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Cytosolic activator of adenylate cyclase: Reconstitution, characterization, and mechanism of action

(erythrocyte ghosts/peripheral membrane protein/thiol group/5'-guanylyl imidodiphosphate/calcium)

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ABSTRACT **Rat liver and isolated hepatocytes contain high levels of a soluble adenylate cyclase stimulator, whereas rat erythrocytes lack this activity. Accordingly, a reconstitution system was developed with adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] from erythrocyte ghosts and the soluble activator from liver cytosol. Pretreatment of erythrocyte ghosts with the cytosolic factor resulted in a 5- to 15-fold activation of adenylate cyclase in the presence or absence of NaF, 5'-guanylyl imidodiphosphate, or isoproterenol and GTP. The sequence of addition of the cytosolic component and the other activators was critical in determining the maximal activity of the enzyme. The cytosolic factor appears to be a heat-labile M_r 105,000 protein, which activates adenylate cyclase in a saturable reaction involving binding of the protein to the erythrocyte ghosts. This molecular interaction was accompanied by stabilization of a labile thiol group that was essential for catalytic activity. The cytosolic component also unmasks latent adenylate cyclase activity in human erythrocyte ghosts and in cytoskeletal preparations from rat erythrocyte ghosts. These observations suggest that the cytosolic activator may also occur as a native, peripheral membrane component of adenylate cyclase systems and may be required for the expression and stabilization of catalytic activity.**

Soluble components from a variety of organs, cell preparations, and cultured cell lines stimulate particulate adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. The relevant literature has been recently summarized elsewhere (1–5). These presumably cytosolic activators differ in their sensitivity to protease digestion and to heat inactivation, and they frequently exhibit differential effects in the presence of guanyl nucleotides, sodium fluoride, or various hormones (1, 4–13). Maturation- and age-dependent changes in the levels of the soluble activators have been detected in rat reticulocytes (4) and lung (14), respectively.

Attempts have been made to separate and purify some of these cytosolic factors. Two active fractions that act synergistically were derived from rat lung supernatants by DEAE-cellulose chromatography (3). Another two factors were separated from rat osteosarcoma cytosol, and their molecular weights were estimated at 55,000 and 29,000 by Sephadex G-100 gel-permeation chromatography (11). A M_r 13,000 heat-stable factor from liver cytosol that potentiates hormonal stimulation of adenylate cyclase in a GTP-like fashion has been purified 1,000-fold (15). The heat-stable and NaF-specific factor from rat brain was purified about 3,000-fold and behaves as a M_r 59,000 monomer (10).

A sensitive reconstitution system has been developed here, utilizing rat erythrocyte ghosts as a source of adenylate cyclase and rat liver cytosol as a source of the cytosolic activator. This assay has been employed to characterize the physical parameters of the cytosolic factor and to study the kinetic characteristics and the molecular mechanism of the activation process. The results demonstrate the existence of a soluble, heat-labile M_r 105,000 protein that enhances severalfold the basal and the variously stimulated adenylate cyclase activities. Moreover, this activator behaves like a peripheral membrane protein and appears to be obligatory for the expression of catalytic activity in certain systems such as human erythrocytes and detergent-extracted rat erythrocyte ghosts. Furthermore, the interaction of this protein activator with adenylate cyclase leads to the protection of a labile thiol group that is required for catalytic activity.

