} uptake; (3) progesterone-induced maturation does not depend on a rise in intracellular Ca^{2+}, since microinjection of EGTA, a calcium chelator, does not affect maturation induced by progesterone.

Oocyte maturation; Calcium; Progesterone; Acylphosphatase

1. INTRODUCTION

Acylphosphatase (EC 3.6.1.7) is a hydrolase, which specifically catalyzes the hydrolysis of the carboxylphosphate bond of acylphosphates. In vitro, it can hydrolyze several natural products such as 3-phosphoglyceroyl phosphate [1], carbamoyl phosphate [2] and succinoyl phosphate [3]. In addition to these soluble, low molecular weight substrates, acylphosphatase can hydrolyze with high affinity the phosphoenzyme intermediate of various membrane pumps, particularly the Ca^{2+}-ATPase [4] and NA^{+}/K^{+}-ATPase [5] from red blood cell membranes, and the Ca^{2+}/Mg^{2+}-ATPase [6] from sarcoplasmic reticulum of skeletal muscle. Acylphosphatase is a widespread cytosolic enzyme found in two isoenzymatic forms in mammalian tissues, one prevalent in skeletal muscle (for the three-dimensional structure see [7]), and a second in red blood cells. The functional properties of the enzyme isoforms purified from skeletal muscle from different vertebrate species have been investigated [8–12]. All these isoforms have similar kinetic properties and a highly conserved primary structure. The isoenzyme from human red blood cells has also been purified and investigated [13]. It exhibits similar substrate specificity but higher catalytic activity when compared to the muscular isoform.

Xenopus laevis oocytes represent a population of cells physiologically arrested in G2/M phase border of the first meiotic division. They can be induced into the mitotic event as unfertilized eggs by in vitro stimulation with different substances. Oocytes are highly responsive to hormones, such as progesterone [14] and insulin [15], phorbol esters [16] and different ions [17]. Intense research has been carried out to elucidate the intracellular events following interaction of progesterone with its receptor. However the precise mechanism that involves oocyte maturation after progesterone treatment is still not clear. It has been generally accepted that progesterone initiates the maturation signal interacting with the oocyte membrane, followed by a rapid decrease in the levels of cAMP [18]. Boyer et al. demonstrated that progesterone stimulation causes a decrease in the phosphorylation of two proteins of 20 and 32 kDa, probably mediated by inactivation of the cAMP-dependent protein kinase [19]. It has been postulated that progesterone maturation could also implicate a ring of cytosolic free calcium release [20] although a rise in free calcium may not be necessary for progesterone-induced maturation [21]. Furthermore we had to consider the fact that a high level of free calcium in the incubation medium itself is able to remove the meiotic blockage, leading to oocyte maturation [22–24].

Correspondence address: J.C. Lacal, Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid, Spain. Fax: (34) (1) 585 47 85.

We have investigated the putative requirement of free Ca^{2+} for progesterone-induced maturation. Purified acylphosphatase from rat skeletal muscle was microinjected in *Xenopus laevis* oocytes and the effect on germinal vesicle breakdown (GVBD) induced by both high extracellular Ca^{2+} levels and progesterone were analyzed. In our study we have shown that acylphosphatase potentiate progesterone action. Furthermore with the injection of enzyme we obtained a decrease in the Ca^{2+}-induced maturation of the oocytes. Analysis of the effect of acylphosphatase and progesterone on $^{45}Ca^{2+}$ intake into the oocytes, showed that both of them caused a significant decrease in the intake of the ion. Finally, we show that progesterone does not need intracellular Ca^{2+} to cause GVBD. Thus, our results suggest that maturation of the oocytes by progesterone is calcium-independent.

2. MATERIALS AND METHODS

2.1. *Oocytes preparation and microinjection*

Stage VI oocytes were obtained by manual dissection from pieces of ovary surgically removed from adult *Xenopus laevis* females as previously described [25]. Oocytes were maintained at room temperature in Ringer's buffer (100 mM NaCl, 1.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM $NaHCO_3$, pH 7.8). Groups of 20–30 oocytes were microinjected as described [25] with indicated amounts of BSA or purified rat skeletal muscle acylphosphatase contained in 50 nl of 20 mM MES pH 7.0, or with 50 nl of either 20 mM MES, pH 7.0 or 20 mM MES, 50 mM EGTA, pH 7.0.

