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FORSKOLIN STABILIZES A FUNCTIONALLY COUPLED STATE BETWEEN ACTIVATED GUANINE NUCLEOTIDE-BINDING STIMULATORY REGULATORY PROTEIN, N_s, AND CATALYTIC PROTEIN OF ADENYLATE CYCLASE SYSTEM IN RAT ERYTHROCYTES

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SUMMARY: Guanine nucleotide-binding stimulatory regulatory protein of adenylate cyclase system, N_s, in rat erythrocytes was activated by the treatment with guanylyl 5'-imidodiphosphate or $NaF-AlCl_3$ in the presence of Mg^{2+}. The activation was counterbalanced to the basal state either by the removal of Mg^{2+} or by the addition of $\beta(\gamma)$-subunit of N protein of this system. The depression from the activated state was markedly protected by the coexistence of forskolin at the time of the deactivation depending on the dose of forskolin. EC_{50} of forskolin for the stabilizing effect was much lower than that for the stimulation of adenylate cyclase activity. These data indicate that forskolin has an effect on the interaction between N_s and catalytic unit of adenylate cyclase system in addition to the direct effect on the catalytic unit.

Forskolin, a diterpene from the root of Coleus forskohlii, has been known to stimulate adenylate cyclase activity (1). It has been proposed that the diterpene has a direct activating effect on the catalytic unit of adenylate cyclase system, since the stimulation is observed in the absence of a functional N_s. However, evidence has been accumulated that forskolin also affects N_s. For instances, i) difference is observed in the kinetics of adenylate cyclase activation by forskolin between the presence and absence of N_s (2,3), ii) binding site of forskolin is increased by activation of N_s (4,5), and iii) forskolin induces the change in the size of adenylate cyclase complex (6). In the present study, we found that the attenuation of the function of N_s by

Abbreviations: N_s, guanine nucleotide-binding stimulatory regulatory protein in adenylate cyclase system; GppNHp, guanylyl-5'-imidodiphosphate; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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either the removal of Mg^{2+} (7) or addition of β-subunit (may contain γ-subunit) of N protein (8) was prevented by forskolin. The results seem to suggest that forskolin stabilizes N_s-activated state of adenylate cyclase system.

MATERIALS AND METHODS

Forskolin was purchased from Calbiochem-Behring, and [^{3}H]ATP from Amersham International Ltd. All other agents were of reagent grade from standard commercial sources.

Male rats of Wistar strain (150-200 g) were killed by exsanguination from the carotid artery under ether anesthesia, and whole blood was collected into a tube containing 10 mM EDTA in saline. Erythrocytes were collected and washed 3 times by centrifugation in the same solution at 500 x g for 10 min. Buffy coat was carefully discarded, and the erythrocytes in the final pellet was hypotonically lysed by the addition of 10 vols. of 5 mM Tris-HCl/1 mM EDTA (pH 7.4). The lysate was centrifuged at 20,000 x g for 10 min, and the precipitate was repeatedly washed with the same solution. Final pellet was suspended with 50 mM Tris-HCl buffer (pH 7.4), and the suspension was used as the membrane preparation in the following experiments. β-Subunits of N protein was purified from the bovine brain by the method of Sternwise et al. (9) with minor modifications (10), and characterized by the inhibition of adenylate cyclase activity as well as by the mobility in SDS-PAGE.

The above-mentioned membrane preparation was incubated at 30°C in the presence of 10 mM $MgCl_2$ either with 10 μM GppNHp for 1 hr or with 5 mM NaF and 20 μM $AlCl_3$ for 10 min to activate N_s. After the activation, the preparation was washed twice with 50 mM Tris-HCl/10 mM $MgCl_2$ (pH 8) by centrifugation at 20,000 x g for 10 min to remove the activators. Deactivation of the N_s-activated adenylate cyclase system in the preparation was undertaken by incubating either with 10 mM EDTA to remove Mg^{2+} (7) or with 25 μg/ml β-subunit of N protein (8) for various periods. In cases indicated, 20 μM forskolin was added to the medium. After the incubation, a mixture for the assay of adenylate cyclase activity was added immediately. The mixture consisted of 50 mM Tris-HCl, 20 mM $MgCl_2$, 0.1 mM ATP, 1 μCi [^{3}H]ATP, 8 mM theophylline, 10 mM creatine phosphate, and 40 unit/ml creatine phosphokinase (pH 8). The reaction was stopped by the addition of the equal vol. of 10 % SDS/10 mM EDTA. [^{3}H]Cyclic AMP produced was separated by Dowex 50 x 4 and neutral alumina column chromatographies (11), and the radioactivity was measured with a liquid scintillation spectrometer. Protein was measured by the method of Lowry et al. (12).

