Inhibition of Adenylyl Cyclase by a Family of Newly Synthesized Adenine Nucleoside 3'-Polyphosphates*

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The synthesis of a number of adenine nucleoside 3’-polyphosphates has been devised via a phosphotriester approach that combines the method of alkoxide activation with the use of 2,2,2-tribromoethyl phosphoromorpholinocloridate as a phosphorylating agent. The family of compounds included 3’ADP, 3’ATP, 2’-deoxy-3’ADP, 2’-deoxy-3’ATP, 2’5’,5’-dideoxy-3’ADP, and 2’5’,5’-dideoxy-3’ATP. Potency as inhibitors of adenylyl cyclases followed the order: 3’-mono- < 3’-di- < 3’-triphosphate and adenosine (Ado) < 2’-d-Ado < 2’,5’,5’-dd-Ado derivatives, with 2’,5’-dideoxy-3’ATP exhibiting an IC50 of ~40 nm. This order was maintained with purified and recombinant forms of the type I enzyme. The nucleoside 3’-phosphates caused noncompetitive inhibition of the type I adenylyl cyclase from bovine brain, consistent with inhibition via the P-site. Inhibition was not due to hydrolytic products because this was minimal and inconsistent with the use of 2,2,2-tribromoethyl phosphoromorpholinocloridate as a phosphorylating agent. The family of compounds included 3’ADP, 3’ATP, 2’-deoxy-3’ADP, 2’-deoxy-3’ATP, 2’5’,5’-dideoxy-3’ADP, and 2’5’,5’-dideoxy-3’ATP. Potency as inhibitors of adenylyl cyclases followed the order: 3’-mono- < 3’-di- < 3’-triphosphate and adenosine (Ado) < 2’-d-Ado < 2’,5’,5’-dd-Ado derivatives, with 2’,5’-dideoxy-3’ATP exhibiting an IC50 of ~40 nm. This order was maintained with purified and recombinant forms of the type I enzyme. The nucleoside 3’-phosphates caused noncompetitive inhibition of the type I adenylyl cyclase from bovine brain, consistent with inhibition via the P-site. Inhibition was not due to hydrolytic products because this was minimal and inconsistent with the use of 2,2,2-tribromoethyl phosphoromorpholinocloridate as a phosphorylating agent. The family of compounds included 3’ADP, 3’ATP, 2’-deoxy-3’ADP, 2’-deoxy-3’ATP, 2’5’,5’-dideoxy-3’ADP, and 2’5’,5’-dideoxy-3’ATP. Potency as inhibitors of adenylyl cyclases followed the order: 3’-mono- < 3’-di- < 3’-triphosphate and adenosine (Ado) < 2’-d-Ado < 2’,5’,5’-dd-Ado derivatives, with 2’,5’-dideoxy-3’ATP exhibiting an IC50 of ~40 nm. This order was maintained with purified and recombinant forms of the type I enzyme. The nucleoside 3’-phosphates caused noncompetitive inhibition of the type I adenylyl cyclase from bovine brain, consistent with inhibition via the P-site. Inhibition was not due to hydrolytic products because this was minimal and inconsistent with the use of 2,2,2-tribromoethyl phosphoromorpholinocloridate as a phosphorylating agent.

Adenylyl cyclase is a family of membrane-bound enzymes that catalyze the formation of 3’,5’-cAMP from ATP and is regulated by numerous neurotransmitters and hormones via cell surface receptors and guanine nucleotide-dependent regulatory proteins (G-proteins). In an isoyme-dependent manner, enzyme activity is also regulated directly, by forskolin, by Ca2+ / calmodulin, and by certain adenosine derivatives via a site that is distinct from the catalytic site. Whereas numerous drugs have been developed that act on the cyclic nucleotide phosphodiesterases, those that act directly on adenylyl cyclases have been less well explored: the main class of such pharmacological agents comprises forskolin and its analogs (3).

