The solubilized catalytic unit (C) of adenylate cyclase from bovine caudate nucleus was separated from the component which mediates activation by guanine nucleotides (G/F) by the method of Strittmatter and Neer (Strittmatter, and Neer, E. J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6344-6348). The separated catalytic unit is much more active in the presence of Mn\(^{2+}\) than when Mg\(^{2+}\) is the only divalent cation. The affinity of the catalytic unit for its substrate, Me\(_3\)ATP, is the same with either cation. Free Mn\(^{2+}\) activates the enzyme at unit even in the absence of ATP. These observations from bovine caudate nucleus was separated from the component which mediates activation by guanine nucleotide-activated forskolin and decays with a half-time of 23 °C. The separated catalytic unit from caudate nucleus can be activated by the plant diterpene, forskolin. Forskolin does not significantly affect the apparent \(K_m\) of the enzyme for ATP. With forskolin, the catalytic unit is active even with Mg\(^{2+}\) as the divalent cation. However, activation by forskolin is synergistic with that of Mn\(^{2+}\) and free Mn\(^{2+}\) still activates the enzyme. Forskolin also potentiates the activation of C by G/F*, but this depends critically on the order in which activators are added. This study shows, by the plant diterpene, forskolin, that a 1000-fold activation of the catalytic unit can be produced by forskolin, G/F*, and Mn\(^{2+}\). Forskolin had no effect on the rate of activation of the G/F unit by guanosine 5\'-(\(\beta,\gamma\)-imino)triphosphate or on the amount of G/F* needed to activate C. G/F is known to mediate the activation of adenylate cyclase by hormone receptors. The activity of the catalytic unit has been considered to reflect passively the activity state of the G/F unit. The studies with forskolin suggest that this may not always be the case and that the state of the catalytic unit itself may be important in determining the overall activity of the adenylate cyclase system.

The hormone-stimulated adenylate cyclase system is made up of at least three separable components: the hormone receptor, the catalytic unit, and the guanine nucleotide binding regulatory unit which couples the receptor to the catalytic unit (for a review, see Ref. 1). Each of these components is probably made up of more than one polypeptide subunit.

Adenylate cyclase can be solubilized from the plasma membrane by detergents. Nonionic detergents, such as Lubrol 12A9 or Triton X-100, solubilize the catalytic and G/F* units as a complex, but hormone responsiveness is lost (1, 2). Recently, the laboratory developed a method for separating the catalytic and G/F* units from bovine cerebral cortex by gel filtration in ammonium sulfate and cholate, an ionic detergent (3). A very similar method was independently described for liver adenylate cyclase by Rose (4). The separate G/F unit could then be activated by the nonhydrolyzable GTP analogue, Gpp(NH)p, in the absence of C and the two components could be subsequently reconstituted into an active complex. This gave the possibility of studying the function of the brain catalytic and G/F units separately.

The separated catalytic unit from brain is activated by at least two known proteins, the G/F unit and calmodulin (3, 5). Its activity is also greatly enhanced by a divalent cation, Mn\(^{2+}\) (3, 6), and, as this study shows, by the plant diterpene, forskolin. The work described in this paper defines some of the interactions of Mn\(^{2+}\), forskolin, and the G/F unit in controlling the activity of the catalytic unit.

Kinetic studies of membrane-bound or soluble adenylate cyclase have suggested that the enzyme is regulated by metal ions at sites distinct from the active site, which requires Me\(_3\)ATP as a substrate (7, 8). The initial studies could not distinguish sites on the G/F unit from sites on the catalytic unit. Using separated components, it has now been possible to show that the function of the G/F unit is modulated by Mn\(^{2+}\) (9, 10). The catalytic unit has a different metal ion requirement. When it is separated from the G/F unit, it is only slightly active with Mg\(^{2+}\) as the only divalent cation but

