Isozyme-dependent Sensitivity of Adenylyl Cyclases to P-site-mediated Inhibition by Adenine Nucleosides and Nucleoside 3’-Polyphosphates*

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Recombinant adenylyl cyclase isozyme Types I, II, VI, VII, and three splice variants of Type VIII were used to examine their isozyme-dependent sensitivity to inhibition by several adenine nucleoside derivatives and by the family of recently synthesized adenine nucleoside 3’-polyphosphates (Désaubry, L., Shoshani, I., and Johnson, R. A. (1996) J. Biol. Chem. 271, 14028–14034). Inhibitory potencies were dependent on isozyme type, the mode of activation of the respective isozymes, and on P-site ligand. For the nucleoside derivatives, potency typically followed the order 2’,5’-dideoxyadenosine (2’,5’-ddAdo) > β-adenosine > 9-(cyclopentyl)-adenine (9-CP-Ade) > 9-(tetrahydrofuryl)-adenine (9-THF-Ade; SQ 22,536), with the exception of Type II adenylyl cyclase, which was essentially insensitive to inhibition by 9-CP-Ade. For the adenine nucleoside 3’-polyphosphates, inhibitory potency followed the order Ado > 2’,dAdo < 2’,5’,dAdo and 3’-mono- < 3’-di- < 3’-triphosphate. Differences in potency of these ligands were noted between isozymes. The most potent ligand was 2’,5’-dd-3’-ATP with IC50 values of 40–300 nM. The data demonstrate that selectivity for some ligands, suggesting the possibility of isozyme-selective inhibitors to take advantage of differences in P-site domains among adenylyl cyclase isozymes. Differential expression of adenylyl cyclase isozymes may dictate the physiological sensitivity and hence importance of this regulatory mechanism in different cells or tissues.

Adenylyl cyclase is potently and directly inhibited by analogs of adenosine via a domain referred to as the P-site from its requirement for an intact purine moiety (1–4). Domains for catalysis and inhibition have been distinguished by use of enzyme purification (5, 6), inhibition kinetics (7, 8), site-specific covalent ligands (9), and selective amino acid substitutions (10). These data suggest that the P-site is distinct from, yet homologous to and interacting with, the catalytic domain. The observation that purified native and recombinant Type I adenylyl cyclases are inhibited by P-site ligands, although exhibiting decreased sensitivity to inhibition (4–6, 11), establishes the locus of the P-site on the enzyme per se and that inhibition is not via cell surface receptors or G-proteins.

P-site-mediated inhibition has been characterized pharmacologically (1, 2, 4, 12–16). Inhibition requires an intact adenine moiety, and potency of inhibition is increased substantially for deoxyribose and especially 3’-phosphorylribose adenine nucleosides. Inhibitory potency follows the order: 3’-mono- < 3’-di- < 3’-triphosphate and adenosine (Ado) < 2’-deoxy (d)1-Ado < 2’,5’-d-ddAdo, with 2’,5’-dd-3’-ATP being the most potent ligand and exhibiting an IC50 ~40 nM (15). In addition, tolerance for large substitutions at the 3’ site and for other ribose modifications has been demonstrated (1, 2, 4).

We reported previously that levels of 2’d-3’-AMP and 3’-AMP varied considerably in different tissues and were dependent on the metabolic state of the animal (17). Moreover, sensitivity of adenylyl cyclases to inhibition by these nucleotides varied among rat tissues. We suggested that diversity in P-site-mediated inhibition depended on the adenylyl cyclase isoform and that this diversity may well dictate differences in pharmacological and possibly physiological influence of this inhibition in the respective tissues (17). This is also suggested from studies in which P-site agonists, e.g. 2’,5’-dAdo or 9-THF-Ade, have been used to alter function in a variety of cell systems, e.g. (14, 18–23). Although effects on cell function and on cellular cAMP levels have been uniformly consistent with P-site inhibition of adenylyl cyclase, potencies of a given compound differed among systems, and unexpected potencies of some ligands were noted in others. Since it was not possible in those earlier studies to establish which adenylyl cyclase isozyme was(were) present, it is possible that variations in expected behavior may well have been due to differences in levels of expression of the several isozymes.

