The Catalytic Mechanism of Mammalian Adenylyl Cyclase

EQUILIBRIUM BINDING AND KINETIC ANALYSIS OF P-SITE INHIBITION

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The mechanism of P-site inhibition of adenylyl cyclase has been probed by equilibrium binding measurements using 2'-[3H]deoxyadenosine, a P-site inhibitor, and by kinetic analysis of both the forward and reverse reactions (i.e. cyclic AMP and ATP synthesis, respectively). There is one binding site for 2'-deoxyadenosine per Cα/Cβ heterodimer; the Kd is 40 ± 3 μM. Binding is observed only in the presence of one of the products of the adenylyl cyclase reaction, pyrophosphate (PPi). A substrate analog, Ap(CH2)pp (α,β-methylene adenosine 5'-triphosphate), and cyclic AMP compete for the P-site in the presence of PPi, but P-site analogs do not compete for substrate binding (in the absence of PPi). Kinetic analysis indicates that release of products from the enzyme is random. These facts permit formulation of a model for the adenylyl cyclase reaction, for which we provide substantial kinetic support. We propose that P-site analogs act as dead-end inhibitors of product release, stabilizing an enzyme-product (E-PPi) complex by binding at the active site. Although product release is random, cyclic AMP dissociates from the enzyme preferentially. Release of PPi is slow and partially rate-limiting.

Adenosine and various analogs of the nucleoside have both stimulatory and inhibitory effects on adenylyl cyclase activity (reviewed in Ref. 1). Londos and Wolff (2) categorized these effects mechanistically, based on their structure–activity relationships. Two types of adenosine-reactive sites were identified: those with strict requirements for the ribose moiety, designated R sites, and those with strict structural constraints for interaction with the purine ring, designated P sites. R sites are the ligand-binding sites of adenosine-specific G protein-coupled receptors, which can either stimulate or inhibit adenylyl cyclase activity indirectly, while P sites, whose occupancy inhibits cyclic AMP synthesis, are structural features of adenylyl cyclases themselves (2–7). The physiological significance of P-site inhibition is unclear, but concentrations of 3'-AMP found in vivo appear sufficient to inhibit adenylyl cyclase activity (8).

P-site inhibition is typically noncompetitive or uncompetitive with respect to substrate ATP, depending on the divalent cation utilized in the assay (Mg2+ usually yielding noncompetitive kinetics; Mg2+ uncompetitive) (3, 4, 9–11). Furthermore, the apparent potency of such inhibitors increases when adenylyl cyclase is activated (3–5, 7, 11). Representative P-site reagents, ordered by potency, include 2',5'-dideoxy-3'-ATP > 2',5'-dideoxy-3'-ADP > 2',5'-dideoxy-3'-AMP > 2'-deoxy-3'-AMP > 3'-AMP > 2'-deoxyadenosine > adenosine (12, 13). Although several mechanisms for P-site inhibition have been proposed, there is as yet no conclusive evidence to support any particular hypothesis.

Using engineered, soluble forms of mammalian adenylyl cyclase, we and others have shown that the conserved cytosolic domains of the enzymes contain the structural components necessary for Gαs and forskolin-stimulated adenylyl cyclase activity, as well as the characteristic features of P-site inhibition (14–18). We have also utilized the competitive substrate analog Ap(CH2)pp to identify a single substrate binding site on the enzyme (19). Binding of Ap(CH2)pp to adenylyl cyclase is completely unaffected by the addition of a potent P-site inhibitor. Neither of the two cytosolic domains of adenylyl cyclase contains a classical nucleotide binding motif, although those domains share approximately 200 amino acid residues of similar sequence. These observations add credence to the possibility that the substrate binding and P sites on the enzyme are structurally distinct.

The experiments described herein further define the nature of the P site and the mechanism of P-site inhibition. P-site inhibition is also used as an tool to facilitate understanding of the reaction catalyzed by adenylyl cyclases.

**EXPERIMENTAL PROCEDURES**

**Materials**—2'-[3H]Deoxyadenosine (7 Ci/mmol) was purchased from ICN and lyophilized regularly to remove [3H]H2O. Ap(CH2)pp was also purchased from ICN. Hexokinase and type XI glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim and Sigma, respectively; these preparations were centrifuged, and the pellet added to the assay. Adenylyl cyclase was purified by expression in Escherichia coli as described (16, 18, 20). Gαs was activated (whenever used) by incubation with 50 mM NaHepes (pH 8:0), 20 mM MgSO4, 1 mM EDTA, 2 mM dithiothreitol, and 400 μM GTPγS at 30 °C for 30 min; free GTPγS was then removed by gel filtration.

