PRO-NUCLEOTIDE INHIBITORS OF ADENYLYL CYCLASES
IN INTACT CELLS

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SUMMARY

Several 9-substituted adenine derivatives with protected phosphoryl groups were synthesized and tested as inhibitors of adenylyl cyclase in isolated enzyme and intact cell systems. Protected 3'-phosphoryl derivatives of 2',5'-dideoxyadenosine (2',5'-dd-Ado) and β-L-2',5'-dideoxyadenosine, protected 5'-phosphoryl derivatives of β-L-2',3'-dideoxyadenosine, and protected phosphoryl derivatives of (R)-9-(2-phosphonomethoxypropyl)-adenine and of 9-(2-phosphonomethoxyethyl)adenine were synthesized. Phosphate protection was afforded by two cyclosaligenyl- or three S-acyl-2-thioethyl-substituents. Protected nucleotides were tested on two mouse preadipocyte cell lines (OB-1771 and F442A), on human macrophages, and on cardiac ventricular myocytes isolated from rat. With preadipocytes and macrophages the compounds were tested for their capacity to block the forskolin-induced increase in [3H]cAMP formation in cells prelabeled with [3H]adenine. A striking selectivity for 2',5'-dd-Ado-3'-phosphoryl derivatives was observed. Cyclosaligenyl-derivatives (IC$_{50}$ ~2 µM) were considerably less potent than S-acyl-2-thioethyl-derivatives. The best studied of these was 2',5'-dd-Ado-3'-O-bis(S-pivaloyl-2-thioethyl)-phosphate, which suppressed forskolin-stimulated [3H]cAMP formation (IC$_{50}$ ~30 nM) and the opening of cAMP-dependent Cl$^-$ channels in myocytes (IC$_{50}$ ~800 nM). None of the protected nucleotides inhibited adenylyl cyclases isolated from rat brain or OB1771 preadipocytes. These compounds exhibit the hallmarks of prodrugs. The data suggest that they are taken up, are deprotected, and are converted to a potent inhibitory form to inhibit [3H]cAMP formation, but only by intact cells. The relatively easy availability of these prodrugs and their very potent inhibition of adenylyl cyclase in intact cell systems should make them useful tools for blocking cAMP-mediated pathways, in biochemical, pharmacological, and potentially therapeutic contexts.
INTRODUCTION

Adenylyl cyclases are potently inhibited by adenine nucleoside polyphosphates [1-7]. These compounds have been useful as reversibly and irreversibly binding ligands [6,8] and for resolving aspects of the three-dimensional structure of the enzyme [9,10], but they are not useful for studies in intact cells because they do not cross cell membranes intact. In general, the problem of membrane permeability has been circumvented by the use of nucleosides in studies with intact cell systems. Consequently, to inhibit cAMP formation in intact cells investigators have relied on adenine nucleosides [e.g. 11-18]. Although cell permeable, adenine nucleosides are much less potent than the corresponding nucleoside polyphosphates and because of the relatively high concentrations needed to inhibit adenylyl cyclases may have unintended side effects [2-4, 6,7,11,19-24]. To circumvent the general problem of poor permeability of nucleotides in intact cells, biodegradable lipophilic groups have been chemically linked to nucleoside phosphate group(s), giving families of pro-nucleotides that are uncharged and membrane permeable [25-29]. These are typically nucleoside monophosphates with a protected phosphate group, with protection being afforded by any of several substituents [30-37].

The protected nucleoside monophosphate has four beneficial characteristics. First, it is cell permeable. Second, the otherwise highly selective and often rate-limiting initial phosphorylation of a nucleoside is bypassed because the nucleoside monophosphate has been transported into the cell [25-31]. The nucleoside monophosphate can then readily be phosphorylated by intracellular enzymes to the corresponding polyphosphate, often the most potent and desired form. Third, its specificity can be controlled by use of specific bases or nucleosides. And fourth, its efficacy to affect cells and tissues can be controlled by the choice and character of the phosphate-protecting moieties. The efficacy of pro-nucleotides relies on their uptake, their intracellular deprotection, either spontaneously [34-36] or enzymatically [27,30-33], their rapid subsequent phosphorylation to the polyphosphate form, as well as the specificity and potency of their interaction with the target protein [25-29, 31,39]. This idea has been particularly successful in the development of prodrug inhibitors of viral reverse transcriptases [e.g. 25-39].
We previously reported the synthesis of a cholesteryl-derivative of 2',5'-dd-3'-AMP as a potential pro-
nucleotide inhibitor of adenylyl cyclase [40]. Although this compound exerted effects on intact cells, it was
difficult to handle because of poor solubility in most solvents usable with cells. Presented here are the
syntheses of protected phosphate derivatives of 9-substituted adenines and their effects on adenylyl cyclase
in isolated enzyme and intact cell systems. From earlier studies three classes of compounds emerged as
potentially useful inhibitors of adenylyl cyclases. One is typified by β-L-2',3'-dideoxyadenosine-
5'-triphosphate (β-L-2',3'-dd-5'-ATP \(^1\); IC\(_{50}\) ~24 nM [6]), a competitive inhibitor of the enzyme. Second, is the
comparably potent 2',5'-dd-3'-ATP \(^1\); IC\(_{50}\) ~40 nM [2,3]), a non-competitive post-transition-state inhibitor.
And third, are the stable acyclic phosphonate derivatives of adenine, PMEApp \(^1\) (IC\(_{50}\) ~175 nM [7]) and
PMPApp \(^1\). These are also known inhibitors of viral reverse transcriptases and prodrug forms of their
respective monophosphates, PMEA and PMPA, have been made [31,39,41,42]. In the studies reported here,
phosphate protection was afforded by two cyclosaligenyl- (cycloSAL) [29,34-36], or three S-acyl-2-thioethyl-
(SATE) [33,39] substituents, with different acyl groups. Processing of the cycloSAL-derivatives occurs by
spontaneous hydrolysis [29,34-36], whereas deprotection of the SATE-derivatives relies on intracellular
carboxyesterases and a subsequent intramolecular reaction [31,32]. A striking selectivity among these
compounds was noted and a class of agents that can be used in intact cell and tissue systems to inhibit cAMP
formation has been identified. Presented here are the first examples of potent prodrugs that can be delivered
to intact cells to inhibit adenylyl cyclase.
EXPERIMENTAL PROCEDURES