2.2. *Maturation assay*

Oocyte maturation was analyzed in 24-well plastic plates containing 2 ml of the appropriate medium. Each group was incubated after microinjection or treatment at room temperature for different times in Ringer's buffer containing indicated concentrations of progesterone or $CaCl_2$. After evaluation of the external phenotype (appearance of the white spot in the animal pole) under a Zeiss stereomicroscope SV8, oocytes were fixed in 10% trichloroacetic acid and monitored for the disappearance of the germinal vesicle (GVBD) by dissection of fixed oocytes.

2.3. *Acylphosphatase activity assay*

Purified active rat skeletal muscle acylphosphatase was prepared according to Ramponi et al. [12]. Oocytes microinjected with acylphosphatase and BSA as indicated above were incubated in Ringer's buffer and homogenated at indicated time points with modified acetone powder method [26] to achieve partial purification of the enzyme. After centrifugation in microfuge at 12 krpm, pellets were dissolved in 0.1 M sodium acetate pH 5.3 and centrifuged to remove precipitated material. Acylphosphatase activity was determined assaying the supernatant with 5 mM benzoyl-phosphate as previously described [27].

2.4. *$^{45}Ca^{2+}$ uptake assay*

Groups of 10 oocytes were microinjected with either BSA or acylphosphatase in duplicate samples. Oocytes were then washed and incubated in Ringer's buffer containing $^{45}Ca^{2+}$ in a final concentration of 30 mM $CaCl_2$, supplemented with or without 1 μg/ml progesterone. After incubation, oocytes were washed three times in Ringer's buffer, lysed in 0.5 M NaOH and counted by liquid scintillation for $^{45}Ca^{2+}$ incorporation. Each point was normalized to the total protein content of the samples. The experiment was repeated three more times with similar results.

3. RESULTS

3.1. *Acylphosphatase activity after microinjection in* X. laevis *oocytes*

The enzymatic activity of acylphosphatase purified from rat skeletal muscle can be readily quantified by an in vitro assay, using benzoyl-phosphatase as substrate [27]. To set up the conditions of an appropriate assay for the exogenous enzyme, we first investigated the levels of endogenous acylphosphatase activity in the soluble fraction of homogenate from *X. laevis* oocytes. As shown in Fig. 1, stage VI oocytes had no detectable hydrolyzing activity on acylphosphates measured with