**Adenylyl Cyclase Assays**—Synthesis of cyclic AMP was measured as described (21) for 10–15 min at 30 °C in a final volume of 100 μL. GTPγS-Gαs was present (400 nM) unless otherwise indicated. Activities are expressed per mg of the limiting adenylyl cyclase domain in the assay (VC1). The other cytosolic domain (IIC2) was present in excess (1 μM) to drive the interaction between the two protein fragments. To determine kinetic constants, the concentration of MgATP was varied from 10 μM to 2.66 mM with a fixed excess of 10 mM Mg2+. Initial velocities were linear with time, and less than 10% of the ATP was consumed at the lowest substrate concentrations. All points were measured in duplicate and experiments were repeated two to four times. Values are reported ± S.E. of the mean.

**Synthesis of ATP from cyclic AMP and PPi**—the reverse reaction, was measured spectrophotometrically in the presence of glucose, hexokinase, NADP, and glucose-6-phosphate dehydrogenase. Reaction velocities were calculated from the linear increase in A405 resulting from the reduction of NADP. Reactions contained 20 mM NaHepes (pH 8.0), 50 mM glucose, 0.8 mM NADP, 3 mM free MgCl2, 1.7 units of hexokinase,

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1 The abbreviations used are: G protein, heterotrimeric guanine nucleotide-binding protein; Gαs, the α subunit of the G protein that stimulates adenylyl cyclase; ApCH2pp, α,β-methylene adenosine 5'-triphosphate; GTPγS, guanosine 5'-γ-thiotriphosphate; PPi, pyrophosphate.
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FIG. 1. Uncompetitive inhibition of G\textsubscript{\alpha}-stimulated adenylyl cyclase activity by the P-site inhibitor 2'-deoxyadenosine. Assays (0.7 mM VC\textsubscript{1}, 1 mM IC\textsubscript{2}) were performed in the presence of 10 mM free Mg\textsubscript{Cl\textsubscript{2}}, 400 mM activated G\textsubscript{\alpha}, 0.02–1.6 mM MgATP, and no inhibitor (○) or 87 mM [■], 175 mM [▲], or 350 mM [●] 2'-deoxyadenosine. *Values for K\textsubscript{d} (340 ± 90 mM) and V\textsubscript{max} (68 ± 7 \mu\text{mol/min/mg}) were determined by Lineweaver-Burk analysis. Replots (inset) of slopes (●) and intercepts (■) versus the corresponding concentrations of 2'-deoxyadenosine were used to determine the K\textsubscript{d} for inhibition.

and 0.3 units of glucose-6-phosphate dehydrogenase in a volume of 500 \mu\text{L}. The concentrations of substrates varied: 1.25–20 mM for cyclic AMP and 0.125–4 mM for Mg\textsubscript{PP\textsubscript{2}}. PP\textsubscript{2} was always added last to avoid precipitation. Reactions were typically started by addition of 0.4 mM VC\textsubscript{1}, 2 mM IIC\textsubscript{2}, and 1 mM GTP-S-G\textsubscript{\alpha} (final concentrations) to the other reaction components (at 30 °C), and absorbance changes were measured for 10–15 min in a Beckman DU650 spectrophotometer with a temperature-controlled cuvette holder. The increase in absorbance of the adenylyl cyclase was subtracted as background (<0.006 OD units/min). Optical densities of greater than 1.5 were excluded from analysis.

Equilibrium Dialysis—Equilibrium dialysis was performed essentially as described (19). To quantify binding of 2'-deoxyadenosine, each chamber contained 20 mM Na\textsubscript{Hepes} (pH 8.0), 2.5 mM Mg\textsubscript{Cl\textsubscript{2}}, 2 mM dithiothreitol, 75 mM NaCl, 2'-[\textsuperscript{3}H]deoxyadenosine (12.5–250 mM), and other additions as indicated. One chamber contained 18 mM VC\textsubscript{1}, 18 mM IIC\textsubscript{2}, and 25 mM GTP-S-G\textsubscript{\alpha}; both of the cytosolic domains of adenylyl cyclase were necessary to observe binding. The opposite chamber contained buffer in lieu of the proteins. Samples were removed after dialysis for 24 h at 4 °C with rotation. Duplicate 15-\muL aliquots from each chamber were analyzed by liquid scintillation spectrometry. Binding data have been normalized to protein concentrations based on the amount of active protein in the preparation, which was determined by titration with GTP-S-G\textsubscript{\alpha} (19). Preparations of VC\textsubscript{1} and IIC\textsubscript{2} were more than 60% active by this criterion.