MATERIALS AND METHODS

Cholera toxin was purchased from Schwarz/Mann, digitonin was from Sigma, and collagenase was obtained from Millipore. [α-^{32}P]ATP and [2,8-^{3}H]cyclic AMP were supplied by New England Nuclear. Adenylate cyclase was assayed at 30°C in the presence of 0.2 mM ATP, 10^7 dpm of [α-^{32}P]ATP, 10^5 dpm of [2,8-^{3}H]cyclic AMP, 7 mM $MgCl_2$, 8 mM phospho*enol*pyruvate, 10 μg of pyruvate kinase, and 50–100 μg of membrane protein in a total volume of 0.1 ml of 50 mM Tris·HCl, pH 7.6. The assay was terminated after 12 min and the product was separated on neutral alumina as described (16). Rat or human erythrocyte ghosts were prepared by repeated hypotonic lysis in 7 mM Tris·HCl, pH 7.6 (16), and were suspended in 50 mM Tris·HCl, pH 7.6. Rat livers were homogenized with a Brinkmann Polytron homogenizer at setting 3.5 for 30 sec. The homogenization was performed in 3 vol of 50 mM Tris·HCl, pH 7.6, containing 0.25 M sucrose, 0.1 mM EDTA, and phenylmethylsulfonyl fluoride at 10 μg/ml. Alternatively, rat livers were perfused *in situ* with 200 ml of the above buffer prior to homogenization. Hepatocytes were prepared by liver perfusion *in situ* at 37°C with Hanks' balanced salt solution containing EGTA followed by the same medium containing $CaCl_2$ and collagenase (17) and were then lysed by sonication. The soluble fraction from the homogenized livers or sonicated hepatocytes was obtained by centrifugation at 20,000 × *g* for 15 min followed by centrifugation of the supernatant for 2 hr at 300,000 × *g*. The final supernatants (8–15 mg of protein per ml) were freed from low molecular weight compounds by gel filtration over a Sephadex G-50 column (Pharmacia) and were used immediately or stored at −90°C for up to a month without any loss of activity. Cytosol from rat or human erythrocytes was prepared by lysing the cells in 4 vol of 5 mM Tris·HCl, pH 7.6, followed by centrifugation at 300,000 × *g* for 1 hr. Rat erythrocyte cytoskeletons were prepared from erythrocyte ghosts by treatment with 10 vol of 10 mM Tris·HCl, pH 7.6, containing

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Abbreviation: p[NH]ppG, 5'-guanylyl imidodiphosphate.

0.1 mM EDTA and 2% Triton X-100 followed by washing the detergent shells with Triton-free buffer (18). Digitonin shells from rat erythrocytes were obtained as described (19). β-Adrenergic receptors were assayed by using the antagonist (±)-[^{125}I]iodocyanopindolol (20). Protein concentration was measured by the Coomassie blue R-250 method (21).

RESULTS

Direct addition of liver cytosol to membranes in the reaction mixture of the adenylate cyclase assay resulted in a maximal 2-fold to 3-fold activation of the enzyme. This type of assay presented several drawbacks because of the presence in liver cytosol of phosphodiesterases, phosphatases, and other components that interfere with the measurement of enzyme activity. Consequently, another type of assay was developed such that liver cytosol was present only during a preincubation step. This approach resulted in considerably greater enzyme stimulation and the presence of interfering factors in the assay was avoided. The cytosol itself did not contain any adenylate cyclase activity before or after the preincubation.

Exposure of rat erythrocyte ghosts to a heat-labile component in liver cytosol resulted in a 10- to 15-fold stimulation of adenylate cyclase activity (Fig. 1*A*). This effect was observed on basal activity and in the presence of NaF, 5'-guanylyl imidodiphosphate (p[NH]ppG), p[NH]ppG and isoproterenol, or GTP and isoproterenol. The effect of the various activators was enhanced about 2-fold in the presence of cytosolic factor (Fig. 1*B*). The cytosolic factor also produced a 7-fold stimulation of adenylate cyclase from rat erythrocytes that had been preactivated with cholera toxin. The heat-labile cytosolic component was entirely sensitive to trypsin, was unaffected by *N*-ethylmaleimide, and was excluded from a Sephadex G-50 column. These results were also observed with cytosol from perfused livers or from hepatocytes, indicating that the adenylate cyclase activator was a cytosolic constituent of parenchymal cells. The stimulatory effect appeared to be saturable with increasing amounts of cytosol (Fig. 2*B*) and could still be observed when the cytosol was diluted up to 1:30 (Fig. 2*A*). The stimulation of enzyme activity in the presence or absence of p[NH]ppG, isoproterenol, or NaF reflected mostly an increase in V_{max} for ATP and $MgCl_2$. The apparent K_m for p[NH]ppG remained unchanged. However, a small but reproducible decrease was observed in the apparent K_m for ATP from 130 μM to 80 μM. Similarly, the apparent K_m for isoproterenol was diminished from 0.13 μM to 0.075 μM. These changes in the kinetics of isoproterenol action were not accompanied by any change in the characteristics of the β-adrenergic receptor, which were determined by using 3-[^{125}I]iodocyanopindolol. The number of binding sites (1.8 pmol/mg of protein) and the K_d (3.5×10^{-11} M) were unaltered. Similarly, the K_d for isoproterenol (4×10^{-7} M) determined by competition was not changed.