The domain through which inhibition of mammalian adenylyl cyclases occurs with adenosine derivatives, excepting the enzyme from sperm, is referred to as the P-site from an evident requirement for a purine moiety (4–11). Key structural requirements for P-site ligands include a requirement for an intact adenine moiety, enhanced inhibitory potency with 2’-deoxy- and especially 2’,5’-dideoxy-ribosyl moieties, and a notably strong preference for a 3’-phosphate (4–8). The most potent ligands have been 2’5’,5’-dideoxyadenosine 3’-monophosphate (2’,5’-dd-3’AMP), the naturally occurring 3’AMP, and 2’,5’-dd-3’AMP (8, 12), and recently we described the effects of the 2’,5’-dd-3’ADP and 2’,5’-dd-3’ATP on brain adenylyl cyclases (13). Noncompetitive inhibition kinetics with these ligands (13–15) and irreversible inactivation studies with P-site-selective covalent affinity probes (16)‡ are consistent with inhibition occurring at a site that is distinct from the catalytic site. As a part of our effort (17) to investigate the structure and properties of this inhibitory site on adenylyl cyclases, we describe here a short and efficient synthesis of a number of adenosine 3’-polyphosphates and the potent inhibition of adenylyl cyclase that these 3’-nucleotides exert. Although the biology and biochemistry of nucleoside 3’-polyphosphates are essentially unexplored in eukaryotic systems, inhibition of adenylyl cyclases by this class of compound may well imply roles as intracellular regulators of this transmembrane signaling system.

EXPERIMENTAL PROCEDURES

Preparation and Assay of Adenylyl Cyclase—Both detergent-solubilized and particulate preparations of adenylyl cyclase from rat and bovine brains were prepared and assayed as described previously (8, 15, 16). Lubrol-PX was filtered through alumina (Neutral, AG7, from Bio-Rad) to remove peroxides. Bovine brain adenylyl cyclase was purified as described by Pfeuffer et al. (18). Inhibition kinetics were determined on enzyme assayed with concentrations of divalent cation fixed in excess of the 5’ATP concentration as described previously (19).

Quantification of Nucleotides by HPLC—Nucleotides were quantified after HPLC as areas under peaks determined with a Waters 996 photodiode array detector and the accompanying Millennium software (v.2.10). Chromatography was on a DEA–5PW column (Altex, 5 μm, 7.5 × 75 mm) developed with sequential step gradients of triethylam-
monium bicarbonate, pH 8.5, to separate the nucleoside 3′-mono-, 3′-di-, and 3′-triphosphates and 2′-3′-AMP and 3′-5′-AMP.

Materials—Recombinant type I enzyme was generously supplied by Drs. R. Taussig and A. G. Gilman. Membranes and membrane extracts were from fall army worm ovarian (S19) cells infected with a type I adenyl cyclase encoding baculovirus. THF was distilled from benzo- phenone. NMR spectra (Fig. 1) were recorded with a Bruker AMX-400 MHz instrument. Phosphoromorpholinochloridate was synthesized with cold acetone and dried with sodium iodide in acetone to a methanol solution of the triethylammonium chloride (3.10 mol, 3H, J = 6.6 Hz, 3H-5), 2.79–2.87 (m, 2H, H-2’ and H-3’), 4.36–4.39 (3H, 1H, H-4’), 4.72–4.79 (m, 1H, H-3’), 6.33 (t, 1H, J = 6.6 Hz, H-1’), 8.06 (s, 1H, H-2), 8.25 (s, 1H, H-8); 31P NMR (D2O) δ -7.05 (ppm, 1H, Jp-p = 21.1 Hz, P-1), -1.77 (J = 21.1 Hz, P-2); FAB-MS 394 (M - H+).

2′-5′-Dideoxyadenosine 3′-Diphosphate—Phosphor triester 3a (1.42 g, 2.5 mmol) reacted with mono(tri-n-butylammonium)-phosphate (30 mmol), as described above, yielding 1.35 mmol (54%) of 2′,5′-dideoxyadenosine 3′-diphosphate 5a (δ 1.1.30 (d, 3H, J = 6.6 Hz, 3H-8), 2.79–2.87 (m, 2H, H-2’ and H-3’), 4.36–4.39 (3H, 1H, H-4’), 4.72–4.79 (m, 1H, H-3’), 6.33 (t, 1H, J = 6.6 Hz, H-1’), 8.06 (s, 1H, H-2), 8.25 (s, 1H, H-8); 31P NMR (D2O) δ -7.05 (ppm, 1H, Jp-p = 21.1 Hz, P-1), -1.77 (J = 21.1 Hz, P-2); FAB-MS 394 (M - H+).