Steady-state Kinetic Model—We derived the rate equation for the reaction scheme presented in Fig. 5 using steady-state assumptions for the catalytic steps and rapid equilibrium for inhibitor binding. The critical features of the model include random release of products and no binding of P-site inhibitor to the free enzyme. The rates of both the forward and reverse reactions are complicated functions, described as $v = [E]\cdot[R][ATP]$, [cyclic AMP], [PP\textsubscript{2}], [2'-deoxyadenosine]), where $v$ is the velocity in n\text{mol/s} and [R] is the total molar concentration of enzyme.

The equations that describe this function are presented in the “Appendix.” Because of their complexity, they have not been rearranged to conform to standard Michaelis-Menten format. Curve fitting of individual experiments and modeling of kinetic data based on the steady-state rate equations were performed using Sigma Plot (Jandel Scientific).

RESULTS

P-site Inhibition of Adenylyl Cyclase—Inhibition of the G\textsubscript{\alpha}-stimulated catalytic activity of our preparation of adenylyl cyclase by the P-site inhibitor 2'-deoxyadenosine is uncompetitive with respect to Mg\textsubscript{ATP} (Fig. 1). These reactions (and those described below) were performed with a limiting concentration of the C\textsubscript{1} domain of type V adenylyl cyclase and an excess of the C\textsubscript{2} domain of type II adenylyl cyclase to drive the protein-protein interaction that is necessary for catalytic activity. Similar uncompetitive kinetics has been observed previously with a more potent P-site inhibitor, 2'-deoxy-3'-AMP, using G\textsubscript{\alpha}-stimulated bovine brain adenylyl cyclase (22) (presumably a mixture of isoforms) or a soluble system in which the two cytosolic domains of the enzyme were linked covalently (15). Although 2'-deoxyadenosine has an unfortunately high K\textsubscript{d} (240 ± 60 mM) compared with 2'-deoxy-3'-AMP (5 \muM, data not shown), we have utilized 2'-deoxyadenosine for this work because of the availability of the compound in radiolabeled form. Adenylyl cyclases (native enzymes or the soluble system utilized here) display noncompetitive or mixed noncompetitive inhibition by P-site analogs with respect to Mn\textsubscript{ATP} (3, 4, 10, 11, 15, 23, 24). We have focused our analysis on G\textsubscript{\alpha}-activated adenylyl cyclase activity with Mg\textsubscript{ATP} as substrate (in the absence of Mn\textsuperscript{2+}) to avoid the complications of mixed inhibition and the use of a second divalent cation (particularly Mn\textsuperscript{2+} in the presence of PP\textsubscript{2}, which has limited solubility).

Equilibrium Dialysis with 2'-[\textsuperscript{3}H]Deoxyadenosine—Uncompetitive inhibition implies that 2'-deoxyadenosine and ATP do not combine with the same form of the enzyme; noncompetitive inhibition is consistent with such a mechanism (but does not demand it). Florio (25) suggested that P-site ligands were dead-end inhibitors that formed a complex with the PP\textsubscript{2}-bound form of the enzyme (see Fig. 5). Alternatively, Johnson and Shoshani (22) suggested that the P-site is distinct from the active site and that both inhibitor and substrate could be bound simultaneously. To distinguish between these possibilities, we utilized equilibrium dialysis to examine directly the requirements for binding of 2'-deoxyadenosine to adenylyl cyclase. This was not possible previously because of limiting quantities of protein. We were unable to detect binding of 80 \muM 2'-deoxyadenosine to 18 \muM VC\textsubscript{1} and IIC\textsubscript{2} in the presence of G\textsubscript{\alpha} and Mg\textsuperscript{2+} or Mn\textsuperscript{2+} (Fig. 2A); similar results were obtained after addition of the substrate analog Ap(CH\textsubscript{2})pp. However, a modest level of binding was detected upon addition of 5 mM ATP under conditions (24-h incubation and very high enzyme concentration) where ATP and the products of the adenylyl cyclase reaction, cyclic AMP and PP\textsubscript{2}, should be in equilibrium. (The equilibrium constant, 0.065 mM (26), implies final concentrations of roughly 0.3 mM ATP and 4.67 mM cyclic AMP and PP\textsubscript{2}.) We thus tested the capacity of cyclic AMP and PP\textsubscript{2} to support binding of 2'-deoxyadenosine. Binding of the P-site inhibitor was readily observed in the presence of PP\textsubscript{2}; this was not true in the case of cyclic AMP. Binding of 2'-deoxyadenosine required both VC\textsubscript{1} and IIC\textsubscript{2} and was not observed with the individual proteins in the presence or absence of G\textsubscript{\alpha} and/or pyrophosphate (Fig. 2A and data not shown).