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* Part of this work was presented at the 1982 FASEB meeting in New Orleans, LA (Bender, J. L., and Neer, E. J. (1982) Fed. Proc. 41, 1408). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^1\) The abbreviations used are: G/F, the component of adenylate cyclase which mediates activation of the enzyme by guanine nucleotides; C, the catalytic component of adenylate cyclase, this component may be made of more than one polypeptide; G/F*, the guanine nucleotide regulatory component which has been fully activated by Gpp(NH)p as described in the under “Materials and Methods”; C-G/F, the complex of catalytic and G/F units; Gpp(NH)p, guanosine 5\'-(\(\beta,\gamma\)-imino)triphosphate; App(NH)p, adenosine 5\'-(\(\beta,\gamma\)-imino)triphosphate; EGTA, ethylene glycol bis(\(\beta\)-aminoethoxy) ether)-\(N,N,N',N'\)-tetraacetic acid.
Adenylate Cyclase Catalytic Unit

Preparation of Isolated Catalytic Unit

Catalytic unit was prepared from bovine brain (400 g), homogenized in 3 ml of 0.3 M sucrose, 10 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol. The homogenate was centrifuged at 100,000 × g for 20 minutes. The supernatant was adjusted to pH 7.0 with 20% Tris-Cl, pH 7.0, and 0.2 M NaOH was added per ml. The mixture was centrifuged at 100,000 × g for 20 minutes. The supernatant contained soluble catalytic unit. It was frozen in liquid nitrogen, then stored at -70°C.

Activated catalytic unit was passaged over a column of Sepharose 6B (commercially available from Pharmacia Fine Chemicals) equilibrated with 30 ml of 0.4 M Tris-Cl, pH 7.4, 20% glycerol, 0.5 mM dithiothreitol, and 0.05 mg/ml heparin. The mixture was centrifuged at 100,000 × g for 20 minutes. The supernatant contained soluble catalytic unit. It was frozen in liquid nitrogen, then stored at -70°C.

Cocoon acid had to be removed from the catalytic fraction before they could be assayed, either by precipitation and resuspension or by dialysis. For precipitation, fractions were mixed with equal volumes of saturated Na2HPO4 adjusted to pH 7.0 with NaOH, and were precipitated at 4°C for 15 minutes. The precipitated fractions were resuspended in 0.4 M Tris-Cl, pH 7.4, 20% glycerol, 0.5 mM dithiothreitol, and 0.05 mg/ml heparin. The mixture was centrifuged at 100,000 × g for 20 minutes. The supernatant contained soluble catalytic unit. It was frozen in liquid nitrogen, then stored at -70°C.

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shown in Fig. 3 will be discussed later). App(NH)p was used instead of ATP to minimize hydrolysis of the nucleotide during the inactivation. The rate of inactivation with 0.25 mM App(NH)p and Mg2+ was only slightly slower than with Mg2+ alone. The decay curves obtained from these experiments do not obey simple first order kinetics. However, the observation that Mn2+ can stabilize the enzyme is consistent with the idea that Mn2+ can interact with C both at the active site and block its activation by G/F*. As shown in Fig. 4, G/F* can activate the catalytic unit even in the presence of MnCl2. The experiments shown were done in glycylglycine/maleate buffers. However, similar results were obtained when Tris buffers were used. We have previously reported that Mn2+ whether Tris or glycylglycine/maleate buffer is used, either in the presence of 20 mM MnCl2, see Methods) were assayed with 15 ul diluted G/F* and 15 mM MgCl2, 106 ug protein. This experiment is representative of three similar experiments. 20 x 1 samples of caudate nucleus catalytic unit precipitated and resuspended in buffer 4 (glycylglycine/maleate/Na MgCl2, see Methods) were assayed with the indicated concentrations of MnCl2 and either 60 ul ATP or 600 ul ATP present in the assay mixture. 20 ul contained 15 ug protein. This experiment is representative of three similar experiments.