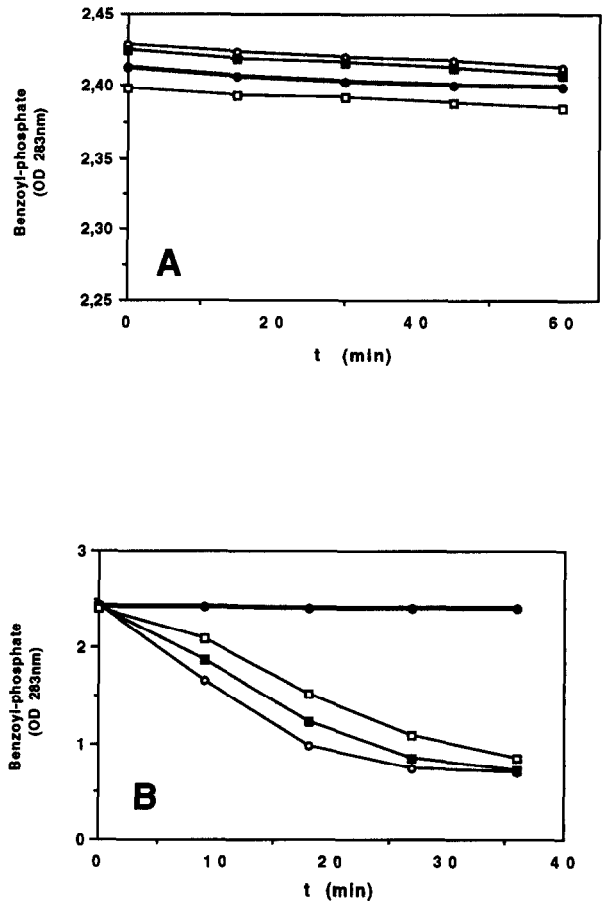

Fig. 1. Acylphosphatase activity assay. Oocytes were treated as described in section 2. Incubations were carried out in Ringer's buffer at room temperature for different times. Acylphosphatase activity was measured using 5 mM benzoyl-phosphate as substrate [27] at indicated time points. The assay was carried out with sets of 5 oocytes incubated for different times after microinjection (see below). Each point was normalized to the total amount of protein and referred to 10 μg of total protein. (A) Basal acylphosphatase activity of the homogenate from oocytes previously microinjected with BSA as control. The activity shown was comparable to the spontaneous hydrolysis of benzoyl-phosphate. (B) Hydrolyzing activity of the homogenate from oocytes previously injected with active acylphosphatase (10 ng/oocyte). (●), spontaneous hydrolysis of the substrate (without enzyme); (○), hydrolysis at 10 min incubation; (■), 1 h incubation; and (□), 18 h incubation.

5 mM benzoyl-phosphate as substrate. After microinjection of the active acylphosphatase purified from rat skeletal muscle (Fig. 1B), the soluble fraction of the oocyte homogenate displayed a readily detectable acylphosphatase activity. This activity was related to the exogenously injected enzyme, since control oocytes microinjected with equivalent amounts of bovine serum albumin (BSA) showed no activity (Fig. 1A). The enzymatic activity of microinjected acylphosphatase was detected in oocyte extracts after 18 h of injection, suggesting that the enzyme was stable and active even at this late time after microinjection.

The biological activity of microinjected acylphosphatase only was also investigated. Different amounts of the enzyme were microinjected into oocytes, and GVBD was followed up to 20 h of injection. Under these conditions, no significant increase in GVBD was observed when compared to control BSA-injected oocytes (Table I), indicating that microinjected acylphosphatase had no significant signalling activity by itself in this system, despite conservation of enzymatic activity.

3.2. *Acylphosphatase blocks Ca^{2+}-induced GVBD*

It has been previously reported that high concentrations of calcium in the incubation medium induce GVBD in *Xenopus laevis* oocytes [22–24]. We have also observed this effect as a dose-dependent induction of GVBD by exogenous Ca^{2+}. At 20 hours incubation the increased rate of GVBD was observed starting at 30 mM Ca^{2+} (Fig. 2), with a value of 75% maturation and increased at 50 mM, reaching 97% GVBD. Similar results were observed also at shorter times of incubation (data not shown). When low amounts (10 ng/oocyte) of acylphosphatase were microinjected, a drastic change in the response rates to the calcium treatment was observed at 20 h of incubation (Fig. 2). Oocytes injected with acylphosphatase showed a reduction of about 60% of GVBD at 30 mM calcium. At concentrations higher than 30 mM Ca^{2+}, maturation values were similar for both acylphosphatase- and BSA-microinjected oocytes (Fig. 2).

Table I

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

	Microinjection (ng/oocyte)	% GVBD
Not microinjected	–	10 ± 2
BSA	10	10 ± 3
	170	10 ± 3
Acylphosphatase	10	10 ± 3
	170	12 ± 5

Oocytes were selected as described and then microinjected with indicated amounts of either control BSA or acylphosphatase in groups of 20–30 oocytes per point. Disappearance of the germinal vesicle was analyzed at 20 h of injection. The data shown are from a single experiment repeated three times with similar results.

