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# Cation and Structural Requirements for P Site-Mediated Inhibition of Adenylate Cyclase

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## SUMMARY

The cation and structural requirements of the intracellular inhibitory "P" site of adenylate cyclase were investigated in human platelet membranes, bovine sperm particles, and detergent-solubilized and purified preparations from rat and bovine brain. Sensitivity of adenylate cyclase to P site-mediated inhibition was enhanced by reversible and irreversible activators of the enzyme. The most effective sensitization of the platelet and brain adenylate cyclases was observed with $Mn^{2+}$ and upon proteolysis with ninhibin in the presence of guanosine 5′-*O*-(3-thiotriphosphate). These resulted in $IC_{50}$ values for (2′,5′dideoxy-adenosine (2′,5′-dd-Ado) and 2′-deoxy-3′-AMP of ~1–2 μM. The data were consistent with the ideas that P site-mediated inhibition of adenylate cyclase is dependent on divalent cation and is a function of enzyme activity. A number of nucleosides and nucleotides were synthesized and used to define structural requirements for P site-mediated inhibition of a detergent-solubilized adenylate cyclase from rat brain. The data suggest a strict requirement for an intact adenine moiety and a β-glycosidic linkage for the ribosyl moiety. 2′-Deoxy-and especially 2′,5′-dideoxy-ribosyl moieties enhanced sensitivity and a strong preference for phosphate at the 3′-position was exhibited. Substitutions at the 5′-ribose position impaired sensitivity. The order of potency and $IC_{50}$ values of the more potent adenosine analogs were 2′,5′-dideoxy-3′-AMP (≃0.1 μM) > 2′-deoxy-3′-AMP (≃1 μM) > 2′,5′-dd-Ado (≃3 μM) > 3′-AMP (≃9 μM) > 2′-deoxy-adenosine (≃15 μM) > adenosine (≃80 μM). Large substitutions at the 3′-ribose position were tolerated, e.g., dApdN di- and $dAp(dN)_4$ penta-nucleotides and succinyl- and *p*-fluoro-sulfonyl-benzoyl- moieties. The purified adenylate cyclase from bovine brain was inhibited by P site agonists with $IC_{50}$ values of 34 and 45 μM for 2′-deoxy-3′-AMP and 2′,5′-dd-Ado, respectively. The data imply, first, that the locus of the P site is the catalytic subunit of adenylate cyclase and, second, that the increased sensitivity observed with $Mn^{2+}$ is due to an effect of the cation on the catalytic subunit. In contrast with adenylate cyclases from other mammalian tissues, the enzyme from bovine sperm exhibited only weak sensitivity to P site agonists; 2′-deoxy-3′-AMP ≃ 2′,5′-dd-Ado > adenosine, each with $IC_{50}$ values > 1000 μM. This lack of effect of P site agonists adds further support to the idea that the catalytic subunit of the sperm enzyme and its regulation are distinct from those of somatic cell adenylate cyclases.

Adenosine is known to affect adenylate cyclase activity through three distinct sites. In addition to the two cell-surface receptors for adenosine, mediating stimulation ($R_a$ or $A_2$) or inhibition ($R_i$ or $A_1$) of adenylate cyclase, an internal site mediating inhibition of the enzyme has been described (e.g., Refs. 1–6). Early studies focused on the effect of adenosine per se, but due to the weak sensitivity to this nucleoside it is uncertain whether adenosine could serve as a naturally occurring agonist at this inhibitory site. This inhibitory site has been referred to as the P site from its apparent requirement for an intact purine moiety, and a number of more potent compounds have been described (e.g., Refs. 3 and 6). Although the physiological importance of inhibition of adenylate cyclase via the P site is unclear, this inhibition is characteristically noncompetitive with respect to metal-ATP, is metal dependent, and is accompanied by a 20-fold increase in the affinity of the enzyme for free metal (e.g., Refs. 1–20). An important aspect of P site-mediated inhibition is that stimulated forms of adenylate cyclase are substantially more sensitive to inhibition than are unstimulated forms. This phenomenon was originally reported by Haslam and Lynham (21) and by McKenzie and Bär (13) and was subsequently observed by us and others (2–6). Available evidence from the work of many investigators suggests that the form of adenylate cyclase most sensitive to inhibition by P site agonists is GTP·$\alpha_s$·C,[2] although this is affected by

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[2] $G_s$ and $G_i$ (equivalent to $N_s$ and $N_i$) are the guanine nucleotide-dependent regulatory subunits of anenylate cyclase that mediate, respectively, stimulation and inhibition of the enzyme by stimulatory and inhibitory hormone receptors. These are heterotrimeric proteins consisting of $\alpha_s\beta\gamma$ or $\alpha_i\beta\gamma$ subunits, respectively. C refers to the catalytic subunit of adenylate cyclase.

**ABBREVIATIONS:** EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Gpp(NH)p;, guanosine 5′(β,γ-imino)triphosphate; 2′,5′-dd-Ado, 2′,5′-dideoxy-adenosine; 2′,5′-dd-3′-FSBA, 2′,5′-dideoxy-3′*p*-fluoro-sulfonylbenzoyladenosine; 2′-d-3′-AMP, 2′-deoxy-3′-adenosine monophosphate

divalent cations and other membrane components may also be involved (e.g., Refs. 10, 13, 14, and 22–24).

This report describes P site-mediated inhibition of adenylate cyclases in platelet membranes and in particulate, detergent-solubilized, and purified preparations from brain. The effectiveness of a variety of reversible and irreversible stimuli are compared in their ability to sensitize the enzymes to inhibition by P site agonists. Evidence is presented that the locus of the P site is on the catalytic unit of adenylate cyclase. We describe the effects of additional, newly synthesized, site-specific, and more potent analogs of adenosine and their use in defining the agonist structural requirements of inhibition.

## Materials and Methods

**Preparation of adenylate cyclases.** Membranes from human platelets were prepared as previously described (4) with 5 mM EDTA present throughout the membrane preparation procedure. The preparation of particulate and detergent-dispersed adenylate cyclase from rat or bovine brain was as previously described (25). The catalytic unit of adenylate cyclase was purified to near homogeneity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, from bovine brain cortex on forskolin- and wheat germ lectin-agarose columns essentially as described by Smigel (26). Washed particles containing adenylate cyclase were prepared from bovine sperm, as previously described (27), in the presence of 1 mM benzamidine and pepstatin, chymostatin, leupeptin, and soybean trypsin inhibitor, each at 10 μg/ml.