Materials - 2',5'-dd-Ado, 2',5'-dd-3'-AMP, 2',5'-dd-3'-ADP, 2',5'-dd-3'-ATP, 2',5'-dd-3'-A4P, β-L-2',3'-dd-Ado, β-L-2',3'-dd-5'-AMP, and β-L-2',3'-dd-5'-ATP were synthesized as previously described [1-4,6]. PMEA was prepared as described by Benzaria et al. [39] and the synthesis of PMPA followed methods of Holy and co-workers [43,44]. The enantiomers, (1S, 2R)- and (1R,2S)-1-(6-aminopurin-9-yl)-2-(hydroxymethyl)-cyclopentane, used as precursors for the synthesis of (1S,2R)- and (1R,2S)-9-(cyclopentyl)-Ade-2'-phosphate-bis(t-Bu-SATE), were generous gifts from Dr. Fritz Theil, ASCA GmbH, Berlin-Adlershof, Germany. The triphosphate forms of these compounds, (1S,2R)- and (1R,2S)-9-(cyclopentyl)-adenosine-2'-triphosphate, were generous gifts of Dr. Martin von Janta-Lipinski, Max-Delbrück Center for Molecular Medicine, Berlin-Buch, Germany. 5'-ATP, cAMP, and adenosine deaminase (bovine spleen; #A5178) were from Sigma. Lubrol-PX, used to solubilize rat brain adenylyl cyclase and in enzyme assays, was from Sigma and before use was filtered through alumina (Neutral, AG7, from Bio-Rad Laboratories) to remove peroxides. [α-32P]-5'ATP (25 Ci / mmol) was from International Chemical and Nuclear Corp. Creatine kinase and creatine phosphate, used in adenylyl cyclases assays to maintain ATP levels, were from Boehringer/Mannheim. 1-Methyl-3-isobutylxanthine (IBMX) was from Aldrich and forskolin was from Calbiochem. Pro-nucleotides used in these studies were synthesized as described below.

2',5'-dideoxyadenosine-3'-O-cyclosaligenyl-phosphates (1 and 2 in Figure 1) - To a stirred solution of 2',5'-dd-Ado (141 mg, 0.60 mmol) in 10 ml of dimethylformamide was added diisopropylethylamine (116 mg, 0.90 mmol, 1.5 eq) followed by the dropwise addition at -40 °C of a solution of the cyclic chlorophosphane (0.90 mmol, 1.5 eq/unsubstituted, 170 mg (for compound 1); 182 mg (for compound 2)), in 1 ml of tetrahydrofuran as per Scheme 1. The cyclic chlorophosphanes were synthesized according to Meier [29,45]. The reaction mixture was stirred for 30 min and tert-butylhydroperoxide (0.2 ml, 1.6 mmol, 2.7 eq, 90 % in tert-butanol) was added. The mixture was stirred for 30 min at room temperature and evaporated to dryness under reduced pressure. The residue was purified by column chromatography with silica gel with a stepwise gradient of 0 to 4% methanol in dichloromethane. The products were isolated as colorless foams.
2',5'-dideoxyadenosine-3'-O-cycloSal-Phosphate (2',5'-dd-3'-AMP-(H-Sal); 1 in Figure 1) - Yield, 190 mg (0.47 mmol, 79%); TLC: Rf = 0.29 (methylene chloride/methanol = 93:7, v/v), Rf = 0.16 (ethyl acetate/methanol = 95:5, v/v). HPLC: Rt = 33.2 min. 1H NMR (DMSO-d6): δ = 8.69, 8.34, 8.32 and 8.12 (s, 2H, H-2 and H-8, two diastereomers), 7.44-7.05 (m, 5H, 3H C6H4 and NH2), 6.89-6.73 (m, 1H, 1H C6H4), 6.33-6.27 (m, 1H, H-1'), 5.58-5.30 (m, 2H, CH2), 5.15 (m, 1H, H-3'), 4.26-4.15 (m, 1H, H-4'), 3.23-3.17 (m, 1H, H-2'), 2.68-2.61 (m, 1H, H-2''), 1.34-1.24 (m, 3H, CH3). 31P NMR (DMSO-d6): δ = -8.56, -9.60.