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RESULTS

Syntheses—The synthesis of nucleoside 3′-polyphosphates has received considerably less attention than have those of the corresponding 5′-polyphosphates because the former are more difficult to synthesize and because their biological roles are not as well described (23–31). To our knowledge the only reported chemical synthesis of 2′-deoxy-nucleoside 3′-triphosphates was that by Jöse and Moffatt (28) and proceeded by homologation of the corresponding nucleoside 3′-monophosphate by use of phosphoramidophosphate. Other nucleoside 3′-polyphosphates have also been prepared by homologation of the respective 3′-monophosphates (23–26, 29). The major drawback with this method is that the synthesis of the phosphoromorphophosphates requires easy accessibility to the corresponding nucleoside 3′-monophosphate. Van Boom and colleagues prepared nucleoside 3′-diphosphates in the thymidine and guanosine series by a phosphor triester approach that involved the use of an activated morpholinophosphonate (27, 30, 31). Unfortunately, such a procedure cannot be applied when the base of the nucleoside is adenine, the 6-amino group being more reactive toward phosphorylating agents than is the 3′-hydroxyl of the nucleoside moiety (32, 33). Recently Uchiyama et al. (33) reported that a facile 3′-phosphorylation of N-unprotected nucleosides could be achieved using magnesium alkoxide formation. We wish to report here that sequential treatment of N-unprotected 2′,5′-dideoxyadenosine derivatives 1 (20, 21) with an equimolar amount of tert-buty lammonium phosphorothionate (34) in THF at 20°C afforded the phosphor triesters 3 in good yields (57 and 82%) (Scheme 1).

In conclusion, the utility and efficiency of the method of the alkaline activation combined with the use of the phosphorylating agent 2 has been demonstrated by its application to the syntheses of various adenosine 3′-polyphosphates in only two steps, avoiding additional steps generally required for such syntheses (protection and deprotection of the exocyclic amino group and condensation of morpholine with the nucleoside monophosphate).

Inhibition of Adenyl Cyclases—The adenosine 3′-polyphosphates and the deoxy derivatives (Fig. 1) are a family of inhibitors of adenyl cyclase in which potency increased with the number of 3′-phosphates and with the removal of the 2′-OH
and 5'-OH groups (Fig. 2). IC$_{50}$ values for inhibition of rat brain adenylyl cyclase by the three series of adenosine 3'-phosphates are presented in Table I and compared with those of the parent nucleosides and with three cyclic nucleotides. The cyclic nucleotides were poor inhibitors. For each parent nucleoside, inhibitory potency increased with the number of 3'-phosphates. Inhibitory potency of the 3'-phosphates followed the order Ado, 2'-d-Ado, 2',5'-dd-Ado. The greatest effects were those caused by the removal of the 2'-hydroxyl group and by the sequential addition of phosphates at the 3' position, each increasing potency many-fold. The combined effect of the removal of the 2'- and 5'-hydroxyl groups and the addition of the 3'-polyphosphate are incorporated in 2',5'-dd-3'-ATP, which exhibited an IC$_{50}$; 40 nM (Table I and Ref. 13). This potency is almost 2 orders of magnitude greater than that of previous ligands and makes 2',5'-dd-3'-ATP the most potent nonprotein regulator of adenylyl cyclase thus far described. It approaches the potency of the stimulatory effect of rGs$_{a}$ on the type I adenylyl cyclase and the stimulatory and inhibitory effects of Gbg$_{a}$ on $\alpha_{s}$-activated-type II and I, respectively (35). The observation that adenosine and 2'-deoxyadenosine 3'-polynucleotides are also very potent inhibitors of adenylyl cyclase is important in that these 3'-nucleotides are known to occur naturally, whereas the 2',5'-deoxyadenosine 3'-polynucleotides are not. Consequently, the adenosine and 2'-deoxyadenosine 3'-polynucleotides are potentially important and unexplored intracellular regulatory nucleotides. The rank order presented in Table I was maintained with the purified native bovine type I and with the recombinant wild type I adenylyl cyclases, though with these enzymes each of the 3'-nucleotides was noticeably less potent than with the enzyme in the crude detergent-dispersed rat brain preparation (13). This is consistent with the loss of potency of 2'-d-3'AMP and 2',5'-dd-Ado we had previously noted upon purification of the bovine brain adenylyl cyclase (8). This may reflect the loss of membrane phospholipid important for tertiary structure or loss of calmodulin in the purified preparations, because Ca$^{2+}$/calmodulin enhances sensitivity to P-site ligands (36).

Mode of Inhibition—Because inhibition by this class of compound occurred with purified enzyme, inhibition is an effect on the enzyme directly. We reported previously that 2',5'-dd-3'ATP and 2',5'-dd-3'ADP elicited linear noncompetitive inhibition of both purified and crude, detergent-extracted preparations of adenylyl cyclases (13). This behavior was fully
consistent with inhibition occurring at the P-site (15). Not surprisingly, the 2'-deoxyadenosine 3'-polynucleotides also caused noncompetitive inhibition (Fig. 3). Thus, all of the adenine 3'-nucleotides synthesized and tested here constitute a class of substantially more potent P-site inhibitors of adenylyl cyclases.

