Pertussis toxin facilitates the progesterone-induced maturation of *Xenopus* oocyte

Possible role of protein phosphorylation

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Progesterone triggers the first meiotic cell division of *Xenopus* oocyte and inhibits cAMP synthesis. The effect of pertussis toxin purified from *Bordetella pertussis* was tested on the maturation of *Xenopus* oocyte. The toxin did not inhibit progesterone-induced resumption of meiosis or the hormone-induced drop in cAMP level. This indicates that progesterone action is not mediated by the N_i subunit of the oocyte adenylate cyclase. Furthermore, pertussis toxin caused a reduction in the time course of maturation correlated with the precocious appearance of an alkali stable 47 kDa phosphoprotein, a marker of the maturation promoting factor (MPF) activity. Pertussis toxin effects mimicked those of 2-glycerophosphate suggesting that both agents act on the steady-state level of phosphorylation implicated in MPF activity.

Oocyte   Progesterone   Pertussis toxin   Phosphorylation

1. INTRODUCTION

Progesterone reinitiates the first meiotic cell division (meiotic maturation) in the full-grown *Xenopus* oocyte by a post-transcriptional mechanism [1]. The action of progesterone can be mimicked by microinjection of the regulatory (R) subunit of cAMP-dependent protein kinase or of the protein inhibitor (PKI) of its catalytic (C) subunit [2,3]. Microinjection of the C subunit inhibits subsequent progesterone-induced maturation [2]. It was therefore concluded that a drop in the activity of the C subunit is a necessary and sufficient step involved in the mechanism of progesterone action. On the other hand, it is well established that the steroid hormone inhibits in vivo [4] as well as in vitro [5-7] the oocyte adenylate cyclase. Therefore, a hypothetical sequence of biochemical events for progesterone action would be: steroid → cAMP → C subunit →

putative phosphorylated protein(s) → MPF (maturation promoting factor).

A major feature in this scheme is the elucidation of the mechanism by which progesterone controls the oocyte adenylate cyclase. Here, we have investigated the effect of pertussis toxin on progesterone-induced maturation. Pertussis toxin isolated from *Bordetella pertussis* abolishes the inhibition of adenylate cyclase controlled by hormones or neurotransmitters in a variety of systems; the toxin acts via ADP ribosylation of the guanine nucleotide inhibitory protein, N_i [8,9].

2. MATERIAL AND METHODS

Xenopus laevis adult females were obtained from SEREA-CNRS (France). [^{32}P]Orthophosphate (carrier free, 10 mCi/ml) was from Amersham (France). Progesterone, purified cholera toxin and 2-glycerophosphate (disodium salt) were

purchased from Sigma. 3-Isobutyl-methylxanthine (IBMX) and sodium orthovanadate were from Aldrich.

Colonies of *B. pertussis* strain 509-22 (Institut Pasteur) grown on Bordet-Gengou agar plates were incubated in 200 ml of Verwey et al. medium [10] and cultured under gentle stirring (48 h, 35°C). The culture was inoculated in a fermentor containing 40 l of medium and grown at 35°C with stirring for 48 h. The supernate was recovered and concentrated 30-fold by ultracentrifugation on Amicon Hollow Fiber Hx1 (DC-2 apparatus, Amicon, Lexington, MA). The retentate contained practically all 'LPF' and 'HSF' activities tested according to standard methods [11]. Toxin purification was performed according to [12] and [11], by affinity chromatography on haptoglobin with the following modifications. A 15-ml aliquot of the crude toxin (~2 mg protein/ml) was absorbed in a plastic column on 30 ml of a gel equilibrated in buffer 1 (0.1 M sodium phosphate, pH 7.0), and prepared by coupling haptoglobin (purified from human plasma) [13] to AH-Sepharose 4B with glutaraldehyde. The column was successively eluted with 100 ml of buffer 1, 100 ml of butter 2: 0.1 M Tris–HCl (pH 10.0), 0.5 M NaCl and 100 ml of buffer 2 containing 3 M KSCN. The effluent eluted by the last buffer contained almost all LPF and HSF activities. It was concentrated 50-fold on Amicon PM-10 membrane. This material (~2.5 ml, 390 μg protein/ml) constituted the purified PT preparation used here.