**Preparation of adenylate cyclase-activating protease (ninhibin)[3] from bovine sperm.** An adenylate cyclase-activating, acrosin-related protease was prepared from bovine sperm as previously described (28). Sperm were obtained either as fresh ejaculate or were washed from epididymides. Essentially, sperm were lysed, homogenized, and centrifuged to obtain a washed particulate fraction, which was then extracted with a buffer containing 10 mM triethanolamine·HCl, 2 mM EDTA, and 500 mM $NH_4HCO_3$. The supernatant fraction resulting from this extraction was concentrated approximately 10-fold by ultrafiltration on Amicon PM-10 membranes. The concentrated extract was then purified by routine biochemical procedures to near homogeneity, exhibiting a single band migrating at 40 kDa on sodium dodecyl sulfate-polyacrylamide slab gels. The crude extract and the purified enzyme were used alternatively for the activation of adenylate cyclase.

**Determination of adenylate cyclase activity.** Adenylate cyclase activities were determined essentially as previously described (4, 27). For adenylate cyclase in platelet membranes, the reaction mixture typically contained 50 mM triethanolamine·HCl, pH 7.5, 1 mM 3-isobutyl-1-methylxanthine, 1 mM dithiothreitol, 0.1 mM cAMP, 0.1 mM EGTA, bovine serum albumin (1 mg/ml), 2 mM purified creatine phosphate, creatine kinase (100 μg/ml), myokinase (100 μg/ml), adenosine deaminase (5 units/ml), 100 μM ATP, 2 mM $MgCl_2$, and [$\alpha$-$^{32}$P]ATP (2 to 5 × $10^6$ cpm), without or with 10 μM GTPγS, in a volume of 100 μl. For the determination of adenylate cyclase in detergent-dispersed preparations from rat brain, the assay was similar to that described above except that cAMP, adenosine deaminase, and myokinase were omitted because they are not necessary in these preparations (29). For proteolytic activation, the adenylate cyclase preparation was incubated with the sperm protease for 15 min at 30° in a reaction mixture that was complete except for [$\alpha$-$^{32}$P]ATP. At the end of this incubation, [$\alpha$-$^{32}$P]ATP was added, and the formation of [$^{32}$P]cAMP was determined after further incubation at 30° (10 min for platelet particles and 15 min for brain enzyme). Reactions of adenylate cyclase from either source were terminated by precipitation with zinc acetate that contained 0.5 mM unlabeled cAMP and sodium carbonate, by the method of Jakobs *et al.* (30). [$^{32}$P]cAMP was isolated by sequential chromatography on Dowex-50 and then $Al_2O_3$ columns, essentially as described by Salomon *et al.* (31). The unlabeled cAMP allowed us to monitor recovery of the labeled cAMP by measurement of absorbance at 259 nm with an aliquot of the sample. Radioactivity was determined by Cherenkov radiation in a liquid scintillation counter.

**Materials.** Several analogs of adenosine were synthesized for these studies. 2′,5′-dd-Ado was prepared by the methods described by Beacham (32) and Wang *et al.* (33). All succinylated compounds were prepared essentially as described by Steiner *et al.* (34), except that triethylamine was used instead of morpholine-*N*,*N*′-dicyclohexylcarboxyamidine. The 3′-phosphorylation of 2′,5′-dd-Ado was by minor modification of the anhydrous condensation method described by Symons (35) for the preparation of 5′-nucleoside monophosphates. The preparation of 2′,5′-dd-3′-FSBA was done with the help of Dr. J. V. Staros (Vanderbilt University) by the method of Pranab *et al.* (36). Except for 2′,5′-dd-Ado, each of the synthesized compounds was purified by preparative high performance liquid chromatography procedures and the structures of all synthesized compounds were verified by mass spectrometry and/or NMR.

[$\alpha$-$^{32}$P]ATP was prepared enzymatically, as described by Walseth and Johnson (37), or was purchased from New England Nuclear (Boston, MA) or from ICN Pharmaceuticals (Irvine, CA). Before use, the myokinase and adenosine deaminase were centrifuged and the supernatant ammonium sulfate was removed. The pelleted enzymes were then resuspended in water or the reaction mixture for use. If these enzymes were used without removal of as much ammonium sulfate as possible, the salt carried into the assay was sufficient to reduce substantially the ability of the sperm protease to activate the platelet adenylate cyclase. DEAE-agarose, Dowex-50, neutral alumina, and all electrophoresis reagents were from Bio-Rad (Richmond, CA). ATP, GTPγS, creatine phosphate, and creatine kinase were obtained from Boehringer Mannheim (Indianapolis, IN). Creatine phosphate was purified as previously described (29). Other reagents were from commercial sources and were of the highest quality available.

## Results

**Various stimuli and P site sensitivity of the platelet adenylate cyclase.** In previous studies with Jakobs and coworkers (4), we reported that adenosine inhibited adenylate cyclase of human platelet membranes with an apparent $IC_{50}$ > 1 mM for basal activity but $IC_{50} \simeq 10$–14 μM for the enzyme activated maximally by GTP and prostaglandin $E_1$. This inhibition was associated with an approximately 20-fold increased apparent affinity for free $Mg^{2+}$ (4). These earlier studies detailed the characteristics of inhibition only by adenosine. However, more potent P site agonists are known and sensitivity to inhibition can be induced by a number of agents in addition to hormone and GTP, possibly by different mechanisms. Table 1 compares the potency of adenosine, 2′-d-3′-AMP, and 2′,5′-dd-Ado as P site inhibitors of human platelet adenylate cyclase and compares the effectiveness of several stimuli to sensitize the enzyme to inhibition by 2′,5′-dd-Ado. With enzyme stimulated by 10 mM $Mn^{2+}$ plus 100 μM forskolin, the rank order of potency was adenosine < 2′-d-3′-AMP < 2′,5′-dd-Ado. Sensitivity of platelet adenylate cyclase to inhibition by 2′,5′-dd-Ado was optimal upon reversible activation with $Mn^{2+}$/forskolin or by irreversible proteolytic activation with ninhibin plus 10 μM GTPγS. The $IC_{50}$ values for 2′,5′-dd-Ado for both stimuli were comparable (<1 μM). Sensitivity to inhibition was somewhat less for enzyme stimulated with 10 μM prostaglandin $E_1$

[3] "Ninhibin" is an adenylate cyclase-activating protease isolated from bovine sperm. It belongs to the acrosin family of trypsin-like, serine proteases. It was given the name ninhibin due to its apparent effect on $N_i(G_i^3)$, and the name is used throughout this manuscript solely to distinguish the activity obtained with the extraction procedure and purification scheme we used from the activity obtained with procedures commonly used in the preparation of acrosin.