3'-Nucleotide Stability—Typical adenylyl cyclase preparations contain a number of phosphohydrolyses and substantial degradation of the adenine nucleoside 3'-polynucleotides might be expected (37, 38). However, when these 3'-nucleotides were incubated with a detergent-dispersed adenylyl cyclase from rat brain under conditions known to elicit excellent sensitivity to P-site-mediated inhibition, with the exception of 3'ATP, there was no significant breakdown of any of the adenosine or 2'-deoxyadenosine 3'-polynucleotides or of 2',5'-dd-3'ATP (Table II).3 Notably, there was some conversion of 3'ADP to 3'ATP (as well as to breakdown products of 3'ATP) and of 2',5'-dd-3'ADP to 2',5'-dd-3'ATP. This phosphorylation of 3'ADP and 2',5'-dd-3'ADP was found to be due to contaminating kinase(s) in the incubation and did not occur in the absence of enzyme. 3'ATP breakdown led to the formation of 3'AMP and 2'AMP in variable ratios, and this occurred also in the absence of enzyme. The hydrolysis of 3'ATP implies its potency (IC50 = 2 μM) was likely an underestimation. In the absence of the adenylyl cyclase preparation led to the formation of 3'AMP and 2'AMP in variable ratios, and this occurred also in the absence of enzyme.

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3 These experiments on nucleotide stability were conducted in the absence of 5'ATP, which is present in adenylyl cyclase reactions. 5'ATP was omitted because it partially comigrates with the 3'-triphosphates we tested, making the quantification of nucleoside 3'-triphosphates more difficult. In other experiments we found no evidence that 5'ATP altered the small appearance of 3'-di- and 3'-monophosphate breakdown products, implying that 5'ATP did not affect the hydrolysis of the 3'-triphosphates.

DISCUSSION

Presented here are the synthesis of a new family of adenosine 3'-polynucleotides and the effects of these 3'-nucleotides on adenylyl cyclase. The characteristics of inhibition conferred

![Fig. 3. Double-reciprocal plot of effects of 2'-d-3'ADP on rat brain adenylyl cyclase. Activities were determined as for Fig. 2, with MnCl2 fixed at 5 mM in excess of the ATP concentrations.](http://www.jbc.org/)

| Nucleotide | Nucleotide | 3'-P | 3'-PP | 3'-PPP |
|------------|------------|------|-------|-------|
| 2'd3'AMP   | 2.6        | 97.4 | 95.8  | 91.3  |
| 2'd3'ADP   | 1.2        | 3.0  | 5.3   |      |
| 2'd3'ATP   | 1.4        | 2.0  |       |      |
| 2',5'dd3'AMP| 21.4       | 78.6 | <0.6  | 89.9  |
| 2',5'dd3'ADP| 1.7        | 7.8  | <0.1  | 98.6  |
| 2',5'dd3'ATP| 1.4        | <0.1 | <0.1  |      |
| 3'ADP      | 2.7        | 2.0  | 73.1  | 22.2  |
| 3'ATP      | 2.3        | 72.9 | 6.1   | 18.8  |

Values are percentages normalized from the areas of the eluted peaks from anion exchange HPLC for each nucleotide added. Nucleotides were exposed for 15 min at 30 °C with normal adenylyl cyclase reaction mixture, including 50 mM triethanolamine·HCl, pH 7.5, 5 mM MnCl2, 100 μM forskolin, 1 mM 3-isobutyl-1-methylxanthine, 1 mM dithiothreitol, 1 mg of bovine serum albumin/ml, 0.1% (v/v) Lubrol-PX, 2 mM creatine phosphate, and 100 μg of creatine kinase/ml, but absent 5'ATP.3 Extracted rat brain protein was 21 μg/tube.
to those of ligands acting on adenylyl cyclases via the P-site. For the parent nucleosides and for their respective 3'-phosphorylated derivatives, potency increased with removal of ribosyl-hydroxyl groups and exhibited the rank order of Ado < 2'-d-Ado < 2',5'-dd-Ado. These compounds exhibited increased inhibitory potency with the successive addition of phosphates to the 3'-ribose position. Although the most potent inhibitor was 2',5'-dd-3'ATP (IC$_{50}$ ~ 40 nM), it is important that the adenosine and 2'-deoxyadenosine 3'-polyphosphates also exhibited potency in the micro- to submicromolar range, respectively. Of these 3'-nucleotides it is probable that all but the 2',5'-deoxyadenosine derivatives are naturally occurring.