Interaction of the Catalytic Unit with Forskolin—Fig. 5 shows the activation of the catalytic unit by the plant diterpene forskolin. Apparent half-maximal activation was at 20 uM forskolin which is in agreement with results in other kinds of preparations reported by Seamon et al. (14). Forskolin does not significantly affect the apparent Km of the isolated catalytic unit for ATP (Fig. 6) whether this is determined with Mg2+ or Mn2+ (0.16 ± 0.03 mM Mg-ATP, 0.25 ± 0.06 mM Mn-ATP; n = 3). While the values for the apparent Km are slightly higher than those given above for the same determinations without forskolin, the difference is not significant. Forskolin (100 uM) stabilized C against inactivation. The
Adenylate Cyclase Catalytic Unit

Figure 5. Effect of forskolin on the catalytic unit. 
25 pl samples of catalytic unit were prepared and resuspended in Buffer 1 (physiological saline containing MgCl2, see Methods) were assayed with the indicated concentrations of forskolin in the assay mixture (---). 25 pl catalytic unit contained 25 g protein. This experiment is representative of three similar ones.

Figure 6. Lineweaver-Burk plot of the ATP dependence of catalytic unit activity with Mg2+ and Mn2+. 
20 pl samples of catalytic unit were prepared and resuspended in Buffer 2 (physiological saline containing 10 mM MgCl2, see Methods) were assayed with 10 μM forskolin (---) or 20 μM forskolin and 5 mM MgCl2 (---). The velocity of the reaction is given in pmol of cyclic AMP (20 pl x 30 min). 20 pl of catalytic unit contained 12 g protein. This experiment is representative of three similar ones.

Figure 7. Lineweaver-Burk plot of the ATP dependence of catalytic unit activity with Mg2+ and Mn2+. 
20 pl samples of catalytic unit were prepared and resuspended in Buffer 3 (physiological saline containing MgCl2, see Methods) were assayed with 10 μM forskolin (---) or 20 μM forskolin and 5 mM MgCl2 (---). The velocity of the reaction is given in pmol of cyclic AMP (20 pl x 30 min). 20 pl of catalytic unit contained 12 g protein. This experiment is representative of three similar ones.

Table I

Reversibility of forskolin activation

| Sample | Activity | pmol cAMP/50 pl x 10 min |
|--------|----------|--------------------------|
| Enzyme from Sepharose 6B column applied to Sephadex G-50 column | 49 ± 9 |
| Sample eluted from Sephadex G-50 column | 50 ± 12 |

Table II

Activation of the catalytic unit by Mn2+, forskolin, and G/F*

Twenty pl of caudate nucleus catalytic unit (C) diluted 1.5 in Buffer 1 was incubated for 30 min at 23 °C with 5 mM MnCl2 (Mn2+), 100 μM forskolin (F), or 20 μM of G/F* (G/F*) as indicated, 5 mM MnCl2 or 100 μM forskolin was also added after incubation, to the assay, as indicated. Twenty pl of diluted catalytic unit contained 1 µg of protein and 20 μM of G/F* contained 2 µg of protein. Assays without G/F* (1-3 and 5) did not lose more than 20% activity during incubation (controls not shown). Incubation with forskolin for an additional 30 min at 23 °C had no effect on activity in assays 8, 10, 11, and 12 (controls not shown). Note that final compositions of assays 6 and 7, of assays 8 and 9, and of assays 10 to 13 are the same. This experiment is representative of four similar ones.

| Sample | Activity | pmol cAMP/10 min x 20 pl |
|--------|----------|--------------------------|
| 1. C | 1 |
| 2. C, Mn2+ | 7 |
| 3. C, F | 65 |
| 4. C, G/F* | 34 |
| 5. C, Mn2+, F | 20 + 3 = 72 |
| 6. C, Mn2+, G/F* | 135 |
| 7. C, G/F* | 119 |
| 8. C, G/F* | Mn2+ , F | 101 |
| 9. C, G/F*, F | 3 + 4 = 99 |
| 10. C, Mn2+, G/F* | 269 |
| 11. C, G/F* | 269 + 4 = 243 |
| 12. C, Mn2+, G/F*, F | 243 |
| 13. C, G/F* , F | 234 |
| 14. C, G/F*, F | Mn2+ | 1200 |
| 15. C, G/F*, F | 5 + 9 = 477 |

half-time for inactivation at 30 °C increased from 5 min in the presence of Mg2+ alone to 17 min in the presence of forskolin and Mg2+ (Fig. 3).