Ovaries were surgically removed from MS 222 (1 g/l, Sandoz) anaesthetized females and transferred in medium A: 88 mM NaCl, 0.33 mM Ca(NO$_3$)$_2$, 1 mM KCl, 0.41 mM CaCl$_2$, 0.82 mM MgSO$_4$, 2 mM Tris–HCl (pH 7.4). Stage VI oocytes [14] were collected after dispase and collagenase digestion as reported in [3].

Usually 30 oocytes per 2 ml medium A were induced to mature by addition of 1 μM progesterone to the external medium or by microinjection into the oocytes of 50 nl of MPF containing cytoplasm preleved from progesterone-matured oocytes.

The criterion for maturation was the apparition of a white spot surrounded by pigment on the colored animal pole of the oocyte.

Oocyte cAMP content was determined by a protein binding assay method as already described [15].

Oocytes were labelled by incubation for 16 h in medium A (50 oocytes/ml) containing 500 μCi/ml ^{32}PO$_4$. They were then thoroughly washed, transferred in medium A and treated by pertussis toxin or 2-glycerophosphate as indicated. At the end of incubation oocytes were homogeneized (usually 3 oocytes/100 μl) in a buffer containing: 75 mM KCl, 50 mM NaF, 10 mM Na$_2$HPO$_4$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM 4-nitrophenylphosphate, 100 μM sodium orthovanadate and 50 mM Tris–HCl (pH 7.4). After centrifugation at $1000 \times g$ for 20 min, proteins in the supernatants were analysed by SDS–polyacrylamide gel electrophoresis on slabs according to Laemmli [16]. Alkali treatment of the gels was performed in 1 M NaOH for 2 h at 55°C. Radioactive bands were detected by autoradiography of the dyed gels.

3. RESULTS

Defolliculated oocytes were incubated for 2 h in the presence of 2 μg/ml of purified pertussis toxin. They were then exposed to 1 μM progesterone. The breakdown of the nuclear envelope was never inhibited. Even at higher doses (up to 40 μg/ml) the toxin was inefficient to block the hormone action. Similarly, no inhibitory effects were observed when oocytes were preincubated for 24 h in the presence of the toxin before addition of progesterone. On the other hand, pertussis toxin never induced maturation in the absence of the hormone.

Unexpectedly, as shown in fig.1, the kinetic of progesterone-induced maturation was shortened after toxin treatment. In 4 experiments performed with oocytes isolated from different females the times for GVBD$_{50}$ (50% of germinal vesicle breakdown) were shortened by $21.1 \pm 6.5\%$ as compared to the times for GVBD$_{50}$ in control oocytes. Microinjection of the toxin (6 ng/oocyte) resulted in a similar decrease of the kinetic of maturation. Although pertussis toxin accelerated the resumption of meiosis, it did not modify the dose response curve of maturation induced by progesterone, suggesting that the toxin does not act via the oocyte cAMP level. We therefore tested directly the effect of pertussis toxin on the oocyte cAMP content. A 5-h incubation in the presence of pertussis toxin had no effect on oocyte cAMP level, neither on the basal level nor on the level
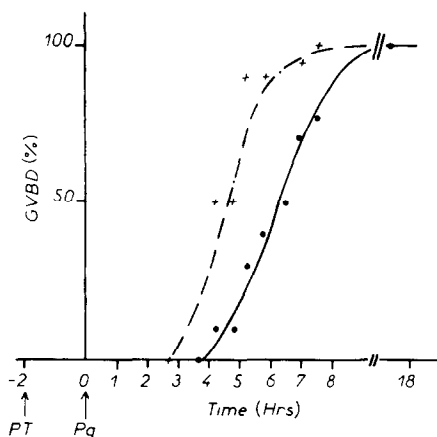
Fig.1. Effect of pertussis toxin on the kinetic of progesterone-induced maturation. Thirty oocytes were incubated in 2 ml medium A in the presence of 2 μg/ml pertussis toxin. After 2 h, 1 μM progesterone was added to the incubation medium. The percentage of maturation was scored at various times after hormonal stimulation in toxin-treated oocytes (· —— +) and in control oocytes (●——●). The figure presents a typical experiment performed on oocytes from the same female.
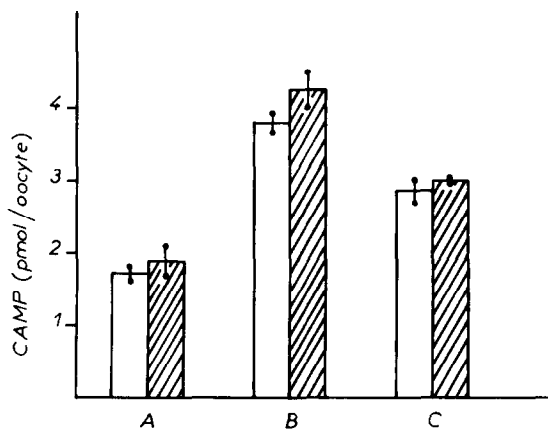