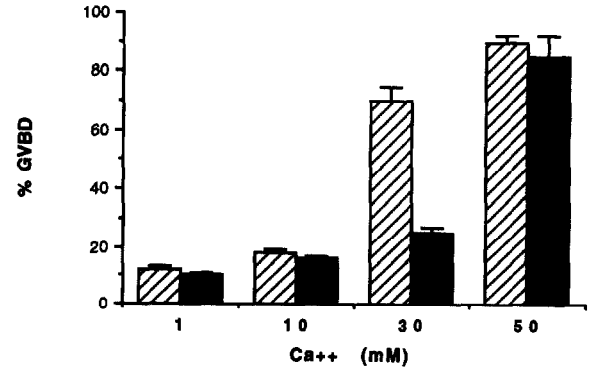

Fig. 2. Effect of microinjection of acylphosphatase on calcium-induced maturation. GVBD was analyzed after 20 h incubation at room temperature. Each assay was obtained adding respectively 1, 10, 30, 50 mM $CaCl_2$ to the Ringer's buffer containing no calcium (see section 2). For each point groups of 20–30 oocytes were injected with either BSA, as control (□) or with 10 ng/oocyte acylphosphatase (■), both proteins in 20 mM MES pH 7.0. These results were found to be representative of a series of three different experiments.

3.3. *Synergism between progesterone and acylphosphatase*

Progesterone treatment can remove the meiotic blockage of unfertilized *Xenopus laevis* oocytes. Using 0.5–1 μg/ml progesterone, GVBD is fully detectable by the external appearance of a little white spot in the animal pole of the oocyte after 7–8 h of in vitro stimulation. On the other hand, a mature oocyte lack germinal vesicle when this is split open after 10% trichloro acetic acid (TCA) fixation. Since acylphosphatase can interfere with induced maturation by Ca^{2+}, we reasoned that microinjection of the enzyme in progesterone-treated oocytes could be an excellent tool to discriminate the putative role of intracellular Ca^{2+} in progesterone action. Thus we investigated whether progesterone was able to induce oocyte maturation at physiological Ca^{2+} concentrations in the presence of acylphosphatase. Oocytes were injected with 17 ng/oocyte of purified rat skeletal muscle acylphosphatase and then treated with 0.5 μg/ml progesterone. GVBD was fully detectable in acylphosphatase-injected oocytes at short times of stimulation (5 h) when compared to control, BSA-injected oocytes (Fig. 3). Microinjection of 170 ng/oocyte of acylphosphatase also enhanced progesterone induced GVBD (data not shown). As acylphosphatase was observed to increase oocyte maturation by progesterone and also to decrease Ca^{2+}-induced GVBD we used this enzyme to examine the role of calcium in the progesterone maturation pathway.

3.4. *Effects on Ca^{2+} homeostasis by acylphosphatase and progesterone*

The above results suggested that acylphosphatase

could somehow interfere with Ca^{2+} entry into the oocyte, since it blocked external Ca^{2+}-induced maturation at 20 hours stimulation. Thus, we directly investigated this hypothesis by measuring $^{45}Ca^{2+}$ uptake in *X. laevis* oocytes after microinjection of either control BSA or acylphosphatase. Fig. 4 shows that, indeed, microinjection of the acylphosphatase drastically inhibited Ca^{2+} uptake. Oocytes treated with progesterone also exhibited a drastic inhibition of Ca^{2+} uptake, consistent with the finding that acylphosphatase potentiated this progesterone action. Thus, both acylphosphatase injection and progesterone treatment show the same effects on extracellular calcium intake by *X. laevis* oocytes.

3.5. *Effects of intracellular calcium release on progesterone-induced maturation*

An increase in intracellular free Ca^{2+} can be achieved either by entering the cell from the extracellular medium or by its release from the intracellular stores. The above results indicate that extracellular Ca^{2+} is not required for oocyte maturation induced by progesterone. Thus, we investigated whether intracellular Ca^{2+} could account for a putative Ca^{2+} requirement in progesterone action. For this reason, we first microinjected EGTA (a Ca^{2+} chelator) into oocytes, to a final intracellular concentration of 5 mM. Oocytes were then treated with a high extracellular calcium concentration (Fig. 5A) or progesterone (Fig. 5B), and then GVBD was determined. Under these conditions, progesterone was equally effective (Fig. 5B) while the effect of Ca^{2+} treatment was reduced by EGTA microinjection, indicating that intracellular Ca^{2+} due to release from the intracellular stores is not relevant for the role of progesterone.