Analysis of 2'-[\textsuperscript{3}H]Deoxyadenosine binding to adenylyl cyclase in the presence of activated G\textsubscript{\alpha}, Mg\textsuperscript{2+}, and 2.5 mM PP\textsubscript{2} revealed a single binding site per C\textsubscript{1}/C\textsubscript{2} heterodimer with a K\textsubscript{d} of 40 ± 3 mM (Fig. 3). Both cyclic AMP and Ap(CH\textsubscript{2})pp inhibited the binding of 2'-deoxyadenosine, implying that these molecules compete with P-site inhibitors for a single binding site (Fig. 2B). Inhibition by the substrate analog, Ap(CH\textsubscript{2})pp, renders highly unlikely the possibility of 2'-deoxyadenosine binding to a site that is distinct from the catalytic site but that still requires PP\textsubscript{2} to be manifest. This is the first direct evidence in support of Florio’s hypothesis (25) of dead-end inhibition of adenylyl cyclase by P-site agents.

Product Inhibition—Binding of a P-site inhibitor that is observable only in the presence of a reaction product suggests that release of product from the enzyme is at least partially rate-limiting. Patterns of inhibition of enzymatic activity by product are useful in determining if release of product is an ordered or random event. Adenylyl cyclase activity is inhibited by both reaction products, and previous studies with the enzyme from Brenthiobacterium liquefaciens demonstrated their random release (27). Our enzyme system also displays random
release of products, since the kinetics of inhibition of enzymatic activity by both cyclic AMP and PP is mixed; this is the pattern predicted by steady-state models. The intersection points for double-reciprocal plots (1/activity versus 1/substrate concentration) at increasing concentrations of either product, PPi (Fig. 4A) or cyclic AMP (Fig. 4B), are above the abscissa and to the left of the ordinate. A purely competitive pattern of inhibition is expected for both products only if rapid equilibrium kinetics applies (28). The slopes of Fig. 4B are not a linear function of cyclic AMP concentration and tend to curve upwards at high concentrations of the cyclic nucleotide. This is also indicative of a steady-state system where the assumption of rapid equilibrium does not apply (28). Finally, both cyclic AMP and PPi can compete with Ap(CH2)pp for binding to adenylyl cyclase (equilibrium dialysis data not shown); this again indicates that release of product is random.

The equilibrium binding studies and patterns of product inhibition described to this point permit formulation of a model for inhibition of Gsα-stimulated adenylyl cyclase activity by P-site inhibitors (Fig. 5). We observe binding of 2'-deoxyadenosine only in the presence of PPi, and therefore the inhibitor is hypothesized to bind to only a single intermediate along the reaction coordinate. This is consistent with the uncompeti-
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**Fig. 5. Model for the mechanism of P-site inhibition.** Odd- and even-numbered rate constants describe the forward and reverse reactions, respectively. Estimates of the values of these rate constants are provided in Table II. The species I represents the P-site inhibitor, 2’-deoxyadenosine. $K_i$ represents the dissociation constant for binding of 2’-deoxyadenosine to the E-PP_i complex.

The reverse reaction is readily reversible (26, 29, 30). The equilibrium constant, measured with the enzyme from B. liquefaciens (26), is 0.065 $M$ (pH 7.3, 25 °C) and thus actually favors ATP synthesis under standard conditions of $1 M$ concentrations of reactants and products. The synthesis of ATP from cyclic AMP and PP_i is activated by both $G_s$ and forskolin, as anticipated (Table I). Secondary plots of the apparent $1/V_{max}$ versus 1/[cyclic AMP] (Fig. 7A) or 1/[PP_i] (Fig. 7B) provide the $V_{max}$ of the system at infinite substrate concentrations (3.9 ± 0.3 $\mu$mol/min·mg [1.8 ± 0.1 s$^{-1}$]) and the apparent dissociation constants for binding of the second substrate ($K_{PP_i} = 12 ± 3 M$, $K_{PP_i} = 0.7 ± 0.3 M$). If steady-state kinetics apply, $K_{AMP}$ and $K_{PP_i}$ cannot be assumed to represent binding constants but instead represent the $K_m$ for cyclic AMP and PP_i, respectively (28).