Fig.2. Effect of pertussis toxin on oocyte cAMP levels. Oocytes were incubated for 5 h in medium A in the presence of 1 mM IBMX (A), in the same medium plus 6 nM cholera toxin (B) or in the same medium plus 6 nM cholera toxin and the addition of 1 μM progesterone 1 h before the end of the incubation (C). Incubations were performed either in the absence (▭) or presence (▨) of 2 μg/ml pertussis toxin. Oocytes were then homogenized and cAMP was determined as described. Data are the means of 2 experimental points assayed in duplicate.

reached after stimulation on the cyclase by cholera toxin (fig.2). In addition, the drop in cAMP level induced by progesterone was not significantly affected by the presence of pertussis toxin (fig.2).

Interestingly, pertussis toxin was also found to accelerate MPF-induced maturation. In two different experiments $GVBD_{50}$ was shortened by 30 min in toxin-treated oocytes as compared to control oocytes. We have recently shown that low M_r inhibitors of phosphoprotein phosphatase, such as 2-glycerophosphate, facilitate progesterone-induced maturation without interfering with the oocyte cAMP level [17]. In order to know if pertussis toxin may work in ovo by a similar mechanism, we compared the effects of 2-glycerophosphate, pertussis toxin or both agents on the kinetics of maturation (fig.3). At the tested doses, 2-glycerophosphate as well as pertussis toxin accelerates the kinetic of progesterone-induced maturation, 2-glycerophosphate being the most efficient, and the effects of the two agents were not
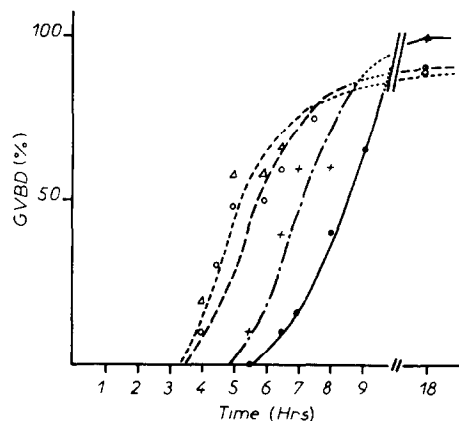

Fig.3. Effect of pertussis toxin and 2-glycerophosphate on the kinetics of progesterone-induced maturation. Batches of 10 oocytes were either incubated in medium A in the presence of 2 μg/ml pertussis toxin (· —— +), incubated in medium A after receiving a microinjection of 50 nl of 50 mM 2-glycerophosphate (○——○), or incubated in the presence of pertussis toxin after the 2-glycerophosphate microinjection (△ --- △). After 2 h, they were stimulated by the addition of 1 μM progesterone to the incubation medium. The percentage of maturation was scored at various times in treated (+, ○, △) and control (●——●) oocytes. The results presented were obtained on oocytes isolated from the same female.

additive. This suggests that pertussis toxin as 2-glycerophosphate interferes with the steady-state level of protein phosphorylation.