2',5'-dideoxyadenosine-3'-O-cycloMeSal-Phosphate (2',5'-dd-3'-AMP-(Me-Sal); 2 in Figure 1) - Yield, 200 mg (0.48 mmol, 80%); TLC: Rf = 0.31 (methylene chloride/methanol = 93:7, v/v), Rf = 0.17 (ethyl acetate/methanol = 95:5, v/v); HPLC: Rt = 34.1 min. 1H NMR (DMSO-d6): δ = 8.65, 8.33, 8.32 and 8.12 (s, 2H, H-2 and H-8, two diastereomers), 7.30-7.03 and 6.72 (m, 5H, 3H C6H3 and NH2), 6.30-6.27 (m, 1H, H-1'), 5.54-5.48 (m, 2H, CH2), 5.18-5.15 (m, 1H, H-3'), 4.22-4.11 (m, 1H, H-4'), 3.29-3.21 (m, 1H, H-2'), 2.67-2.60 (m, 1H, H-2''), 2.27-2.15 (m, 3H, CH3 saligenyl), 1.37-1.25 (m, 3H, CH3). 31P NMR (DMSO-d6): δ = -8.98, -9.02.

2',5'-dideoxyadenosine-3'-O-bis(S-acyl-2-thioethyl)-Phosphates (2',5'-dd-3'-AMP-bis(SATE); 3, 4, and 5 in Figure 1) - To a stirred solution of 2',5'-dd-Ado (100 mg, 0.42 mmol) in a mixture of tetrahydrofuran and dimethylformamide (1.5 ml, 2:1, v/v) was added 1H-tetrazole (88 mg, 1.26 mmol, 3.0 eq) followed by the dropwise addition at room temperature of a solution of the bis(S-2-hydroxyethylthioacyl)-N,N-diisopropylphosphoramidite, where the -acyl- group was -acetyl-, -pivaloyl-, or -benzoyl- (0.51 mmol, 1.25 eq of -acetyl-, 188 mg (for compound 3); -pivaloyl-, 230 mg (for compound 4); or -benzoyl-, 252 mg (for compound 5)), in 0.5 ml of tetrahydrofuran. The S-acyl-2-thioethanols and bis(S-2-hydroxyethylthioacyl)-N,N-diisopropylphosphoramidites were synthesized according to Lefebvre et al. [33]. The reaction mixture was stirred for 45 min and then cooled to -40°C and tert-butylhydroperoxide (0.44 ml, 1.26 mmol, 3.0 eq, 90% in tert-butanol) was added. The reaction mixture was stirred for 30 min at room temperature and then diluted with 30 ml of dichloromethane. It was subsequently washed with 10 ml of a 10% aqueous sodium sulfite solution and then 10 ml of water. The organic phase was dried with sodium sulfate and was
evaporated to dryness under reduced pressure. The residue was purified by column chromatography on silica
gel with a step gradient of 0 to 4% methanol in dichloromethane. The products were isolated as colorless
foams.

2',5'-dideoxyadenosine-3'-O-bis(S-acetyl-2-thioethyl)-phosphate (2',5'-dd-3'-AMP-bis(Me-SATE); 3 in Figure 1) - Yield, 100 mg (0.19 mmol, 46%); TLC: Rf = 0.37 (methylene chloride/methanol = 93:7, v/v),
Rf = 0.16 (ethyl acetate/methanol = 95:5, v/v). HPLC: Rt = 34.2 min. ¹H NMR (DMSO-d₆): δ = 8.35
and 8.16 (2s, 1H each, H-2 and H-8), 7.30 (br s, 2H, NH₂), 6.32 (t, J = 6.9 Hz, 1H, H-1'), 4.96-4.94 (m, 1H, H-3'),
4.23-4.19 (m, 1H, H-4'), 4.51-4.07 (m, 4H, 2×CH₂O), 3.26-3.15 (m, 1H, H-2' partially obscured by t, J = 6.3 Hz, 3H, CH₃), 1.34 (d, J = 6.4 Hz, 3H, CH₃). ³¹P NMR (DMSO-d₆): δ = -1.47.

2',5'-dideoxyadenosine-3'-O-bis(S-pivaloyl-2-thioethyl)-phosphate (2',5'-dd-3'-AMP-bis(t-Bu-SATE); 4 in Figure 1) - Yield, 190 mg (0.31 mmol, 75%); TLC: Rf = 0.45 (methylene chloride/methanol = 93:7, 
v/v), Rf = 0.27 (ethyl acetate/methanol = 95:5, v/v). HPLC: Rt = 42.8 min. ¹H NMR (DMSO-d₆): δ = 8.56
and 8.35 (2s, 1H each, H-2 and H-8), 6.37 (t, J = 6.7 Hz, 1H, H-1'), 4.95 (m, 1H, H-3'), 4.25-4.22 (m, 1H, H-4'),
4.14-4.06 (m, 4H, 2×CH₂O), 3.59 (br s, 2H, NH₂), 3.18-3.13 (m, 1H, H-2' partially obscured by t, J = 6.2 Hz, 2H, 2×CH₂S), 2.68-2.64 (m, 1H, H-2''), 1.35 (d, J = 6.4 Hz, 3H, CH₃), 1.18 and 1.16 (2s, 9H each, 2
C(CH₃)₃). ³¹P NMR (DMSO-d₆): δ = -1.47.