TABLE 1

**Specificity and sensitivity of P site-mediated inhibition of adenylate cyclase from human platelets**

Platelet adenylate cyclase was assayed as described under Materials and Methods. Concentrations of the indicated additions were 10 mM $MnCl_2$, 10 mM $MgCl_2$, 100 μM forskolin, 10 μM GTPγS, or 1 μM prostaglandin ($PGE_1$). The ATP-regenerating system was 2 mM creatinine phosphate and 100 μg of creatinine kinase/ml, except as indicated. Values are averages from two (± range) or three (± SEM) experiments as indicated in parentheses, each conducted in duplicate.

| Assay Condition | Compound | $IC_{50}$ μM |
|---|---|---|
| $Mn^{2+}$/forskolin | Adenosine | 16.3 ± 0 (2) |
| $Mn^{2+}$/forskolin | 2′-d-3′-AMP | 2.2 ± 0.7 (3) |
| $Mn^{2+}$/forskolin | 2′5′-dd-Ado | 0.84 ± 0.06 (3) |
| $Mn^{2+}$/Forskolin (no ATP-regenerating system) | 2′-d-3′-AMP | 9.1 ± 0.1 (2) |
| $Mg^{2+}$/Forskolin | 2′,5′-dd-Ado | 5.5 ± 2.7 (2) |
| $Mg^{2+}$/GTPγS | 2′,5′-dd-Ado | 1.7 ± 0.4 (2) |
| $Mg^{2+}$/$PGE_1$/GTP | 2′,5′-dd-Ado | 2.23 ± 0.01 (2) |
| $Mg^{2+}$/GTPγS/ninhibin | 2′,5′-dd-Ado | 0.71 ± 0.22 (2) |

plus 10 μM GTP ($IC_{50}$ ≃2.2 μM), or with 10 μM GTPγS ($IC_{50}$ ~1.7 μM), and was least potent with 100 μM forskolin ($IC_{50}$ ~5.5 μM). Two additional observations are worth noting. First, the sensitivity to inhibition was greatest when ATP concentrations were effectively maintained during the reaction (compare $IC_{50}$≃2.2 μM for 2′-d-3′-AMP with creatine phosphate and creatine kinase and $IC_{50}$≃9.1 μM without the regenerating system). And second, the presence or absence of adenosine deaminase (5 units/ml) and myokinase (100 μg/ml), which are often used in adenylate cyclase assays, had no effect on the sensitivity of the platelet adenylate cyclase to inhibition by either 2′-d-3′-AMP or 2′,5′-dd-Ado (data not shown). The differences in effectiveness of the various stimuli to induce sensitivity to P site-mediated inhibition suggest that the enzyme may be sensitized by more than one mechanism. Presumably, sensitivity is dependent upon conformational changes being induced in the catalytic subunit of the enzyme, optimally induced either by $Mn^{2+}$/forskolin acting directly on C or by hormone/GTP or GTPγS, through the formation of the GTP·$\alpha_s$·C complex. If this is correct, it might be expected that proteolytically activated enzyme would exhibit the greatest sensitivity to inhibition by 2′,5′-dd-Ado. Proteolytic activation of adenylate cyclase in the presence of GTPγS is thought to be due to clipping of $G_{i\alpha}$, thereby allowing the catalytic subunit to become fully activated by $G_{s\alpha}$ (38, 39), i.e., fully in the form GTPγS·$\alpha_s$·C. Activation by $Mn^{2+}$/forskolin may cause a conformational change in the catalytic subunit, comparable to that induced by the formation of the GTP·$\alpha_s$·C complex.

Although adenosine and 2′,5′-dd-Ado are established P site agonists, 2′,5′-dd-Ado is not naturally occurring and sensitivity to adenosine is inadequate for it to be considered seriously as a potentially physiologically important P site agonist. Because 2′-d-3′-AMP is naturally occurring and has been shown to inhibit several adenylate cyclases, the sensitivity of the platelet enzyme to 2′-d-3′-AMP was determined (Table 1). The $IC_{50}$ value for adenosine (≃16 μM) was comparable to that previously reported for enzyme that had been stimulated with maximal concentrations of prostaglandin $E_1$/GTP (~10–14 μM) (4). Although the $IC_{50}$ we observed for 2′-d-3′-AMP was lower than that reported for the adenylate cyclases of the fat body of the silkworm ($IC_{50}$~13 μM with 2 mM $Mn^{2+}$; $IC_{50}$~4 μM with 2 mM $Mn^{2+}$ plus 5 mM NaF) (9) and of bovine thyroid membranes ($IC_{50}$~70 μM with 0.2 mM $Mn^{2+}$ plus 30 μM Gpp (NH)p) (12), sensitivity to inhibition by 2′-d-3′-AMP was in a range consistent with a potential physiological role for the P site to modulate hormonally stimulated adenylate cyclase.

**Various stimuli and P site sensitivity of solubilized adenylate cyclase from brain.** Although platelet adenylate cyclase was highly sensitive to inhibition by specific analogs of adenosine, making it a useful test system for probing P site-mediated inhibition of the cyclase, its susceptibility to inactivation upon detergent solubilization (40) and the difficulty in obtaining large amounts of enzyme make it less than ideal for developing biochemical probes specific for this inhibitory site. Use of the adenylate cyclase of brain averts some of these problems.

The detergent-dispersed enzyme from brain exhibits a high specific activity that can be further substantially increased by a variety of stimuli, e.g., $Ca^{2+}$/calmodulin, $Mn^{2+}$, stable guanine nucleotides, and proteolysis. The high activity makes the enzyme generally useful for evaluating enzyme inhibition, but stimulated forms are particularly useful as a test system for inhibition by P site agonists. The detergent-dispersed enzyme from rat brain exhibited the expected activation by 10 μM GTPγS (4-fold) and the expected inhibition by 100 μM EGTA (approximately 80%) (Fig. 1). The inhibition by EGTA is presumably due to the chelation of $Ca^{2+}$ and the consequent functional removal of calmodulin (39, 41).[4] However, in the presence of GTPγS and EGTA, this solubilized form of the enzyme exhibited a striking sensitivity to proteolytic activation (Fig. 1). The sperm extract increased activity 45-fold in the experiment shown here and up to 100-fold in others. [For comparison, proteolytic activation of the platelet adenylate cyclase was typically 10-fold or less in the presence of GTPγS and EGTA (38, 39).] The magnitude of the effect is likely due to a combination of effects, one being the effect of EGTA to suppress basal activity without significantly altering the maximal activity obtained upon proteolytic activation, resulting in a greater degree of stimulation. In addition, $G_s$ would be activated by GTPγS and, thereby, presumably cause the formation of the stable GTPγS·$\alpha_s$·C complex, a form that should be optimally sensitive to P site-mediated inhibition.

