**The Catalytic Unit of Ram Sperm Adenylate Cyclase Can Be Activated through the Guanine Nucleotide Regulatory Component and Prostaglandin Receptors of Human Erythrocyte**

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Ram sperm adenylate cyclase is insensitive to fluoride, guanine nucleotides, cholera toxin, and hormones, and appears devoid of the guanine nucleotide regulatory component. In this paper, we demonstrate that human erythrocyte membranes are capable of restoring guanine nucleotide regulation and fluoride sensitivity to ram sperm adenylate cyclase. The reconstitution process in the presence of guanyl-5'-yl imidodiphosphate or NaF is time-dependent, directly proportional to the quantity of erythrocyte protein added to the reconstitution system, and is only observed when Mg-ATP is used as substrate. Furthermore, the guanyl-5'-yl imidodiphosphate-reconstituted activity can be activated by prostaglandin E1 or prostaglandin E2 through the binding sites normally present in human erythrocyte membranes. It therefore appears that reconstitution of the adenylate cyclase system can be readily performed in normal membranes which are deficient in regulatory component, and not only using defective cultured cell lines.

Recent studies have shown that adenylate cyclase from mature mammalian spermatozoa is a membrane-bound enzyme (5-7), yet it cannot be stimulated by either hormones, fluoride (6, 8, 9), guanine nucleotides, or cholera toxin (10). Activity is optimal in the presence of a large excess of Mn$^{2+}$ (over the concentration of ATP) as the necessary cation, while the activity is 10 to 20-fold lower when Mn$^{2+}$ is replaced by Mg$^{2+}$ (6, 8, 9, 11). Therefore, the adenylate cyclase of mature ram sperm appears similar to that of the AC mutant of the S49 lymphoma cell line, a system where cyclase is also responsive to hormones, fluoride, GTP, and cholera toxin (12). Indeed, the AC mutant lacks a specific protein component, referred to as G/F or N, which is necessary for the stimulation of adenylate cyclase by guanine nucleotides and fluoride (13, 14). Addition of N component restores the ability of these substances to stimulate this cyclase system. Various studies have shown that the N component can be prepared from wild type lymphoma cells (13-15), from liver membranes (16), or even more readily, from human erythrocytes (17-21). Indeed, human erythrocytes appear to be relatively enriched in N component (22), as compared to catalytic site and hormonal receptors.

In the present paper, we show that the ram sperm adenylate cyclase can acquire a normal sensitivity to fluoride, Gpp(NH)p, cholera toxin, and Mg$^{2+}$ by complementation with the guanine nucleotide regulatory component of human erythrocyte. Furthermore, this system can be activated by prostaglandins, most probably by complementation with the prostaglandin receptors from erythrocytes.

**EXPERIMENTAL PROCEDURES**

**Materials**—ATP, GTP, creatine phosphate, creatine kinase, and cholera toxin were obtained from Sigma. Guany-5'-yl imidodiphosphate and NaF were purchased from Boehringer Mannheim. All other chemicals were from E. Merck, Darmstadt. [32P]ATP (22 Ci/mmol) was obtained from Amersham, Radiochemical Centre, United Kingdom. Cyclic [8-'H]AMP (10 Ci/mmol) was obtained from Commissariat à L'énergie Atomique, Saclay, France.

**Ram Sperm Preparation**—Semen was diluted in 20 volumes of 150 mM NaCl and washed twice by centrifugation at room temperature at 2,000 rpm for 10 min. The pellets were homogenized in 1 mM NaHCO$_3$ centrifuged in a SS34 Sorval rotor for 10 min at 20,000 rpm at 4°C, resuspended in 1 mM NaHCO$_3$, and finally stored in liquid nitrogen.

**Human Erythrocyte Membrane Preparation**—Human erythrocyte membranes were prepared by the procedure of Stock and Kast (23) as reported previously for the complementation assay (17-22). Adenylate cyclase activity in our preparation was comparable to the activity reported by Johnaon et al. (17), Nielsen et al. (20), and Roden et al. (21). It was not detectable when tested in the absence of activator, and attained 0.3 to 0.5 pmol of cyclic AMP formed/mg of protein/min at 30°C in the presence of 10 mM Gpp(NH)p or 10 mM NaF. In all the complementation experiments described here, the final activities were corrected for the contribution of cyclase activity originating from the erythrocyte membranes.