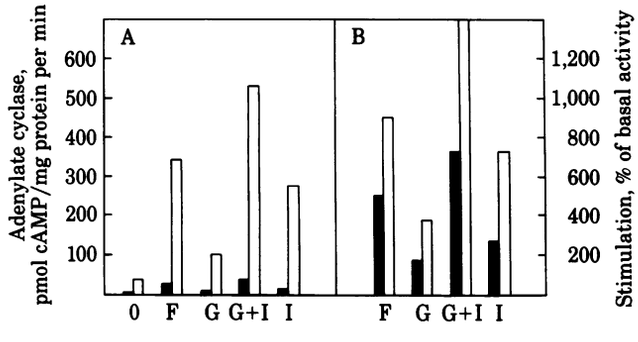

FIG. 1. Stimulation of adenylate cyclase from rat erythrocyte ghosts by preincubation with rat liver cytosol. A suspension of rat erythrocyte ghosts (0.5 ml, 3.5 mg of protein per ml) was incubated with 0.5 ml of unheated (open bars) or boiled (closed bars) rat liver cytosol in the presence of 5 mM $MgCl_2$. After 10 min at 23°C 10 ml of ice-cold 50 mM Tris·HCl, pH 7.6, with 5 mM $MgCl_2$ was added and the membranes were retrieved by centrifugation for 10 min at 40,000 × *g* and resuspension in the same buffer. (*A*) Between 100 and 120 μg of membrane protein was used for the adenylate cyclase assay in the absence of any activators (0) or with 12 mM NaF (F), 10 μM p[NH]ppG (G), 10 μM p[NH]ppG and 10 μM isoproterenol (G + I), or 10 μM GTP and 10 μM isoproterenol (I). (*B*) Percent stimulation compared to basal activity in the absence of G, F, or I.

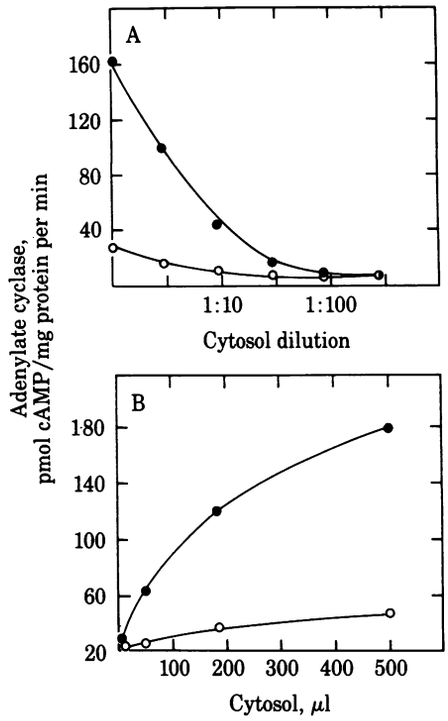

FIG. 2. Dependence of adenylate cyclase activation on the concentration of liver cytosol. Boiled (○) and unheated (●) cytosol were diluted in liver homogenization buffer. Enzyme activation was performed as described in the legend to Fig. 1, using a constant volume of different dilutions of cytosol. The enzyme was assayed in the presence of 10 μM p[NH]ppG and 10 μM isoproterenol. Data are plotted with increasing cytosol dilution (*A*) or with increasing cytosol concentration (*B*).

The relationship of the cytosolic activator to other activators of adenylate cyclase was determined by varying their sequence of addition (Table 1). Maximal stimulation was possible only when the interaction of the cytosolic factor with adenylate cyclase preceded the addition of p[NH]ppG and isoproterenol or NaF. Reversing the order of the additions or the simultaneous addition of all activators resulted in considerably less stimulation.