Proteolytic activation of the solubilized adenylate cyclase was found to be irreversible and the activated enzyme was stable and exhibited maximum sensitivity to inhibition by P site agonists (Fig. 2). Treatment with ninhibin increased sensitivity of the brain enzyme to inhibition by 2′,5′-dd-Ado ~50-

[4] This effect of EGTA was not mimicked by 30 μM trifluoroperazine, which also is known to block calmodulin-mediated activation of adenylate cyclase. Trifluoroperazine did not lower basal activity but blocked the stimulation by GTPγS and did not affect the maximal activity obtained with the sperm protease plus GTPγS (not shown).

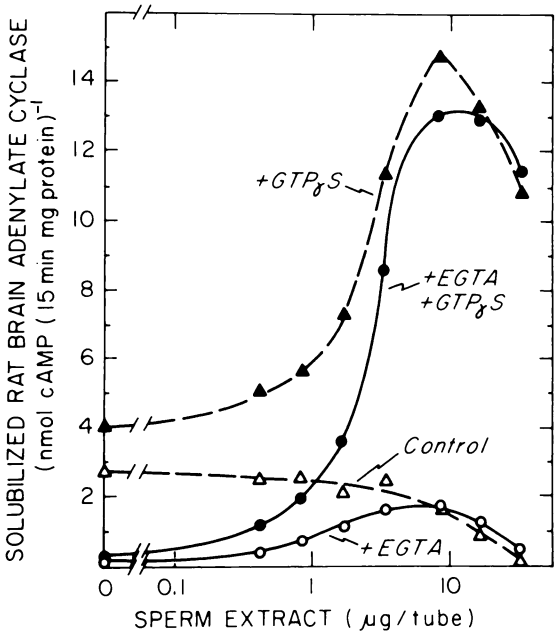


**Fig. 1.** Stimulation of a detergent-dispersed adenylate cyclase from rat brain by a protease extracted from bovine sperm. A sperm extract containing ninhibin activity was prepared as under Materials and Methods and varying amounts were incubated with a Lubrol-PX-dispersed adenylate cyclase from rat brain for 15 min at 30°, in the presence of a reaction mixture that was complete except for [$\alpha$-$^{32}$P]ATP. [$\alpha$-$^{32}$P]ATP was then added and adenylate cyclase activity was determined in a second incubation for 15 min at 30°, in the presence of 100 $\mu$M ATP and 2.1 mM $MgCl_2$. Additions were GTP$\gamma$S, 10 $\mu$M and EGTA, 100 $\mu$M.

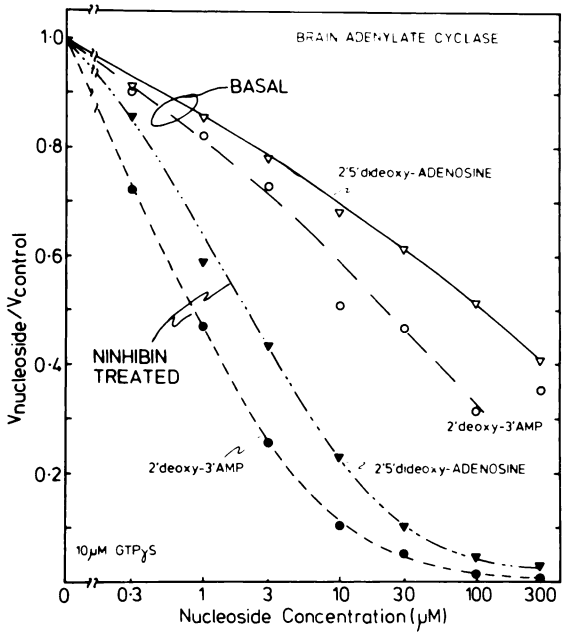


**Fig. 2.** Effect of proteolytic treatment to enhance sensitivity of the brain adenylate cyclase to inhibition by 2′,5′-dd-Ado and by 2′-d-3′-AMP. Detergent-dispersed adenylate cyclase from rat brain was preincubated for 15 min at 30°, with or without ninhibin, with the indicated nucleoside concentrations and with a reaction mixture that was complete except for [$\alpha$-$^{32}$P]ATP. [$\alpha$-$^{32}$P]ATP was then added and adenylate cyclase activity was determined in a second incubation for 15 min at 30°. Ninhibin was prepared as described under Materials and Methods. EGTA (100 $\mu$M), GTP$\gamma$S (10 $\mu$M), and $MgCl_2$ (2.1 mM) were present in all tubes. Basal activity, 0.39 nmol of cAMP(15 min·mg protein)$^{-1}$; $IC_{50}$, 2′,5′dd-Ado, 100 $\mu$M; 2′-d-3′-AMP, 23 $\mu$M. Activity after proteolytic treatment, 33.4 nmol of cAMP(15 min·mg protein)$^{-1}$; $IC_{50}$, 2′,5′-dd-Ado, 2 $\mu$M; 2′-d-3′-AMP, 0.8 $\mu$M.