Forskolin may activate the catalytic unit by dissociating an inhibitory subunit of C. To test for this possibility, we incubated the catalytic unit with 100 μM forskolin at 23 °C for 20 min, then passed it over a Sepharose 6B column equilibrated with Buffer 5 (see "Materials and Methods") containing 0.1% Lubrol 12A9 and 50 μM forskolin. The apparent Stokes radius of the isolated catalytic unit (relative to marker enzymes of known Stokes radius) was 70 Å whether or not the gel filtration was performed with forskolin in the buffer. This suggests that forskolin does not cause the dissociation of a large enzyme component. Dissociation of a small enzyme component might not affect the elution position of the catalytic unit, but a small component would be separated from C by the gel filtration. Therefore, we took the fractions containing the peak of enzymatic activity from Sepharose 6B and separated away the forskolin by passing the enzyme over a column of Sephadex G-50 equilibrated in Buffer 5 with 0.1% Lubrol 12A9 but without forskolin. If an inhibitory unit had been separated by the original Sepharose 6B gel filtration, we would expect the enzyme to be activated persistently and no longer responsive to forskolin. This was not the observed result as shown in Table I. Activation by forskolin was reversed by removal of forskolin on Sephadex G-50. The enzyme was then again sensitive to stimulation by forskolin.

Synergistic Activation of the Catalytic Unit by Mn2+, G/F, and Forskolin—Mn2+ (5 mM) and forskolin (100 μM) together activate the catalytic unit 2.6 ± 0.1-fold (n = 9) over the sum of the individual activities (Table II, lines 2, 3, and 5). Forskolin also slightly increases the affinity of the catalytic unit for free Mn2+. The half-maximal stimulation occurs at 0.25 mM Mn with forskolin (Fig. 7) compared with 0.5 mM Mn without forskolin (Fig. 2). However, this small difference in affinity does not explain the synergism between Mn2+ and forskolin which occurs at saturating concentrations of both ligands and substrate.
Adenylate Cyclase Catalytic Unit

Activation by forskolin was also synergistic with that produced by interaction of the catalytic unit with G/F*. However, this was critically dependent on the time when forskolin was added. It was essential that forskolin be present in the 30-min incubation at 23 °C during which G/F* combined with the catalytic unit. When the reconstitution took place with forskolin, the activity of the stimulated complex was 2.7 ± 0.2-fold greater than the sum of activity with forskolin or G/F* (Table II, lines 3, 4, and 9). On the other hand, if the catalytic unit and G/F* were allowed to combine and forskolin was added afterwards, the final activity was only equal to the sum of the individual ones (Table II, lines 3, 4, and 8). The results are the same regardless of the concentration of forskolin (Fig. 8).

Stabilization of the catalytic unit by forskolin would not explain these results since assays without G/F or forskolin did not lose more than 20% of their activity during the incubation at 23 °C.

Since forskolin has to be present during the activation of

**Fig. 7.** Effect of forskolin on Mn**++** stimulation of catalytic unit.

20 μL samples of caudate nucleus catalytic unit precipitated and resuspended in Buffer 4 (glycylglycine/maleic acid/MgCl₂, see Methods) were assayed with the indicated concentrations of MnCl₂, 100 μM forskolin, and 60 μM (——) or 600 μM (—–) ATP present in the assay mixture. 20 μL catalytic unit contained 7 μg protein. This experiment is representative of three similar ones.

