2',5'-Dideoxyadenosine 3'-Polyphosphates Are Potent Inhibitors of Adenylyl Cyclases*

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Laurent Désaubry, Ilana Shoshani, and Roger A. Johnson†

From the Department of Physiology and Biophysics, State University of New York, Health Sciences Center, Stony Brook, New York 11794-8661

2',5'-Dideoxyadenosine 3'-di- and triphosphates were tested as inhibitors of brain adenylyl cyclases. With an IC50 ~ 40 nM, 2',5'-dideoxy-3'-ATP is the most potent non-protein synthetic regulator of adenylyl cyclases thus far described. Neither 2',5'-dideoxy-3'-ADP nor 2',5'-dideoxy-3'-ATP inhibited activity by competition with substrate, and the linear noncompetitive inhibition observed was consistent with interaction via a distinct domain. The availability of this ligand will permit the development of a variety of probes that will be extremely useful in investigating adenylyl cyclase structure and the role(s) that this class of compound may play in physiologically regulating cell function.

Adenylyl cyclase is a family of membrane-bound enzymes that catalyze the formation of 3'-5'-cAMP from 5'-ATP. In mammalian systems, the 10 known adenylyl cyclase isozymes are regulated by numerous hormones and neurotransmitters via cell surface receptors linked via stimulatory (Gs) and inhibitory (Gi) guanine nucleotide-dependent regulatory proteins (G-proteins), as well as by numerous other agents also of physiological and biochemical interest. These include agents or enzymes that act on hormone receptors, specific bacterial toxins that act on Gs and Gi, and agents that act directly on adenylyl cyclase. Direct activation can be caused by forskolin with all but one isozyme and by Ca2+/calmodulin with four isozymes. The enzyme is also inhibited directly by certain adenosine derivatives which act via a distinct domain, referred to as the "P"-site from a requirement for a purine moiety (1-8). Of the mammalian adenylyl cyclases that have been tested, all save the enzyme from sperm have been found to be susceptible to P-site-mediated inhibition (5, 9-12). Although P-site ligands act directly on adenylyl cyclase, enzyme stimulated by hormones (via Gs/α) or Mn2+ are most sensitive to inhibition, and P-site ligands may be viewed as attenuating the enzyme's susceptibility to such activation (5, 8, 13-18).

Although the three-dimensional structure of adenylyl cyclase is not known, the deduced primary sequence suggests a membrane topology exhibiting a repeated structure of six membrane spanning regions followed by a large cytosolic domain, giving twelve membrane spanning regions and two cytosolic domains (19). The two cytosolic domains (C1 and C2) are homologous with each other and with the established catalytic domain of guanylyl cyclases (20), supporting the idea that each contains a nucleotide binding region. However, it is not known whether C1, C2, or both form the catalytic site, nor whether one or both domains participate in inhibition by P-site ligands. Inhibition kinetics and irreversible inactivation studies with P-site-selective covalent affinity probes are consistent with inhibition occurring at a site that is distinct from the catalytic site (13-15). Shared key structural features for substrate and for P-site ligands include a requirement for adenine, enhanced efficacy with 2'-deoxyadenosine derivatives compared with those of adenosine, and a requirement (catalysis) or preference (inhibition) for phosphate (1-5). Given the shared requirements of these ligands, some similarities in the binding domains for each may be expected. Lacking have been high affinity labeled ligands suitable for binding to either or both domains.

The most potent inhibitory ligands have been 2',5'-dideoxyadenosine 3'-monophosphate (2',5'-dd-3'-AMP) and the naturally occurring 3'-AMP and 2'-3'-AMP, with IC50 values in the micromolar range (5, 22). In the synthesis of 2',5'-dd-3'-AMP, referred to in a previous study (5), a small amount of a more potent inhibitory peak also was detected in HPLC eluates. Upon ashing, this unexpected inhibitor was found to contain two phosphates per adenine (23) and the most likely product was 2',5'-dd-3'-ADP. Subsequently, we developed a synthesis for the 3'-polyphosphorylated derivatives of 2',5'-ddAdo (24). Reported here are the effects of these agents on adenylyl cyclase and the mode of their inhibition.