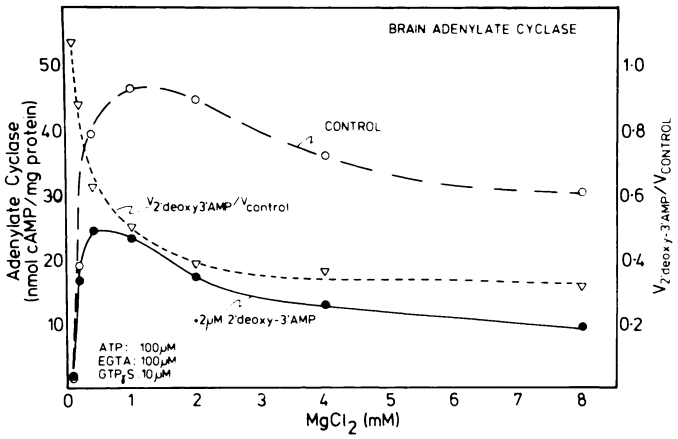


**Fig. 3.** Magnesium dependence for inhibition of adenylate cyclase by 2′-d-3′-AMP. Solubilized adenylate cyclase from rat brain was preincubated for 15 min at 30° with ninhibin and a reaction mixture that was complete except for [$\alpha$-$^{32}$P]ATP and with 2 $\mu$M 2′-d-3′-AMP and varying concentrations of $MgCl_2$ (in excess of ATP and EGTA concentrations) as indicated. [$\alpha$-$^{32}$P]ATP was then added and adenylate cyclase activity was determined in a second incubation for 15 min at 30°. EGTA (100 $\mu$M), 10 $\mu$M GTP$\gamma$S, and 100 $\mu$M ATP were present in all tubes.

fold and by 2′-d-3′-AMP ~30-fold (Fig. 2). Moreover, sensitivity to inhibition by both compounds was generally comparable to that of the platelet enzyme, although in comparison with the platelet adenylate cyclase 2′-d-3′-AMP ($IC_{50}$ ≃1 $\mu$M) was a slightly more potent inhibitor of the brain adenylate cyclase than was 2′,5′-dd-Ado ($IC_{50}$ ≃3 $\mu$M) (Fig. 2). This potency of 2′-d-3′-AMP was also observed when the brain enzyme was stimulated by $Mn^{2+}$, or $Mn^{2+}$/forskolin (data not shown). Inhibition by 2 $\mu$M 2′-d-3′-AMP increased with increasing concentrations of $Mg^{2+}$ (Fig. 3), but under no circumstances was the sensitivity to inhibition with $Mg^{2+}$ as great as with $Mn^{2+}$ (data not shown). Thus, consistent with observations made with adenylate cyclases from other sources (e.g., Refs. 1–4, 7–12, 14, 18, and 19), inhibition of the brain enzyme by 2′-d-3′-AMP was also metal dependent and sensitivity of the solubilized brain enzyme was maximally increased in a reversible manner with $Mn^{2+}$ or in an irreversible manner by proteolytic activation in the presence of $Mg^{2+}$/GTP$\gamma$S.

**Structure-activity relationship for P site inhibition.** Given the high activity of the detergent-solubilized and proteolytically activated adenylate cyclase from rat brain and its sensitivity to inhibition by 2′,5′-dd-Ado and 2′-d-3′-AMP, this form of the enzyme was used as a test system to evaluate the structural requirements for inhibition of adenylate cyclase via the P site. The effects of many nucleosides and nucleoside phosphates were tested and the concentration-dependence curves obtained with some of these are shown in Fig. 4. $IC_{50}$ values obtained from similar experiments with other compounds are given in Table 2. Fig. 5 shows the structures of the principal P site agonists. For these experiments, adenylate cyclase was activated by pretreatment with ninhibin plus GTP$\gamma$S in the presence of $Mg^{2+}$. Comparable sensitivity and rank order of potency were obtained when adenylate cyclase was stimulated with $Mn^{2+}$ plus forskolin. Values for adenosine or 2′-deoxy-adenosine were unchanged by the inclusion of the adenosine deaminase inhibitor erythro-hydroxy-nonyl-ade-

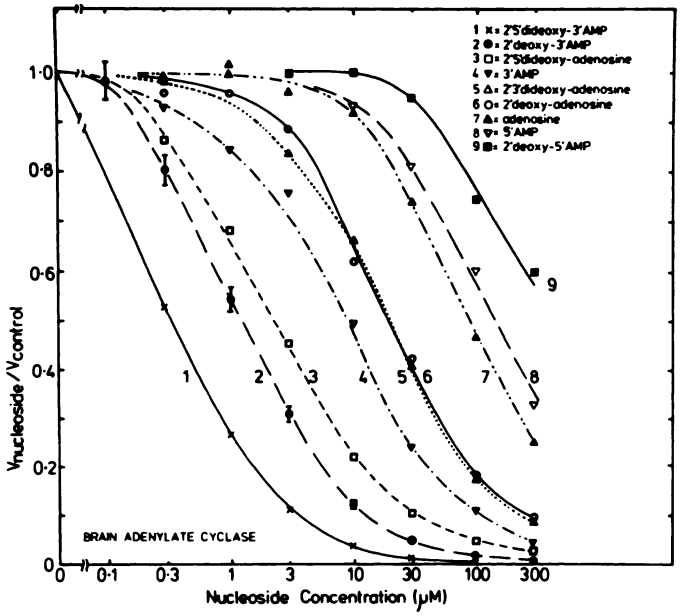


**Fig. 4.** Structure-activity relationships for the inhibition of a proteolytically activated adenylate cyclase from rat brain. Detergent-solubilized adenylate cyclase from rat brain was pretreated with ninhibin and 10 μM GTPγS for 15 min at 30° with a reaction mixture that was complete except for [α-$^{32}$P]ATP and with the indicated concentrations of nucleoside or nucleoside phosphate. [α-$^{32}$P]ATP was added and adenylate cyclase activity was determined in a second incubation for 15 min at 30°. EGTA (100 μM), 10 μM GTPγS, and 2.1 mM $MgCl_2$ were present in all tubes. Values represent averages from at least two normalized experiments for each compound, each assayed in duplicate. For 2′-d-3′-AMP (*curve 2*), values are averages from eight experiments ± SEM. $IC_{50}$ values from these and other compounds are shown in Table 1.

nine, suggesting that the weak sensitivity to these compounds was not due to their being deaminated in the incubation.

