MODE OF INTERACTION BETWEEN FORSKOLIN AND MANGANESE ION IN ACTIVATING CATALYTIC UNIT OF ADENYLALE CYCLASE FROM RAT BRAIN

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Rat brain adenylate cyclase was solubilized with a combination of 0.7% sodium cholate and 0.6 M ammonium sulfate, and fractionated by addition of solid ammonium sulfate. The precipitate at 35% ammonium sulfate saturation contained neither guanine nucleotide-binding regulatory protein (G protein) nor calmodulin, and was used as the catalytic unit of the enzyme system. This catalytic unit was activated synergistically by forskolin and Mn$^{2+}$. An apparent $K_m$ value for Mg-adenosine triphosphate (ATP) of the catalytic unit was about 80 μM in the basal state, while it increased in concurrence with the increase in the enzyme activity when forskolin was added to the assay system. The increase in the $K_m$ value depended on the forskolin concentration up to 1 μM, above which the value converged on ca. 200 μM. Furthermore, activation of the catalytic unit by forskolin was more marked at higher concentration of Mg-ATP. On the other hand, Mn$^{2+}$ suppressed the increase in the $K_m$ value for Mg-ATP by forskolin, though the value in the basal state was not changed by Mn$^{2+}$ alone. These findings indicate that the activation of the catalytic unit by forskolin is accompanied by the change in the affinity for Mg-ATP and Mn$^{2+}$ modifies the change.

Keywords—adenylate cyclase; adenylate cyclase system catalytic unit; forskolin, manganese ion; rat brain

Adenylate cyclase system is composed of at least three types of separable components; catalytic unit, guanine nucleotide-binding regulatory protein(s) (G protein(s)), and receptor(s) for the effector,$^{1,2}$ and is regulated by an interaction of these components. On the other hand, the catalytic unit itself has regulatory sites such as divalent cation-binding site, through which the activity of the enzyme is directly regulated independently of the control from the receptor.$^{3-5}$

Recently, forskolin, a hypotensive diterpene from the root of *Coleus forskohlii*, has been reported to stimulate both membrane-bound$^{6,7}$ and detergent-solubilized adenylate cyclase.$^{8}$ Since activation of this enzyme by forskolin can be found in cyc-$S49$ lymphoma cells which lack the functional G protein,$^{9}$ the catalytic unit itself is assumed to be a target of the diterpene. The assumption is supported by the finding that the catalytic unit separated from G protein by forskolin–Sepharose affinity chromatography is also activated by this diterpene.$^{10}$ However, mechanism of the direct activation of the catalytic unit by forskolin and relation between forskolin and divalent cation actions remain uncertain.

In this study, we used a preparation of the catalytic unit of adenylate cyclase solubilized from rat brain with sodium cholate and ammonium sulfate and fractionated by ammonium sulfate precipitation. The preparation was free from G protein and calmodulin. This paper presents evidence that activation of the catalytic unit by forskolin is accompanied by an increase in an apparent $K_m$ value for Mg-adenosine triphosphate (ATP). We also show that the rate of activation by forskolin is dependent on the substrate concentration. Furthermore, we demonstrate that the
effect of forskolin to increase the $K_m$ value is modified by Mn$^{2+}$.

MATERIALS AND METHODS

Materials — ATP, guanosine 5'-($\beta$, $\gamma$-imino)-triphosphate (Gpp(NH)p), adenosine 3',5'-cyclic monophosphate (cyclic AMP), creatine phosphokinase, phosphocreatine, dithiothreitol, bovine serum albumin, and Tris were purchased from Sigma; forskolin from Calbiochem; and ATP-$2^\text{-3}$H from the Radiochemical Centre, Amersham. Trifluoperazine was kindly donated from Yoshitomi Pharmaceutical Co. All other reagents were of analytical grade from standard commercial sources.

Preparation of Adenylate Cyclase from Rat Brain — All procedures were carried out at 4°C. Frozen and pooled rat brains were homogenized in 5 volumes of an ice-cooled 250 mM sucrose solution containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% ethanol (pH 7.4) using a Porter-Elvehjem homogenizer. The homogenate was centrifuged at 10000 × g for 10 min to remove cell nuclei and debris. Then, the post-nuclear supernatant was centrifuged at 100000 × g for 30 min. Adenylate cyclase was solubilized from this precipitate by suspending in 50 mM Tris-HCl buffer containing 250 mM sucrose, 15 mM MgCl$_2$, and 1 mM dithiothreitol (pH 8.0), and by adding sodium cholate and ammonium sulfate to the suspension to the concentration of 0.7% and 0.6 M, respectively, as described by Ross.$^{11}$ After standing for 20 min, the suspension was centrifuged at 100000 × g for 30 min. To the supernatant, solid ammonium sulfate was added with stirring to 35% saturation, and the mixture was centrifuged at 20000 × g for 30 min. The pellet was suspended in 50 mM Tris-HCl buffer (pH 8.0) which contained 3.5 mg/ml lecithin for the reconstitution of membranous condition.

