Activation of adenylate cyclase by sperm membranes

The role of guanine nucleotide binding proteins

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Adenylate cyclase *Guanine nucleotide binding protein* *Sperm*

1. INTRODUCTION

Adenylate cyclase from mature mammalian spermatozoa is a membrane-bound enzyme [1–3]. It exhibits characteristics similar to that of the variant cyc$^-$ S49 lymphoma, which is deficient of active guanine nucleotide binding stimulatory component [4]. The sperm cyclase is unresponsive to hormones [2,5], fluoride [2,5], guanine nucleotides or cholera toxin [6], and is highly responsive to Mn^{2+} as opposed to Mg^{2+} [2,5,7,8]. In contrast to cyc$^-$ the sperm cyclase does not respond to forskolin [9].

Human red blood cell (HRBC) membranes appear to be relatively enriched in the stimulatory GS component as compared to catalytic component (C) and the hormonal receptors [10]. These membranes have been used for the purification of the stimulatory G_S and the inhibitory G_I, guanine nucleotide binding proteins [11,12].

Mixing of sperm membranes with HRBC membranes results in an adenylate cyclase activity that is far greater than the calculated additive activities of these individual membranes [9,13]. The observed superadditive activity has been interpreted as a complementation of G_S-deficient sperm adenylate cyclase by G_S of HRBC membranes [9,13].

Here, we show that sperm catalytic component does not interact with G_S from human HRBC membranes. The observed superadditive activity is caused by sperm factor which is different from its catalytic component. This factor is probably similar to the sperm factor that activates brain and platelets adenylate cyclase [14,15]. Addition of the sperm factor to cyc$^-$ S49 lymphoma membranes neither elicits superadditive response, nor affects the inhibition of forskolin response by GTPγS. Reconstitution of cyc$^-$ membranes by G_S restores the ability of the sperm factor to induce superadditive response to GTP and GTPγS. Treatment of platelet membranes with NEM which eliminate the inhibition of adenylate cyclase by the α_2-adrenergic agonists, does not prevent the stimulation induced by sperm factor.

2. MATERIALS AND METHODS

2.1. *Preparation of sperm membranes*

Sperm membranes were prepared from thawed bovine and freshly collected ram semen as in [13]. Frozen bovine semen was obtained from 'Hasherut' artificial insemination and breeding center, Hafaitz Haim. Frozen semen was thawed as in [16]. Under these conditions, the viability and fertility of the bovine sperm is preserved [16].

Abbreviations: GTPγS, guanosine 5′-[γ-thio]triphosphate; NEM, *N*-ethylmaleimide; PGE_1, prostaglandin E_1; epi, epinephrine

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Fresh ram semen was obtained from the Volkani Center (Institute of Animal Sciences, Bet Dagan). For reasons of availability, most of the experiments were performed with bovine sperm.

2.2. *Preparation of other membranes*

Plasma membranes of S49 cyc⁻ lymphoma were prepared as in [17]. HRBC membranes were prepared from outdated blood obtained from hospital blood bank. G_S was extracted in the presence of lubrol Px 1.2% and NaCl 100 mM as in [11]. Human platelet membranes were prepared as in [18], and treated with NEM as in [19].

2.3. *Adenylate-cyclase determinations*

Adenylate cyclase assays were performed at 30°C for 40 min, for the following membranes: HRBC, sperm, and HRBC–sperm mixture, and for 30 min for other membranes. The assay volume was 50 μl and it contained 0.5 mM [α^{32}P]ATP 4–1 × 10^6 cpm/tube, and the ingredients as in [17]. Reconstitution of cyc⁻ S49 membranes with G_S extract and the assay of the reconstituted system, were performed as in [11]. Purification of cyclic [^{32}P]AMP was as in [20]. Each of the experiments shown were repeated at least twice and comparable results were obtained. Standard deviation of triplicate determinations were <7% of the means. The experiments described in fig.2 were performed using both bovine and ram sperm. Comparable results were obtained for both sperm sources. Protein was estimated as in [21] using bovine serum albumin as a standard.

2.4. *Materials*

[α-^{32}P]ATP and c[^{3}H]AMP, were purchased from the Radiochemical Center (Amersham); Lubrol-Px was purchased from Sigma and was deionized with a mixture of Dowex 1 and 50. Other chemicals were of highest purity available.