**Fig. 8.** Synergetic activation of the catalytic unit by G/F* as a function of forskolin concentration. Cerebral cortical G/F* was diluted 10-fold into Buffer 1 (see "Materials and Methods"). Catalytic unit from caudate nucleus was diluted 5-fold into Buffer 5. Equal volumes of G/F* and catalytic unit were mixed and incubated at 23 °C for 30 min with (•—•) or without (△—△) the indicated concentrations of forskolin. Forskolin at the indicated concentrations was added after reconstitution to the set of samples incubated without the diterpene. As a control, diluted catalytic unit was mixed with an equal volume of Buffer 5 and incubated at 23 °C for 30 min with the indicated concentrations of forskolin. **Fig. 9.** Effect of forskolin on the rate of G/F* activation by Sp(1)MgCl₂. Cerebral cortical G/F* was diluted 10-fold into Buffer 4. 20 μL diluted G/F* was incubated for the indicated times with 25 μM Sp(1)MgCl₂ in the presence (—–) or absence (△—△) of 100 μM forskolin. Samples were chilled on ice and 15 μL caudate nucleus catalytic unit diluted 1:1 in Buffer 5 was added. 100 μM forskolin was also added to samples previously incubated without diterpene. Samples were then immediately assayed. 15 μL diluted catalytic unit contained 3 μg protein and 25 μL diluted G/F* contained 3 μg protein. The differences in activity between G/F*-activated catalytic unit and catalytic unit alone are presented. This experiment is representative of three similar ones.

**Fig. 10.** Effect of forskolin on the G/F* dose response of the catalytic unit.

Cerebral cortical G/F* was diluted to the protein concentrations indicated in Buffer 5. 25 μL of caudate nucleus catalytic unit diluted 1:1 in Buffer 5 was added to 24 μL diluted G/F* in the presence (•—•) or absence (△—△) of 100 μM forskolin. Samples were incubated at 23 °C for 30 minutes, then immediately chilled and assayed. 25 μL 1:15 diluted catalytic unit contained 3 μg protein. This experiment is representative of three similar ones.
The activity of the catalytic unit stimulated synergistically by G/F* and forskolin can be increased still further by the addition of Mn"++. This final activity is again greater than the sums of the individual stimulations (Table II). It is only the "superactive" state of the enzyme which can respond to Mn"++. Once the C-G/F* complex has formed, activation by Mn"++ only produces the increment in activity that it would have, if it had been added to the catalytic unit with forskolin (compare lines 10 and 11 with the sum of 4 and 5).

Reversal of the Forskolin-induced Active State—Our previous experiments (Table I) as well as those of others (14, 25) showed that the action of forskolin is reversible. We wished to determine whether the "superactive" state induced by forskolin persists even in its absence. To answer this question, we activated the catalytic unit with G/F* without or with forskolin generating the states shown in lines 8 and 9 of Table II. In the former situation, we added forskolin to the sample after activation by G/F*. We then passed the two preparations over Sephadex G-50 to remove free forskolin. The stripped enzyme samples were incubated at 23 °C for the times noted in Fig. 11. They were assayed with or without readdition of forskolin.

Fig. 11 shows that the "superactive" state induced by forskolin does persist for a measurable time even in the absence of forskolin. When forskolin is removed from the "superactive" enzyme, the activity drops to a level similar to that of a C-G/F* complex. This indicates that the columns do indeed remove forskolin from the sample. However, readdition of forskolin fully restores the original, synergistic, forskolin-induced activity. In contrast, the control C-G/F* complex to which forskolin was added after activation of the catalytic unit by G/F*, can only be reactivated to its initial, lower level. As can be seen from the figure, the capacity of the C-G/F* complex to respond to forskolin in a synergistic fashion decays at 23 °C with a half-time of about 20 min.

