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Regulation of the Catalytic Component of Adenylate Cyclase

Potentiative Interaction of Stimulatory Ligands and 2′,5′-Dideoxyadenosine

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SUMMARY

Both adenosine and the P site agent 2′,5′-dideoxyadenosine (DDA) reversibly inhibit adenylate cyclase activity in two different preparations of the enzyme that lack the stimulatory guanine nucleotide-binding protein, G/F: plasma membranes from the *cyc*$^-$ variant of S49 lymphoma cells and the resolved catalytic component (C) from rabbit liver. P site agents do not compete with any of the activators of C (Mn^{2+}, G/F, forskolin), nor do they diminish the potency of any activator of C. Rather, activation of C increases the effectiveness of P site agents and causes up to a 10,000-fold increase in the potency of DDA. The ability of G/F or forskolin to potentiate P site inhibition is also noted at concentrations much lower than those required for the stimulation of adenylate cyclase activity. These data are inconsistent with a simple two-state allosteric model for the regulation of the activity of C and demand the postulation of either a distinct, inhibited conformation of the enzyme or the existence of a dead-end complex with adenosine bound to the catalytic site.

INTRODUCTION

While adenosine and many purine-modified adenosine analogues can either stimulate or inhibit adenylate cyclase activity, ribose-modified analogues such as DDA[4] act only to inhibit. Distinct characteristics of the inhibition mediated by this purine-specific site, referred to as the P site, include (a) action independent of guanine nucleotides, (b) sensitivity to inhibitors of adenosine transport when studied in intact cells, and (c) persistence of inhibition in detergent-solubilized preparations (see ref. 2 for review). These properties have suggested to several investigators that the P site does not represent a cell-surface receptor for adenosine, but rather that it is an intracellularly oriented site that is closely associated with adenylate cyclase itself (2). Adenylate cyclase, as usually assayed, consists of at least two different proteins in addition to hormone receptors. The catalytic unit (referred to as "C") is relatively inactive in the presence of Mg^{2+} and is insensitive to guanine nucleotides or fluoride. It can be activated by either Mn^{2+} or forskolin. However, since C has not yet been purified, it is uncertain whether this activity represents one or more proteins. The stimulatory GTP-binding protein, G/F, mediates stimulation of C by guanine nucleotides and fluoride, and is a required intermediary for the stimulation of C by hormone-receptor complexes (see ref. 3 for review). It is likely that a distinct inhibitory GTP-binding protein also can regulate the activity of C (4). Several groups have suggested that the P site is a functional part of C (5–8), although Wolff *et al.* (9) concluded that G/F probably has a role in P site action. Neer and Salter (10) recently demonstrated P site-mediated inhibition in a preparation of bovine cerebral cortical C that was free of G/F. In this communication, we confirm that P site inhibition is observed in a similarly prepared C fraction from rabbit liver as well as in plasma membranes of the *cyc*$^-$ variant of S49 lymphoma cells, both of which are deficient in G/F.

It has been noted that P site-mediated inhibition is greater when adenylate cyclase is activated, but these studies have generally been performed using G/F-mediated stimulatory inputs (6, 9, 11, 12). By using both G/F-free preparations, we show that P site-mediated inhibition of C is not competitive with respect to activating ligands of the catalytic unit. In fact, inhibition is markedly potentiated by all stimulatory ligands tested. These data are inconsistent with a simple, two-state allosteric

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[4] The abbreviations used are: DDA, 2′,5′-dideoxyadenosine; C, catalytic component of adenylate cyclase; G/F, GTP-binding regulatory component of adenylate cyclase; *cyc*$^-$, S49 lymphoma cell variant that is deficient in G/F; GTPγS, guanosine-5′-(3-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate.

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model for the regulation of the catalytic protein. They argue that P site agents bind preferentially to the active state of C and inhibit its activity, either by promoting a further allosteric transition to an inactive state or by binding to the active site noncompetitively with respect to ATP. Each of these mechanisms predicts testable effects on ligand binding to C.

MATERIALS AND METHODS

G/F was purified from rabbit liver, as described by Sternweis *et al.* (13), through chromatography on heptylamine-Sepharose. Active fractions were diluted with two volumes of 20 mM Tris-Cl/1 mM EDTA/1 mM dithiothreitol/10 mM NaF/6 mM $MgCl_2$/25 mM NaCl/0.2% Lubrol 12A9 (pH 8.0) and applied to a 5-ml column of DEAE-Sephacel that had been equilibrated in the same buffer without $MgCl_2$ or NaF and that contained only 0.1% Lubrol. G/F was eluted in the latter buffer containing 225 mM NaCl. G/F obtained by this procedure had a specific activity of 700 units/mg (13). To activate purified G/F stably, the protein was incubated at 30° for 75 min in 30 mM sodium Hepes (pH 8.0)/215 mM NaCl/50 mM $MgCl_2$/0.2% Lubrol/1 mM dithiothreitol/50 μM GTPγS. Activation was terminated by dilution with 20 volumes of buffer at 0° containing no $MgCl_2$ or GTPγS, a 20-fold molar excess of GDP, and a 2-fold molar excess of EDTA. For assay, activated G/F was added to cyc^- membranes (40 μg) or liver catalytic protein (10 μg) is siliconized tubes and incubated for 30 min at 0° prior to assay.