**Fig. 6. Inhibition of adenyl cyclase activity by 2’-deoxyadenosine and the products, cAMP and PP_i.** Assays (4 nM VC1; 1 $\mu$M IIC2) were performed in the presence of 10 mM free MgCl$_2$, 400 nM activated $G_{con}$, and 1 mM MgATP. A, reactions containing 0–1.6 mM MgPP_i, were assayed in the presence of no inhibitor (●) or 30 $\mu$M (■), 75 $\mu$M (▲), or 150 $\mu$M (▼) 2’-deoxyadenosine. B, reactions containing 0–8 mM MgAMP were assayed in the presence of no inhibitor (●) or 50 $\mu$M (■), 150 $\mu$M (▲), or 320 $\mu$M (▼) 2’-deoxyadenosine.
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TABLE I

| Activity (μmol/min-mg) | 1 μM Gs | 50 μM forskolin | 1 μM Gs + 50 μM forskolin |
|-----------------------|--------|----------------|--------------------------|
| No activator          | 0.09 ± 0.001 | 1.46 ± 0.003 | 1.46 ± 0.07 |
| 1 μM forskolin        | 3.43 ± 0.05 |

We have fit data presented in Figs. 1, 4, 6, 7, and 8 to the steady-state rate equation shown in the "Appendix" to obtain estimates of $K_i$ and the rate constants $k_i$ through $k_{12}$. These constants were used to plot simulations of the data, which are shown in Fig. 10, and these lines were used to calculate the values shown in the columns labeled "Simulated fit." The rate constants used to simulate experimental data are: $k_1 = 2.62 \times 10^6$ M s$^{-1}$; $k_{22} = 89.5 s^{-1}$; $k_{59} = 1 s^{-1}$; $k_{26} = 11.1 s^{-1}$; $k_{58} = 2.78 \times 10^4$ M s$^{-1}$; $k_{16} = 0.39 s^{-1}$; $k_{99} = 142$ s$^{-1}$/M; $k_{11} = 56 s^{-1}$; $k_{20} = 3.54 \times 10^4$ M/s; $K_i = 150$ μM. Experimental values for the forward and reverse reactions are reported as the average and the S.E. of the mean for two to four experiments. 2’-dAdo, 2’-deoxyadenosine.

TABLE II

| Kinetic parameters: experimental data versus simulated fits to the steady-state rate equation |
|-------------------------------------|
| Forward reaction | Experimental data | Simulated fit |
| $K_{cat}$ (Figs. 1 and 4) | 0.34 ± 90 mM | 0.26 mM |
| $V_{max}$ (Figs. 1 and 4) | 30 ± 3 s$^{-1}$ | 27 s$^{-1}$ |
| $K_{MPP}$ (Fig. 1) | 240 ± 60 μM | 256 μM |
| $K_{MPP}$ (Fig. 4A) | 0.31 ± 0.02 mM | 0.16 mM |
| $K_{AMP}$ (Fig. 4B) | 2.3 ± 0.4 mM | 2.7 mM |

| Reverse reaction | Experimental data | Simulated fit |
|------------------|------------------|---------------|
| $K_{cat}$ (Fig. 8A) | 0.39 ± 0.09 mM | 0.37 mM |
| $V_{max}$ (Fig. 8A) | 1.0 ± 0.1 s$^{-1}$ | 0.90 s$^{-1}$ |
| $K_{MPP}$ (Fig. 8B) | 8.7 ± 0.4 mM | 8.8 mM |
| $V_{max}$ (Fig. 8B) | 1.04 ± 0.05 s$^{-1}$ | 1.33 s$^{-1}$ |
| $K_{MPP}$ (Fig. 8B) | 280 ± 30 μM | 284 μM |
| $K_{AMP}$ (Fig. 7) | 0.12 ± 0.04 mM | 0.16 mM |
| $K_{AMP}$ (Fig. 7) | 2.3 ± 0.8 mM | 2.7 mM |

$*$ Values of $K_i$ and $V_{max}$ for the reverse reaction are the apparent values obtained with the specified concentration of the second substrate and do not represent the $K_{i}$ or maximal $V_{max}$ of the system with infinite substrate concentrations. The calculated $V_{max}$ of the reverse reaction is 1.8 ± 0.1 s$^{-1}$ versus 2.1 s$^{-1}$ for the simulated fit.

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Adenylyl cyclase contains a single ATP binding site that may lie at the interface between the C1 and C2 domains of the protein (19, 31). Potent P-site inhibitors have no effect on the binding of the substrate analog 2’-deoxyadenosine (Table III). However, the patterns of inhibition by the P-site analog remain unchanged (not shown). The $K_i$ for binding of 2’-deoxyadenosine to adenylyl cyclase in the presence of PPi is also lowered compared with the $K_{i}$ alone (21 μM, Fig. 9). Increased activation of adenylyl cyclase by the combination of forskolin and Gs thus further stabilizes the enzyme-2’-deoxyadenosine-PPi complex.