We had reported previously the existence of a functionally relevant interaction between membrane-associated components of the adenylate cyclase system and erythrocyte cytoskeletons prepared by detergent extraction (18, 19, 22). Here we show that the stimulatory effect of the cytosolic factor persisted when detergent extraction followed the activation step. Moreover, the activation of the enzyme could be reversed by exposing the erythrocyte ghosts to low-ionic-strength buffer in the presence of EDTA (Table 2). The enzyme could then be restimulated by exposure to the cytosolic activator (Table 2). Because relatively large volumes of buffer were used to reverse the enzyme activation, it was not possible to detect any stimulatory activity that might have been released from the activated ghosts. This possibility was assessed by employing small volumes of a chaotrope (0.6 M KI, Table 3). This treatment in-

Table 1. Sequential activation of adenylate cyclase by the cytosolic factor and by NaF or p[NH]ppG and isoproterenol

Combination number	First preincubation		Second preincubation		Additions in assay		Adenylate cyclase activity*	
	0	A	Control	Cytosol	0	A	F	G+I
1	+	−	+	−	+	−	4	4
2	+	−	+	−	−	+	105	30
3	+	−	−	+	+	−	23	16
4	+	−	−	+	−	+	310	250
5	−	+	+	−	+	−	80	22
6	−	+	+	−	−	+	84	26
7	−	+	−	+	+	−	145	35
8	−	+	−	+	−	+	215	80

Aliquots (0.5 ml) of a rat erythrocyte ghost suspension containing 50 mM Tris·HCl, pH 7.6, 2 mg of protein, and 10 mM $MgCl_2$ were preincubated with (A) or without (0) activator for 10 min at 30°C (first preincubation). Then 10 ml of ice-cold 50 mM Tris·HCl containing 10 mM $MgCl_2$ was added, the samples were centrifuged for 10 min at 40,000 × *g*, and the pellets were resuspended in 0.5 ml of buffer. These pellets were then treated with boiled (Control) or unheated cytosol (Cytosol) as described in the legend to Fig. 1 (second preincubation). Subsequently they were assayed for adenylate cyclase activity in the presence (A) or absence (0) of activator. The activator (A) denotes either 12 mM NaF (F) or 10 μM p[NH]ppG and 10 μM isoproterenol (G + I) as specified in the last column.

*pmol cAMP/mg of protein per min.

activated adenylate cyclase but did not reduce the activity of the cytosolic factor (data not shown). The degree of stimulation of adenylate cyclase by the KI extract of prestimulated erythrocyte ghosts is about one-third of the degree of stimulation obtained with cytosol. This is probably due to the relatively low binding capacity of erythrocyte ghosts for the stimulatory factor. The fact that KI extracts of control erythrocyte ghosts display some stimulatory activity may be due to the release of endogenous activator.

The cytosolic factor was capable of unmasking latent NaF- and p[NH]ppG-responsive adenylate cyclase activities in the detergent residue of rat erythrocyte ghosts (erythrocyte shells) (Table 4). Similarly, the ability of liver cytosol to restore latent enzyme activity was confirmed in human erythrocyte ghosts, which otherwise have virtually no detectable enzyme activity (Table 4). In contrast, cytosol prepared from rat or human erythrocytes did not produce any significant stimulation of adenylate cyclase from either rat or human erythrocyte ghosts.

Sucrose density gradient velocity sedimentation (Fig. 3) and gel-permeation chromatography on Ultrogel AcA 34 (LKB) (Fig. 4) separated the activator from the major protein peaks. Table 5 summarizes the physical parameters that were derived from Fig. 3 and Fig. 4, indicating the presence of one major activator with an apparent M_r of 105,000.

The cytosolic activator of adenylate cyclase also stabilized the enzyme against spontaneous inactivation. Studying the mechanism of this stabilization provided a clue to the mechanism of the concomitant stimulation. Incubation of rat erythrocyte ghosts in 50 mM Tris·HCl, pH 7.6, at 23°C, but not at 0°C, for 20 min resulted in a significant loss of enzyme activity (Table 6). Inactivation was augmented by $MgCl_2$ and was blocked by EGTA in the presence or absence of $MgCl_2$. Exogenously added $CaCl_2$ reversed the effect of EGTA. The cytosolic factor