2',5'-dideoxyadenosine-3'-O-bis(S-benzoyl-2-thioethyl)-phosphate (2',5'-dd-3'-AMP-bis(Ph-SATE); 5 in Figure 1) - Yield, 170 mg (0.26 mmol, 63%); TLC: Rf = 0.33 (methylene chloride/methanol = 93:7, 
v/v), Rf = 0.19 (ethyl acetate/methanol = 95:5, v/v). HPLC: Rt = 41.6 min. ¹H NMR (DMSO-d₆): δ = 8.31
and 8.14 (2s, 1H each, H-2 and H-8), 6.82-7.87, 7.69-7.64, 7.55-7.50 (m, 2Cl₄H₄), 3.70 (br s, 2H, NH₂),
6.30 (t, J = 6.9 Hz, 1H, H-1'), 4.97 (m, 1H, H-3'), 4.29-4.19 (m, 5H, H-4' and 2×CH₂O), 3.41 (t, J = 6.1 Hz, 4H, 2×CH₂S), 3.26-3.13 (m, 1H, H-2'), 2.62-2.54 (m, 1H, H-2''), 1.29 (d, J = 6.8 Hz, 3H, CH₃). ³¹P NMR (DMSO-d₆): δ = -1.44.
9-(2,5-Dideoxy-β-L-erythro-pentofuranosyl)adenine (β-L-2',5'-dd-Ado) - By use of the procedure previously described in the D-series by Désaubry et al. [40], β-L-2'-deoxyadenosine (0.7 g, 2.8 mmol) was dissolved in pyridine (10 ml) and treated with diphenyldisulfide (0.76 g, 3.5 mmol) and tributylphosphine (0.87 ml, 3.5 mmol) (Scheme 2). After 24 h, 10 ml of methanol was added, the solution was concentrated to dryness and co-evaporated with toluene, then was co-evaporated with methanol. The resulting material was subjected to column chromatography on silica gel with a stepwise gradient of 0 to 12% methanol in dichloromethane to give the thio-intermediate (0.70 g, 73%). The thio-intermediate (0.7 g, 2.04 mmol) was dissolved in a mixture of methanol-isopropanol (1:1, v/v, 10 ml) and treated with Raney Nickel (1 g). The suspension was refluxed for 10 days, then Nickel was removed and washed by three portions of boiling methanol. The combined filtrates were concentrated to dryness and the residue was purified by column chromatography on silica gel with an isocratic gradient of 10% methanol in dichloromethane to give β-L-2',5'-dd-Ado (282 mg, 59%).

β-L-2',5'-dd-Ado - Rf (MeOH/CH2Cl2, 15/85) = 0.33; mp: 173° C; UV (EtOH 95) λmax 260 nm (ε, 14600), λmin 227 nm (ε, 2200); 1H NMR (DMSO-d6) δ = 8.28 (s, 1H, H-8), 8.13 (s, 1H, H-2), 7.22 (br s, 2H, NH2-6), 6.26 (t, 1H, H-1', J = 6.7 Hz), 5.30 (br s, 1H, OH-3'), 4.19 (m, 1H, H-3'), 3.9-3.8 (m, 1H, H-4'), 2.8-2.7 (m, 1H, H-2'), 2.3-2.2 (m, 1H, H-2''), 1.24 (d, 3H, CH3-5', J = 6.4 Hz); 13C NMR (DMSO-d6) δ = 156.9 (C-6), 153.4 (C-2), 150.0 (C-4), 140.3 (C-8), 120.0 (C-5), 83.6 (C-1'), 83.2 (C-4'), 75.5 (C-3'), 39.3 (CH2-2'), 19.9 (CH3-5'); MS FAB>0 m/z 236 (M+H)+, 136 (BH2)+, MS FAB<0 m/z 234 (M-H); [α]D20 = -39 (c, 0.96, DMSO).

9-[2,5-Dideoxy-3-O-[bis(S-pivaloyl-2-thioethyl)-phosphate]-β-L-erythro-pentofuranosyl]-adenine (6 in Figure 1) - Adapting the procedure for the synthesis of -O-bis(S-acyl-2-thioethyl)-pro-nucleosides [33], sublimed 1H-tetrazole (61 mg, 0.88 mmol) was added to a stirred solution of β-L-2',5'-dd-Ado (69 mg, 0.29 mmol) in a mixture of tetrahydrofuran and dimethylformamide (1.5 ml, 2:1, v/v), followed by the dropwise addition of a solution of bis(S-2-hydroxyethylthioacyl)-N,N-diisopropyl-phosphoramidite (-pivaloyl- 160 mg, 0.42 mmol, for compound 6 in Figure 1) in 0.5 ml of tetrahydrofuran at room temperature. After 45 min, a
3 M solution of tert-butylhydroperoxide (0.293 ml, 0.88 mmol) in toluene was added. After further stirring at room temperature for 30 min, the mixture was diluted with dichloromethane and was washed successively with 10% aqueous sodium sulfite solution and then with water. The organic layer was dried over sodium sulfate, filtered, and evaporated to dryness under reduced pressure. Purification of the residue by column chromatography on silica gel with a stepwise gradient of 0 to 4% methanol in dichloromethane afforded 9-{2,5-dideoxy-3-[bis(S-pivaloyl-2-thioethyl)phosphate]-β-L-erythro-pentofuranosyl}adenine (β-L-2',5'-dd-3'-AMP-bis(t-Bu-SATE); compound 6 in Figure 1; 65 mg, 56%) as a colorless syrup.