DISCUSSION

Adenylyl cyclase is a single ATP binding site that may lie at the interface between the C1 and C2 domains of the protein (19, 31). Potent P-site inhibitors have no effect on the binding of the substrate analog 2’-deoxyadenosine to this site (19). This result is reflected in the uncompetitive nature of P-site inhibition with respect to MgATP. A classical uncompetitive inhibitor does not bind to the free enzyme; rather, the inhibitor binds to the enzyme only after substrate is bound.

Several mechanisms for P-site inhibition have been suggested. The first involves formation of a dead-end complex, initially proposed by Wolin (27) to describe inhibition of adenyl cyclase from B. liquefaciens by adenosine. It was suggested that competitive inhibition by adenosine reflected the formation of an enzyme-ATP-adenosine complex at the catalytic site.
that was not in rapid equilibrium with active forms of the enzyme. Florio (25) and Florio and Ross (7) suggested a related mechanism for mammalian adenylyl cyclase, a dead-end complex containing enzyme, PPi, and P-site inhibitor. An alternative mechanism was later proposed by Johnson and Shoshani (22), in which P-site inhibitor and substrate bind simultaneously and display modest synergy as inhibitors. This is certainly consistent with the notion that both molecules bind to the same site.

To discern the reaction intermediate(s) involved in P-site interactions, we have used equilibrium dialysis to detect requirements for binding of 2'-deoxyadenosine. Interactions were not detected with enzyme alone, nor in the presence of Ap(CH2)pp or cyclic AMP. By contrast, binding was readily detected in the presence of the product PPi; one molecule of 2'-deoxyadenosine bound per C1/C2 heterodimer with a Kd of 40 ± 3 μM. Cyclic AMP or Ap(CH2)pp reduced or abolished binding. These observations provide clear evidence to support the hypothesis that P-site analogs act as dead-end inhibitors of product release.

Analysis by us and others of both mammalian and bacterial adenylyl cyclases indicates that the release of product is random (22, 27). This fact and the binding data permit formulation of the reaction scheme shown in Fig. 5. Our remaining kinetic data support this mechanism and provide additional information about product release. Dixon plots describing the inhibition of the forward reaction by both 2'-deoxyadenosine and products show parallel or intersecting lines for cyclic AMP or PPi, respectively. Parallel lines indicate that cyclic AMP and P-site inhibitors act in a mutually exclusive fashion, each inhibitor reducing the effectiveness of the other. This is certainly consistent with the notion that both molecules bind to the same site.

The Dixon plot that describes the interaction between 2'-deoxyadenosine and PPi, (lines intersecting above the abscissa; Fig. 6A) indicates that both molecules are bound to the enzyme simultaneously and display modest synergy as inhibitors. This result was demonstrated previously by Florio with forskolin-activated adenylyl cyclase activity in cyc− S49 cell membranes.
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Table III
Kinetic parameters for Gs- and forskolin-stimulated adenylyl cyclase

| Reaction | $K_{m,ATP}$ | $V_{max}$ | $K_a(app)$ | $K_{m,cAMP}$ | $K_{m,PPi}$ | $V_{max}$ | $K_a(app)$ |
|----------|-------------|-----------|------------|--------------|-------------|-----------|------------|
| Forward  | $0.25 \pm 0.02$ | $100 \pm 30$ | $60 \pm 16$ | $16 \pm 3$ | $0.34 \pm 0.05$ | $3.3 \pm 0.02^a$ | $39 \pm 9^b$ |
| Reverse  | $1.8 \pm 0.2^a$ | $73 \pm 9^b$ |

$^a V_{max}$ and the apparent $K_a$ for 2'-deoxyadenosine measured with respect to cyclic AMP (see Fig. 7B).

$^b V_{max}$ and the apparent $K_a$ for 2'-deoxyadenosine measured with respect to MgPPi (see Fig. 7A).

Fig. 9. Binding of 2'-deoxyadenosine to Gs- and forskolin-activated adenylyl cyclase.

Equilibrium dialysis measurement of 2'-deoxyadenosine binding to 18 μM VC1 and IIC2 in the presence of Gs (25 μM) and G-3'-[piperidino(propionyl)]forskolin (100 μM). Reactions contained 5–100 μM 2'-deoxyadenosine and no other addition (●) or 2.5 mM PPI (○) and were incubated for 24 h at 4 °C as described under "Experimental Procedures." $R$ is defined in the legend of Fig. 3.

