Oocyte adenylyl cyclase contains N_i, yet the guanine nucleotide-dependent inhibition by progesterone is not sensitive to pertussis toxin

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Membranes were obtained from *Xenopus laevis* oocytes after removal of follicular cells by collagenase treatment. [^{32}P]ADP-ribosylation with pertussis toxin showed them to contain a single $M_r = 40000$ substrate for this toxin that co-migrates on sodium dodecylsufate-polyacrylamide gel electrophoresis with pure human erythrocyte N_i, the inhibitory regulatory component of adenylyl cyclase. [^{32}P]ADP-ribosylation of oocyte membranes with cholera toxin also showed presence of a single substrate but of $M_r = 42000$. These results indicate, that the adenylyl cyclase system of oocytes, like that of somatic cells and unlike that of spermatozoids, contains the catalytic unit C and both of the known regulatory N components.

The possible susceptibility to pertussis toxin of the guanine nucleotide-dependent inhibition of oocyte adenylyl cyclase by progesterone was investigated. This action of progesterone is mediated by a membrane bound receptor as opposed to a receptor of cytosolic or nuclear localization. However, the inhibitory effect of progesterone was unaffected by pertussis toxin, even though the oocyte membrane N_i was fully ADP-ribosylated with pertussis toxin, as revealed by lack of further [^{32}P]ADP-ribosylation on subsequent re-incubation with pertussis toxin. These results indicate that the action of progesterone, in spite of being nucleotide-dependent, is either not mediated by N_i, suggesting the existence of an additional nucleotide regulatory component, or if mediated by N_i, involves a mode of regulation of this coupling protein that is different from that by which all other inhibitory hormones act on adenylyl cyclase.

Progesterone *Oocyte* *Inhibitory regulatory component (N_i)* *Adenylyl cyclase* *Pertussis toxin*

1. INTRODUCTION

Ovarian oocytes are arrested in the first meiotic prophase and need to be triggered to complete meiosis before fecundation by a spermatozoon can take place. Sustained high levels of cAMP apparently play a role in maintaining this physiological meiotic arrest state (for review see [1]). In amphibian oocytes, the resumption of meiosis results from a progesterone-induced reduction in oocyte cAMP levels [2–6]. Interestingly, however, this effect of progesterone is not mediated by a classical steroid hormone receptor, but by a plasma membrane-bound receptor [7,9] which directly attenuates the activity of the adenylyl cyclase system by a guanine nucleotide-dependent mechanism [10–13]. Since all hormones thus far known to inhibit adenylyl cyclase in a guanine nucleotide-dependent manner do so by activating a coupling protein called N_i [14–17], we wondered whether or not progesterone acts by a similar mechanism on the amphibian oocyte. Related to this question was also the question whether or not

Abbreviations: N, nucleotide binding regulatory component of adenylyl cyclases; N_s, stimulatory N; N_i, inhibitory N; GMP-P(NH)P, guanyl-5′-yl imidodiphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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the adenylyl cyclase system of the oocyte contains an N_i. Even though it has been shown that the oocyte adenylyl cyclase responds to cholera toxin [6,12] and hence contains an N_s, the stimulatory regulatory component of the system, the possible existence of an N_i has not as yet been investigated. We now report that N_i is indeed a component of the oocyte adenylyl cyclase and that pertussis toxin, which blocks receptor-mediated inhibition of adenylyl cyclase in all other systems studied [18–24] does not affect progesterone inhibition.

2. MATERIALS AND METHODS

2.1. *Isolation of oocytes*

Adult *Xenopus laevis* females, obtained from South African Snake Farm, Capetown, Republic of South Africa and maintained with constant 12 h light and 12 h dark periods at 22°C, were anesthetized by hypothermia and pieces of ovary were removed surgically. The tissue was incubated with 0.2% collagenase for 3 h at 25°C in amphibian saline: 10 mM Tris-HCl, pH 7.6, 0.82 mM $MgSO_4$, 0.74 mM $CaCl_2$, 1.0 mM KCl, 0.33 mM $Ca(NO_3)_2$, 88 mM NaCl, 2.4 mM $NaHCO_3$, and 10 μg/ml each of penicillin and streptomycin sulfate. Large follicle free oocytes were then separated by passing the suspension through a Nitex screen [25] with 700 μm pores. The retained oocytes were subsequently separated manually under the microscope to select only those cells having more than 1.2 μm in diameter (stage VI oocytes according to [26]).

2.2. Preparation of oocyte membranes

The oocyte enzyme present in membrane rich fractions were prepared by homogenization in one volume of a solution containing 0.88 M sucrose, 50 mM Tris-HCl, pH 8.0, 1 mM DTT and 1 mM EDTA using a Dounce homogenizer and 10 strokes with pestle A and 10 strokes with pestle B. The homogenate was then centrifuged at $1000 \times g$ for 15 min, the supernatant was recentrifuged at $20\,000 \times g$ for 20 min and finally at $105\,000 \times g$ for 2 h to obtain a particulate fraction. The fluffy layer of material present on top of the high speed pellet contained the adenylyl cyclase activity [27] and was used in these experiments.