3. RESULTS

3.1. *The catalytic component of sperm adenylate cyclase does not interact with G_S*

In agreement with [9,11] mixing of sperm membranes with HRBC membranes causes a superadditive stimulation of adenylate cyclase (fig.1). Nevertheless, we have failed to apply successfully standard reconstitution protocols [4] for the sperm

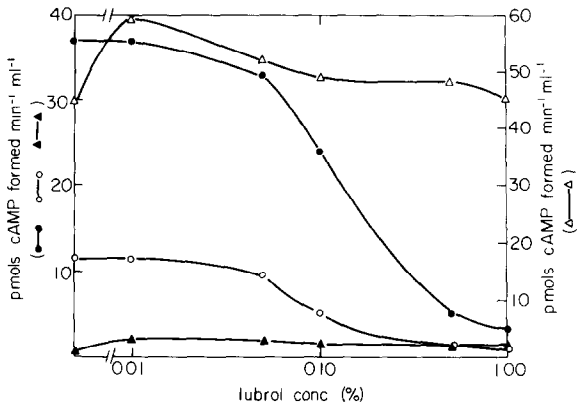

Fig.1. Effect of varying concentrations of lubrol on adenylate cyclase activity of sperm and HRBC membranes: (○—○) HRBC membranes (160 μg/assay); (▲—▲) sperm membranes (4 μg/assay); and (●—●) mixture of HRBC and sperm membranes were assayed in the presence of forskolin (0.1 mM) and GTPγS (100 μM) for 40 min at 30°C. Lubrol, at the indicated concentrations (final), was added directly to the assay mixture. The activity of sperm in the presence (△—△) of $MnCl_2$ (20 mM) was also determined.

HRBC system. Addition of HRBC detergent extract, containing active G_S but inactive C, failed to complement any G_S mediated responses in sperm membranes. Furthermore, addition of increasing concentrations of Lubrol to mixture of sperm and HRBC membranes, progressively decreased and finally abolished the observed superadditive activity, in a mode similar to that of adenylate cyclase in HRBC membranes alone (fig.1), yet, Mn^{2+}-dependent activity of sperm cyclase was unaffected by the same concentrations of lubrol (fig.1). The above observations suggested that superadditive cyclase activity observed in the mixture of sperm and HRBC membranes probably represents activation of the catalytic component of HRBC cyclase by sperm membranes. To directly examine this hypothesis, the following experiment was conducted: HRBC membranes were incubated at 35°C for 60 and 150 min. The forskolin plus GTPγS response of the exposed membranes was decreased by 72 and 78%, respectively, as compared to control (fig.2). The superadditive activity of the mixture of sperm and HRBC membranes decreased in similar proportion; 66 and 78% of the control, respectively. The same results were obtained when GTP, GTPγS or forskolin were used

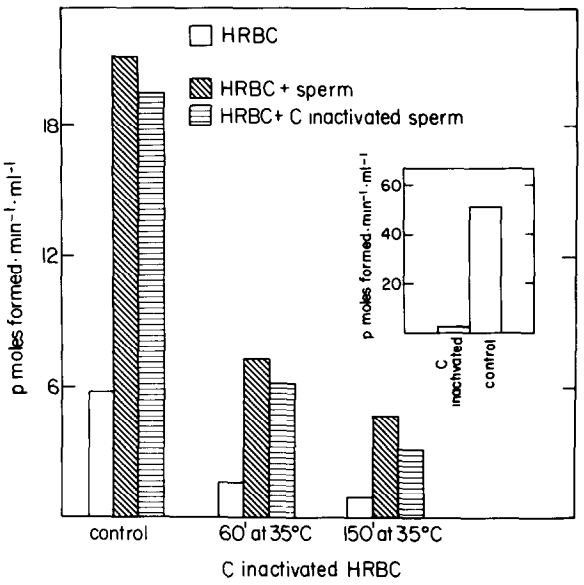

Fig.2. The stimulation of adenylate cyclase activity caused by sperm is proportional to the amount of active catalytic units in HRBC membranes. HRBC membranes at 16 mg/ml were exposed to 35°C for 60 or for 150 min, or kept on ice. These 3 preparations of HRBC membranes (160 μg/assay) were assayed alone, and in a combination with sperm and sperm C-inactivated membranes (4 μg/assay). The activators used were forskolin (10 μM) plus GTPγS (100 μM). Inset: Inactivation of the catalytic component of sperm adenylate cyclase. Sperm membranes (400 μg/ml) were exposed to 45°C for 60 min and then assayed in the presence of 20 mM Mn^{2+}. Control membranes were kept on ice and then assayed as above.