Table 2. Reversal of activation and restimulation of adenylate cyclase activity

Treatment	Adenylate cyclase activity, pmol cAMP/mg protein per min
1. Erythrocyte ghosts + liver cytosol	260
2. Erythrocyte ghosts + boiled cytosol	98
3. Treatment 1, then reversal buffer	88
4. Treatment 3, then liver cytosol	240
5. Treatment 3, then boiled cytosol	109

Erythrocyte ghosts (1 ml) were maximally activated with the cytosolic factor as indicated in the legend to Fig. 1. They were subsequently incubated at 30°C for 15 min in the presence of 20 ml of a solution containing 1 mM EDTA, 1 mM dithiothreitol, and 5 mM Tris·HCl, pH 7.6 (reversal buffer). Then they were restimulated in the presence of unheated or boiled liver cytosol. The enzyme was assayed with 10 μM p[NH]ppG and 10 μM isoproterenol.

Table 3. Release of adenylate cyclase activator by KI from prestimulated erythrocyte ghosts

Erythrocyte ghost extract	Adenylate cyclase activity, pmol cAMP/mg protein per min
KI + prestimulated ghosts (unheated cytosol)	112
KI + prestimulated ghosts (boiled cytosol)	55
KI + control ghosts (unheated cytosol)	70
Tris·HCl + prestimulated ghosts	62
No additions	48

An erythrocyte ghost suspension (1 ml, 3 mg of protein per ml) was prestimulated in the presence of 1 ml of unheated or boiled liver cytosol as explained in the legend to Fig. 1. The ghosts were washed twice at 4°C with 20 vol of 50 mM Tris·HCl, pH 7.6. The ghosts were then suspended in 2 ml of 0.6 M KI containing 5 mM dithiothreitol. After 15 min at 0°C the KI extract was centrifuged for 1 hr at 300,000 × *g* and the supernatant was desalted on a Sephadex G-50 column to remove KI. A parallel experiment was run in which 50 mM Tris·HCl, pH 7.6, with 5 mM dithiothreitol was used in the extraction step in the absence of KI. The various extracts were used to preactivate fresh erythrocyte ghosts as detailed for Fig. 1. Adenylate cyclase was assayed with 10 μM p[NH]ppG and 10 μM isoproterenol.

Table 4. Unmasking of latent adenylate cyclase activity in rat erythrocyte detergent shells and human erythrocyte ghosts by liver cytosol

Enzyme source	Adenylate cyclase activity, pmol cAMP/mg protein per min		
	0	G	F
Triton shells (boiled cytosol)	—	—	—
Triton shells (unheated cytosol)	7	12	15
Digitonin shells (boiled cytosol)	—	—	—
Digitonin shells (unheated cytosol)	15	60	110
Human erythrocyte ghosts (boiled cytosol)	—	—	5
Human erythrocyte ghosts (unheated cytosol)	4	25	40

Detergent shells were prepared by extraction of a rat erythrocyte suspension (0.5 mg protein/ml) with 2% Triton X-100 or with 2% digitonin. The resulting cytoskeletal shells were washed and resuspended in 50 mM Tris·HCl, pH 7.6, at 1.5 mg of protein per ml. Human erythrocytes were separated from leukocytes and from reticulocytes by centrifugation (23) and ghosts were subsequently prepared. The authenticity of cAMP as a product was established by Dowex/alumina purification and by sensitivity to beef heart cyclic nucleotide phosphodiesterase. The activation and assay were conducted as described in the legend to Fig. 1 with boiled or unheated cytosol. Additions in the assay: 0, none; G, 10 μM p[NH]ppG; F, 12 mM NaF. A — indicates activity was <0.5 pmol cAMP/mg of protein per min.