2.3. ADP-ribosylation of pertussis toxin

Pertussis toxin was preactivated by incubating it in 20 mM DTT for 30 min at 32°C [17]. Oocyte membranes (60 μg protein) were subjected to two successive incubations. The first incubation (32°C, final volume 60 μl) was carried out in the presence of 1 mM NAD^+ with or without 10 μg/ml pertussis toxin in medium containing 100 mM potassium phosphate, pH 7.5, 1 mM ATP, 0.5 mM GTP, 15 mM thymidine, 5 mM DTT, 1 mM EDTA. After 30 min to 4 h the incubations were stopped and the membranes washed twice by sedimentation ($40\,000 \times g$ for 10 min) with 50 mM sodium-Hepes, pH 8.0, and resuspended in the same medium at a final concentration of 6 mg/ml. In some experiments aliquots of the thus treated membranes (60 μg) were then subjected to a second ADP-ribosylation incubation under the same conditions as the first, except that 10 μM [^{32}P]NAD^+ (300 Ci/mol; synthesized according to [28] were used and incubations were for 30 min at 32°C. At the end of the second incubation, the reactions were stopped by the addition of 1 ml of ice-cold 20% trichloroacetic acid, followed by centrifugation at 3000 rpm for 30 min. The pellets were washed once with 1.5 ml cold anhydrous diethyl ether to remove remaining trichloroacetic acid.

2.4. SDS-PAGE

The ether-washed membrane pellets were resuspended in sample buffer and electrophoresed on 10% polyacrylamide gel slabs according to Laemmli [29] containing 1% sodium dodecyl sulfate and Pyronin Y as the tracking dye. Electrophoresis was at room temperature using a constant voltage of 100–150 V (~ 30 mA of initial current). Gels were subsequently stained with Coomassie brilliant blue, dried and subjected to autoradiography at −70°C for 3 days.

3. RESULTS AND DISCUSSION

The first question studied was whether the oocyte adenylyl cyclase system would contain an N_i through which the guanine nucleotide-dependent inhibition of activity could come about. Functional studies did not lend a clue, for addition of GTP analogs such as GMP-P(NH)P at very low concentrations or for very short times had either no effect or simply stimulated the activity of the

oocyte membranes. Testing for inhibitory effects of GMP-P(NH)P in the presence of forskolin also did not result in inhibition of adenylyl cyclase activity [30]. GMP-P(NH)P never showed an inhibitory effect such as seen in other systems where a transient inhibitory action of the analog has been interpreted to be the expression of N_i activity [31]. We therefore resorted to the use of the toxin from *Bordetella pertussis,* which has been shown by Ui and collaborators to ADP-ribosylate a membrane polypeptide of M_r 40 000–41 000 [18,19] that is the α-subunit of N_i [16,17]. As illustrated in fig. 1, treatment of oocyte membranes with pertussis toxin in the presence of [^{32}P]NAD$^+$ resulted in incorporation of radioactive label in a polypeptide that had the same migration as [^{32}P]ADP-ribosylated α-subunit of pure N_i [32].

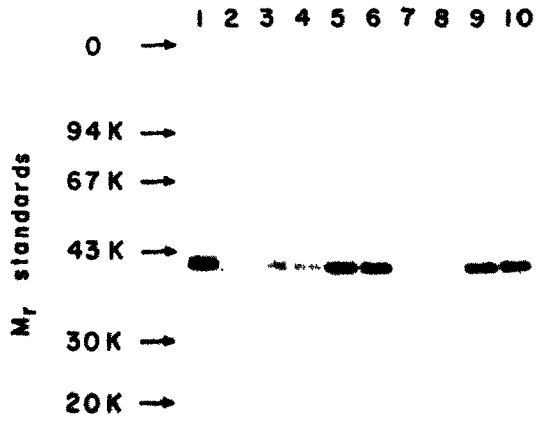

Fig. 1. Incorporation of [^{32}P]ADP-ribose into a protein of oocyte membranes and effect of pretreatment of such membranes with pertussis toxin on subsequent labelling with [^{32}P]NAD$^+$. Lanes 3 through 10: Oocyte membranes were subjected to two consecutive treatment incubations. The first was carried out in the presence of 1 mM NAD$^+$ and 10 μg/ml pertussis toxin for either 30 min (lanes 3–6) or 4 h (lanes 7–10); the second incubation was for 30 min in the presence of 10 μM [^{32}P]NAD$^+$ without (lanes 3,4 and 7,8) and with (lanes 5,6 and 9,10). Treated membranes were then subjected to SDS–PAGE followed by Coomassie blue staining and autoradiographic analysis of the gel. The rest of the conditions are described under Experimental Procedures. Lanes 1 and 2: Autoradiography of 11 ng of human erythrocyte N_i (purified as described [29] and incubated as described above for the second incubation of the oocyte membranes in the presence (lane 1) and absence (lane 2) of pertussis toxin [29]. Molecular mass markers were: phosphorylase B (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (33 000), soybean trypsin inhibitor (20 000), and α-lactalbumin (14 400).