The model shown in Fig. 5 is consistent not only with the kinetic data shown above but also with classical features of P-site inhibition. These inhibitors display a strict requirement for an adenine ring (2, 5, 12), consistent with binding at the catalytic site. Crystallographic data and evidence from mutagenesis suggest that the active site lies at the interface of the C1 and C2 domains (19, 31). A mutation that dramatically lowers the IC50 for P-site inhibition (K923A in type I adenylyl cyclase) is also located at this interface (31, 32). P-site inhibitors containing a 3'-ribose phosphate (e.g. 2'-deoxy-3'-AMP) have a greatly increased potency (12), and this suggests that the enzyme-PPi-inhibitor complex might resemble a structure close to a product-like transition state, accommodating both PPs and a nucleotide containing a 3'-phosphoryl substituent. Furthermore, the catalytic site must be capable of accommodating several phosphates and large substitutions at the 3'-ribose position, since 3'-polyphosphates are more potent than other P-site inhibitors, and other modifications at this position are easily tolerated (12, 13). Isoform-dependent sensitivity of adenylyl cyclases to various adenosine analogs may reflect differences in the capacities of their active sites to accommodate the 3'-moieties of these analogs (33).

Activated forms of adenylyl cyclase are more sensitive to P-site inhibition than are nonstimulated forms of the enzyme (3–5, 7, 11). This result is explained by a dead-end inhibitor model. To observe P-site inhibition of the forward reaction, release of PPi from the enzyme must be at least partially rate-limiting, otherwise the enzyme-PPi complex would never be present at sufficient concentrations to bind inhibitor. Note that $k_3$ and $k_{11}$ in our model are approximately equal (Table II). Any alteration of the enzyme that slows reaction steps prior to the release of product, such that product release is no longer rate-limiting, should decrease the potency of P-site inhibitors. This is the equivalent of saying that a higher concentration of a P-site inhibitor will be required to trap the E-PPi complex if it is present in lower steady-state concentrations. For example, if, under basal conditions, the rate of synthesis of cyclic AMP and PPs is slow relative to the rate of release of these products, enzyme-PPi will not accumulate and P-site inhibitors will be impotent. Activation of adenylyl cyclase by forskolin or Gs may increase the rate of synthesis of cyclic AMP and PPi ($k_{3}$), leading to higher steady-state concentrations of enzyme-PPi, and thus increased potency of P-site inhibitors.

Alternatively, if the enzyme-PPi-P-site inhibitor complex mimics a transition state and activators stabilize the transition state of the enzyme, they will increase the affinity of adenylyl cyclase for P-site inhibitors. Binding and kinetic data for Gs- and forskolin-activated adenylyl cyclase in the presence of saturating concentrations of PPi (33) suggest that both mechanisms serve to increase the potency of P-site inhibitors. Thus, addition of forskolin to Gs-activated adenylyl cyclase causes a 3-4-fold increase in $V_{max}$ of the forward and reverse reactions. The apparent $K_a$ for 2'-deoxyadenosine is decreased (4-fold) as expected. In addition, the $K_a$ for binding of 2'-deoxyadenosine is also decreased (2 fold) in the presence of both activators (Fig. 9).
suggesting that activators stabilize the enzyme-PP$_i$-P-site inhibitor complex. If this complex represents a product-like transition state, the binding data imply that G$_{st}$ and forskolin increase the catalytic activity of adenylyl cyclase by stabilizing the transition state of the enzyme.

A related feature of P-site inhibition is the decreased potency of these compounds when analogs of ATP are used as substrates (22). These analogs have either a decreased affinity for the enzyme or they impair the rate of cyclization. Thus, the release of product is no longer rate-limiting compared with the other steps of the reaction, and the potency of P-site inhibitors decreases. The large number of P-site phenotypes obtained as a result of mutagenesis (32) can also be explained by any decrease in $k_{cat}$ (or increase in $K_d$ for ATP) such that the rate-limiting step occurs prior to release of product.