Although the tertiary structure of adenylyl cyclases 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 (39). The two cytosolic domains (C1 and C2) are homologous with each other and with the established catalytic domain of guanylyl cyclases (40), supporting the idea that each contains a nucleotide binding region. It is easy to speculate that each of these two cytosolic domains binds one of the nucleotides, one site for catalysis (5'ATP) and a distinct site for inhibition (3'ATP), but this is uncertain. It is known that expression of either C1 or C2 domain alone is insufficient to catalyze effectively the formation of 3':5'-cAMP and that co-expression of independently vectored cytosolic domains substantially improves the catalytic efficiency (41).

Available evidence suggests that for most adenylyl cyclases, catalysis and inhibition by P-site ligands occur at distinct sites. This conclusion is supported principally by studies of irreversible inactivation with P-site targeted ligands and by enzyme kinetics (15, 16). Inactivation by 2',5'-dd-3'FSBA (16) occurred in the presence of 5'ATP but not in the presence of 2',5'-dd-Ado, a modestly potent P-site ligand. We suggested that this was consistent with P-site ligands binding at a site distinct from and independent of that of the substrate. This is nominally supported by inhibition kinetics, which have consistently exhibited straightforward noncompetitive behavior (4–8, 13–15), except for enzyme activated by pretreatment with GTP-yS$_{332}$ when uncompetitive inhibition was noted (15). Both uncompetitive and noncompetitive inhibition typically imply inhibitor binding to the enzyme in the presence of substrate and at a site distinct from the catalytic site (45), but other mechanisms may lead also to this apparent kinetic behavior as we have discussed previously (15). The principal argument is that P-site ligand may form an inactive metal-PP$_{i}$-adenosine complex at the catalytic site through the binding of the P-site ligand and the substrate (15). With MnCl$_{2}$ fixed at 5 mM in excess of the ATP concentrations, inorganic ammonium tetraphosphate and inorganic sodium pyrophosphate. Lower panel, double-reciprocal plot for inhibition of rat brain adenylyl cyclase by inorganic manganese triphosphate.

**FIG. 4. Inhibition of rat brain adenylyl cyclase by inorganic polyphosphates.** Upper panel, enzyme velocities with polyphosphate relative to control velocities. Activities were determined as for Fig. 2, with MnCl$_{2}$ fixed at 5 mM in excess of the ATP concentrations. a, inorganic ammonium tetraphosphate; b, inorganic sodium triphosphate; o, inorganic sodium pyrophosphate. Lower panel, double-reciprocal plot for inhibition of rat brain adenylyl cyclase by inorganic manganese triphosphate.
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4 Activation of Gs (e.g., GTPγS, or GTPγS via hormone receptors) in an adenyl cyclase preparation has the effect of markedly lowering the enzyme's $K_m$ for Mg$^{2+}$.

5 The nomenclature for guanosine or adenine 3'-polyphosphates have been known for several decades, they have received relatively little attention in mammalian systems (50-58). Nucleoside 3'-polyphosphates have been of interest in the regulation or prokaryotes because guanosine 5'-bispolyphosphate can serve as an activator. This is because there are few if any adenine nucleotides that interact with only one protein or protein type, we suspect that adenosine 3'-polyphosphates may be involved in the regulation of development. For guanosine 3'-polyphosphates, the enzyme's $K_m$ for Mg$^{2+}$.

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The likelihood that adenosine 3'-polyphosphates may also occur naturally is suggested. Although these more rigorous studies have been done in bacterial systems, the reported presence of both guanosine and adenosine 3'-5'-bis-polyphosphates in cultured mammalian cells suggests that they may be involved in comparable responses also in these more complex systems (58). The concentration of pppApp was estimated to be 7–7% of that of GTP, or in the low micromolar range, and similar results were obtained with Chinese hamster ovary and BHK-21 cells (58). This could result in 3'-ATP levels consistent with the $K_m$ for inhibition of adenyl cyclase. Thus, an increase in the levels of adenosine or 2'-deoxyadenosine 3'-polyphosphates would precipitate P-site-mediated inhibition of adenyl cyclase, lowered cellular levels of 3:5-cAMP, and all the downstream effects this would cause. The effectiveness of this form of regulation may differ among tissues, reflecting differential expression of adenyl cyclase isozymes and their respective sensitivity to P-site-ligands.

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