Resolved C was prepared from rabbit liver essentially as described (14), except that 3 mM 2-mercaptoethanol was substituted for dithiothreitol and Tris-Cl was substituted for sodium Hepes in the gel filtration step. The C-containing fractions from the AcA34 column were diluted with three volumes of 0.9 mM dimyristoylphosphatidylcholine in 0.1 M sodium Hepes (pH 8.0)/1 mM EDTA/2 mM 2-mercaptoethanol. The activity of this preparation was stable for at least 1 month at −80°. Plasma membranes were prepared from S49 lymphoma cells as described previously (15). Specific activity of adenylate cyclase in cyc^- membranes or in hepatic C have varied less than 2-fold over about 18 months.

Adenylate cyclase activity was assayed at 30° in a final volume of 0.1 ml containing 0.5 mM [α-^{32}P]ATP (20–100 cpm/pmole), bovine serum albumin (0.1 mg/ml), pyruvate kinase (10 μg/ml), 6 mM phosphoenolpyruvate, 50 mM sodium Hepes (pH 8.0), 0.4 mM 3-isobutyl-1-methylxanthine (to block both R site activities and phosphodiesterase), 1 mM EDTA, adenosine deaminase (2 units/ml), and various concentrations of $MgCl_2$ or $MnCl_2$ (concentrations in the text refer to excess over 1 mM EDTA (see refs. 14 and 15). Forskolin was added in 0.1 assay volume of either methanol or 50% aqueous dimethyl sulfoxide. Solvent controls were used throughout. Assays were generally carried out over 20 min, and activity was constant over this interval in the presence or absence of adenosine, DDA, or activators. Data shown are the means of duplicate or triplicate samples that varied by less than 10%, usually by less than 5%.

Protein was determined by the method of Lowry *et al.* (16) or Schaffner and Weissman (17), using bovine serum albumin as standard. DDA was obtained either from P-L Biochemicals or ICN, forskolin from Calbiochem, and adenosine and adenosine deaminase from Sigma Chemical Company. DDA showed a single ultraviolet-absorbing spot after thin-layer chromatography on silica gel G in ethyl acetate/2-propanol/saturated NH_4OH/water (27:23:4:4) (18). This system separates DDA, adenosine, 2′- and 5′-deoxyadenosine, and adenine. [^{3}H] Dihydroforskolin was prepared by reductive tritiation under $^3H_2$ of 10 mM forskolin in ethyl acetate, using Pd on charcoal at room temperature for 24 hr. It was more than 98% pure on silica gel G chromatography in chloroform/methanol/water (75:25:1), with or without pretreating the plates with $AgNO_3$. Sources of other reagents have been listed previously (14, 15).

RESULTS

P site agents inhibit the catalytic component of adenylate cyclase. Plasma membranes of cyc^- S49 lymphoma cells and a chromatographically prepared fraction from rabbit hepatic plasma membranes were used to study P site-mediated inhibition of the catalytic protein of adenylate cyclase in the absence of G/F. Both preparations lack both G/F activity and the 45,000 M_r GTP-binding subunit of G/F (3, 14, 19). Inhibition of resolved hepatic C and cyc^- membranes by 1 mM DDA are shown in Tables 1 and 2 in comparison with the corresponding G/F-replete preparations, plasma membranes from rabbit liver and wild-type S49 cells. DDA inhibits adenylate

TABLE 1

Inhibition by DDA of adenylate cyclase activity in plasma membranes from rabbit liver and wild-type S49 lymphoma cells

This large comparative survey has been performed twice with similar results. Specific activities for S49 cell membranes are typical for large-scale preparations. Data for hepatic membranes are typical of our preparations and are similar to those of others (ref. 6, for example).

Addition to assay	Adenylate cyclase		% Inhibition
	Control	DDA (1 mM)	
	pmoles/min/mg		
Wild-type S49 cells			
Mg^{2+} (9 mM)	4.5	1.1	76
GTP (10^{-5} M)	8.4	2.2	74
(−)-Isoproterenol (10^{-5} M)	9.4	2.2	77
(−)-Isoproterenol + GTP	100	13	87
(−)-Isoproterenol + GTPγS (10^{-5} M)	110	13	88
NaF (10 mM)	160	26	84
Mn^{2+} (2.5 mM)	7.3	2.7	63
Mn^{2+} + forskolin (0.3 mM)	380	8.0	98
Mg^{2+} + forskolin	270	24	91
Rabbit liver			
Mg^{2+}	18	3.5	80
GTP	21	4.0	81
Glucagon (10^{-5} M)	32	5.6	82
Glucagon + GTP	80	7.6	91
Glucagon + GTPγS	160	9.3	94
NaF	100	9.9	90
Mn^{2+}	20	3.7	82
Mn^{2+} + forskolin	290	12	96
Mg^{2+} + forskolin	220	29	87

TABLE 2

Inhibition of adenylate cyclase activity by DDA in preparations that lack functional G/F

G/F* refers to G/F that was preactivated with GTPγS as described in Methods. More extensive examples of the activities outlined are shown in the figures.