The above data suggest (a) a strict requirement for an intact adenine moiety (compare potencies of compounds **1**, **3**, **6**, **7**, **27**, and **35**, and **16**, **29**, and **30**); (b) a requirement for a β-glycosidic linkage for the ribosyl moiety (compare compounds **1** and **2**); (c) a substantial enhancement of the inhibitory potency by 2′-deoxy- and especially 2′,5′-dideoxy-ribosyl moieties (compare compounds **1**, **3**, **12**, and **14**); and (d) a strong preference for phosphate at the 3′-position (compare compounds **1**, **8**, and **9**, **3** and **16**, **14** and **15**). These general requirements are consistent with those determined with particulate enzyme from other tissues (e.g., Refs. 1, 2, 6–9, 16, and 42) and these similarities suggest that this model for evaluation of P site agonists is valid. Of the compounds tested, 2′,5′-dideoxy-adenosine 3′-phosphate was the most potent P site agonist. Large substitutions at the 3′-position are clearly tolerated, as evidenced particularly by the inhibitory effectiveness of the deoxy-adenosine di- and pentanucleotides (compare compounds **39–45**), of 2′,5′-dd-3′-FSBA (compound **20**), and of 2′,5′-dideoxy-3′-succinyl-adenosine (compound **19**). The 2′,5′-dd-3′-FSBA was prepared as a potential covalent labeling agent and the 3′-succinyl analogs were prepared as precursors for potential affinity agaroses. The inhibition by the deoxy-adenosine dinucleotides was not due to their being partially hydrolyzed to 2′-d-3′-AMP or 2′-deoxy-adenosine, because neither compound could be detected subsequent to incubation of the dinucleotides in the cyclase assay.

**Locus of the P site.** The above observations with the brain and platelet adenylate cyclases add further support to the idea that only stimulated forms of the enzyme are sensitive to inhibition by adenosine and its more potent analogs. However, it is unclear which components of the enzyme complex are actually required for the sensitized form. Because detergent-solubilized forms of the enzyme exhibit all of the characteristics of P site inhibition seen with intact membrane preparations, those components necessary for manifesting the inhibition are presumably also solubilized. Available evidence suggests that the P site is likely located on the catalytic unit (C) but that sensitivity to inhibition is influenced by association of $G_s\alpha$. This suggestion is derived from observations of Premont *et al.* (14), who demonstrated P site-mediated inhibition of enzyme deficient in functional $G_s$ protein, and from observations of Florio and Ross (11) with S49/*cyc*$^-$ enzyme, which contains no $G_{s\alpha}$ but which exhibited increased sensitivity to inhibition by 2′,5′-dd-Ado upon reconstitution with activated $G_s$ from liver. That the P site is likely localized on C was substantiated further by Yeager *et al.* (20) and Minocherhomjee *et al.* (24), who demonstrated inhibition of a purified $Ca^{2+}$ calmodulin-sensitive adenylate cyclase from brain by adenosine.

We have studied the locus of the P site further, using two forms of adenylate cyclase. One is the enzyme from bovine sperm that is known not to contain $G_s$ protein and the other is an enzyme from bovine brain that we purified to apparent homogeneity. Although the procedure we used differs from that of Yeager *et al.* (20), in that affinity chromatography was on forskolin-agarose instead of calmodulin-agarose, the actual form(s) of brain cyclase purified may be the same. In agreement with the data of Smigel (26), this purified form exhibited a molecular size of approximately 120 kDa and also showed sensitivity to stimulation by $Ca^{2+}$/calmodulin. Table 3 compares the $IC_{50}$ values of 2′-d-3′-AMP and 2′,5′-dd-Ado at three stages of purification of this enzyme. 2′-d-3′-AMP was typically slightly more potent than 2′,5′-dd-Ado throughout purification. Sensitivity to inhibition by both agonists decreased by perhaps 1 order of magnitude by purification of the enzyme. The $IC_{50}$ of 45 μM for 2′,5′-dd-Ado we observed compares with an $IC_{50}$ of 24 μM reported by Yeager *et al.* (20) for a $Ca^{2+}$/calmodulin-sensitive adenylate cyclase from bovine brain. Thus, the catalytic unit per se is the locus of P site-mediated inhibition, although maximally enhanced potency likely depends on other factors.

In contrast with the marked sensitivity to P site agonists of the adenylate cyclases from platelets and rat or bovine brain (above), the enzyme from bovine sperm was poorly inhibited (Fig. 6). Although exhibiting roughly the same rank order of potency as the solubilized brain enzyme (i.e., 2′-d-3′-AMP ≃ 2′,5′-dd-Ado > adenosine), a 50% reduction in activity required greater than 1000 μM 2′-d-3′-AMP. Even though $Mn^{2+}$ is required for activity of the sperm adenylate cyclase, this cation did not sensitize the enzyme to inhibition by these agents, as it had with particulate and/or solubilized adenylate cyclases from many other sources. This lack of effect of the more potent inhibitory agonists is consistent with the findings of Henry et al. (43) and argues either that the catalytic unit of the sperm adenylate cyclase is distinct from the enzyme from platelets and brain or that it lacks some factor or binding domain required specifically to mediate or to enhance inhibition by these agents. Both are likely correct. The sperm adenylate cyclase has a smaller molecular size than the brain enzyme and it lacks a coupled $G_s$ protein. Moreover, addition of $G_s$ to the

TABLE 2

**Specificity for P site-mediated inhibition of solubilized adenylate cyclase from rat brain**

$IC_{50}$ values were determined with adenylate cyclase that had been proteolytically activated by pretreatment with ninhibin and GTPγS or activated by assay with 10 mM $Mn^{2+}$ (indicated by [c]), as described under Materials and Methods. An $IC_{50}$ given as greater than some value indicates only that this was the highest concentration tested, in some instances exhibiting inhibition at 300 μM but less than 50%, whereas for other there was no detectable effect, e.g., d(GAAAA) was not inhibitory at 30 μM. For some compounds higher concentrations were not tested because amounts of the compounds were limiting and for others solubility was limiting. The following compounds were newly tested in this study: **2, 10, 13, 15, 17–20, 23, 27, 28, 35–46**.