EXPERIMENTAL PROCEDURES

Preparation and Assay of Adenylyl Cyclase—Detergent-solubilized preparations of adenylyl cyclase from rat and bovine brains were prepared and assayed as described previously (5, 14, 15). Bovine brain adenylyl cyclase was purified as described by Pfeuffer et al. (25). Inhibition kinetics were determined on enzyme assayed with concentrations of divalent cation fixed in excess of the ATP concentration as described previously (26). Inhibition was determined on enzyme that was activated in the presence of Mn2+ and forskolin.

Estimation of Bound Phosphate—Forskolin-stimulated phosphatase was determined on ashed samples by the malachite green method of Stull and Buss (23). Adenine was estimated by UV-absorption at 259 nm following purification on high performance liquid chromatography.

Materials—2',5'-dd-3'-ADP and 2',5'-dd-3'-ATP were prepared as described previously (24). Lubrol PX (from Sigma) was filtered through alumina (Neutral, AG7, from Bio-Rad Laboratories) to remove peroxides. Recombinant Type I enzyme was generously supplied by Drs. R. Tausig and A. G. Gilman. Enzyme was purified by forskolin affinity chromatography from extracts of fall army worm ovarian (Sf9) cells infected with a Type I adenylyl cyclase encoding baculovirus (27). Other reagents were of the highest quality from commercial sources.

RESULTS AND DISCUSSION

2',5'-Dideoxyadenosine 3'-phosphates are a family of inhibitors of adenylyl cyclase in which potency increased with the number of 3'-phosphates (Fig. 1). IC50 values for inhibition of

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† To whom correspondence should be addressed. Tel.: 516-444-3040; Fax: 516-444-3432; E-mail: rjohnson@ccmail.sunysb.edu.

‡ To whom correspondence should be addressed. Tel.: 516-444-3040; Fax: 516-444-3432; E-mail: rjohnson@ccmail.sunysb.edu.
Adenylyl Cyclase Inhibition by 2',5'-Dideoxy-3'-ATP

The enzyme by these nucleotides are presented in Table I and are compared with those of the parent nucleoside and with known ligands. 2',5'-dd-3'-ADP and 2',5'-dd-3'-ATP exhibited IC₅₀ values of 0.1 μM and 0.04 μM, respectively. Potency of the 3'-monophosphate (2',5'-dd-3'-AMP, IC₅₀ = 0.5 μM) was enhanced approximately 5-fold by the addition of the second phosphate at the 3'-position (2',5'-dd-3'-ADP) and an additional two-plus-fold by the addition of the third phosphate (2',5'-dd-3'-ATP). Thus, the most effective inhibitory ligands of adenylyl cyclase are effected by the removal of both 2'- and 5'-hydroxyl groups from adenosine and the addition of polyphosphate at the 3'-position (Table I). The rank order noted here was maintained with the purified native bovine type I and with the recombinant wild type I adenylyl cyclases, although with these enzymes each of the 3'-nucleotides was noticeably less potent than with the enzyme in the crude detergent-dispersed rat brain preparation (Table II). The reason for the loss of inhibitory potency of these 3'-nucleotides upon purification of adenylyl cyclase is unknown but is consistent with previously noted observations with 2'-d-3'-AMP and 2',5'-dd-3'-Ado with the bovine brain enzyme (5). The reduced sensitivity may be due simply to the changes in structure of the enzyme upon isolation and removal of the native phospholipid environment.