Addition to assay	Adenylate cyclase		% Inhibition
	Control	DDA (1 mM)	
	pmoles/min/mg		
Cyc^- S49 cells			
Mg^{2+} (25 mM)	1.5	1.3	13
Mn^{2+} (2.5 mM)	13	5.9	55
Mn^{2+} + forskolin (0.3 mM)	650	16	98
Mg^{2+} + forskolin	180	15	92
G/F* (3.3 μg/ml)	710	34	95
Rabbit Hepatic C			
Mg^{2+}	2.6	2.0	23
Mn^{2+}	29	13	54
Mn^{2+} + forskolin	200	18	91
Mg^{2+} + forskolin	180	38	79
G/F*	130	12	90

cyclase activity in each preparation in the presence of any of several activating ligands, including those whose stimulation is mediated by G/F (GTPγS, NaF, glucagon, or isoproterenol) and those that do not require G/F in order to stimulate [Mn^{2+}, forskolin (19, 20)]. Inhibition was not dependent on guanine nucleotides, was not antagonized by 3-isobutyl-1-methylxanthine (a potent R site blocker), and was observed whether activation was readily reversible (agonist plus GTP, Mn^{2+}, forskolin) or quasi-irreversible (NaF, GTPγS). In the hepatic C preparation, inhibition was also independent of the presence of phospholipid, in contrast to the requirement for lecithin for optimal coupling of G/F (21). As shown previously (2), P site agents are not competitive with substrate. When activities were measured over the range of 5 μM–1 mM Mn^{2+}-ATP or Mg^{2+}-ATP, DDA either did not alter the K_m or decreased it slightly. From these data, we conclude that the inhibition caused by DDA is a typical P site response. Adenosine itself, while somewhat less potent than DDA, shares all of the features of DDA that are described below. The data presented below are taken from experiments performed using either *cyc*$^-$ plasma membranes or hepatic C. However, all experiments have been performed at least once with each preparation, and the two preparations yield identical results.

Functional interaction of P site agents with activators of C. In plasma membranes, inhibition by P site agents is generally more effective, more potent, or both, when assayed in the presence of activators of the enzyme (5, 6, 11, 12). This pattern appears to hold when P site inhibition is examined in the absence of G/F, inasmuch as DDA is least effective under conditions where C is slightly stimulated (Mg^{2+} or Mn^{2+} alone; Table 2). The complete DDA concentration curves, shown in Fig. 1, indicate a striking effect of the activation of C upon the potency of inhibition by DDA. Basal activity is poorly inhibited, and inhibition is limited by the solubility of DDA. In the presence of Mn^{2+}, which produces a 10-fold stimulation of activity, DDA is at least 10 times more potent. With Mn^{2+} plus forskolin, which together activate C several hundred-fold, the potency of DDA is more than 1000-fold greater than in the presence of Mg^{2+} alone, and maximal inhibition is usually 98–99%. DDA displays similarly high potency and completeness of inhibition in the presence of GTPγS-preactivated G/F and Mg^{2+}

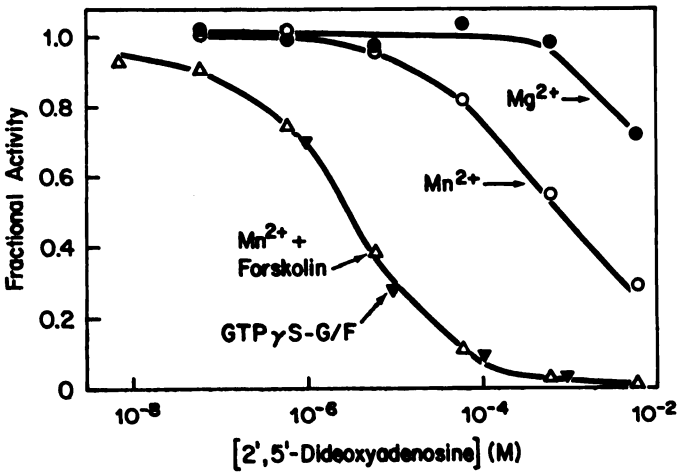

FIG. 1. *Effect of activators of the catalytic component on the inhibitory potency of DDA*

Adenylate cyclase activity in *cyc*$^-$ membranes was assayed as described in the presence of 26 mM $MgCl_2$ (●), 3.5 mM $MnCl_2$ (○), $MnCl_2$ plus 0.3 mM forskolin (△), or $MgCl_2$ plus GTPγS-activated G/F (332 ng/assay) (▼). For the assay of activity with $MgCl_2$ alone, the concentration of ATP was lowered to 0.25 mM in order to increase the specific activity of the isotope. Specific enzymatic activities (picomoles per minute per milligram) in the absence of DDA were $MgCl_2$, 2.4; $MnCl_2$, 20; $MnCl_2$ plus forskolin, 530; G/F, 510.