To evaluate this diversity in response, we are reporting the first investigation of P-site-mediated inhibition of several recombinant types of adenylyl cyclase. This study compares potency of several P-site ligands to inhibit Type I (24), Type II (25), Type VI (26), Type VII (27), and three splice variants of Type VIII (28, 29) adenylyl cyclases. These adenylyl cyclase isozymes were selected because they represent forms of the enzyme exhibiting distinct regulatory characteristics (30). For example, all are activated by Gα but Types I and VIII are

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activated also by Ca\(^{2+}\)/calmodulin. When Type I enzyme is activated by G\(_{\beta\gamma}\), it is inhibited by G\(_{\beta\gamma}\) (11, 31, 32), whereas when Type II adenylyl cyclase is activated by G\(_{\alpha}\), its activity is increased further by G\(_{\beta\gamma}\) (31, 32). The Type VI enzyme responds to G\(_{\alpha}\) (26) but is not activated by Ca\(^{2+}\)/calmodulin or G\(_{\beta\gamma}\).

### EXPERIMENTAL PROCEDURES

**Preparation and Assay of Adenylyl Cyclases from Rat and Bovine Brain**—Both detergent-solubilized and particulate preparations of adenylyl cyclase from rat and bovine brains were prepared and assayed as described previously (4, 5, 8, 9, 17). Enzyme assays were conducted in duplicate or triplicate. IC\(_{50}\) values were derived from inhibition curves comprising six to eight concentrations of inhibitor in two to eight experiments for each condition.

**Preparation of Expressed Recombinant Adenylyl Cyclases**—Membranes and membrane extracts were prepared as described previously (25, 26, 32) from fall army worm ovarian (Sf9) cells infected with baculovirus encoding Type I, Type II, or Type VI adenylyl cyclase or vector alone (thyroid peroxidase controls). Cells were lysed by N\(_2\) cavitation, and the particulate fraction was collected by centrifugation. The membranes from Sf9 cells encoding Type I adenylyl cyclase (32) were resuspended in a buffer containing 20 mM HEPES, pH 8, 1 mM EDTA, 6% sucrose, 5 mM MgCl\(_2\) and 1 mM dithiothreitol. Membranes from Sf9 cells encoding Type II, Type VI, and thyroid peroxidase (22, 26) were resuspended in a buffer containing 20 mM HEPES, pH 8, 1 mM EDTA, 2 mM dithiothreitol, 200 mM sucrose, 1 mM phenethylsulfonyl fluoride, and 2 \(\mu\)g/ml each of leupeptin and aprotinin. Detailed procedures for the preparation of Type II and Type VI enzyme have been described (33). All membranes were diluted at least 10-fold with a buffer containing 20 mM HEPES, pH 8, and 5% glycerol before being diluted further into the adenylyl cyclase assay containing the same buffer.

**Expressed Enzymes**—Type VII (27) and the splice variants of Type VIII (29) were expressed in HEK 293 cells (ATCC CRL 1573) as described previously. HEK cells expressing Type VII enzyme were washed in phosphate-buffered saline (prepared from Sigma tablets P-4417), scraped from the dishes into a small portion of the buffered saline, collected by centrifugation, and then stored at \(-80^\circ\) C. Membranes were prepared following lysing of the cells by N\(_2\) cavitation as described previously (28). 9-Cyclopentyl-9H-adenine Methanesulfonate (9-CP-Ade)—9-Cyclopentyl-9H-adenine was synthesized by amination of the corresponding 6-chloropurine that was itself prepared by alkylation, as adapted from Montgomery and Temple (34). A mixture of 6-chloropurine (5.57 g, 36 mmol), cyclopentyl bromide (6.56 g, 44 mmol), and K\(_2\)CO\(_3\) in dimethyl sulfoxide (80 ml) was stirred at ambient temperature for 3 days. The reaction medium was diluted with water (700 ml), extracted with EtOAc (3 \(\times\) 150 ml), washed with brine, dried over \(\text{MgSO}_4\), and purified by flash chromatography on silica gel; elution was with EtOAc:hexane (9:1) yielding 4.10 g (51%) of 9-Chloro-9-cyclopentyl-9H-purine. A solution of 6-chloro-9-cyclopentyl-9H-purine (0.67 g, 3 mmol) and ammonia (20 mmol) in ethanol was heated at 90 \(^\circ\) C for 24 h. The medium was evaporated in vacuo, and the residue was dispersed in brine (20 ml), extracted with EtOAc (3 \(\times\) 50 ml), washed with brine, dried over \(\text{MgSO}_4\), and purified by flash chromatography on silica gel, eluting with EtOAc:MeOH (95:5), yielding 0.36 g (59%) of 9-CP-Ade. The methanesulfonate salt was prepared by adding one equivalent of methanesulfonic acid to a solution of the free base in isopropyl alcohol. The crude salt was recrystallized from isopropyl alcohol.