\[ β-L-2',5'-dideoxyadenosine-3'-O-bis(S-pivaloyl-2-thioethyl)-phosphate \] (6 in Figure 1)

\[ R_f (\text{MeOH}/\text{CH}_2\text{Cl}_2, 10/90) = 0.39; \text{UV (EtOH 95)} \lambda_{max} 259 \text{ nm (ε, 14200)}, 236 \text{ nm (ε, 10300)}, \lambda_{min} 240 \text{ nm (ε, 10100)}; ^1\text{H NMR (CDCl}_3\) δ = 8.28 (s, 1H, H-8), 7.87 (s, 1H, H-2), 6.30 (t, 1H, H-1', J = 6.8 Hz), 5.58 (br s, 2H, NH₂-6), 4.9-4.8 (m, 1H, H-3'), 4.3-4.2 (m, 1H, H-4'), 4.1-4.0 (m, 4H, 2×CH₂O), 3.2-3.0 (m, 5H, 2×CH₂S and H-2'), 2.7-2.6 (m, 1H, H-2''), 1.40 (d, 3H, CH₃-5', J = 6.6 Hz), 1.17 and 1.16 (2s, 18H, 2×(CH₃)₃C); ^13\text{C NMR (CDCl}_3\) δ = 206.1 (2′CO), 155.8 (C-6), 153.5 (C-2), 150.1 (C-4), 139.6 (C-8), 120.9 (C-5), 84.8 (C-1'), 82.1 (C-4'), 66.8 (2×CH₂O), 66.7 (C-3'), 47.0 (2×C(CH₃)₃), 38.1 (CH₂-2'), 28.9 (2×CH₂S), 27.7 (2×(CH₃)₃C), 19.6 (CH₃-5'); ^31\text{P NMR (CDCl}_3\) δ = -2.05; MS FAB>0 m/z 604 (M+H)⁺, 136 (BH₂)⁺, MS FAB<0 m/z 458 (M-t-BuSATE)-, 134 (B)-. HPLC: elution with a linear gradient of 0% to 80% acetonitrile in 20 mM triethylammonium acetate (pH 7) over 30 min at 1 ml/min, Rt = 25.8 min (99.67% area); isocratic elution with 45% acetonitrile in 20 mM triethylammonium acetate (pH 7) at 1 ml/min, Rt = 13.7 min (99.70% area). HRSM Calcd for C₂₄H₃₉N₅O₇PS₂ (M+H)⁺: 604.2113. Found : 604.2029.

\[ β-L-2',3'-dideoxyadenosine-5'-O-bis(S-acetyl-2-thioethyl)-phosphate \] (7, 8, and 9 in Figure 1)

To a stirred solution of β-L-2',3'-dd-Ado (44 mg, 0.187 mmol) in a mixture of tetrahydrofuran and dimethylformamide (1.5 ml, 2:1, v/v) was added 1H-tetrazole (39 mg, 0.56 mmol, 3.0 eq) followed by the dropwise addition at room temperature of a solution of bis(S-2-hydroxyethylthioacyl)-N,N-diisopropylphosphoramidite, where the -acyl- group was -acetyl- for compound 7, -pivaloyl- for compound 8,
or \(-benzoyl\)- for compound 9. For \(\beta\)-L-2',3'-dideoxy-adenosine-5'-O-bis(S-pivaloyl-2-thioethyl)-phosphate (\(\beta\)-L-2',5'-dd-3'-AMP-bis(t-Bu-SATE); compound 8 in Figure 1) bis(S-2-hydroxyethylthio pivaloyl) N,N-diisopropylphosphoramidite (127 mg, 0.28 mmol, 1.5 eq) in 0.5 ml of tetrahydrofuran was added. The reaction mixture was stirred for 45 min and then cooled to -40°C and tert-butylhydroperoxide (45 ml, 0.45 mmol, 2.4 eq, 90% in tert-butanol) was added. The reaction mixture was stirred for 30 min at room temperature and then diluted with 30 ml of dichloromethane. It was then washed with 10 ml of a 10% aqueous sodium sulfite solution and then with 10 ml of water. The organic phase was dried with sodium sulfate and evaporated to dryness under reduced pressure. The residue was purified by column chromatography with silica gel with a step gradient of 0 to 4% methanol in dichloromethane. The product (compound 8) was isolated as a colorless foam. Syntheses of \(\beta\)-L-2',3'-dideoxyadenosine-5'-O-bis(S-acetyl-2-thioethyl)-phosphate (\(\beta\)-L-2',5'-dd-3'-AMP-bis(Me-SATE); compound 7 in Figure 1) and \(\beta\)-L-2',3'-dideoxyadenosine-5'-O-bis(S-benzoyl-2-thioethyl)-phosphate (\(\beta\)-L-2',5'-dd-3'-AMP-bis(Ph-SATE); compound 9 in Fig. 1) followed from the respective bis(S-2-hydroxyethylthio acyl)-N,N-diisopropylphosphoramidite in a similar fashion.