The possibility that P site agents inhibit adenylate cyclase by competing with activating ligands for binding to C or by decreasing the affinity of C for such ligands was tested by determining the effect of DDA on the concentration dependence of stimulation by forskolin or by GTPγS-preactivated G/F. The results obtained for Mn^{2+} plus forskolin, using hepatic C, are shown in Fig. 2A. Stimulation by forskolin reaches a maximum and then declines, as observed by Seamon and Daly (20)[5]. Inhibition by DDA is apparent at all concentrations of forskolin and, at a fixed concentration of DDA, the fractional inhibition actually increases with increasing forskolin concentration. The concentration of forskolin that stimulates half-maximally (EC_{50})[6] is not increased by DDA, but rather is decreased 4-fold (at 10^{-4} M DDA in the presence of Mn^{2+}). This effect is more obvious in the experiment of Fig. 2B, where DDA caused a 10-fold decrease in the EC_{50} for forskolin in *cyc*$^-$ membranes in the presence of Mg^{2+}. The data in Fig. 3A show that the potency of DDA is likewise progressively increased by increasing concentrations of forskolin. At a maximally effective concentration of forskolin (10^{-4} M), DDA was 50-fold more potent than at the threshold concentration (10^{-6} M) and about 1000-fold more potent than in the presence of Mn^{2+} alone. Figure 3B displays two measures of the potency of DDA as a function of forskolin concentration. Comparison of these parameters with the concentration curve for activation of adenylate cyclase indicates that forskolin greatly increases the potency of DDA at concentrations that activate C only slightly, if at all.

A similar, but not identical, relationship between the potencies of DDA and activator was observed if G/F-mediated stimulation of C was considered. Results obtained with the GTPγS-activated form of G/F are shown in Fig. 4. As was the case with forskolin, inhibition by DDA was not abolished by increasing the concentration of activated G/F, and the EC_{50} for G/F was not increased

[5] Since the concentration of forskolin that produces maximal activation is close to its limit of solubility in aqueous solution, it is likely that the decrease in activity seen at higher concentrations is artifactual. There is less of a decrease if forskolin is added in 50% aqueous dimethylsulfoxide rather than in methanol, and the decrease is observed at higher concentrations of forskolin.

[6] In this paper, "EC_{50}" refers to the concentration of an activating ligand that stimulates activity to one-half of the maximal activity observable with that particular ligand. The IC_{50} for DDA often cannot be defined experimentally in an analogous manner because the concentrations of DDA necessary to achieve complete inhibition of basal and Mn^{2+}-stimulated activities exceed its solubility. Since concentration curves for DDA in the presence of different activators are shifted in a parallel manner, "potency" is used here to refer to the concentration of DDA necessary to achieve any given level of fractional inhibition.

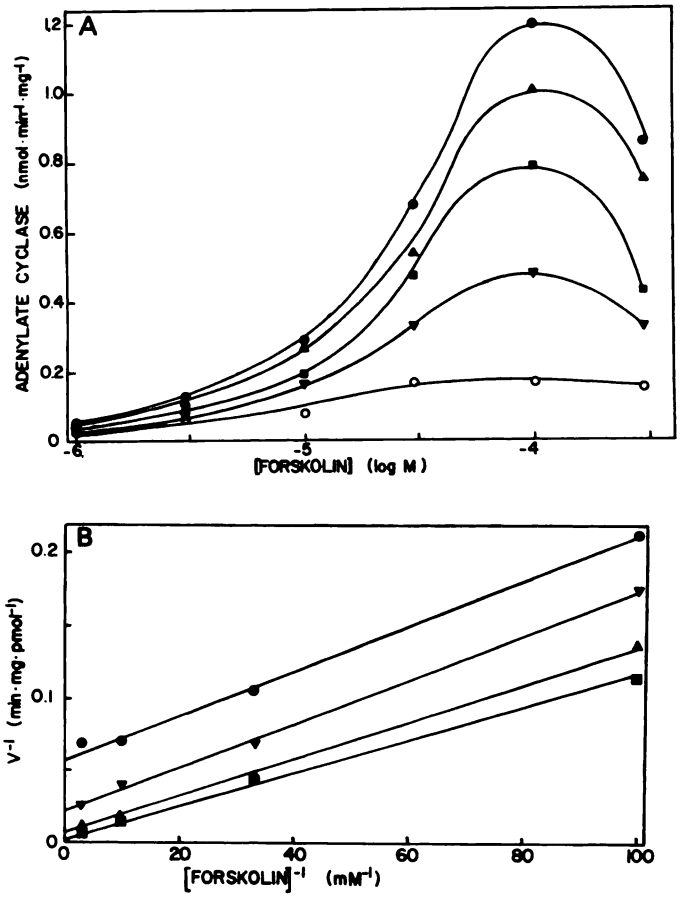

FIG. 2. *Inhibition by DDA of forskolin-stimulated adenylate cyclase activity in the absence of G/F*

A. Adenylate cyclase activity in the resolved hepatic C preparation was assayed as described in the presence of 3.5 mM $MnCl_2$. Forskolin was added in 10 μl of 75% methanol, and all assays contained 7.5% methanol. DDA was present in the following concentrations: 0 (●), 10^{-6} M(▲), 10^{-5} M (■), 10^{-4} M (▼), 10^{-3} M (○). This experiment (quantitation of DDA inhibition using Mn^{2+} plus forskolin) has been repeated twice with hepatic C and four times with *cyc*$^-$ membranes, yielding similar results in each case.