RESULTS AND DISCUSSION

Rat erythrocyte membrane was treated with either GppNHp or NaF-$AlCl_3$ to activate N_s of adenylate cyclase system, and then washed twice with 50 mM Tris/10 mM $MgCl_2$ (pH 8) to remove the activator. Almost full activation of the system was attained by the treatment. As shown in Table 1, the activated state was maintained at least for 30 min in the presence of Mg^{2+}, while was returned to the basal level by the addition of EDTA to remove Mg^{2+}. The result coincides with previous papers indicating an essential role of Mg^{2+} in

Table 1: Changes in adenylate cyclase activity after addition of EDTA in the membrane pretreated with either GppNHp or NaF-$AlCl_3$

Incubation period (min)	Adenylate cyclase activity (pmol/min/mg protein)			
	GppNHp-treated membrane		$NaF-AlCl_3$-treated membrane	
	-EDTA	+EDTA	-EDTA	+EDTA
0	38.5	38.5	30.0	30.0
5	36.3	28.0	31.5	21.6
10	37.5	21.5	30.1	20.4
20	37.5	19.8	31.1	15.4
30	37.8	12.0	30.3	12.0

Adenylate cyclase system in the erythrocyte membrane (basal activity 2,5 pmol/min/mg protein) was activated by an incubation with either 10 μM GppNHp for 1 hr or with 5mM NaF-20μM $AlCl_3$ for 10 min. After removing the activators, the membrane was incubated in the presence or absence of 10 mM EDTA for indicated periods and measured for adenylate cyclase activity.

the activation of N_s (6,7). Such a deactivation of adenylate cyclase system by Mg^{2+} deprivation, however, was not due to the denaturation of N_s or the catalytic unit of the system, since the adenylate cyclase activity was reenhanced by the addition of either GppNHp or NaF-$AlCl_3$ in the presence of Mg^{2+} even after the treatment with EDTA for 30 min. Addition of β-subunit of N protein to the Ns-activated state also caused the deactivation (data not shown).

The deactivation of GppNHp- or NaF-$AlCl_3$-preactivated adenylate cyclase activity by the addition of EDTA was prevented by 20 μM forskolin, as shown in Fig. 1 (A and B). Forskolin also prevented the decrease in the preactivated adenylate cyclase activity caused by the addition of β-subunit of N protein (Fig. 2). These result seem to indicate that forskolin has the stabilizing effect on the N_s-activated state of adenylate cyclase system in addition to the known direct stimulating effect on the catalytic protein of the system, because, without such a stabilizing effect, the preactivated adenylate cyclase activity should be decreased by the addition of EDTA or β-subunit to a level of the direct activation as indicated at far right in Figs. 1 and 2.

To ascertain the stabilizing effect of forskolin, the erythrocyte membrane was treated first with GppNHp, and then with EDTA and various concentrations of forskolin. Adenylate cyclase activity was measured after adjustment of the