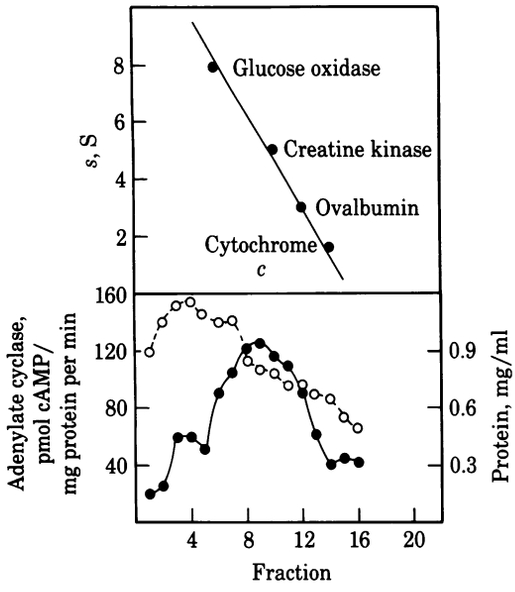

FIG. 3. Sucrose-density gradient velocity sedimentation of the rat liver cytosolic activator of adenylate cyclase. Cytosol (2 ml) was applied to a 30-ml sucrose gradient (5–20%). The samples were centrifuged in a TV-850 DuPont vertical rotor for 4 hr at 50,000 rpm and 2-ml fractions were collected. The various fractions were assayed for their ability to stimulate rat erythrocyte ghost adenylate cyclase in the presence of 10 μM p[NH]ppG and 10 μM isoproterenol as described for previous figures. The sedimentation coefficient varied by less than 6.5% in five separate experiments. ○, Protein; ●, activity. (*Upper*) Sedimentation coefficients of proteins of known molecular weight in a parallel gradient.

as well as dithiothreitol and reduced glutathione (GSH) were able to prevent enzyme inactivation. The reducing agents were also able to partially restore lost activity (data not shown). When

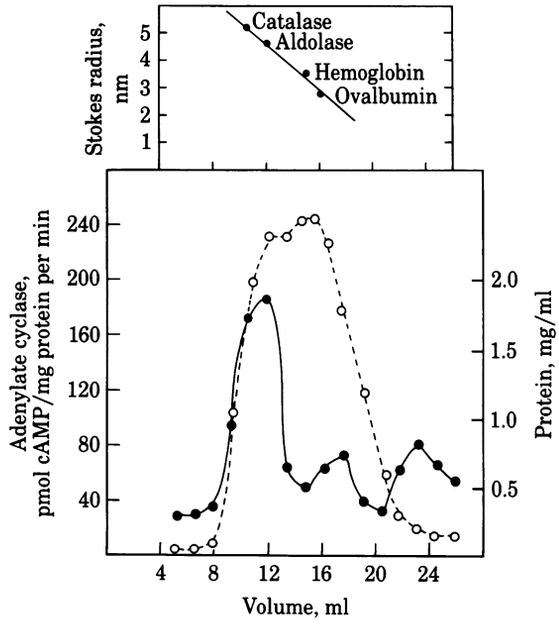

FIG. 4. Gel-permeation chromatography of the rat liver cytosolic activator of adenylate cyclase. Cytosol (0.8 ml) was applied to a 24.5-ml Ultrogel AcA 34 column, which was eluted with 25 mM Tris·HCl, pH 7.6. Fractions (0.85 ml) were collected and assayed for activity as described in the legend to Fig. 3. The position of the major peak of activity was invariant in four different experiments. ○, Protein; ●, activity. (*Upper*) Stokes radii of standard proteins chromatographed on the same column.

Table 5. Molecular parameters of the soluble adenylate cyclase activator from rat liver cytosol

Sedimentation coefficient, $s_{20,w}$, S	5.5
Stokes radius, α, nm	4.5
Molecular weight, M_r*	105,000
Frictional ratio, f/f_0†	1.45

* M_r was calculated according to the equation

$$M_r = \frac{6\pi N \eta_{20,w}}{1 - \bar{v}\rho_{20,w}} \cdot \alpha s_{20,w},$$

in which N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20°C, $\rho_{20,w}$ is the density of water at 20°C, and $\bar{v}$ is the partial specific volume of the protein, which was assumed to be 0.735 ml/g.
† $f/f_0 = \alpha[4\pi N/3M_r\bar{v}]^{1/3}$.

enzyme activity was already protected by chelating Ca^{2+} or by the addition of the cytosolic component, thiol-reducing agents did not produce any additional effect. This suggested that all the indicated methods of stabilizing enzyme activity involved essential thiol group(s) of adenylate cyclase. By comparing the effects of the cytosolic activator in the presence of $MgCl_2$ and in the presence of EDTA, it was concluded that apparent activation of adenylate cyclase represented a combined effect of enzyme stabilization and true stimulation.