4. DISCUSSION

Oocytes from *X. laevis* are arrested at the G2/M border of the first meiotic prophase. Release from prophase and entry into the cell cycle can be physiologically triggered off by progesterone or insulin, with the induction of the germinal vesicle breakdown (GVBD). This maturation process is similar, from the biochemical point of view, to that observed when cultured cells are stimulated by growth factors. Among the best characterized alterations found during maturation of *X. laevis* oocytes, the most significant ones include activation of a cascade of serine-threonine kinase, including *cdc2*, MAP kinase and S6 kinase II, as well as a reduction of the cAMP levels with the consequent reduction on protein kinase A activity [18]. Recently, it has been reported that phosphorylation/dephosphorylation events associated with the regulation of the Maturation-Promoting Factor plays a critical role in the transition from G2 to M phase [18].

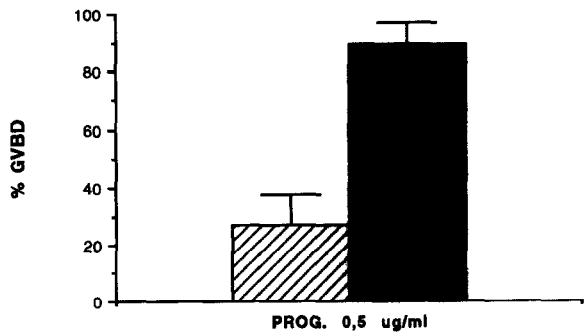

Fig. 3. Effect of acylphosphatase on progesterone induced GVBD. Oocytes were selected as described, and microinjected with either 17 ng/oocyte BSA (□), or 17 ng/oocyte of acylphosphatase (■) and then treated with progesterone (0.5 μg/ml). The rate of GVBD was estimated after 5 h of incubation in the presence of progesterone. The results shown were obtained from three different experiments.

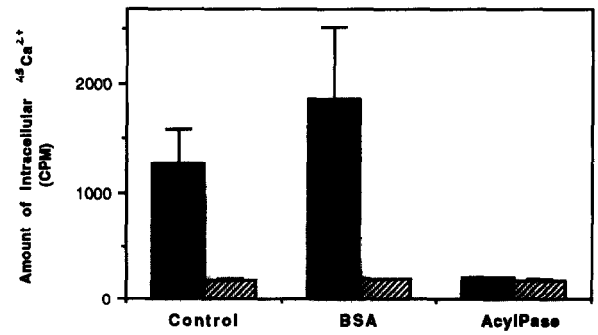

Fig. 4. Effect of progesterone treatment or acylphosphatase microinjection on $^{45}Ca^{2+}$ uptake. Different sets of 20–30 oocytes were either not microinjected (control), or microinjected with either BSA or acylphosphatase, as indicated in section 2. These sets of oocytes were either treated with 1 μg/ml of progesterone (□) or were left untreated (■). Analysis of $^{45}Ca^{2+}$ uptake was performed as described in section 2. Each point was normalized to the total protein content of the sample. The experiment was repeated three times with similar results.

Apart from the intensive research on phosphorylation/dephosphorylation events in *X. laevis* maturation, there is abundant evidence which suggests that the regulation of ionic fluxes also plays an important role in this process. Thus, it has been shown that changes in the ionic composition of the incubation medium can modulate oocyte maturation [21]. In particular, an increase in the extracellular Ca^{2+} concentration alone, can induce GVBD [22–24]. However, a rise in cytosolic calcium in progesterone-induced maturation is still controversial [21].

Regulation of the ionic content in most cells is accomplished by specialized enzymatic systems. Among these, Na^+/K^+-ATPase, Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase belong to the E_1E_2 type of transport ATPases which form a phosphoderivative of aspartic acid residues, an acylphosphate, as its functional intermediate [28–30]. The Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase are membrane systems that pump Ca^{2+} ions out off the cytoplasm into the endoplasmic reticulum, coupling calcium transport to ATP hydrolysis. These pumps allow the cell to maintain the intracellular calcium concentration