Oocytes were prelabelled by $^{32}PO_4$ for 16 h. One batch of oocytes was then incubated in the presence of pertussis toxin and another batch received a microinjection of 2-glycerophosphate. At various times thereafter endogenous phosphorylated proteins were analysed. Neither the level nor the profile of protein phosphorylation was modified after 2, 4 or 6 h treatment of oocytes with either agent as compared to those of control oocytes.

It has been shown that a burst of protein phosphorylation occurs concomitantly with MPF appearance [18]. This burst took place earlier in 2-glycerophosphate as well as in pertussis toxin-treated oocytes induced to mature by progesterone as compared to control maturing oocytes (not shown). Neither qualitative nor quantitative dif-
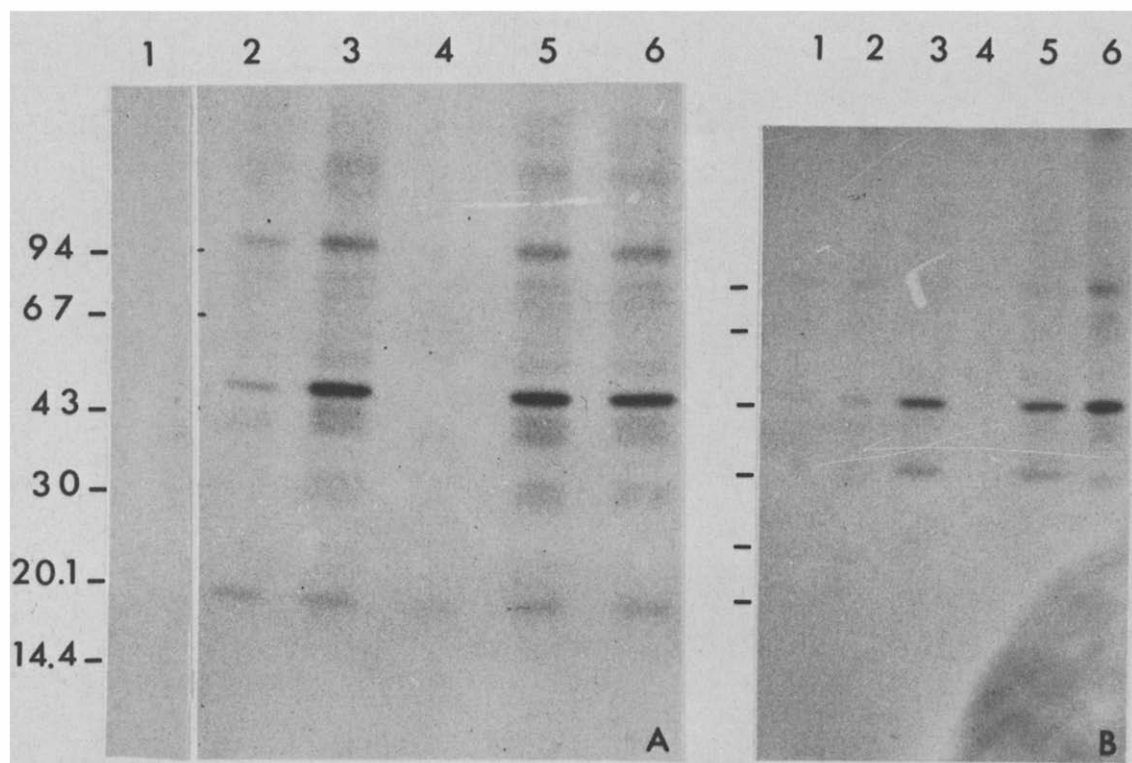