**Adenosine 3′-Diphosphate (3′-ADP) and Adenosine 3′-Triphosphate**

(Fig. 1. Scheme for synthesis of 3′-ADP and 3′-ATP. The symbols \(\Delta\) indicate sonication.

FIG. 1. Scheme for synthesis of 3′-ADP and 3′-ATP. The symbols \(\Delta\) indicate sonication.

FIG. 2. Elution profile of reaction products from the synthesis of 3′-ADP and 3′-ATP. A portion of the reaction mixture after 10-h sonication from the synthesis shown in Fig. 1 was subjected to high performance anion exchange chromatography, as described under “Experimental Procedures.” Elution was with a discontinuous gradient of triethylammonium bicarbonate (Et\(_3\)NH\(_2\)CO\(_3\)), as indicated.

FIG. 3. Inhibition of recombinant forms of adenylyl cyclase isozymes by 3′-d-3′-AMP. Activities were determined with 100 \(\mu\)M ATP, 5 mM MnCl\(_2\), and 100 \(\mu\)M forskolin. (3′-ATP)—3′-ADP was synthesized by a modification of the methods described by Mitchel et al. (35) and Sheridan et al. (36) (Fig. 1). The principal modifications are the use of sonication to facilitate the reaction, which otherwise would have been allowed to continue for 150 h (35), and the use of anion exchange chromatographic techniques to separate the several polyphosphorylated derivatives of adenosine obtained. This modified procedure represents a one-step preparation of nucleoside 3′-di- and 3′-triphosphates from the corresponding monophosphate and is characterized by a short reaction time and convenient reaction conditions.

A suspension of 3′-AMP (1 g, 2.8 mmol) and phosphoramic acid (1.63 g, 16.8 mmol) in 50 ml of formamide was sonicated under an argon atmosphere overnight. The temperature was maintained at 20–25 \(^\circ\) C by putting the ultrasonication bath in a cold box at 4 \(^\circ\) C. The medium was cooled to 5 \(^\circ\) C, diluted with 2 liters of cold water (5 \(^\circ\) C), and neutralized with triethylamine (2.8 ml, 20 mmol). Reaction products were purified on QAE-Sephadex (HCO\(_3\) form) with a linear gradient of triethylammonium bicarbonate (0.01–0.3 M) followed by an isocratic elution with 0.3 M triethylammonium bicarbonate and then another linear gradient with triethylammonium bicarbonate (0.3–1 M) (Fig. 2). The appropriate fractions were lyophilized and then coevaporated several times with methanol, yielding 380 \(\mu\)mol (14%) of 3′-ADP and 14.6 \(\mu\)mol 3′-ATP. Both nucleotides were isolated as their respective sodium salts by precipitation in 1 M sodium iodide in acetone from the methanol solution of the triethylammonium nucleotide. The precipitate was centrifuged and washed three times with cold acetone and dried in vacuo giving the sodium salts of 3′-ADP and 3′-ATP. No impurities were noted.
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Activation of adenylyl cyclase isozymes by different stimulatory agents and IC₅₀ values for inhibition by 2'-d-3'-AMP

Table I

| Enzyme type | Mn²⁺ | Mn²⁺ | Mg²⁺ | Mg²⁺ |
|-------------|------|------|------|------|
| Velocity   | IC₅₀ | Velocity | IC₅₀ | Velocity | IC₅₀ |
| pmol/(min mg) | µM | pmol/(min mg) | µM | pmol/(min mg) | µM |
| I           | 669 ± 47 | 3.9 | 3,513 ± 155 | 5.9 | 160 ± 36 | 32 |
| II          | 143 ± 5  | 9.6 | 1,080 ± 226 | 23.8 | 21 ± 4 | 9.6 |
| VI          | 77 ± 15  | 17  | 1,343 ± 250 | 10.5 | 3 ± 1 | ND |
| Rat brain   | 1.2     |      | 3.3       |      |        |      |

a ND, not determined.