DISCUSSION

In this study, we have investigated several aspects of the function of the separated catalytic unit of adenylyl cyclase from bovine caudate nucleus: the interaction with Mn"++, activation by forskolin, and the role of both of these activators in modulating the response of the catalytic unit to activation by G/F*.

Separation of the catalytic unit from the G/F unit (3) and, in the case of brain tissue, from calmodulin (5) changes its metal ion requirement. However, the affinity of the enzyme for its substrate, Me-ATP, is the same whether activity is measured with Mg"++ or Mn"++. The apparent K_m of the isolated catalytic unit for ATP is similar to values determined for the holoenzyme (1) or membrane-bound C from cyc S49 cells (12) suggesting that neither the interaction of the catalytic unit with the G/F unit nor association with a membrane affects this parameter. The activation of the enzyme by Mn"++ could be because Mn-ATP is a better substrate than Mg-ATP. Our studies do not rule out this possibility but they demonstrate that in addition a large part of the activation of the catalytic unit by Mn"++ is due to the interaction of this metal ion with a regulatory site on the enzyme. The evidence for this is that free Mn"++ continues to activate the enzyme at
concentrations greater than are required to convert all available ATP to Mn-ATP.

The affinity of the isolated catalytic unit for Mn\(^{2+}\) is not high (0.5 mM). However, it is hard to know what the affinity of the site might be in the membrane. Adenylate cyclase from various regions of the brain is inhibited by EGTA (11, 26-29). This laboratory has shown that EGTA inhibition can be partially reversed by Cs\(^+\) but virtually entirely by Mn\(^{2+}\), suggesting that EGTA may inhibit by chelating bound Mn\(^{2+}\) (11). Studies with membrane-bound brain enzyme suggested that membrane structure must be somewhat disrupted to allow access of EGTA to the metal ion (30). If a metal ion binding site is indeed sequestered in the membrane, its affinity for metal in the native enzyme might be quite different from that observed in a solubilized, separated component.

The isolated catalytic unit is stabilized by Mn\(^{2+}\) even in the presence of Mg\(^{2+}\) and in the absence of substrate. However, addition of the substrate analogue, App[NH]p, enhances the stabilization. Stabilization by App[NH]p is minimal in the presence of Mg\(^{2+}\) alone. The observation that free Mn\(^{2+}\) stabilizes the enzyme is consistent with the idea that there is a regulatory Mn\(^{2+}\) site on the enzyme. We cannot, at present, say whether the regulatory metal ion site is on the polypeptide bearing the active site or on another component of the catalytic unit.

It has been reported by several laboratories that Mn\(^{2+}\) uncouples the catalytic unit from the G/F unit preventing activation of the enzyme by Gpp(NH)p (14, 24). We have not been able to uncouple the enzyme with Mn\(^{2+}\) up to 20 mM either in studies with isolated components or in membrane preparations. Mn\(^{2+}\) activates the C-G/F* complex equally well whether added before or during reconstitution. At present, we have no explanation for the difference between our results and those of others.

The plant diterpene, forskolin, is a recently discovered activator of adenylate cyclase (14). Forskolin increases the V\(_{\text{max}}\) of the catalytic unit without affecting the apparent K\(_{\text{m}}\) for Mg-ATP or Mn-ATP. It slightly increases the affinity of the Mn\(^{2+}\) site for the Mn\(^{2+}\). Forskolin stabilizes the catalytic unit against inactivation at 30 °C. Although forskolin clearly affects the function of the component which we have separated from the G/F unit and from calmodulin, we cannot say that it acts on the catalytic polypeptide itself since we do not yet know the polypeptide composition of the catalytic unit.