We next investigated whether the effect of progesterone to inhibit the oocyte adenylyl cyclase system is interfered with by pertussis toxin treatment. Initial experiments showed that progesterone-mediated inhibition of the *Xenopus* oocyte adenylyl cyclase in well washed membranes appeared to be unaffected by pertussis toxin treatment.

In order to interpret the results of such an experiment, however, it was first necessary to determine conditions leading to the ADP-ribosylation of essentially all the N_i molecules in the membrane

Table 1

Lack of effect of ADP-ribosylation of oocyte N_i by the toxin of *Bortdetella pertussis* on oocyte adenylyl cyclase activity

Oocyte membranes were pretreated for 90 min at 32°C as described in the legend to the figure in the presence of 1 mM NAD$^+$ without or with 10 $\mu g \cdot ml^{-1}$ pertussis toxin, washed with 50 mM sodium Hepes, pH 8.0 in the cold, resuspended in the same medium and then incubated for adenylyl cyclase activity for 25 min at 32.5°C using a final volume 100 μl containing 60 μg of membrane protein, 0.1 mM [α-^{32}P]-ATP (1000 cpm $\cdot$ pmol^{-1}; synthesized as in [40], 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM [^{3}H]cAMP (10 000 cpm), 2 mM 3-isobutyl-1-methylxanthyne, 1 mM DTT and an ATP regenerating system [41]. The reactions were stopped and the [^{32}P]cAMP formed was isolated by a modification [42] of the method of Salomon et al. [43]. The values are means of triplicates that agreed with 10%.

Additions pretreatment of membranes	Incubation conditions	Adenylyl cyclase activity (pmol cAMP $\cdot$ mg^{-1} protein)
1 mM NAD$^+$	Basal	4.2
	25 μM GMP-P(NH)P	89.2
	25 μM GMP-P(NH)P plus 2.5 μM progesterone	46.8
1 mM NAD$^+$ plus 10 $\mu g \cdot mg^{-1}$ pertussis toxin	Basal	4.6
	25 μM GMP-P(NH)P	95.1
	25 μM GMP-P(NH)P plus 2.5 μM progesterone	49.2

[22,33]. We therefore subjected membranes to an extensive treatment with pertussis toxin in the presence of a supersaturating concentration of NAD^+ (1 mM) and then, after washing to remove excess NAD^+, tested in a second incubation with pertussis toxin and [^{32}P]NAD^+ whether vacant sites had remained. The results of such an experiment are also shown in fig. 1. Control membranes that had been subjected to the first incubation with 1 mM NAD^+ either for 30 min (lanes 5 and 6) or 4 h (lanes 9 and 10) contained a M_r = 40 000 pertussis toxin substrate when subsequently incubated with [^{32}P]NAD^+. In contrast, when the first incubation was carried out in the additional presence of 10 μg/ml pertussis toxin, substantial loss of the [^{32}P]NAD^+ label was evident after 30 min (lanes 3 and 4) and total loss after 4 h (lanes 7 and 8). Other experiments showed that ADP-ribosylation by pertussis toxin under these conditions was actually complete by 90 min at 32°C.

Table 1 illustrates the adenylyl cyclase activities in control and pertussis toxin-treated oocyte membranes and shows that toxin treatment had no effect on either basal or GMP-P(NH)P-stimulated adenylyl cyclase activities and that the treatment also did not interfere with the inhibitory action of progesterone.

In a previous publication we reported that cholera toxin treatment of the oocyte adenylyl cyclase system greatly increases the capacity of GTP to stimulate the enzyme and that such treatment does not prevent the inhibition caused by the progesterone in the presence of either GTP or the nonhydrolyzable analog GMP-P(NH)P [12]. In agreement with the presence of a functional response to cholera toxin, and a stimulating

Table 2

The effect of exogenous regulatory subunits of adenylyl cyclase on oocyte membrane adenylyl cyclase and the inhibitory reponse to progesterone

N_s and N_i, purified from human erythrocytes as described [32], were preincubated with oocyte membranes at the protein ratios shown on the table at 4°C for 30 min in medium containing 0.2% Lubrol PX, 1% ethylene glycol, 0.5 mM β-mercaptoethanol, 0.5% bovine serum albumin and 25 mM sodium Hepes, pH 8.0, at oocyte membrane concentration of 3.0 mg protein · ml^{-1}. 20 μl aliquots of these mixtures were then used to measure their adenylyl cyclase activity as described in the legend to table 1 with the additives indicated on the table. Values are means of triplicates which agreed within 10%.