\(\beta\)-L-2',3'-dideoxyadenosine-5'-O-bis(S-pivaloyl-2-thioethyl)-phosphate (7 in Figure 1) - Yield, 60 mg (0.099 mmol, 53 %); TLC: \(R_f\) = 0.32 (methylene chloride/methanol = 93:7, v/v), \(R_t\) = 0.14 (ethyl acetate/methanol = 95:5, v/v). HPLC: \(R_t\) = 41.1 min. \(^1\)H NMR (DMSO-\(d_6\)): \(\delta = 8.40\) and 8.28 (2s, 1H each, H-2 and H-8), 7.96 (br s, 2H, NH2), 6.28 (t, \(J = 5.2\) Hz, 1H, H-1'), 4.31 (m, 1H, H-4'), 4.19-4.08 (m, 2H, 2H-3'), 4.01-3.93 (m, 4H, 2\times\text{CH}_2\text{O}), 3.06 (t, \(J = 6.1\) Hz, 4H, 2\times\text{CH}_2\text{S}), 2.51 (m, 2H, 2 H-5'), 2.18-2.11 (m, 2H, 2 H-2'), 1.16 and 1.15 (2s, 9H each, 2 C(CH_3)_3. \(^{31}\)P NMR (DMSO-\(d_6\)): \(\delta = -0.59\).

(R)-bis(S-acetyl-2-thioethyl)-9-(2-phosphonomethoxypropyl)adenine (10, 11, and 12 in Figure 1) - For the synthesis of the \(-pivaloyl\)- derivative (11 in Figure 1) a mixture of mono triethyl-ammonium \(N^6\)-(4-monomethoxytrityl)-9-(2-phosphonomethoxypropyl)adenine (86 mg, 0.13 mmol), synthesized according to Holy and co-workers [43,44], and S-pivaloyl-2-thioethanol (105 mg, 0.65 mol, 5.0 eq) were co-evaporated three times with 5 ml of pyridine each and dissolved in 1.5 ml of dry pyridine. 1-(Mesitylene-sulfonyl)-3-nitro-1H-
1,2,4-triazole (193 mg, 0.65 mmol, 5.0 eq) was added and the reaction mixture was stirred for 3 days at room temperature. The reaction was stopped by the addition of 20 ml of saturated aqueous sodium bicarbonate and the resulting mixture was extracted with 30 ml of methylene chloride. The organic phase was dried by filtration through MgSO₄ and the filtrate was evaporated to dryness. The residue was purified by flash chromatography (20 g silica gel) with a step gradient of 0 to 4% methanol in methylene chloride. The appropriate fractions were combined and evaporated to dryness. The residue was dissolved in 5 ml of acetic acid:water:methanol = 8:1:1 (v:v:v). The reaction mixture was stirred for 24 h at ambient temperature. The solvent was evaporated in vacuo and the residue was co-evaporated twice with 5 ml of toluene, twice with 5 ml of methanol, and then with 5 ml of methylene chloride. The residue was purified by flash chromatography (15 g silica gel) with a step gradient of 0 to 20% methanol in methylene chloride yielding (R)-bis(S-pivaloyl-2-thioethyl)-9-(2-phosphonomethoxypropyl)adenine (t-Bu-SATE-PMPA; compound 11 in Figure 1; 48 mg, 0.083 mmol, 64%) as a colorless oil. Syntheses of (R)-bis(S-acetyl-2-thioethyl)-9-(2-phosphonomethoxypropyl)adenine (Me-SATE-PMPA; compound 10 in Fig. 1) and (R)-bis(S-benzoyl-2-thioethyl)-9-(2-phosphonomethoxypropyl)adenine (Ph-SATE-PMPA; 12 in Figure 1) followed from the respective S-acyl-2-thioethanol in a similar fashion.

(R)-bis(S-pivaloyl-2-thioethyl)-9-(2-phosphonomethoxypropyl)adenine (11 in Figure 1) - TLC: R₂ = 0.28 (methylene chloride/methanol = 93:7, v/v), R₂ = 0.08 (ethanol acetate/methanol = 95:5, v/v). HPLC: Rt = 40.6 min. ¹H NMR (DMSO-d₆): δ = 8.14 and 8.06 (2s, 1H each, H-2 and H-8), 7.23 (br s, 2H, NH₂), 4.25-4.18 (m, 2H, NCH₂), 3.99-3.85 (m, 7H, CH₃CH₂, PCH₂, 2 CH₂O), 3.07-3.00 (m, 4H, 2 × CH₂S), 1.17 (s, 18H, 2 C(CH₃)₃), 1.09 (d, J = 6.2 Hz, 3H, CH₃). ³¹P NMR (DMSO-d₆): δ = 22.97.

(R)-bis(S-acetyl-2-thioethyl)-9-(2-phosphonomethoxyethyl)adenine (13, 14, and 15 in Figure 1) - The syntheses of the series bis(Me-SATE)-PMEA, 13; bis(t-Bu-SATE)-PMEA, 14; and bis(Ph-SATE)-PMPA, 15; followed Benzaria et al. [39] and as described above for the respective PMPA derivatives, but from N⁶-(4-mono-methoxytrityl)-9-(2-phosphonomethoxyethyl)adenine.