The most interesting and potentially instructive aspect of the interaction of forskolin with the catalytic unit is the synergistic activation which we observe among forskolin, Mn\(^{2+}\), and G/F*. Forskolin and Mn\(^{2+}\) together activate the catalytic unit 2.6 times more than the sum of the individual activities. Forskolin also potentiates the activation of the catalytic unit by G/F, but this depends on forskolin’s being present during the enzyme reconstitution. If forskolin is added after the C-G/F complex has formed, then the effects are only approximately additive. A simple interpretation would be that association of the catalytic unit with the G/F unit blocks the access of forskolin to a site which must be occupied for potentiation to occur. This notion is consistent with the observation that synergism is not observed in Lubrol 12A9-solubilized bovine brain enzyme even when forskolin is present during the activation by Gpp(NH)p (data not shown). In Lubrol 12A9-solubilized enzyme, the components are not dissociated and therefore the site may not be accessible.

The state of the reconstituted enzyme induced by forskolin persists for a time even in the absence of the diterpene and relaxes back to the conformation of the C-G/F complex obtained when the reconstitution takes place without forskolin. Like other conformational changes in the adenylate cyclase system, the process is rather slow with an approximate half-time of 20 min at 23 °C.

The “superactive” state induced by forskolin and G/F* allows a further activation by Mn\(^{2+}\) (compare lines 10 and 11 with 12 and 13 in Table II). Overall, we can activate the catalytic unit about 1000-fold by adding forskolin, Mn\(^{2+}\), and G/F* in the right order. The specific activity of the fully activated enzyme (based on the amount of protein in the catalytic unit preparation) is 0.1 μmol of cAMP/mg/min.

We do not yet know the mechanism of the forskolin-induced potentiation of the G/F activation of the catalytic unit. Seamon and Daly have recently observed synergistic activation of adenylate cyclase by Gpp(NH)p and forskolin in membranes from tissues which lack a mechanism for hormonally induced inhibition of adenylate cyclase (31). One could postulate that we have removed or inactivated an inhibitory guanine nucleotide regulatory unit in the course of preparing G/F. However, the fact that we can see either additive or synergistic effects depending on the order of addition of the components argues against this interpretation. Other possibilities are that forskolin might act by removing an inhibitory peptide from the catalytic unit or that it might increase activity by recruiting a population of silent catalytic units. We could find no evidence of the former possibility in direct experiments and the arguments outlined below suggest this is not the explanation. Either of the proposals could explain synergism of forskolin and Mn\(^{2+}\) or forskolin and G/F*. However, both seem unlikely in view of the finding that the combination of Mn\(^{2+}\), G/F, and forskolin during enzyme reconstitution gives activity which is again a multiple of the individual synergistic activations. Thus, if forskolin and Mn\(^{2+}\) specifically recruited one population of catalytic units and forskolin plus G/F recruited a second population, one would expect that all three together would only activate those two populations. To account for the multiplicative effect when all three activators are present together, one would have to postulate a third population of catalytic units which are only active with all three stimulators. A similar argument applies to putative inhibitors. Although such theories cannot be ruled out, they do not seem, at present, to be the simplest interpretation of the data. We conclude therefore that forskolin induces a variety of conformational states of the enzyme which are reflected in different activities and different responsiveness to the G/F unit (the physiological activator) and to metal ions.

The overall activity of the adenylate cyclase system has been thought to reflect the activation state of the G/F unit which is in turn controlled by the hormone receptor. In this view, the control goes only from outside the cell to inside. If the forskolin site should, in fact, turn out to be the site for an as yet unrecognized intracellular modulator of adenylate cyclase, then these studies suggest that the catalytic unit itself may be another point at which the cellular response to hormones is regulated.