**Adenylate Cyclase Assay**—Incubations were performed at 30°C for the indicated time periods in a final volume of 50 μl containing 0.5 mM [γ-32P]ATP (2.10$^{6}$ cpm), 3 mM MgCl$_2$ (except when otherwise indicated), 1 mM EDTA, 1 mM cyclic AMP, 50 mM Tris-HCl, pH 7.6, and an ATP-regenerating system consisting of 25 mM phosphocreatine and 1 mg/ml of creating phosphokinase, and the indicated amount of proteins. The reaction was terminated by a modification (25) of the procedure of White (26). Protein was estimated by the procedure of Lowry et al. (27) using bovine serum albumin as a standard.

**Treatment with Cholera Toxin**—Sperm membrane (1 mg/ml) or human erythrocyte membranes (3 mg/ml) were incubated with 50 mM Tris-HCl, pH 7.6, containing 1 mM diithiothreitol, 1 mM NAD, 1 mM ATP, 19 μM GTP, and 10 μg/ml of cholera toxin previously incubated in the presence of 20 μM diithiothreitol for 30 min at 37°C (28). After a 20-min incubation at 30°C, the samples were diluted 25-fold in cold 50 mM Tris-HCl, pH 7.6, and centrifuged at 20,000 rpm for 15 min at 4°C. The pellets were reconstituted in 50 mM Tris-HCl, pH 7.6. Controls were incubated in the same conditions but in the absence of cholera toxin.
Complementation of Ram Sperm—A titration in which increasing amounts of human erythrocyte membranes were combined with a fixed amount of sperm membranes is shown in Fig. 1. In the absence of erythrocyte membranes, the ram sperm adenylate cyclase activity was 5 pmol of cyclic AMP formed/mg of protein/min and was not increased by addition of either Gpp(NH)p, hormones, or NaF. When erythrocyte membranes were added to the assay mixture, basal activity was not modified; however, stimulation of adenylate cyclase activity by NaF and Gpp(NH)p was clearly restored. It reached a maximum, 55- and 24-fold stimulation for NaF and Gpp(NH)p activation, respectively, when 160 μg of human erythrocyte membranes were added to 3 μg of sperm membrane protein.

When the amount of human erythrocyte membranes was kept constant while the amount of ram sperm protein was varied from 0.1 to 10 μg/assay, the reconstituted activity assayed in the presence of 10 μM Gpp(NH)p was proportional to the amount of ram sperm protein. This activity reached a plateau corresponding to a 23-fold enhancement of cyclase activity (Fig. 2).

Time Course of Reconstitution—The time course of reconstitution of ram sperm membranes in the presence of Gpp(NH)p is shown in Fig. 3. Sperm membranes (4 μg/assay) were incubated for various periods of time at 30 °C in the presence of 10 μM Gpp(NH)p and all the other assay reagents and in the absence or presence of human erythrocyte membranes (107 μg/assay). Fig. 3 clearly demonstrates that a 5-min
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**Fig. 4. Effects of various concentrations of MgCl₂ and MnCl₂ on the adenylate cyclase activity.** The adenylate cyclase activities were measured as described under "Experimental Procedures" in the presence of 0.5 mM ATP and 1 mM EDTA. Various concentrations of cation were added (0 to 25 mM). The adenylate cyclase activity was measured in the presence of 4 μg/assay of sperm membranes, and in the absence of MgCl₂ (○), or the presence of 10 μM Gpp(NH)p (△, *) for 20 min at 30 °C. Activity was measured in the absence (---) or in the presence of 80 μg of protein/assay of human erythrocyte membranes (-- - -). Activities are expressed in picomoles of cyclic AMP formed/mg of sperm protein/min at 30 °C.

**Table I**

|                       | Control sperm | Human erythrocyte | Cholera toxin-treated human erythrocyte |
|-----------------------|---------------|-------------------|----------------------------------------|
| Basal activity        | 92.5          | 90                | 1785 (19)                              |
| + 10 μM Gpp(NH)p      | 80            | 1088 (18)         | 5260 (57)                              |
| Cholera toxin-treated sperm | 90      | 90                | 1678 (19)                              |
| Basal activity        | 90            | 90                | 4984 (55)                              |
| + 10 μM Gpp(NH)p      | 85            | 1550 (17)         |                                        |

 lag occurred before Gpp(NH)p started to stimulate cyclase activity; activation was completed in 20 min, and cyclase activity was constant thereafter for at least 40 min. Control sperm adenylate cyclase activity was also linear with time during the same period. The same lag in activation was observed in the presence of Na fluoride.