(1R,2S)- and (1S,2R)-9-(cyclopentyl)-Ado-2'-phosphate-bis(t-Bu-SATE) (16 and 17 in Figure 1) – To a
stirred solution of (1R, 2S)-1-1-(6-aminopurin-9-yl)-2-(hydroxymethyl)cyclopentane (50 mg, 0.21 mmol) in a mixture of tetrahydrofuran and dimethylformamide (1.75 ml, 0.75:1, v/v) was added 1H-tetrazole (59 mg, 0.84 mmol) followed by the dropwise addition at room temperature of a solution of the bis(S-2-hydroxyethylthiopivaloyl)-N,N-diisopropylphosphoramidite (120 mg, 0.25 mmol) in 0.5 ml of tetrahydrofuran. The reaction mixture was stirred at room temperature for 45 min and then cooled to -80°C for 15 min and tert-butylhydroperoxide (0.07 ml, 0.63 mmol, 90% in tert-butanol) was added. The reaction mixture was then stirred for 30 min at room temperature and diluted with 50 ml of dichloromethane. It was subsequently washed with 25 ml of a 10% aqueous sodium sulfite solution and then 25 ml of water and finally with 25 ml of brine. The organic phase was dried with sodium sulfate and was evaporated to dryness under reduced pressure. The residue was purified by chromatography on a Chromatotron with a step gradient of 0 to 5% methanol in chloroform. The product was isolated as a colorless oil: yield 84 mg (65%). TLC: Rf = 0.61 (chloroform/methanol = 9:1, v/v). 1H NMR (CDCl3): δ = 1.22 {s, 18H, 2×CH(CH3)3}, 1.75-1.92 (m, 2H, H-4′), 2.07-2.11 (m, 2H, H-3′), 2.29-2.34 (m, 2H, H-5′), 2.73-2.82 (m, 1H, H-2′), 3.01-3.09 (m, 4H, J = 6.6 Hz, 2×CH2S), 3.71-3.82 (m, 2H, H-6′), 3.89-4.01 (m, 4H, 2×CH2O), 5.07-5.17 (q, 1H, J = 7.4 Hz, H-1′), 6.37 (bs, 2H, NH2), 7.86 and 8.33 (2s, 1H each, H-2 and H-8). 31P NMR (CDCl3): δ = -0.95. Synthesis of t-butyl-SATE derivative of the other diastereomer followed from the respective (1S, 2R)-1-1-(6-aminopurin-9-yl)-2-(hydroxymethyl)cyclopentane in a similar fashion, yield 59 mg (57%, colorless oil).

**General methods for compound 6** - 1H and 13C NMR spectra were recorded at 400 MHz with proton decoupling, at ambient temperature, with a Brüker DRX400. Chemical shifts are given in δ-values referenced to the residual solvent peak: chloroform (CDCl3) at 7.26 ppm and 77.6 ppm or dimethylsulfoxide (DMSO-d6) at 2.49 ppm and 39.5 ppm, relative to tetramethylsilane (TMS). Coupling constants, J, are reported in Hz. Deuterium exchange, decoupling and COSY experiments were performed in order to confirm proton assignments. 2D 1H-13C heteronuclear COSY were recorded for the attribution of 13C signals. 31P NMR spectra were recorded at ambient temperature at 400 MHz with proton decoupling. Chemical shifts are reported relative to external H3PO4. FAB mass spectra were recorded in the positive-ion and negative-ion
modes on a JEOL SX 102 with thioglycerol/glycerol (1:1, v/v, G-T) as matrix. Melting points were
determined in open capillary tubes on a Gallenkamp MFB-595-010M apparatus and are uncorrected. UV
spectra were recorded on a Uvikon 931 (Kontron) spectropolarimeter. Specific rotations were measured in a
1 cm cell on a Perkin-Elmer Model 241 spectropolarimeter settled on the sodium D line. Elemental analyses
were carried out by the "Service de Microanalyses du CNRS, Division de Vernaison (France)". Thin layer
chromatography was performed on precoated aluminum sheets of silica gel 60 F254 (Merck, Art. 9885).
Visualization of products was accomplished by UV absorbance followed by charring with 10% ethanolic
sulfuric acid with heating and/or by spraying with Hanes molybdate reagent. Column chromatography was
carried out on silica gel 60 (Merck, Art. 9385). HPLC analyses were with a reverse-phase analytical column
(Nucleosil, C18, 150 x 4.6 mm, 5 µm) equipped with a prefilter, a precolumn (Nucleosil, C18, 5 µm) and a
photodiode array detector (detection at 260 nm). All moisture-sensitive reactions were carried out under
rigorous anhydrous conditions under argon atmosphere with oven-dried glassware. Solvents were dried and
distilled before use and solids were dried over P2O5 under reduced pressure at room temperature.

General methods for compounds 1 - 5, and 7 - 17 - 31P (101 MHz) and 1H NMR (250 MHz) spectra
were recorded at ambient temperature with a Brüker AC250. 1H NMR chemical shifts are given in δ (ppm)
referenced to the residual solvent peak [dimethyl sulfoxide (DMSO-d6) at 2.49 ppm] relative to
tetramethylsilane (TMS). 31P NMR spectra were recorded in the proton-decoupled mode and 31P chemical
shifts are reported in ppm with phosphoric acid (H3PO4) as external reference. Coupling constants, J, are in
Hz. Column chromatography was carried out on silica gel 60 (particle size 0.040-0.063 mm; EM Science,
Merck, Darmstadt). Analytical thin-layer chromatography, used to monitor all reactions, was performed on
silica gel 60 PF254, with fluorescent indicator, on aluminum-backed plates (0.2 mm; Merck, Darmstadt), and
UV absorbance was used to visualize products. Phosphorus containing compounds were detected by spraying
with Hanes molybdate reagent. Sugar containing compounds were detected by spraying with anisaldehyde
reagent, each time followed by heating. High-performance liquid chromatography (HPLC) was carried out
on a Waters system that included a Model 996 photodiode array detector and analysis was with
Millennium32 software. Separations were by reverse phase chromatography (Ultrasphere, C18, 250 x 4.6 mm, 5 µm) with linear gradients of 0 to 100% acetonitrile in water over a 40 min period at a flow rate of 1 ml/min. Solvents, pyridine, tetrahydrofuran, and dimethylformamide, were of analytical quality absolute from Fluka. 1H-tetrazole and 1-(mesitylenesulfonyl)-3-nitro-1H-1,2,4-triazole were from Aldrich.