B. Inhibition by DDA is not competitive with respect to forskolin. Adenylate cyclase activity in *cyc*$^-$ membranes was assayed in the presence of 8.5 mM $MgCl_2$, 7.5% methanol, and the indicated concentrations of DDA and forskolin. The reciprocal of reaction velocity is plotted as a function of the reciprocal of forskolin concentration. DDA concentrations and EC_{50} values for forskolin, determined from the extrapolated *x*-intercepts, were as follows: 2.5×10^{-7} M DDA (■), EC_{50} = 300 μM; 2.5×10^{-5} M DDA (▲), EC_{50} = 190 μM; 2.5×10^{-4} M DDA (▼), EC_{50} = 69 μM; 2.5×10^{-3} M DDA (●), EC_{50} = 28 μM. Data obtained in the absence of DDA were not distinguishable from those obtained at 2.5×10^{-7} M DDA. This experiment has been performed three times with similar results.

by DDA. G/F also caused a marked increase in the potency of DDA at concentrations that activated C only slightly (Fig. 4B). We conclude from these data that DDA does not interact competitively with either forskolin or G/F.

In the presence of increasing concentrations of Mn^{2+} alone, the activity of C increased quite sharply 6- to 10-fold to a maximum at 2–3 mM and then declined gradually, reaching a plateau at about 50% of maximal activity at concentrations above 5 mM (data not shown). DDA (1 mM) inhibited by about 50% throughout the range of 2–20 mM Mn^{2+}, the decline and plateau phases. DDA also did not increase the concentration of Mn^{2+} at which half-maximal activity was observed. As shown in Fig. 5, increasing stimulation by Mn^{2+} potentiated inhibition by DDA, as was the case with forskolin or G/F. Complete inhibition could not be obtained with reasonable concentrations of DDA in this experiment, even at the highest concentrations of Mn^{2+}. These data are consistent with those of Johnson *et al.* (11), who studied the interactions of divalent cations and adenosine on the substrate kinetics of adenylate cyclase in G/F-replete platelet membranes.

Since basal and stimulated activities responded so differently to P site inhibition, we tested the notion that basal and forskolin-stimulated activities might reflect multiple populations of DDA-inhibitable and noninhibitable enzyme. The ability of forskolin to protect basal catalytic activity against thermal denaturation is depicted in Fig. 6. *Cyc*$^-$ plasma membranes were incubated at 37° with or without 225 μM forskolin. Samples were taken periodically, washed to remove forskolin, and assayed for basal adenylate cyclase activity in the presence of 10 mM $MgCl_2$. In the absence of forskolin, activity decayed at 37° with a $t_{1/2}$ of 1.8 min (0.9–2.1 min in three experiments), whereas forskolin prevented significant loss of basal activity for more than 10 min. Since forskolin can stabilize basal activity, it is likely that basal and forskolin-stimulated activity arise from the same enzyme.

Subsaturating amounts of forskolin and DDA also acted synergistically to stabilize C, as shown in the experiment depicted in Fig. 7. In this case, the unperturbed denaturation rate ($t_{1/2}$ = 1.7 min) was decreased slightly and insignificantly by DDA ($t_{1/2}$ = 1.9 min) and somewhat more by forskolin ($t_{1/2}$ = 3.3 min). However, both agents together further stabilized C ($t_{1/2}$ = 6.1 min). Although at long times there was divergence from first-order decay, a single denaturation rate appeared to account for about 90% of the total activity. At the concentration of forskolin used in this experiment, activity would have been inhibited about 70–80% by the concentration of DDA that was used. These data suggest further that DDA and forskolin can bind simultaneously to a single pool of C.

DISCUSSION

We have studied P site-mediated inhibition of the catalytic unit of adenylate cyclase by adenosine and DDA in two different preparations that have been shown previously not to contain functional G/F (3). These data, and the data of Neer and Salter (10) and Johnson (8), indicate that G/F is not required for P site inhibition. The 41,000 M_r inhibitory GTP-binding protein (4) was also markedly depleted (<7%) in our preparation of hepatic C (22), suggesting that it, too, is unnecessary for P site responses. We conclude that the P site is either on the catalytic unit itself or on an unidentified, closely linked protein, although purified catalytic protein will be needed to locate the P site precisely.