| | Compound | $IC_{50}$ (μM) | | Compound | $IC_{50}$ (μM) |
|---|---|---|---|---|---|
| 1 | β-Adenosine (Ado) | 82 | 24 | Tubercidin (7-deaza-Ado) | >300 |
| 2 | α-Ado | >300 | 25 | Puromycin | >300 |
| 3 | 2′-d-Ado | 15 | 26 | Nebularine (9-β-ribofuranosylpurine) | >300 |
| 4 | 3′-d-Ado (cordycepin) | 13 | 27 | 6-Cl-purine-riboside | >300 |
| 5 | 5′-d-Ado | 17 | 28 | 2′-d-5′-methylthio-Ado | >300 |
| 6 | 2′-d-Inosine | >300 | 29 | 2′-d-3′-CMP | >300 |
| 7 | 2′-d-Guanosine | >300 | 30 | 2′-d-3′-GMP | >300 |
| 8 | 3′-AMP | 8.9 | 31 | 3′,5′-ADP[c] | 50 |
| 9 | 5′-AMP | 150 | 32 | 2′,3′-isopropylidene-Ado | >300 |
| 10 | 5′-AMPF[a] | >100 | 33 | 8-Br-Ado | >300 |
| 11 | 2′-d-5′-AMP | >300 | 34 | $N^6$ (phenylisopropyl)Ado | >300 |
| 12 | 2′,3′-dd-Ado | 9 | 35 | cytosine | >300 |
| 13 | 2-Cl-2′,3′-dd-Ado[b] | >10 | 36 | cyclic ADP-ribose[b] | >50 |
| 14 | 2′,5′-dd-Ado[a] | 2.7 | 37 | Ado5′-PP-ribose | >50 |
| 15 | 2′,5′-dd-3′-AMP[a] | <0.1 | 38 | $P^1P^4$diAdo-5′tetraP | >300 |
| 16 | 2′-d-3′-AMP | 1.2 | 39 | dApdG[c] | 17 |
| 17 | 2′-d-5′-succinyl-Ado[a] | 28 | 40 | dApdA[c] | 22 |
| 18 | 2′,d-3′-succinyl-Ado[a] | 63 | 41 | dApdC[c] | 47 |
| 19 | 2′,5′-dd-3′-succinyl-Ado[a] | 7.7 | 42 | dApdT[c] | 11 |
| 20 | 2′,5′-dd-3′p-FSBA[a] | 30 | 43 | d(AGGGG)[d] | 23 |
| 21 | 3′,5′-cAMP | >300 | 44 | d(AAAAA)[d] | 100 |
| 22 | 2′-d-3′,5′cAMP | >300 | 45 | d(GAAAA)[d] | >30 |
| 23 | A-2′-p-5′-A-2′-p5′-A[c] | >30 | 46 | A-2′-p-5′-A-2′-p-5′-A-2′p-5′-A[d] | >30 |

[a] These compounds were synthesized by us and were purified by preparative high performance liquid chromatography procedures, and the structures were verified by mass spectrometry and NMR as appropriate.

[b] 2-Cl-2′,3′-dd-Ado was a gift of Dr. Dennis Carson (Scripps Clinic) and cyclic ADP-ribose was a gift of Dr. Timothy Walseth (University of Minnesota).

[c] These dinucleotides were each of the form 2′deoxy-3′p5′-2′deoxy N, where N was guanosine, adenosine, cytosive, or thymidine, and they did not contain either 3′ or 5′ terminal phosphate.

[d] Enzyme activated by assay with 10 mM $Mn^{2+}$.

| NUCLEOSIDE | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| adenosine | -OH | -OH | -OH |
| 2′dAdo | -H | -OH | -OH |
| 3′dAdo | -OH | -H | -OH |
| 5′AMP | -OH | -OH | $-OPO_3$ |
| 3′AMP | -OH | $-OPO_3$ | -OH |
| 2′3′ddAdo | -H | -H | -OH |
| 2′5′ddAdo | -H | -OH | -H |
| 2′d3′AMP | -H | $-OPO_3$ | -OH |
| 2′d5′AMP | -H | -OH | $-OPO_3$ |
| 2′5′dd3′AMP | -H | $-OPO_3$ | -H |

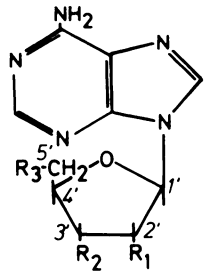


**Fig. 5.** Structures of the principal P site agonists.

sperm enzyme does not impart sensitivity to $G_s$-mediated regulation. Hence, the catalytic subunit of the sperm enzyme probably does not contain a GTP·$\alpha_s$ binding domain nor is its conformation altered by $Mn^{2+}$ to elicit sensitivity to P site agonists.

**Potential physiological sources of P site agonists.** Of the more potent P site agonists tested, several are naturally occurring (e.g., oligo deoxynucleotides, 3′-AMP, 2′-d-3′-AMP) and are of potential physiological interest. Conceiveably, these compounds could be formed by any of several possible mechanisms, including the actions of unique cyclic nucleotide phosphodiesterases, unique adenosine kinases, degradation of nucleic acids, or likely others. In experiments aimed at identifying such possibilities, we observed the formation of 2′-d-3′-AMP and 3′-AMP from poly(dA·dT) and poly(A), respectively, in spleen homogenates and, with homogenates of spleen, liver, and brain, 2′-d-3′-AMP was formed from deoxyribonucleotide dimers (data not shown). Moreover, in preliminary studies we measured levels of 2′-d-3′-AMP and 3′-AMP in extracts from various rat tissues and found them to be in the range consistent with the $IC_{50}$ values reported here. Hence, activities appropriate for the formation of the more potent P site agonists exist and may also exist under *in vivo* conditions.

TABLE 3

**Relative sensitivity of bovine brain adenylate cyclase, at various stages of enzyme purification, to inhibition by 2′-3′-AMP and 2′,5′-dd-Ado**

Adenylate cyclase from bovine brain cortex was extracted and purified from washed particles that had been pretreated for 30 min at 30° with 50 mM triethanolamine·HCl, pH 7.4, 250 mM sucrose, 3 mM dithiothreitol, 2.5 mM benzamidine, 5 mM $MgCl_2$, and 100 μM Gpp(NH)p. Activities were determined with 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, and 50 or 100 μM forskolin. Values are the averages ± SEM from three or four experiments, each conducted in duplicate.

| | $IC_{50}$ (μM) | |
|---|---|---|
| | 2′-d-3′-AMP | 2′-5′-dd-Ado |
| Lubrol-PX extract | 5.6 ± 1.7 | 6.1 ± 1.7 |
| Forskolin-Affigel 102 | 16 ± 7 | 16 ± 4 |
| Wheat germ lectin-Sepharose 6MB | 34 ± 11 | 45 ± 3 |

## Discussion

Inhibition by adenosine and certain ribose-modified analogs is a characteristic of most, but not all, adenylate cyclases. The