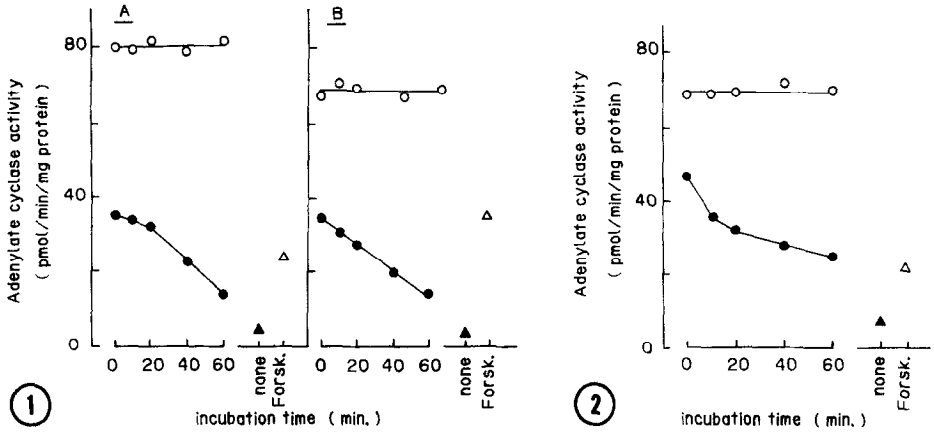

Fig. 1. Effect of forskolin on the stability of N_s-activated state of adenylate cyclase system against Mg^{2+} deprivation. Erythrocyte membrane was treated with 10 mM $MgCl_2$ and either GppNHp (A) or NaF-$AlCl_3$ (B) to activate N_s, and then with 10 mM EDTA either in the presence (○) or absence (●) of 20 μM forskolin for indicated periods. None, basal activity; Forsk, activity with 20 μM forskolin added directly to the assay system.

Fig. 2. Effect of forskolin on the stability of N_s-activated state of adenylate cyclase system against addition of β-subunit of N protein. Erythrocyte membrane was treated with GppNHp to activate N_s, and then with the β-subunit either in the presence (○) or absence (●) of 20 μM forskolin for indicated periods. Others are the same as in Fig. 1.

concentration of forskolin to 20 μM throughout the samples. As shown in Fig. 3, the protective effect of forskolin against the deactivation by EDTA was

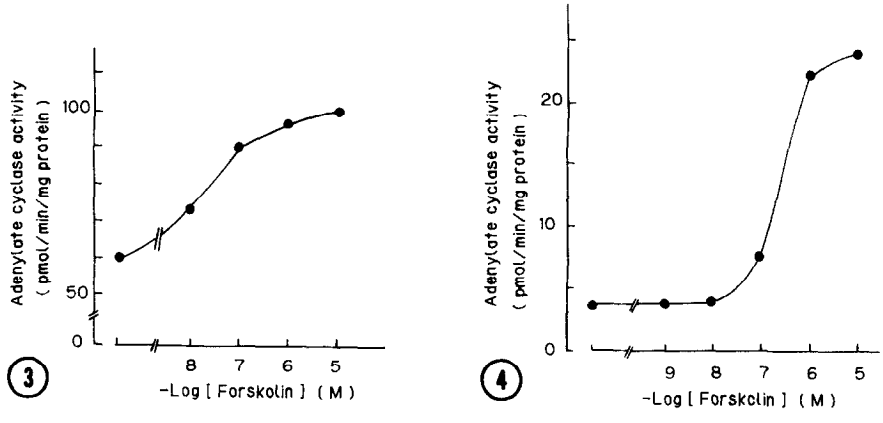

Fig. 3. Stabilization of N_s-activated state of adnylate cyclase system versus concentration of forskolin. Erythrocyte membrane treated with GppNHp to activate N_s was incubated with 10 mM EDTA and indicated concentrations of forskolin for 60 min. Adenylate cyclase activity was measured after adjustment of the concentration of forskolin to 20 μM throughout the samples.

Fig. 4. Stimulation of adenylate cyclase activity versus concentration of forskolin. Adenylate cyclase activity in the erythrocyte membrane was measured at indicated concentrations of forskolin.

concentration-dependent. EC_{50} for such a stabilizing effect of forskolin is calculated as approximately 50 nM from the figure. Fig. 4 shows the direct effect of forskolin for activation of adenylate cyclase. It is evident from the figure that no significant activation of the activity can be attained by forskolin at the concentration of 50 nM. In other words, the concentration of forskolin required for the activation of the catalytic unit is much higher than that for the stabilization of the activated state observed here.

It was reported that purification of the catalytic unit from GppNHp-activated membrane by forskolin-Sepharose affinity chromatography led to co-purification of an α-subunit of N_s (13). Thus, the N_s-activated state of adenylate cyclase system observed here may be ascribed to a complex formation between the α-subunit and catalytic unit, and the addition of EDTA or a β-subunit of N protein may dissociate the complex. Such a consideration may be in line with findings that the high affinity forskolin binding sites are associated with a complex of the catalytic protein and the activated N_s protein from rat brain (4,14) and human platelets (5).

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