Measurements of Adenylate Cyclase Activity and Protein — Adenylate cyclase activity was measured according to the method of Salomon et al.$^{12}$ using ATP-$3^\text{H}$, with some modifications.$^{13}$ The standard assay mixture consisted of 25 mM Tris-HCl, 12.5 mM MgCl$_2$, 1 mM cyclic AMP, 10 mM theophylline, 14.5 mM phosphocreatine, 250 unit/ml creatine phosphokinase, and 0.03–1.25 mM (10$^6$ cpm) ATP-$3^\text{H}$, pH 8.0, in a final volume of 400 µl. The assay was started by addition of the catalytic unit preparation. Standard incubation was performed at 30°C for 20 min. In cases indicated, various concentrations of MnCl$_2$ and/or forskolin were added to the assay mixture. Protein was determined by the method of Lowry et al.$^{14}$ using bovine serum albumin as the standard.

RESULTS

Activation of Catalytic Unit of Rat Brain Adenylate Cyclase by Forskolin

Fig. 1 shows that $K_m$ value for Mg-ATP and maximal velocity of the catalytic unit of adenylate cyclase prepared from rat brain are 87 µM and 19.0 pmol/min/mg protein, respectively, in the basal state. This figure also shows that both of these kinetic parameters are increased by addition of forskolin in a concentration-dependent manner in a range of 0.05–10 µM forskolin. Thus, it is noticeable that the activation of the catalytic unit by forskolin is accompanied by an apparent decrease in the affinity for the substrate. However, the $K_m$ value converged gradually on ca. 200 µM as the concentration of forskolin exceeded 1.0 µM.

Dependence of Forskolin Activation of the Catalytic Unit on the Mg-ATP Concentration

Table I shows relative ratios of activation of the catalytic unit by increasing concentration of forskolin at various concentrations of Mg-ATP, taking the activity without forskolin at each of the Mg-ATP concentration as 1. As shown in this table, the activation by forskolin was higher at higher concentration of Mg-ATP. These findings indicate that the mode of activation of the catalytic unit by forskolin is dependent on the substrate concentration.

Modulation of the Forskolin-Induced Change in the $K_m$ Value for Mg-ATP of the Catalytic Unit by Mn$^{2+}$

Since Mn$^{2+}$ is known to stimulate directly the
Forskolin and Mn on Adenylate Cyclase

![Graph showing the effect of forskolin and Mg-ATP on adenylate cyclase activity]

**FIG. 1. Double Reciprocal Plots of Adenylate Cyclase Activity vs. Mg-ATP Concentration in the Presence of Various Concentrations of Forskolin.**

The amount of the catalytic unit used for the activity measurement was 200 μg as protein. The same amount was used throughout.

catalytic unit of adenylate cyclase, its effect was examined on our preparation from rat brain both in the presence and absence of forskolin. Fig. 2 shows apparent synergism between forskolin and Mn$^{2+}$ in the activation of the catalytic unit, agreeing with the previously reported results. It was of interest that the shift of the $K_m$ value for Mg-ATP of the catalytic unit by forskolin was no more observed in the coexistence of Mn$^{2+}$, as shown in Fig. 3. In other words, increasing effect of forskolin on the $K_m$ value was canceled by Mn$^{2+}$. It is likely from these results that Mn$^{2+}$ has not only the directly activating effect on the catalytic unit of rat brain adenylate cyclase but also the modulating effect on the forskolin-induced changes in the catalytic unit. Fig. 4 shows that the increase in the concentration of Mn$^{2+}$ gradually decreases the apparent $K_m$ value for Mg-ATP to the basal value in spite of the presence of 10 μM forskolin. The figure also indicates that Mn$^{2+}$ itself has no effect on the $K_m$ value.