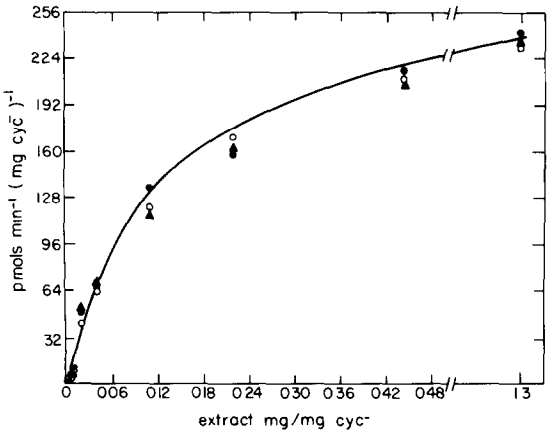

Fig.3. Progressive heat inactivation of the catalytic component of HRBC membranes does not affect the content of G_S. Heat exposed and control HRBC membranes were extracted with lubrol and their G_S content was determined by reconstituting G_S mediated fluoride response in cyc$^-$ S49 membranes. Increasing concentrations of G_S extract (from 0.012–4.00 mg/ml) were reconstituted into cyc$^-$ membranes into cyc$^-$ membranes (320 mg/ml) as explained in section 2.2. To maintain identical reconstitution conditions the dilutions were made into an extract in which G_S was heat inactivated by exposure to 60°C for 60 min. The symbols used denote: (●—●) control membranes; (○—○) membranes exposed for 60 min; (▲—▲) membranes exposed for 150 min.

as activators (not shown). In contrast, G_S content of exposed and non-exposed HRBC membrane was not affected by the heat inactivation treatment. This was demonstrated by extracting G_S from these membranes and measuring G_S mediated reconstitution of fluoride response in cyc$^-$ S49 membranes (fig.3). Both exposed and non-exposed membranes have the same amount of active G_S in their detergent extracts (fig.3). Exposure of sperm membranes to 45°C for 60 min inactivates >95% of its Mn^{2+}-dependent adenylate cyclase activity (fig.2). Yet, the ability of the sperm membranes to stimulate adenylate cyclase activity in HRBC membranes is preserved (fig.2). Therefore, the superadditive stimulation of adenylate cyclase observed when sperm and HRBC membranes are combined is caused by a factor present in sperm membranes which is different from its catalytic component, and which acts upon adenylate cyclase of HRBC membranes. It is likely that this factor is similar, or even identical, to the sperm factor that activates brain [14] and platelets [15] adenylate cyclase.

3.2. *Stimulation induced by sperm factor requires the presence of G_S*

Mixing of heat-inactivated sperm membranes (at the same concentrations used for the activation of HRBC membranes) with cyc$^-$ S49 lymphoma neither elicits superadditive cyclase activity nor affects the inhibition of forskolin response by GTPγS. The latter is probably mediated by G_I [22,23]. The responses to both forskolin and forskolin plus guanine nucleotides are even decreased by about 20% (table 1). In contrast, reconstitution of cyc$^-$ with G_S also restores the superadditive activity induced by sperm. Both GTP and GTPγS

Table 1

Reconstitution of cyc$^-$ with membranes with G_S restores the stimulation of adenylate cyclase by sperm

G_S extract added (mg/mg cyc$^-$)	C-inactivated sperm	GTP	GTPγS	Forskolin + GTP	Forskolin + GTPγS
–	+	0.6	0.3	85.5	65.3
–	–	0.4	0.4	109.8	97.1
0.005	+	4.8	15.0	85.5	65.3
0.005	–	0.8	7.1	99.7	74.7
0.03	+	8.2	58.8	66.8	145.3
0.03	–	1.6	26.8	99.2	191.4
0.15	+	25.4	109.6	96.1	337.3
0.15	–	3.7	47.3	126.0	415.0
0.3	+	36.4	177.6	129.5	425.0
0.3	–	11.1	71.7	139.2	525.2
0.5	+	31.6	148.3	141.3	472.7
0.5	–	8.1	80.7	147.4	513.2

G_S extract of HRBC membranes was prepared and then reconstituted into cyc$^-$ S49 membranes, as in section 2.2. Dilutions of G_S extract were made with heat-inactivated extract (60 min at 45°C) in order to maintain identical experimental conditions. Sperm adenylate cyclase was inactivated by incubation of sperm membranes at 45°C for 60 min. The concentrations of cyc$^-$ and of sperm membranes used were 50 and 4 μg/assay, respectively. The concentrations of guanine nucleotides and of forskolin were 100 and 10 μM, respectively.