The most striking aspect of our data is the large

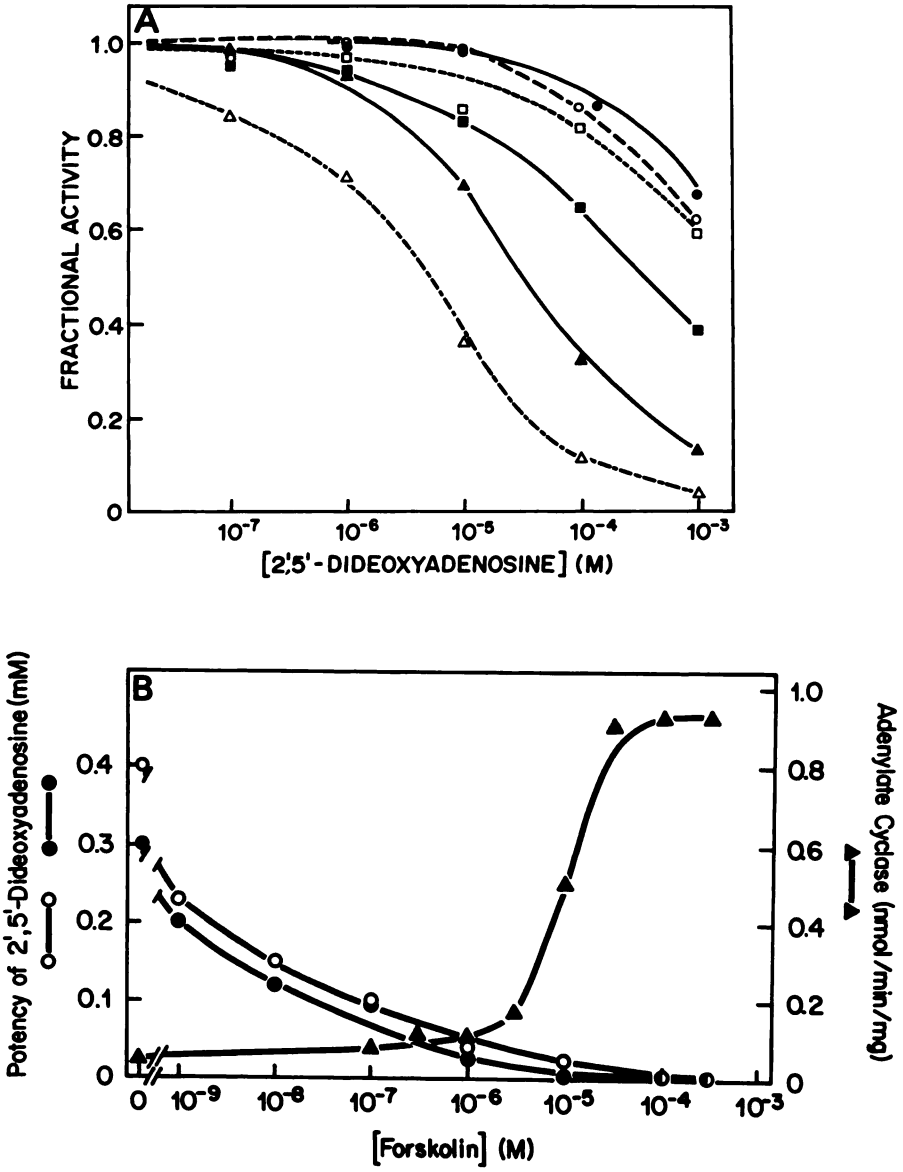

FIG. 3. *Effect of forskolin on the inhibitory potency of DDA*

A. Adenylate cyclase activities in *cyc*$^-$ plasma membranes were assayed as described in the presence of 3.5 mM $MnCl_2$, DDA at the concentrations shown, and several concentrations of forskolin (0, ●; 10^{-8} M, ○; 10^{-7} M, □; 10^{-6} M, ■; 10^{-5} M, ▲; 10^{-4} M, △). Specific activities (picomoles per minute per milligram) at each concentration of forskolin, in the absence of DDA, were as follows: no forskolin, 12.9; 10^{-8} M, 13.0; 10^{-7} M, 14.8; 10^{-6} M, 20.1; 10^{-5} M, 61.8; 10^{-4} M, 324.

B. The data shown in A and other data from the same experiment are replotted in terms of two measures of the potency of DDA: the concentration of DDA required to achieve one-half of the maximal observed inhibition (●), and the concentration required to reduce the absolute activity by 15% (○). The effect of increasing concentrations of forskolin on adenylate cyclase activity (▲) is shown for comparison.

potentiation of P site inhibition that was caused by activators of C. It is also noteworthy that this potentiation was observed with all activators tested, whether activation was rapidly reversible (Mn^{2+}, forskolin) or poorly reversible (activated G/F). The potency of DDA could be increased by a factor of nearly 10^4 by the addition of activators (Figs. 1, 3, and 4). Conversely, the potency with which an activating ligand alters P site action is also much greater than its potency to activate the enzyme (Figs. 3 and 4). In contrast to the interpretation of Tolkovsky (23), we believe that inhibition at the P site markedly increases the apparent coupling to C of all activating ligands, including G/F, rather than uncoupling such interactions.

Although our preparations of C are quite crude, our data cannot be explained by the existence of two populations of adenylate cyclase, one sensitive to activators and to P site inhibitors and another much less so. Such an explanation is inconsistent with the ability of activators to increase markedly the potency of DDA at concentrations that do not increase adenylate cyclase activity. It would also predict that the 6- to 10-fold activation by optimal amounts of Mn^{2+} would increase the potency of DDA nearly as much as does activation by forskolin or

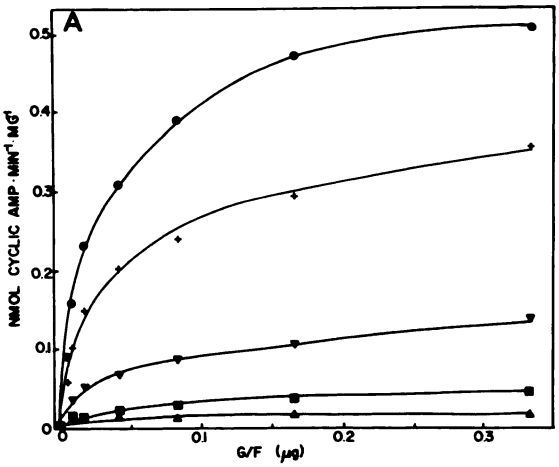

FIG. 4. *Inhibition by DDA of G/F-stimulated adenylate cyclase activity in cyc⁻ membranes*