Table II

| Enzyme type | 2'-d-3'-AMP | 2',5'-dAD | β-Ado | 9-(Cyclopentyl)-adenine | 9-(Tetrahydrofuryl)-adenine |
|-------------|-------------|-----------|-------|------------------------|----------------------------|
|             | µM          |           |       |                        |                            |
| I           | 15         | 17        | 1,343 | 1,343                  | 3,263                      |
| II          | 15         | 17        | 3,3   | 117                    | 1,343                      |
| VI          | 15         | 17        | 9     | 117                    | 1,343                      |
| VIIIA       | ND         |           |       | ND                     |                            |
| Thyroid peroxidase | 4.1 | 2.3 | 58 | 200 | ND |

a Determined with 500 µM MnATP and 5 mM MnCl₂ as substrate and activating cation.
b ND, not determined.

d By high performance liquid chromatography on a Spherosil TSK DEAE-5PW column eluted with a gradient of triethylammonium bicarbonate. The following spectra are for 3'-ADP. 1H NMR (D₂O) δ 3.81 (d, 2H, J = 2.8 Hz, H-5') and 3.59 (d, 2H, J = 2.8 Hz, H-5'), 4.36 (m, 1H, H-3'), 4.72 (m, 1H, H-4'), 3.52 (m, 1H, H-5'), 8.05 (s, 1H, H-2'), 4.82 (s, 1H, H-6'), 4.72 (s, 1H, H-6'), 8.70 (s, 1H, H-2'), 1.06 (s, 3H, H-3'), 1.52 (s, 3H, H-3'), 2.06 (s, 3H, H-3'), 2.58 (s, 3H, H-3'). 31P NMR (D₂O) δ -5.94 (dd, J₆₋₅ = 7.6 Hz, J₉₋₅ = 20.6 Hz, P-1), -1.34 (d, J = 20.5 Hz, P-2). NMR spectra were recorded with a Bruker AC250 at 250 MHz for proton spectra and 101 MHz for 31P spectra, with a 95% solution of H₃PO₄ as external standard.

Materials—[α-32P]ATP was purchased from ICN Pharmaceuticals. Lubrol-PX was filtered through alumina (Neutral, AG7, from Bio-Rad) to remove peroxides. Dimethyl sulfoxide was redistilled under partial vacuum over CaH₂. 2',5'-dAD and the 3'-polyphosphates of 2',5'-dAD and 2'-dAD were synthesized by methods reported previously from this laboratory (9, 15, 16). 9-THF-Ade (SQ 22,536) was a gift from Salvo Lucania, Bristol-Myers-Squibb, Pharmacology Research Institute, P.O. Box 4000, Princeton, NJ 08543.

RESULTS

Activation of Adenylyl Cyclases and Sensitivity to P-site Ligands—Previously reported data suggested tissue-dependent differences in inhibitory potency of P-site ligands and also showed that activation of the enzyme by various agents resulted in different sensitivity of the enzyme to inhibition (17). To ascertain whether these differences would be reflected in specific isozymes, the potency of 2'-d-3'-AMP was evaluated on recombinant bovine Type I, Type II, and Type VI adenylyl cyclases (Fig. 3 and Table I). IC₅₀ values for inhibition of the rat brain enzyme by 2'-d-3'-AMP are shown for comparison. As expected, various stimulatory agents resulted in substantially different activities, with the greatest activity being uniformly obtained when assays were conducted with 5 mM Mn⁺² and 100 µM forskolin. Reaction velocities with Mg⁺² and forskolin were roughly similar to those noted with Mn⁺² alone, and membranes assayed with Mg⁺² exhibited the lowest activities. Furthermore, the Type I enzyme was most potently inhibited, whether activation was with Mn⁺² (IC₅₀ ~4 µM) or Mn⁺² and forskolin (IC₅₀ ~6 µM). This greater sensitivity of the Type I enzyme is consistent with previously published data with enzyme derived from a number of tissues (1–4, 13, 14, 17). The small increase in IC₅₀ upon addition of forskolin is also consistent with earlier experiments with the bovine brain enzyme (5) but is in contrast with behavior of hepatic adenylyl cyclase (7) and the Type VI enzyme. Although forskolin stimulated each adenylyl cyclase, its effect to enhance potency of P-site ligands varied among isozymes. Hence, potency is uncoupled from enzyme activity, suggesting that the processes of catalysis and inhibition might not be causally linked.

Cell-permeable P-site Ligands—One goal of these studies has been to identify P-site ligands that could be used pharmacolog-
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Table III

Efficacy of adenine nucleoside 3’-polyphosphates to inhibit several expressed forms of adenylyl cyclase

| Nucleotide | Rat brain* | Enzyme type | A | B | C |
|------------|------------|-------------|---|---|---|
| 3’-AMP     | 8.9A       | I           | 40 | 90 | 45.9 |
| 3’-ADP     | 3.9C       | II          | 13.9 | 25 | 5.8 |
| 2’-d-3’-AMP | 1.2A       | VI          | 6.44 | 27.9 | 9.1 |
| 2’-d-3’-ADP | 0.14C     | VII         | 2.75 | 2.21 | 0.42 |
| 2’-d-3’-ATP | 0.09C     | VIIIA       | 0.70 | 0.60 | 0.26 |
| 2’,5’-dd-3’-AMP | 0.46B | A          | 1.94 | 2.66 | 0.60 |
| 2’,5’-dd-3’-ADP | 0.10B | B          | 0.25 | 1.12 | 0.53 |
| 2’,5’-dd-3’-ATP | 0.04B | C          | 0.17 | 0.28 | 0.15 |

* A, from Ref. 4. B, from Ref. 15. C, from Ref. 16.