DISCUSSION

A heat-labile, trypsin-sensitive M_r 105,000 macromolecule from rat liver cytosol can appreciably stimulate adenylate cyclase activity in rat erythrocyte ghosts. This activator enhances the basal enzyme activity and the activity assayed in the presence of NaF, p[NH]ppG, isoproterenol and p[NH]ppG, isoproterenol and GTP, or after activation by cholera toxin. The two-step activation and assay procedure developed here obviates the need for the presence of liver cytosol during the enzyme assay and results in a greater degree of stimulation. It also allows the sequential treatment of adenylate cyclase with several activators in order to determine the optimal order of additions. Moreover, the preactivation phenomenon indicates that the cytosolic factor produces a persistent effect, which may be mediated by a covalent change in the enzyme or by a high-affinity binding

Table 6. Stabilization of a thiol group by the cytosolic activator

Additions	Adenylate cyclase, % of control activity
None	35
5 mM $MgCl_2$	20
1 mM EDTA	80
1 mM EGTA + 5 mM $MgCl_2$	72
1 mM EGTA + 5 mM $MgCl_2$ + 0.1 mM free Ca^{2+}	20
5 mM $MgCl_2$ + 10 mM dithiothreitol	92
5 mM $MgCl_2$ + 10 mM GSH	84
1 mM EDTA + 10 mM GSH	90
5 mM $MgCl_2$ + liver cytosol	250
1 mM EDTA + liver cytosol	280
5 mM $MgCl_2$ + 10 mM GSH + liver cytosol	290

Rat erythrocyte ghosts (1 ml, 2.5 mg of protein per ml) were incubated in 50 mM Tris·HCl, pH 7.6, for 20 min at 23°C in the presence of various additions. The erythrocyte ghosts were then washed at 0°C in 15 vol of 50 mM Tris·HCl, pH 7.6, and assayed for adenylate cyclase activity in the presence of 10 μM p[NH]ppG and 10 μM isoproterenol. The results are expressed as percent of enzyme activity of membranes that were treated as above except that the 20-min incubation was performed at 0°C.

reaction. The latter possibility is favored by the fact that the activation process is apparently saturable with increasing cytosol concentration, by the finding that treatment with low-ionic-strength buffer reverses the activation and by the observation that the chaotropic agent, KI, releases an adenylate cyclase stimulator from preactivated erythrocyte ghosts. This implies that the cytosolic factor can also exist as a peripheral membrane protein.

The cytosolic factor can unmask adenylate cyclase activity in digitonin shells of rat erythrocytes and in human erythrocyte ghosts. The fact that the enzyme activity in these preparations is minimal or nondetectable except in the presence of the soluble activator suggests that this molecule may normally be required for the expression of catalytic activity. Furthermore, it may already be present as a native component of adenylate cyclase systems that display significant activity in the absence of added cytosol. Accordingly, digitonin treatment may release the endogenous pool of this factor from rat erythrocyte ghosts, resulting in an absolute dependence of catalytic activity on the exogenous cytosolic activator.

The cytosolic activator of adenylate cyclase seems to exert its effect mainly on the catalytic function of the enzyme. This observation is supported by the lack of any change in the apparent affinity of the enzyme complex for p[NH]ppG and by the absence of any effect on the number of β-adrenergic receptors or their affinity for an agonist or an antagonist.

The activation of adenylate cyclase by the cytosolic factor alters an essential thiol group in the catalytic moiety, apparently reducing its susceptibility to spontaneous oxidation. Interestingly, the data suggest that the reactivity of the thiol group has a strict dependence on membrane-bound Ca^{2+} and is enhanced by $MgCl_2$. Dithiothreitol or GSH at millimolar concentrations can also block or partially reverse the Ca^{2+}-mediated enzyme inactivation.

The relatively sensitive assay developed here for the soluble adenylate cyclase activator can be used to compare tissue levels of this factor and to study possible changes in the regulatory activity in the same tissue. The fact that rat and human erythrocyte lysates lack detectable levels of this factor renders the latent adenylate cyclase from these sources particularly useful for reconstitution experiments. It is hoped that such experiments will further our understanding of the regulation of adenylate cyclase activity by cytosolic or peripheral membrane macromolecules.

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