Fig.4. Precocious presence of the alkali-stable 47-kDa phosphoprotein in treated oocytes. Oocytes were labelled for 16 h with $^{32}PO_4$. One batch was then incubated in the presence of 2 μg/ml pertussis toxin, another batch received a microinjection of 50 nl of a 50 mM 2-glycerophosphate solution. Incubation of labelled control oocytes was conducted in parallel. After 2 h incubation three oocytes from each batch were homogenized and the remaining oocytes were stimulated by 1 μM progesterone. At GVBD$_{50}$ in each batch, 3 matured (i.e., with a white spot) and 3 maturing (i.e., without a white spot) oocytes were separately homogenized. Analysis of phosphorylated proteins was performed by gel electrophoresis. Aliquots of the different 1000 $\times$ g supernatants each containing 300 000 cpm of total radioactivity were applied on the slab. The figure presents the autoradiogram of the alkali-treated gel from experiments performed on two different females (A, B). (A) Lanes 1–3, control oocytes (1, unstimulated; 2, maturing; 3, matured – GVBD$_{50}$ was 4 h 15 min); lanes 4–6, pertussis toxin-treated oocytes (4, unstimulated; 5, maturing; 6, matured – GVBD$_{50}$ 3 h 30 min). (B) Lanes 1–3, same as (A) – GVBD$_{50}$ was 5 h 45 min; lanes 4–6, 2-glycerophosphate-treated oocytes (4, unstimulated; 5, maturing; 6, matured – GVBD$_{50}$ was 3 h 40 min). The scale on the left shows the positions of the M_r markers run simultaneously with each slab.

ferences in the phosphorylation pattern were seen before the burst, indicating that both agents did not act on an initial step in the maturation process. In contrast, we observed that an alkali stable 47-kDa phosphoprotein known to be a marker of MPF activity [19] appeared earlier in pertussis toxin- as well as in 2-glycerophosphate-treated maturing oocytes than in untreated maturing oocytes (fig.4).

These results suggest that pertussis toxin, directly or indirectly, may reach an intracellular target similar to that of 2-glycerophosphate, most probably a phosphoprotein phosphatase/kinase associated with MPF activity.

4. CONCLUSION

Our results do not favour the view that progesterone decreases the *Xenopus* oocyte cAMP concentration via an interaction with the N_i subunit of the adenylate cyclase since pertussis toxin does not block the effect of progesterone, neither on the cAMP level (fig.2) nor on the maturation process (fig.1). These in vivo results confirm the recent report by Olate et al. [20]; these authors show that pertussis toxin does not modify in vitro, the oocyte cyclase activity (basal, GppNHp-stimulated or progesterone-inhibited) although it ADP-ribosylates a 40-kDa protein analog to N_i.

It also seems improbable that progesterone acts directly on the N_s subunit since the hormone was reported to reverse the effect of cholera toxin and GppNHp [4–7]. It may therefore be assumed that the steroid hormone inhibits the oocyte adenylate cyclase through another mechanism at the level of the catalytic subunit. We have recently shown that a phosphoprotein, substrate of the cAMP-dependent protein kinase and the protein phosphatase-1 is capable to in ovo inhibit the oocyte adenylate cyclase [21]. It may then be possible that this phosphoprotein represents an initial target for progesterone with secondarly would change the cyclase activity.

The finding that pertussis toxin facilitates the kinetics of maturation without interfering with the cAMP levels of the oocyte suggests that the toxin may regulate, besides N_i, other cellular proteins which would be implicated in later steps of the maturation process. In fact in other cells, pertussis

toxin was reported to regulate via ADP ribosylation proteins from the family of the GTP binding proteins involved in various cellular processes [22,23]. We show here that pertussis toxin, as 2-glycerophosphate, may act on the phosphorylation/dephosphorylation step associated with MPF activity, which is cAMP-independent [18,24]. We are now testing the hypothesis that a kinase and/or phosphatase activity may be regulated in oocyte by a G-type protein sensible to ADP-ribosylation by pertussis toxin.

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