Table 2

Elimination of epinephrine mediated inhibition in human platelets does not affect the stimulation of adenylate cyclase by sperm

Ligand	Control			NEM-treated		
	Adenylate cyclase activity (pmol.min^{-1}.mg platelets^{-1})					
	No sperm	5 μg/assay	15 μg/assay	No sperm	5 μg/assay	15 μg/assay
Basal	17	28	60	3	5	10
GTP	18	31	65	4	6	12
GTPγS	157	260	344	42	68	98
PGE_1 + GTP	138	146	180	39	47	49
PGE_1 + GTP + epi	104	109	166	37	45	51
Forskolin	322	320	390	120	122	130
Forskolin + GTPγS	509	542	612	172	221	246

Human platelets (HP) were treated with 0.9 mM NEM by a procedure which eliminates the inhibitory pathway [19]. Control and NEM-treated HP membranes (50–60 μg/assay) were assayed for adenylate cyclase activity in the presence of the following ligands: GTP (100 μM), PGE_1 (10 μM), epinephrine (30 μM), forskolin (10 μM), GTPγS (100 μM), in the absence and presence of the indicated concentrations of C-inactivated sperm membranes.

responses are superadditive in the presence of sperm membranes (table 1). However, while the reconstitution of cyc^- cyclase is dependent on the concentration of added G_S, sperm-mediated activation is fully restored even at the lowest G_S concentration used. This observation may indicate that the sperm membrane concentration used in this experiment (4 μg/assay) contains an excess of the stimulatory factor. Indeed, sperm membranes at the same concentration range (3–6 μg/assay) were sufficient to saturate sperm mediated activation of HRBC membranes [13].

To examine if the presence of functional G_I is required for the stimulatory effect of sperm factor the following experiment was conducted. Human platelets (HP) membranes were treated with NEM, under conditions that eliminate the inhibition of adenylate cyclase by α_2-adrenergic agonists [19]. The NEM treatment partially inactivates adenylate cyclase activity. Responses to the various activators used are decreased by 62–78% as compared to untreated membranes (table 2). Nevertheless, the activation of the cyclase through the stimulatory pathway is unaffected in treated membranes. In fact, the fold of cyclase stimulation for each of the activators used is higher in treated membranes than in untreated, and the epinephrine-mediated inhibition of PGE_1 + GTP response is completely suppressed. This may be due to selective inactivation of G_I. Combining sperm membranes with NEM-treated and non-treated HP membranes results in stimulation of adenylate cyclase. In both types of membranes, the fold of cyclase stimulation for the various activators indicated in table 2, is comparable. Therefore, it is clear that NEM-mediated inactivation of G_I does not affect the ability of sperm factor to stimulate adenylate cyclase. At the high concentration of sperm used (15 μg/ml), the inhibition by epinephrine of PGE_1 + GTP response in control membranes is considerably reduced (table 2). This observation agrees with [15], where the sperm factor at high concentration (23 μg/assay) was suggested to abolish G_I-mediated inhibition of adenylate cyclase [15].

4. DISCUSSION

These data indicate that the superadditive adenylate cyclase activity observed when sperm and HRBC membranes are combined is due to stimulation of HRBC adenylate cyclase by sperm, and not due to reconstitution of sperm catalytic component by G_S, as suggested in [9,13]. This conclusion is based on the findings that addition of sperm membranes to HRBC membranes, in which the catalyst was progressively heat-inactivated, reduces the combined superadditive activity by the same extent, even though the G_S content of the heat exposed membranes is unaffected.

Addition of solubilized G_S to sperm membranes, under a variety of conditions, fails to reconstitute any of G_S-mediated responses. Furthermore, while addition of a low concentration of lubrol inactivates the catalytic activity of HRBC cyclase in the same mode as other G_S-stimulated adenylate cyclase enzymes [24], sperm Mn^{2+}-dependent activity is unaffected. Therefore, it is conceivable that the sperm adenylate cyclase enzyme belongs to a class of adenylate cyclase enzymes that is not regulated by guanine nucleotide regulatory proteins. Such notion is supported also by the findings that sperm enzyme is unresponsive to forskolin [9] and has different thermal stability than several other adenylate cyclase enzymes which are all regulated by guanine nucleotide binding proteins [25].

The fact that heat inactivation of sperm adenylate cyclase does not prevent the stimulation of HRBC membranes indicates that the stimulation is mediated by a factor unrelated to the sperm adenylate cyclase catalytic component.

The addition of G_S to cyc^- is required for the expression of the sperm factor action. This finding suggests that sperm factor action is not a simple activation of the catalyst. Moreover, selective inactivation of G_I by NEM does not affect the stimulatory action of this factor. Therefore, it is conceivable that the presence of active G_I is not required for the factor action. Further experiments using more selective blockers of G_I action, like pertussis toxin (IAP) will be required to clarify this observation. High concentrations of sperm factor may suppress G_I mediated inhibition. This may suggest that the factor is composed of different components, or may have a concentration-dependent action. Further elucidation of factor action must await its purification and the study of its action on resolved components of adenylate cyclase.

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