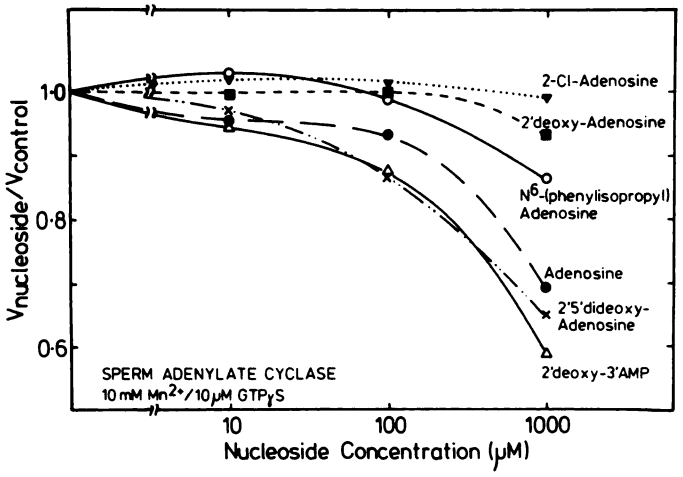


**Fig. 6.** Lack of P site sensitivity of the adenylate cyclase of bovine sperm. Washed particles from bovine sperm were prepared as under Materials and Methods. Adenylate cyclase activity was determined with a 15-min incubation at 37°, with 0.1 mM ATP, 10 mM $MnCl_2$, 10 μM GTPγS, and an ATP-regenerating system consisting of 2 mM creatine phosphate, 100 μg creatine kinase, and 100 ml myokinase. Initial control velocity was 278 pmol of cAMP(15 min·tube)$^{-1}$ and with 10% dimethylsulfoxide was 274 pmol of cAMP(15 min·tube)$^{-1}$. Nucleosides were added at the indicated concentrations, with dilutions being made in water except for $N^6$-(phenylisopropyl)-adenosine, which was diluted in 10% dimethylsulfoxide. Values are the averages of duplicate determinations from one of two similar experiments.

rank order of potency is generally 2′,5′-dd-3′-AMP > 2′-d-3′-AMP > 2′,5′-dd-Ado > 3′-AMP > 2′-deoxyadenosine > adenosine. Large substitutions at the 3′-ribose position are tolerated. These include the potentially useful 3′-succinyl-adenosine analogs (for linkages to immobilizing matrices) and 3′-*p*-fluoro-sulfonyl-benzoyl-adenosine (for covalent attachment to the binding domain), and the potentially interesting di- and pentanucleotide analogs. Substitutions at the 5′-ribose position typically impair sensitivity and modifications in the adenine moiety are not tolerated, with the possible exception of 2-halo additions (42). Hence, the adenosine P site is actually a misnomer and might be more correctly referred to as the "A" (adenine) or "deoxy-adenosine phosphate" site (dAP site).

In general, activation of adenylate cyclase sensitizes the enzyme to inhibition by P site agonists. With particulate, solubilized, or purified preparations from platelets or brain, both reversible and irreversible forms of stimulation of adenylate cyclase were found to sensitize the cyclase to inhibition. However, the efficacy of the various stimuli differed. The orders of effectiveness for sensitizing both the platelet and brain adenylate cyclases were essentially the same; for platelet enzyme it was ninhibin/GTPγS ≃ $Mn^{2+}$/forskolin > hormone/GTP > forskolin and for brain enzyme it was ninhibin/GTPγS ≃ $Mn^{2+}$ ≃ $Mn^{2+}$/forskolin. These results suggest that more than one form of adenylate cyclase is sensitive to P site-mediated inhibition, but a characteristic common to each is the requirement for divalent cation. Proteolytic or hormonal activation of adenylate cyclase substantially increases the apparent affinity of the cyclase for divalent cation and this is especially evident for $Mg^{2+}$. That is, treatment of adenylate cyclase with hormone and GTP or with protease in the presence of GTPγS facilitates the formation of $\alpha_s \cdot C$. The association of this complex (GTPγS·$\alpha_s$) with C (GTPγS·$\alpha_s \cdot C$) increases the apparent affinity for divalent cation ($Me^{2+}$) binding to C and/or $\alpha_s$ (e.g., GTPγS·$\alpha s \cdot C^{Me}$). Thus, the most sensitive configurations of adenylate cyclase are those induced by $Mn^{2+}$, especially in the presence of $G_{s\alpha}$, yielding the GTPγS·$\alpha_s \cdot C^{Me}$ complex. (Data regarding any possible role of $G_i\alpha$ to modulate P site sensitivity have not been reported.) The binding of divalent cation may play the most significant role. P site-mediated inhibition is enhanced with increasing metal concentration, more potently with $Mn^{2+}$ than $Mg^{2+}$, but has been reported to be increased by $Ca^{2+}$ (18), nominally an inhibitory cation. Occupation of the divalent cation binding domain is not only required for inhibition, but the apparent affinity of cation is enhanced by P site-mediated inhibition (4). Divalent cation may be viewed as inducing a conformational change in the catalytic subunit of the enzyme required for binding of P site agonists. That is, only forms of adenylate cyclase in which a divalent cation binding domain is occupied are also capable of binding a P site agonist and, hence, are capable of being inhibited (e.g., $C \rightarrow C^{Me} \rightarrow C_P^{Me}$). Although this appears to be correct for the brain and platelet adenylate cyclases, it apparently does not occur with the sperm enzyme.

Although the physiological significance of P site-mediated inhibition of adenylate cyclase is unclear, the data presented here suggest that 2′-d-3′-AMP and 3′-AMP, in particular, may be sufficiently potent inhibitors of the enzyme to be considered physiologically relevant and these compounds may derive from nucleic acids. Cellular levels of 2′-d-3′-AMP have been reported to be in the range of 0.8 to 8 nmol/g of tissue for toad erythrocytes and spleen (44), values with which our preliminary data are consistent. Hence, adenylate cyclase may be viewed as being rendered sensitive to inhibition by stimulation and the sensitized enzyme may, thus, be exposed to inhibition by on-board cellular constituents other than and substantially more potent than adenosine. Alternatively, the levels of such P site agonists may fluctuate, thereby varying the effectiveness of stimuli of adenylate cyclase.

The present studies define more clearly the requirements for inhibition of platelet and brain adenylate cyclases by what we term P site (or, perhaps better, dAP site) agonists, with regard to agonist structure, metal ion requirements, and enzyme configuration. A number of obvious questions remain. One is the character of the interactions of the components of the adenylate cyclase system and co-factors that bring about inhibition. Perhaps more important, though, are the potential physiological roles of the P site, which remain unclear. Uncertain are (a) whether 2′-d-3′-AMP or 3′-AMP are the only and most potent physiologically relevant, naturally occurring P site agonists; (b) the physiological source of these compounds; (c) the nature of regulation of their synthesis and/or degradation that may help to integrate cellular processes; and (d) whether there exist naturally occurring antagonists for this site.

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