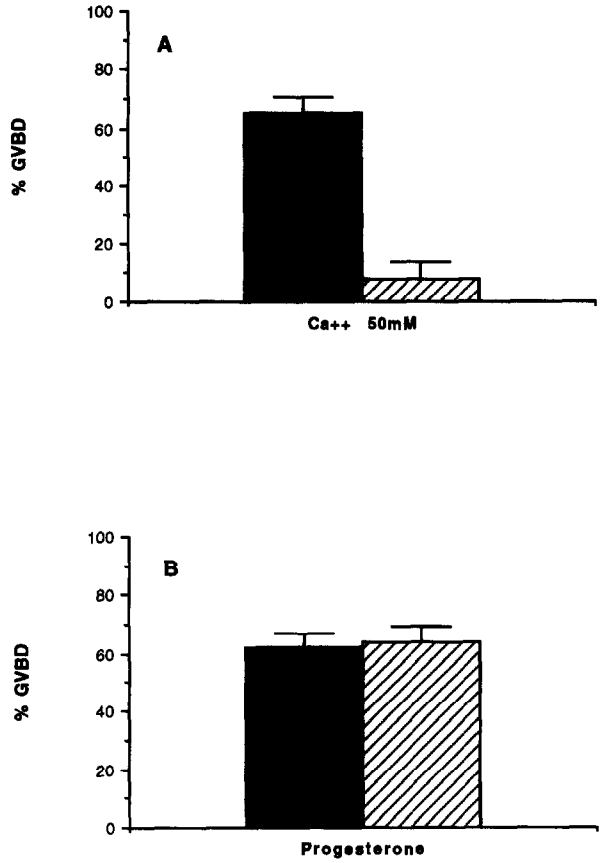

Fig. 5. Effects of microinjecting EGTA on progesterone- and calcium-induced oocyte maturation. Oocytes were microinjected with 50 nl of 20 mM MES pH 7.0 (■) and with a buffer containing 50 mM EGTA (□). The estimated final intracellular concentration of EGTA was 5 mM. Oocytes were then treated with 50 mM $CaCl_2$ (A) or progesterone (1 μg/ml) (B). GVBD was analyzed 20 h after treatment. The results were obtained from three different experiments. Maturation rates are expressed as total percentage of GVBD above basal levels.

below or near the micromolar range in spite of the very high extracellular concentration of free calcium (in the millimolar range) [31]. Other mechanisms are also available to regulate intracellular calcium levels. All of them need the action of Na^+/K^+-ATPase which is important for the control of the whole ionic homeostasis of the cell [32].

Acylphosphatase is a cytosolic enzyme which has been shown to specifically hydrolyze the carboxylphosphate bond of acylphosphate. The action of acylphosphatase on the phosphorylated intermediate of various membrane pumps was shown, particularly on the Na^+/K^+-ATPase [5] the Ca^{2+}-ATPase [4] and the Ca^{2+}/Mg^{2+}-ATPase [6].

Acting on these transporting systems, acylphosphatase may regulate the ionic intracellular environment of the cell, with a particular importance regarding calcium homeostasis. This enzyme may act either directly or indirectly regulating calcium levels: directly by acting on the calcium pumps, indirectly by acting on the Na^+/K^+-ATPase.

We have attempted to clarify the role of calcium ions in progesterone-induced GVBD in *X. laevis* oocytes, by microinjecting purified active acylphosphatase (rat skeletal muscle isoform) under appropriate conditions. Our results demonstrate that acylphosphatase reduces GVBD induced by high extracellular Ca^{2+} concentrations, indicating that indeed this enzyme directly or indirectly affects the entry of extracellular Ca^{2+}. We also have shown a synergistic effect of acylphosphatase for the biological activity of progesterone in this system. Finally, we conclude that Ca^{2+} is not required for progesterone function since (1) acylphosphatase and progesterone induce a blockage of extracellular Ca^{2+} uptake, and (2) progesterone does not require Ca^{2+} release from intracellular stores.

The above results also indicate that microinjection of exogenous acylphosphatase into *X. laevis* oocytes can be used to unmask the putative physiological role of regulated ionic pumps into signal transduction mechanisms, such as those triggered by hormones. Using this approach, we have been able to demonstrate that progesterone action is independent of Ca^{2+}.

Further research using this system will be useful to discriminate the involvement of other ionic fluxes in the biological function of progesterone and other physiological hormones in the oocyte system.

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