2',5'-dd-3'-ATP (IC₅₀ = 40 nm) is almost two orders of magnitude more potent than previous ligands in inhibiting native adenylyl cyclase. The most potent non-protein regulator of adenylyl cyclases thus far described. Since inhibition was also observed with purified adenylyl cyclase, it is clear that 2',5'-dd-3'-ATP acts directly on the enzyme, exerting inhibition independent of either hormone receptor, stimulatory or inhibitory G-protein, or G-protein subunit. It approaches the potency of the stimulatory effect of Gₛα on the type I adenylyl cyclase and the stimulatory and inhibitory effects of βγ on α₅-activated types II and I, respectively (27).

Structurally, 2',5'-dd-3'-ATP and 2',5'-dd-3'-ADP share some properties with 5'-ATP. To ascertain whether either 3'-nucleotide might inhibit activity through simple competition with 5'-ATP at the catalytic site, inhibition kinetics were evaluated (Fig. 2). In this experiment, inhibition by 2',5'-dd-3'-ATP of the forskolin affinity-purified enzyme from bovine brain was found to be noncompetitive with respect to substrate. Moreover, inhibition was linear (Fig. 2, inset) in that replots of slopes and intercepts were also linear with inhibitor concentration. This linear noncompetitive behavior was also seen with the crude, detergent-extracted adenylyl cyclase from rat brain (not shown) and also with 2',5'-dd-3'-ADP (not shown). This behavior argues strongly that inhibition occurred at a site distinct from catalysis and is fully consistent with inhibition occurring at the P-site. P-site-mediated inhibition of adenylyl cyclases is characteristically noncompetitive with respect to substrate, whether MnATP or MgATP (1, 3, 7, 8, 13, 14, 16, 28). Thus, the evidence supports the conclusion that adenylyl cyclases contain distinct and interacting adenosine nucleotide binding domains for catalysis (5'-ATP) and inhibition (2',5'-dd-3'-ATP). The straightforward and linear kinetic behavior was somewhat surprising with the crude detergent extracts as we had expected that phosphohydrolases might have contributed to

![Figure 1](image1.png)

**Fig. 1.** Inhibition of rat brain adenylyl cyclase by 2',5'-dideoxyadenosine and its 3'-phosphorylated derivatives. Activities were determined in the presence of 5 mM MnCl₂, 100 μM 5'-ATP, 1 mM 3-isobutyl-1-methylxanthine, 100 μM forskolin, 1 mg of bovine serum albumin per ml, 3 mM dithiothreitol, 0.1% (w/v) Lubrol PX, 50 mM MOPS buffer, and an ATP-regenerating system including 4 mM phosphoenolpyruvate and 100 μg of pyruvate kinase per ml.

![Figure 2](image2.png)

**Fig. 2.** Kinetics of inhibition of purified bovine brain adenylyl cyclase by 2',5'-d3'-ATP. Activities were determined in the presence of 5 mM MnCl₂ in excess of the ATP concentration, 100 μM forskolin, and an ATP-regenerating system including 2 mM creatine phosphate and 100 μg of creatine kinase per ml.
significant breakdown of the 3'-polyphosphates (21). However, we noted no meaningful breakdown of either nucleotide during assay incubations either with purified or with the crude Lubrol PX-extracted adenylyl cyclase.

The inhibitory potency of 2',5'-dd-3'-ATP suggests that derivatives of it may be useful as a labeled ligand to probe the P-site binding domain on adenylyl cyclase. This could circumvent one of the major constraints in the identification of amino acids involved in the binding of P-site ligands. Presently one must rely on inhibition of catalysis, even for evaluating efficacy of site-directed mutations of expressed protein. With inhibition as end point one is by definition not directly quantifying a single domain on this enzyme, but is rather measuring a downstream event. Changes in catalysis could occur independent of changes in the binding of inhibitory ligand, simply through modification of a domain that influences coupling between inhibitory and catalytic domains. The affinity of 2',5'-dd-3'-ATP is sufficient to allow a binding assay to be developed, and this could significantly aid studies in this direction.

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