REFERENCES
1. Ross, E. M., and Gilman, A. G. (1980) Annu. Rev. Biochem. 48, 533-564
2. Neer, E. J., Echeverria, D., and Knox, S. (1980) J. Biol. Chem. 255, 9782-9789
3. Strittmatter, S., and Neer, E. J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6344-6348
4. Ross, E. M. (1981) J. Biol. Chem. 256, 1949-1953
5. Salter, R. S., Krinks, M. H., Klee, C. B., and Neer, E. J. (1981) J. Biol. Chem. 256, 9830-9833
6. Ross, E. M., and Gilman, A. G. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3715-3719
7. Garbers, D. L., and Johnson, R. A. (1979) J. Biol. Chem. 250, 8449-8456
8. Londos, C., and Preston, M. S. (1977) J. Biol. Chem. 252, 5961-5969
9. Ceci, S. Y., Broaddus, W. C., and Maguire, M. E. (1980) Mol. Cell. Biochem. 33, 67-92
10. Iyengar, R. (1981) J. Biol. Chem. 256, 11042-11050
11. Neer, E. J. (1979) J. Biol. Chem. 254, 2089-2096
12. Ceci, S. Y., and Maguire, M. E. (1982) Mol. Pharmacol. 22, 297-273
13. Somkuti, S. G., Hildebrandt, J. D., Herberg, J. T., and Iyengar, R. (1982) J. Biol. Chem. 257, 6387-6393
14. Seamon, K. B., Padgett, W., and Daly, J. W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3963-3967
15. Seamon, K., and Daly, J. W. (1981) J. Biol. Chem. 256, 9799-9801
16. Gill, D. M., and Meren, R. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3053-3054
17. Cassel, D., and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 9699-9703
18. Hoffmann, F. M. (1979) J. Biol. Chem. 254, 255-258
19. Neer, E. J., and Salter, R. S. (1981) J. Biol. Chem. 256, 12102-12107
20. Krishna, G., Weiss, B., and Brodie, B. B. (1968) J. Pharmacol. Exp. Ther. 163, 379-385
21. Rao, G. S., Del Monte, M., and Nadler, M. L. (1971) Nature New Biol. 232, 253-255
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
23. Bailey, J. L. (1967) Techniques in Protein Chemistry, p. 340, American Elsevier Publishing Company, New York
24. Limbird, L., Hickey, A. R., and Lefkowitz, R. J. (1979) J. Biol. Chem. 254, 2677-2683
25. Insel, P., Stengel, A. D., Ferry, N., and Hanoune, J. (1982) J. Biol. Chem. 257, 7485-7490
26. Bradham, L. S. (1972) Biochim. Biophys. Acta. 276, 434-443
27. Johnson, R. A., and Sutherland, E. W. (1973) J. Biol. Chem. 248, 5114-5121
28. Brostrom, C. O., Huang, Y.-C., Breckenridge, B. M., and Wolff, D. J. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 64-68
29. Cheung, W. Y., Bradham, L. S., Lynch, T.-J., Lin, Y. M., and Tallant, E. A. (1975) Biochem. Biophys. Res. Commun. 66, 1055-1062
30. Neer, E. J., and Echeverria, D. (1981) Biochem. Pharmacol. 30, 2488-2491
31. Seamon, K. B., and Daly, J. W. (1982) J. Biol. Chem. 257, 11591-11596
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J. Biol. Chem. 1983, 258:2432-2439.
Additions and Corrections

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Regulation of purine biosynthesis and interconversion in the chick.

Mary M. Welch and Frederick B. Rudolph

Page 13253, the following statement should be added to the footnote:

A preliminary report of this study was made at the IV International Symposium on Human Purine and Pyrimidine Metabolism at Maastricht, The Netherlands, June 1982, which will be included in the Proceedings of the Conference.

Page 13255, next to last paragraph:

Portions of the last two sentences were omitted. The correct sentences should read:

To determine if the concentration of these compounds changes during the increase in purine biosynthesis that follows β-estradiol treatment, soluble pool sizes of adenine, guanine, and hypoxanthine were determined. While the cellular pool sizes varied somewhat, there was no significant change in the levels of adenine, guanine, and hypoxanthine after stimulation with β-estradiol compared to the control.

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Properties of the adenylate cyclase catalytic unit from caudate nucleus.

Judith L. Bender and Eva J. Neer

Page 2432:

The following should be added to the § footnote:

... and by Grant BC 380 from the American Cancer Society.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.