A. Hepatic G/F was activated as described and added to *cyc*⁻ membranes (41 μg) in siliconized tubes. After 30 min at 0°, adenylate cyclase activity was assayed as described in the presence of 3 mM $MnCl_2$ and 40 mM NaCl. The amount of G/F shown is that added to a 0.1-ml assay volume. DDA was present during the assay in the following concentrations: 0 (●); 10^{-6} M (+); 10^{-5} M (▼), 10^{-4} M (■), 10^{-3} M (▲). Activities are expressed per milligram of *cyc*⁻ protein.

B. Data from a separate but similar experiment, using lower concentrations of G/F, are replotted to display the changes in the potency of DDA, expressed as the concentration required to inhibit activity by 15% (○). In the absence of G/F, 1 mM DDA inhibited only 10%. Activity in the absence of DDA (▲) is also shown. Experiments relating the potencies of activated G/F and DDA using *cyc*⁻ membranes have been performed more than eight times, using either Mg^{2+} or Mn^{2+}, with similar results. A similar relationship of potencies is observed using hepatic C, although the efficiency of activation of hepatic C by G/F is often less than that observed with *cyc*⁻ membranes.

activated G/F. It is inconsistent with the ability of forskolin to protect basal activity against thermal denaturation (Fig. 6) and with the potentiation of the protective effect of forskolin by DDA (Fig. 7). Last, it seems unlikely that plasma membranes from wild-type or *cyc*⁻ S49 cells, resolved hepatic C that has been extensively manipulated in cholate, hepatic and platelet (11) plasma membranes, and a Lubrol extract of *cyc*⁻ membranes (data not shown) would all contain similar proportions of these two hypothetical species.

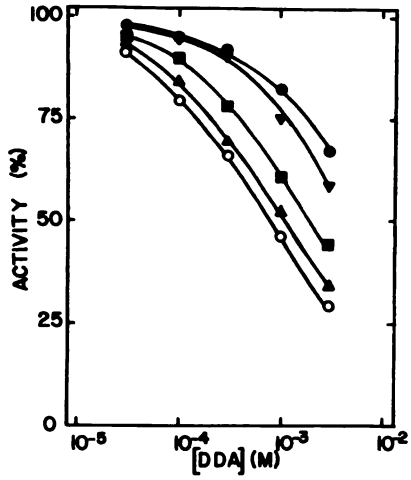

FIG. 5. *Mn^{2+} increases the inhibitory potency of DDA*

Adenylate cyclase was assayed in *cyc*⁻ membranes as described, except that 50 mM Tris-Cl (pH 8.0) was substituted for Hepes and the concentration of ATP was reduced to 50 μM. DDA and $MnCl_2$ were present as indicated. Activities in the absence of DDA at each concentration of Mn^{2+} were as follows (picomoles per minute per milligram): 0.1 mM (●), 1.5; 0.2 mM (▼), 3.6; 0.5 mM (■), 8.0; 1.0 mM (▲), 11.0; 3.0 mM (○), 12.9. This experiment has been performed three times with similar results, although basal specific activity can vary as much as 4-fold among preparations.

The large apparent positive heterotropic interaction of activators and P site agents provokes speculation about its possible molecular basis. The simplest scheme to describe both activation and inhibition of C would be a two-state allosteric model, in which unliganded C exists in an inactive "basal" state and the binding of activators

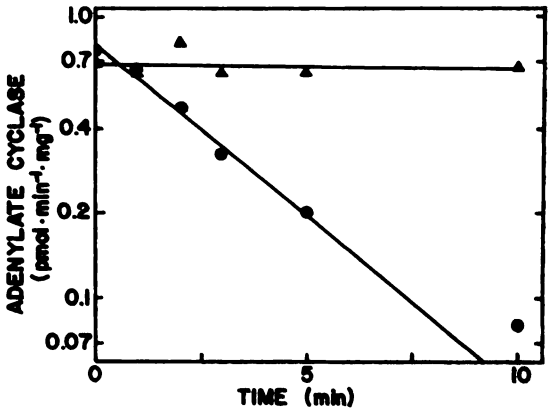

FIG. 6. *Forskolin stabilizes basal adenylate cyclase against thermal denaturation*

Cyc⁻ membranes (1.8 mg/ml) were incubated at 37° in 20 mM Hepes (pH 8)/3 mM $MnCl_2$/0.3 mM EDTA/50 μM ascorbic acid for the indicated intervals in the presence of either 225 μM forskolin (▲) or of vehicle alone (3.8% dimethyl sulfoxide) (●). Aliquots were then diluted 60-fold into ice-cold 20 mM sodium Hepes (pH 8.0) and washed twice in the same buffer. Resuspended pellets were assayed for adenylate cyclase activity with 9.5 mM $MgCl_2$ and 5×10^{-5} M [α-^{32}P]ATP. The low concentration of ATP helped achieve a specific activity of 400 cpm/pmole of ATP. Separate experiments using 0.5 mM ATP yielded similar results. Complete removal of forskolin by washing is indicated by the similarity of control and forskolin-treated zero-time activities. In a parallel experiment, removal of [14,15-^{3}H]dihydroforskolin by the same procedure was found to be >98%.