**Effect of Cations—**It is known that sperm adenylate cyclase can be activated by either Mg²⁺ or Mn²⁺ (6, 8, 9, 11), the ram sperm enzyme being 15-fold more active in the presence of Mn-ATP than in the presence of Mg-ATP. As shown in Fig. 4A, the enzyme activity assayed in the presence of 0.5 mM ATP and 1 mM EDTA increased when Mg concentration varied from 0 to 20 mM, reaching a plateau only at high Mg concentration. This dose-response curve was the same whether 10 μM Gpp(NH)p was added to the assay medium or not. When 80 μg of human erythrocyte membrane protein were added to the assay medium, the basal activity was not changed, while that assayed in the presence of Gpp(NH)p was dramatically increased. Furthermore, the slope of the dose-response curve for Mg was modified, the maximal activity being attained at a much lower total Mg concentration (3 mM instead of 20 mM). The dose-response curve for Mn (Fig. 4B) was similar to that of Mg for the basal sperm activity, maximal activity being attained at a concentration of 25 mM total Mn. However, at all Mn concentrations, the activity was not modified when either Gpp(NH)p or sperm membrane or both were added. It therefore appears that reconstitution of a Gpp(NH)p-sensitive adenylate cyclase was only observed when Mg was the co-substrate of the reaction; it is not possible however to decide whether Mg was necessary only for the expression of the reconstituted system or for the reconstitution process per se.

**Effect of Cholera Toxin on Reconstitution—**Like the AC⁺ lymphoma cell line system, sperm adenylate cyclase is unresponsive to cholera toxin, whatever the preincubation medium or cholera toxin concentration used (Table I). It was therefore interesting to test whether preactivation of the coupling factor from human erythrocyte membranes with cholera toxin could lead to a permanently activated state of N that might be detectable after the reconstitution process. When the human erythrocyte membranes were preactivated by cholera toxin as described under "Experimental Procedures," the reconstitution process could be observed either for the basal activity or in the presence of Gpp(NH)p, leading to an increase in adenylate cyclase activity of 20-fold and 60-fold, respectively (Table I). The data also show that even after prior treatment with cholera toxin, addition of Gpp(NH)p (10 μM) during the cyclase assay caused a further enhancement (3-4-fold) of the reconstituted enzyme activity. Conversely, preincubation of sperm membrane with cholera toxin did not activate the cyclase system, whether or not the erythrocyte membranes were added afterwards.

**Effect of Prostaglandins on Adenylate Cyclase—**Fig. 5 shows the effect of PGE₁, and PGE₂ on ram sperm adenylate cyclase before and after reconstitution with human erythrocyte membranes. Ram sperm adenylate cyclase activity was not enhanced by either prostaglandin; rather, it was decreased (40% inhibition) at high concentration (0.1 mM) of prostaglandins. This finding is in agreement with results observed with human, bull, and monkey spermatozoa (6, 8, 9). In contrast,
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FIG. 5. Effects of various concentrations of PGE₁ and PGE₂ on adenylate cyclase activity. The adenylate cyclase activities were measured, as described under "Experimental Procedures," in the presence of 50 μM Gpp(NH)p and in the presence of various concentrations of PGE₁ (○, △) and PGE₂ (□, △). Adenylate cyclase activity was measured in the presence of sperm membranes (○, △) or in the presence of sperm membranes and human erythrocyte membranes (□, △). The inset represents the sperm adenylate cyclase activity prior to reconstitution and as a function of prostaglandin concentrations.

FIG. 6. Influence of sperm membranes on the stimulation by PGE₁ on the reconstituted system. Various concentrations of sperm membrane proteins were assayed in the presence of 50 μM Gpp(NH)p, in the absence (○, △) or presence of human erythrocytes (□, △), and in the absence (○—○, △—△) or in the presence of 10 μM PGE₁ (△—△, △—△). Incubation was performed at 30 °C for 40 min.

the reconstituted, Gpp(NH)p-stimulated enzyme was enhanced in a dose-dependent manner by PGE₁ and PGE₂; half-maximal activation was attained at 0.1 μM for PGE₁ and 0.8 μM for PGE₂. Fig. 6 demonstrates that the PGE₁ response and the Gpp(NH)p "reconstituted" activity were parallel as a function of the concentration of sperm protein. This indicates that prostaglandin activates sperm adenylate cyclase throughout binding sites present on the erythrocyte membrane.