**DISCUSSION**

The catalytic unit of adenylate cyclase system has been less characterized than other components, because of its instability in the course of purification. The preparation in this study obtained from rat brain by ammonium sulfate precipitation from cholate-ammonium sulfate

**TABLE I. Relative Ratio of Activation of the Catalytic Unit of Rat Brain Adenylate Cyclase by Forskolin at Various Concentration of Mg-ATP**

<table>
<thead>
<tr>
<th>Concentration of Mg-ATP (μM)</th>
<th>Relative ratio of activation by forskolin $^a$</th>
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<tbody>
<tr>
<td>0.1</td>
<td>1.00</td>
</tr>
<tr>
<td>0.1</td>
<td>1.68</td>
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<tr>
<td>1.0</td>
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<td>12.0</td>
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<tr>
<td>1250</td>
<td>12.7</td>
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</table>

$^a$ The values were calculated taking the adenylate cyclase activity without forskolin as 1 at each of Mg-ATP concentrations.
solubilize was neither stimulated by 0.05 mM Gpp(NH)p nor inhibited by 0.5 mM ethylene glycol di(aminooethyl)tetraacetic acid (EGTA) and 0.05 mM trifluoperazine, though it is well known that brain adenylate cyclase system is stimulated by guanine nucleotide and calmodulin. Therefore, this preparation was assumed to be free from functional G protein and calmodulin, and used as the catalytic unit, though the purification was only partial. Similar results were reported for a preparation obtained from bovine caudate nucleus by the similar procedure.

Our results indicate that activation of the catalytic unit of rat brain adenylate cyclase by forskolin causes not only an increase in the maximal velocity of the cyclic AMP production but also in an apparent $K_m$ value for Mg-ATP. Since the concentration of Mg$^{2+}$ is much higher than that of ATP, most of ATP in our experimental system can be assumed to be in the form of Mg$^{2+}$-ATP. The $K_m$ value in the basal state, i.e. without activator, was about 80 $\mu$M which agrees with the value reported for hepatic adenylate cyclase, while the value in the state fully activated by forskolin was about 200 $\mu$M. It is unlikely that the change in the $K_m$ value is due to fluctuation of Mg-ATP concentration by ATP-utilizing enzymes other than adenylate cyclase, since ATP-regenerating system is always added to the assay system for adenylate cyclase activity.

Furthermore, it was found in the present study

![Graph](image)

**FIG. 2.** Activation of the Catalytic Unit by Forskolin in the Presence of Various Concentrations of Mn$^{2+}$

Adenylate cyclase activity of the catalytic unit was measured in the presence (●) or absence (○) of 50 $\mu$M forskolin, with 62.5 $\mu$M Mg-ATP and indicated concentration of Mn$^{2+}$

![Graph](image)

**FIG. 3.** Double Reciprocal Plots of Adenylate Cyclase Activity vs. Mg-ATP Concentration in the Presence of 1 mM Mn$^{2+}$ and Various concentrations of Forskolin
that the activation of the catalytic unit by forskolin was more efficient at higher concentration of ATP. This result seems to coincide with the above-mentioned increase in the apparent $K_m$ value for Mg-ATP by forskolin. Therefore, it may be inferred from these findings that the binding of forskolin to its specific site(s) on the catalytic unit causes a conformational change in the substrate site.

On the other hand, the increase in the apparent $K_m$ value for Mg-ATP of the catalytic unit accompanying to the forskolin activation was suppressed by addition of Mn$^{2+}$ depending on the Mn$^{2+}$ concentration. It is well known that Mn$^{2+}$ has prominent stimulatory effect on adenylate cyclase activity among divalent cations including Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$, and that Mn$^{2+}$ acts directly the catalytic site.\(^{5}\) It has also been reported that Mn$^{2+}$ and forskolin activate adenylate cyclase synergistically, though the mechanism has not been elucidated.\(^{6}\) In relation to these previous findings, the results in the present study is of interest, since the change in the $K_m$ value by Mn$^{2+}$ would explain the synergistic activation of the catalytic unit by the combination with forskolin, provided the decrease in the $K_m$ value means the increase in the affinity for the substrate. The effect of Mn$^{2+}$ to modify the forskolin action may be specific for this divalent cation, because all experiments in this study were performed in the presence of much higher concentration of Mg$^{2+}$. It may be necessary to refer to the finding that Ca$^{2+}$, on the contrary, inhibits forskolin stimulation of adenylate cyclase.\(^{4}\)

Recently, Awad et al.\(^{10}\) reported that forskolin induced marked increase in an apparent $K_m$ value for Mg-ATP in human platelet membrane adenylate cyclase, while no such a change was observed in the value for Mn-ATP of solubilized brain enzyme in the presence of 2 mM excess Mn$^{2+}$. Their results concerning the change in the $K_m$ value and the effect of Mn$^{2+}$ on it seem to be in line with ours. On the other hand, Bender and Neer\(^{20}\) reported that forskolin did not significantly affect the apparent $K_m$ values for Mg- and Mn-ATP of the isolated catalytic unit from bovine caudate nucleus, apparently contradicting with our results. At present, we have no appropriate explanation for the contradiction, though there may be some species difference.

In conclusion, our findings may afford an evidence for the interaction of forskolin and Mn$^{2+}$ in the regulation of the function of the catalytic unit itself of rat brain adenylate cyclase through the change in the affinity to the substrate.

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**REFERENCES**