The inhibition of the forward reaction by P-site inhibitors is noncompetitive or mixed noncompetitive with respect to MnATP (3, 4, 9–11, 15, 24). Mn$^{2+}$ may induce conformational changes of the enzyme that are normally associated with PP$_i$ binding and permit inhibitor binding to the free enzyme. P-site analogs containing covalent attachment groups can inhibit the enzyme irreversibly in the presence of Mn$^{2+}$, both in the presence and absence of ATP (34), consistent with the possibility of binding of these compounds to both E-PP$_i$ and the free enzyme under these conditions. However several lines of evidence may suggest otherwise. Potent P-site inhibitors are not capable of competing with a substrate analog for binding to the free enzyme in the presence of G$_{st}$ and Mn$^{2+}$, even at concentrations 80 times higher than the $K_d$ for inhibition (19). Additionally, we have not observed inhibitor binding to G$_{st}$-activated adenylyl cyclase in the presence of Mn$^{2+}$ (and absence of PP$_i$), with the caveat that 2'-deoxyadenosine is a weak inhibitor, and we may not be able to detect binding if the affinity is low. Mn$^{2+}$ also causes other changes in the enzyme, including reduction of the $K_d$ for a substrate analog, Ap(CH$_2$)$_2$pp, by 20-fold (compared with Mg$^{2+}$) (19). Binding studies with more potent radiolabeled inhibitors (or structural studies) would be useful in determining the relationship between Mn$^{2+}$ and P-site inhibition.

**APPENDIX**

The steady-state rate equation is given by

$$\frac{\delta P}{\delta t} = k_2[EQ] + k_1*[EP] - k_{1*}*[cAMP]+[E0]*[ES]*[EQP]*[EQ]*[EP]*[EPI]$$

$$- k_{1*}[PP_i]+[E0]*[ES]*[EQP]*[EQ]*[EP]*[EPI] \quad (\text{Eq. 1})$$

and is the simultaneous solution of the following algebraic rate equations where the concentration of total enzyme and the intermediate species $E$-ATP, $E$-cAMP-PP$_i$, $E$-cAMP, $E$-PP$_i$, and $E$-PP$_i$-I are represented by $[E0]$, $[ES]$, $[EQ]$, $[EPI]$, and $[PI]$, respectively, and are defined as follows.

$$[ES] = \frac{k_{1*}[cAMP]+[E0]*[EQP]*[EQ]*[EP]*[EPI]}{k_1*[ATP] + k_2 + k_3} \quad (\text{Eq. 2})$$

$$[EQP] = (k_1+k_2*[ATP]+[E0]*[EQ]*[EPI])*B + (k_{1*}[cAMP]*[EP]+(k_{1*}[PP_i]*[EPI])D \quad (\text{Eq. 3})$$

$$[E] = (k_3+k_1+k_2*[ATP]+[E0]*[EP]*[EPI]/(B+D+G) + (k_{1*}[cAMP]*[EP]+(D+G))$$

$$+ (k_{1*}[cAMP]+[E0]*[EPI]/(B+D+G))$$

$$- (k_{1*}[cAMP]+k_{1*}[ATP]+[E0]*[EPI]/(B+D+G))$$

$$- (k_{1*}[cAMP]+k_{1*}[ATP]+[E0]*[EPI]/(B+D+G))$$

$$+ (k_{1*}[cAMP]+[E0]*[EPI]/(B+D+G))$$

$$+ (k_{1*}[cAMP]+k_{1*}[ATP]+[E0]*[EPI]/(B+D+G))$$

$$+ (k_{1*}[cAMP]+k_{1*}[ATP]+[E0]*[EPI]/(B+D+G))$$

$$+ (k_{1*}[cAMP]+k_{1*}[ATP]+[E0]*[EPI]/(B+D+G))$$

(Eq. 4)
The complex functions designated B, D, G, X, Y, and Z are defined as:

\[
B = k_1*[ATP] + k_2 + k_3
\]  \hspace{1cm} (Eq. 7)

\[
D = (k_1+k_2*[ATP]) - k_3 + k_4 + h_4 + k_5 + k_7
\]  \hspace{1cm} (Eq. 8)

\[
G = (k_5+k_7*[ATP]/[D-B]) - (h_4 + k_5*[PPi]/[D])
\]  \hspace{1cm} (Eq. 9)

\[
X = ((k_7+k_3*[ATP]/[B-D]) - (k_1+k_3*[ATP]/[B-D]))\]

\[
+ (k_2*[PPi]*k_3*[ATP]/[D]-h_4)
\]  \hspace{1cm} (Eq. 10)

\[
Y = (k_1+k_3*[ATP]/[B-D]) + (k_4*[PPi]-h_4)
\]  \hspace{1cm} (Eq. 11)

\[
Z = Y + ((-h_4+k_3*[cAMP]/[D]) + (k_5*[cAMP]/[D] + (k_7*[PPi]*k_3*[cAMP]/[D] + h_4)
\]  \hspace{1cm} (Eq. 12)

Simulations of this rate equation are shown in Fig. 10 with the rate constants given in Table II.

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