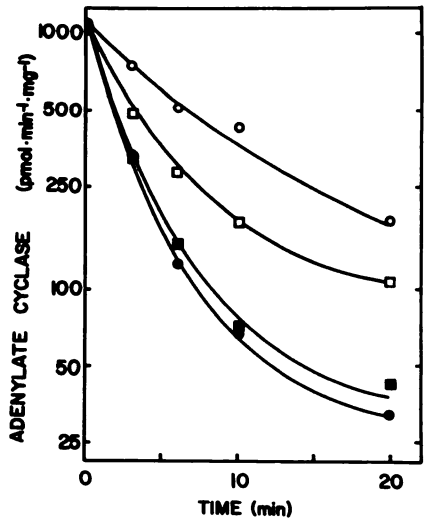

FIG. 7. *DDA enhances the stabilization of adenylate cyclase by forskolin*

Cyc⁻ membranes (2.3 mg/ml) were incubated at 37° for the indicated intervals. Incubation media contained 250 μM DDA (■), 10 μM forskolin (□), both agents together (○), or neither agent (●). Dimethyl sulfoxide (2.7%) was present in each case. Incubations were terminated by 50-fold dilution into ice-cold 20 mM Hepes (pH 8.0). After two washes in this buffer, adenylate cyclase was assayed in the presence of 2 mM $MnCl_2$ and 0.2 mM forskolin. These data are typical of the results of three other experiments.

promotes conversion to an active state, C* (i.e., a stimulatory ligand binds preferentially to the C* state). In this scheme, a P site agent could inhibit either by competing with activating ligands at a single site or by binding to a separate site and promoting conversion to the basal state. If P site inhibition is allosteric, however, either two-state mechanism is clearly inadequate. Simple competition is ruled out by the data of Fig. 7 that suggest that DDA and activator can bind simultaneously to C. It is also inconsistent with the failure of these agents to increase the EC_{50} for activating ligands (Figs. 2 and 4), by the failure of activating ligands to decrease the potency of P site agents (Figs. 1, 3, 4, and 5), and by the increased fractional inhibition by DDA with increasing concentrations of activating ligands (Fig. 2). These latter observations suggest similarly that a P site agent does not simply favor the basal conformation of C, since this scheme would also yield apparently competitive inhibition. This apparent competition would derive from the fact that relative stabilization of the basal state by P site agents would increase the free energy of the C → C* transition and, hence, the concentration of activating ligand required to promote that transition. We are therefore unable to reconcile our data or those of others with a simple two-state allosteric model for the regulation of C.

The apparent synergism of activators and P site inhibitors indicates that P site inhibition is more complex, and two plausible explanations for this behavior exist. If P site agents act allosterically, then they must promote a transition to a distinct inhibited state of C, C^i. This scheme, dealt with elsewhere in more detail (3, 22), allows stimulatory ligands and P site agents to bind to each state of C, with all three being in equilibrium:

$$C \overset{K_1}{\rightleftharpoons} C^* \overset{K_2}{\rightleftharpoons} C^i$$

Since K_1 and K_2 are by definition less than 1 in the absence of activator or inhibitor, the free energy of the C* → C^i transition is less than that for C → C^i. Since binding of activators increases K_1, activators will "sensitize" the enzyme to inhibition, an effect previously referred to by Johnson *et al.* (11). An intuitively simple, but non-unique, case of this scheme simply says that P site agents can bind to and inhibit only the activated form of C. Numerical simulations of this model, based on our data, agree quantitatively with the observed potentiative interactions between P site agents and activating ligands of C (22).

An alternative to an allosteric mechanism is suggested by studies of the soluble adenylate cyclase from *Brevibacterium liquifaciens*, which is significantly inhibited by adenosine only when it is activated by pyruvate (24). Wolin (25) proposed that this apparently uncompetitive inhibition by adenosine reflects the formation of an abortive enzyme-ATP-Me^{2+}-adenosine complex at the catalytic site that is not in rapid equilibrium with active forms of the enzyme. The potentiation of P site inhibition by activators, according to this model, suggests that catalysis by activated C is much faster than dissociation of the dead-end complex, causing accumulation of that complex at high turnover rates. A smaller fraction of total C would accumulate as the dead-end complex when catalytic turnover is slow.

Both the three-state allosteric mechanism and the active site-directed mechanism can account well for our data and those of others. A two-state allosteric model cannot. Both mechanisms also make testable predictions regarding the binding of P site agents to C. The three-state allosteric model predicts that the affinity of C for inhibitor is at least 100 times greater in the presence of an effective activating ligand than in its absence (22). If this prediction is confirmed, it will allow characterization of C as a protein whose labeling by a P site analogue will be specifically enhanced by activators. The active site-directed scheme predicts specific inhibitory effects of pyrophosphate and detailed kinetics for the reverse reaction. Both of these explanations for our findings on P site inhibition should thus be experimentally approachable.

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