Values are IC50 values (μM) and were derived graphically from inhibition curves comprising at least six concentrations of inhibitors, each assayed in duplicate or triplicate, in two to four separate experiments. Interexperiment variation is less than 50%. Activities were determined in the presence of 5 mM MnCl2, 100 μM forskolin, and 500 μM MnATP, except those taken from previously published work with enzyme from rat brain, for which MnATP was 100 μM.

Fig. 5. Inhibition of Type VI adenylyl cyclase by adenine nucleoside 3’-polyphosphates. Activities were determined with 100 μM ATP, 5 mM MnCl2, and 100 μM forskolin.

IC50 values for the Type II enzyme were between the 3’-di- and 3’-tri phosphate derivatives, whereas for the Type VI enzyme it was between the 3’-mono- and 3’-diphosphate derivatives (Table III and Fig. 5). For enzyme Types VI, VII, and all three splice variants of Type VIII, IC50 values were little affected by the addition of the third 3’-phosphate group. The data suggest that inhibition by this class of ligand is a universal feature of adenylyl cyclase isozymes but that the structure of the respective isozymes influences potency of ligands, whether with ribosyl 2’- and 5’-hydroxyl or 3’-mono-, 3’-di-, or 3’-triphosphate groups.

DISCUSSION

To begin establishing a basis for the known tissue dependence for inhibition of adenylyl cyclases by P-site ligands, the efficacy of several ribose-modified and 3’-polyphosphorylated adenosines has been tested on several recombinant forms of this enzyme family. The rationale for the study is that the physiological importance of this regulatory mechanism in a given tissue may be reflected in the sensitivity of the adenylyl cyclase expressed in that tissue. The degree of inhibition would depend on levels of P-site ligand, and these may change as a...
result of changes in cell function. Our additional consideration has been that ligands may be identified exhibiting selectivity toward individual isozymes.

The influence of P-site inhibition in a given tissue may also be influenced by factors affecting activity of adenylyl cyclases. Although the data did not conform to the notion that sensitivity to inhibition by P-site ligands increased as enzyme activity increased (7), they indicated that different means of activation of adenylyl cyclase led to differences in the IC50 values for inhibition (cf. Table I). For example, enzyme activity with Mn2+ + forskolin was uniformly greater than that with Mn2+ alone, whereas the presence of forskolin led to increased sensitivity to inhibition by 2‘-d-3‘-AMP (Table I) or 2‘,5‘-dAdo (not shown) with the Type VI enzyme but decreased sensitivity with Types I and II enzyme. Arguably the enzyme conformation resulting from activation by Mn2+ must differ from those resulting from Mn2+ + forskolin, Mg2+, GTPαs, GTPγs, and appropriate combinations of these. Different conformations will exhibit greater or lesser sensitivity to inhibition by these agents, and this influence will be isozyme-dependent and may affect the physiological role of this inhibition.

Potency of P-site ligands obviously depends also on ligand structure. This was particularly evident with the ribose-modified adenosine derivatives as the comparison was extended to several isozymes (Table II). For example, Type II adenylyl cyclase, unique in its regulation by G-protein subunits and susceptibility to phosphorylation by protein kinase C (31–33), was insensitive to inhibition by 9-CP-Ado and was poorly sensitive to 9-THF-Ado. The differences in P-site potency among the isozymes were more acutely noted with ribose-modified adenine nucleosides than with the 3′-nucleotides. The addition of the 3′-phosphates contributes substantial binding energy (10 kcal/phosphate; Ref. 37) and may minimize the effects of differences in isozyme structure on ligand binding; that is, the differences among isozymes, noted by IC50 values for 9-CP-Ado (Fig. 4), may be masked by the increased binding affinities of the nucleoside 3′-polyphosphates (Fig. 5). An avenue for the development of isozyme-selective ligands may be to exploit the differences established here with the ribose-modified adenine nucleosides and the dramatic inhibitory potency of the adenine nucleoside 3′-polyphosphates, thereby also aiding investigation of enzyme structure and mechanisms of catalysis and inhibition.

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