DISCUSSION

Ram sperm adenylate cyclase is similar to that of the AC- lymphoma cell line mutant described by Ross et al. (14). Both systems are insensitive to NaF, Gpp(NH)p, and cholera toxin, indicating that they are devoid of a functional guanine nucleotide regulatory component.

The present report demonstrates that human erythrocyte membranes are capable of restoring guanine nucleotide regulation and fluoride sensitivity to the ram sperm membrane adenylate cyclase system. The reconstituted system presents the following characteristics. (i) The reconstitution is time-dependent, a lag of 5 to 6 min being observed in the presence of Na fluoride and Gpp(NH)p at 30 °C. (ii) The reconstitution process is proportional to erythrocyte membrane protein added in the presence of Gpp(NH)p or fluoride. The sperm membrane system appears to possess a great excess of catalytic sites/mg of protein as compared to the AC- cell line system, since the reconstitution experiment performed with the same human erythrocyte membrane preparation gave different optimal protein ratios for the different systems: 1 to 100 for sperm membrane to human erythrocyte membranes versus 1 to 5 for AC- lymphoma cell line (18). This is in agreement with the fact that the specific activity of AC- cyclase, assayed in the presence of MgATP, is much lower than that of ram sperm (14). (iii) The reconstitution was only observed when MgATP was used as substrate response of adenylate cyclase activity as a function of MgCl₂ concentration was shifted toward lower concentrations. No reconstitution could be observed in the presence of Mn-ATP. One might hypothesize that the metal binding site is present normally in the ram sperm and that its affinity for Mg²⁺ is enhanced after the addition of the coupling factor(s). Alternatively, Mn²⁺ ions might somehow uncouple the catalytic site from regulatory components as reported in other systems (29, 30); or else the high affinity for Mg²⁺ in the reconstituted system might directly reflect the insertion of a saturating amount of functional N from human erythrocyte membranes, while the low affinity apparent before reconstitution would reflect an abnormal, defective, or poorly functional N. (iv) The reconstituted guanine nucleotide-sensitive, ram sperm adenylate cyclase is activated by prostaglandins, probably through the binding sites normally present in the human erythrocyte membranes (22). In contrast, when epinephrine, human chorionic gonadotropin, or follicle-stimulating hormone were added in the final assay system, they failed to further stimulate the activity in the presence of Gpp(NH)p (data not shown). (v) Finally, pretreatment of the N component of human erythrocyte membranes by cholera toxin greatly enhanced the ability of the factor to activate ram sperm adenylate cyclase, whether Gpp(NH)p was present or not, in the assay. In the present system, as well as in that described by Nielsen et al. (20) and Lad et al. (21), the mechanism of the reconstitution process was not directly probed. In particular, it is not possible at the present time to decide whether a true membrane fusion occurred or whether components from one membrane migrated to the other. The reconstituted system exhibited a prostaglandin response; this might indicate, but does not prove, that the sperm cyclase itself was transferred to the erythrocyte membrane. The fact that cholera toxin was active only when preincubated with erythrocyte membranes, and not with sperm membranes, is direct evidence that the N component does originate from the erythrocytes.

Inasmuch as the adenylate cyclase activity of human erythrocyte membranes can be considered as negligible (15-22), the present findings constitute a functional demonstration that N can be activated by cholera toxin in the absence of a fully functional cyclase and that the "activated" N can be transferred to an acceptor system. Noteworthy is the fact that the cyclase reconstituted with cholera toxin-treated N was still sensitive to the further addition of Gpp(NH)p to the assay.
medium. Similar findings have been reported by Cassel and Selinger (31), Cassel and Pfeuffer (32), Birnbaumer et al. (33), and Abramowitz et al. (34) with membranes from turkey erythrocytes, pigeon erythrocytes, and rat liver. They are compatible with the hypothesis that cholera toxin can exert a stimulatory effect upon adenylate cyclase by a mechanism other than, or in addition to, the classical inhibition of GTPase activity (31).

In this paper we demonstrated that ram sperm membrane adenylate cyclase activity is deficient in guanine nucleotide regulatory protein and can be complemented with an N donor in the absence of detergent. After reconstitution with cholera toxin-treated human erythrocyte, the sperm adenylate cyclase activity was activated 60-fold with respect to basal activity. Ram sperm adenylate cyclase can be used as an acceptor adenylate cyclase deficient in N, and is analogous to the AC-system potentially very useful tool along with other, more sophisticated systems (14, 15, 35, 36) for elucidating many of the open questions concerning the role of the various subunits of the cyclase system.

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