Incubation conditions	N protein added		Adenylyl cyclase activity (pmol cAMP · mg^{-1} protein
	Type	ng N / mg protein	
Basal	None	-	9.1
	N_i	47	9.3
	N_s	55	11.0
4 μM Progesterone	None	-	8.6
	N_i	47	8.5
	N_s	55	9.9
10 μM GMP-P(NH)P	None	-	109
	N_i	47	119
	N_s	55	164
10 μM GMP-P(NH)P plus 4 μM progesterone	None	-	59
	N_i	47	62
	N_s	55	111

response to guanine nucleotides, we found that incubation of oocyte membranes with cholera toxin and [^{32}P]NAD$^+$ under the same conditions as those used for [^{32}P]ADP-ribosylation of N_i but increasing potassium phosphate (pH 7.5 to 0.4 M), resulted in the labelling of a polypeptide band of M_r = 42 000 (not shown). No components larger than M_r = 42 000 were labelled, indicating that the oocyte N_s resembles N_s of human erythrocytes in that it does not show size heterogeneity [32,34].

We tested the effect of the addition of exogenous N_s and N_i, purified from human erythrocytes [32] on the activity of the oocyte enzyme and its responses to guanine nucleotides and progesterone. Table 2 shows that the addition of purified N_i and N_s did not affect the basal activity of the enzyme. However, exogenous N_s significantly stimulated the activity measured in the presence of GMP-P(NH)P. Under the same conditions, N_i had a negligible effect. Progesterone inhibition of the activity stimulated by GMP-P(NH)P was lower on a percent basis (32%) when exogenous N_s was present than when it was absent (45%). However, the absolute decrease of activity produced by progesterone was approximately the same in both cases (53 and 49 pmol cAMP formed per mg protein). A simple interpretation of this finding would suggest that progesterone does not inhibit the activation of adenylyl cyclase caused by exogenous N_s. Exogenous N_i had no effect on progesterone inhibition.

In summary, the results obtained on testing the possible mechanism of action of progesterone indicate that, even though its action is dependent on guanine nucleotides, it is not sensitive to pertussis toxin as is that of other hormones such as opiod peptides [22], somastostatin [23], and adrenaline [24] that act on N_i. Although this does not prove that the action of progesterone does not involve N_i, the experiments do demonstrate that its action is not like that of other inhibitory hormones and indicate unequivocally the existence of a guanine nucleotide-dependent receptor-mediated inhibitory regulation of adenylyl cyclase that is distinct from the two heretofore recognized modes of action of hormone receptors.

Spermatozoa from all species spanning from sea urchin up to man have a very active adenylyl cyclase system that, like that of other cells, is membrane bound, but differs radically from its counterpart in somatic cells in that it does not respond to NaF stimulation, is 10–20 times more active when MnATP instead of MgATP is used a substrate and it is not stimulated by NaF or guanine nucleotides [35–38] (for review see [39]). Due to the difficulty of gaining access to sufficient quantities of mammalian oocytes, little if anything is known about their adenylyl cyclase system. However, using amphibian oocytes, some insight into the structure of the adenylyl cyclase system in oocytes has been gained. Thus, studies from our laboratory [12,13,27] as well as those from others [10,11] on *Xenopus* oocytes have indicated that the adenylyl cyclase system of these cells may be quite similar to that of somatic cells in that it uses MgATP effectively [27], it is stimulated by NaF and guanine nucleotides [10–12] and is affected by cholera toxin [10–12]. The present report complements the characterization of this oocyte system in that we confirmed directly the presence of a cholera toxin substrate of M_r = 42 000, and for the first time we demonstrate that oocytes contain a polypeptide that is substrate for the ADP-ribosylating activity of pertussis toxin. This substrate has a M_r (40 000–41 000) that is the same as that of the α-subunit of N_i derived from human erythrocytes [32]. The fact that exogenously added N_s purified from human erythrocytes enhances the adenylyl cyclase activity of oocytes membranes shows further that this system resembles that of somatic cells. We did not find conditions, however, where we could see an effect of exogenously added N_i. In fact, a clear expression of the endogenously present pertussis toxin substrate was also not seen. Regardless, it is clear that the oocytes have an adenylyl cyclase system similar in constitution to that of somatic cells, i.e., formed of N_s, N_i and a catalytic unit C, and that the mode of action of the oocyte maturing hormone progesterone and its receptor is distinct from that of all other known hormones that affect adenylyl cyclase activities. It is to be expected that this different mode of receptor regulation of adenylyl cyclase will apply to other as yet unrecognized receptors as well.

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