**Cell cultures** - OB1771 and F442A cells were grown to confluence in either 3.5 cm, 5 cm, or 10 cm culture dishes at 37°C in an atmosphere of 5% CO₂. Culture medium was DMEM low glucose (GibcoBRL) supplemented with 8% (w/v) fetal bovine serum, and 1% (v/v) antibiotic antimycotic solution (Sigma Chemical Co.; A5955; a 100-fold concentrated stabilized solution of penicillin, streptomycin, and amphotericin B), as previously described [18].

Human macrophages were prepared from leukocyte packs obtained from the Long Island Blood Service as described by Cheng et al. [46]. Briefly, cells were diluted with phosphate-buffered saline and then were separated by layering over Ficoll-Paque (Pharmacia) and centrifugation. Mononuclear cells were collected and washed three times with phosphate buffered saline to remove platelets. Cells were collected by centrifugation and were resuspended in cold RPMI-1640 medium with L-glutamine (Invitrogen Life Technologies 11875-093) supplemented with 7% human AB serum and 1% antibiotics to a density of ~5 x 10⁶ per ml. Cells were plated onto tissue culture dishes that had been precoated with heat-inactivated calf serum and were incubated for 90 min at 37°C. Dishes were washed three times with RPMI-1640 at 37°C to remove unattached cells. Cells adhering to the dishes were confirmed to be > 95% monocytes. To obtain macrophages, monocytes were cultured for 5 to 10 days in RPMI-1640 medium supplemented with 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid and 7% human AB serum, with medium changes every other day.

Alternatively, macrophages were derived from THP-1 human monocytes (ATCC TIB 202) that were grown in RPMI-1640 medium as above. For experiments on the metabolic fate of protected nucleotides, cells were grown in serum free medium (PFHM-II, protein free hybridoma medium, Invitrogen Life Technologies (Gibco) 12040-077).
Cell homogenates and membrane preparations - OB1771 cells were grown to confluence in ten 10 cm culture dishes as above. To remove culture medium, cells were washed twice with 5 ml of a solution containing 250 mM sucrose, 3 mM dithiothreitol, and 20 mM glycylglycine, pH 7.5, then 3 ml of this buffer was added and dishes were scraped to harvest cells. Plates were washed with an additional 2 ml of buffer and was combined with the harvested cells. The mixture was homogenized on ice with 20 strokes of a glass-Teflon homogenizer. The resulting suspension was used as such in experiments with whole homogenates. The remaining suspension was centrifuged at 550 x g (2000 rpm in a Beckman JA17 rotor) for 15 min at 4°. The resulting supernate was removed and was centrifuged at 50,000 x g (20,000 rpm in a Beckman JA 20.1 rotor) for 20 min at 4°. The resulting pellets were resuspended in 1 ml of medium containing 3 mM dithiothreitol, 250 mM sucrose, and 20 mM glycylglycine, pH 7.5. These suspensions were then used for assay of adenylyl cyclase activity.

[^H]cAMP formation in cells prelabeled with [^H]adenine - This procedure followed suggestions of Salomon [47]. Macrophages or OB1771 cells were grown in 35 mm culture dishes, growth medium was removed from each dish and replaced with 1 ml of medium containing [^H]adenine to a final concentration of 5 µCi per ml. After 2 hr at 37°, [^H]adenine-containing medium was removed by aspiration and cells were washed twice with 2 ml of serum-free medium. Additions were then made in serum-free medium supplemented with bovine serum albumin (1 mg/ml) and 100 µM 3-isobutyl-1-methylxanthine, usually together with other additions, e.g. nucleosides or pro-nucleotides. Typically cells were incubated for an additional 15 min and then 30 µM forskolin was added for a 15 min test period. The medium was aspirated and 1 ml of a solution containing 0.3 M perchloric acid and 0.5 mM cAMP was added. Unlabeled cAMP protects [^H]cAMP formed and allows the quantifying of its recovery. Cells were scraped from the dish, triethanolamine•HCl was added to 50 mM, and the solution was then neutralized on ice by the addition of a small portion of 4.2 M ice-cold KOH to give a final pH between 7 and 7.5. Samples were centrifuged to remove cellular debris and potassium perchlorate. [^H]cAMP was separated by sequential chromatography on Dowex-50 and alumina columns [48]. A portion of the eluate from the alumina column was removed and
used to quantify the recovery of cAMP and the remainder was used to quantify the formation of [3H]cAMP by scintillation spectrometry.

**Adenylyl Cyclase Preparation and Assay** - Adenylyl cyclase from rat brain was prepared as a detergent-dispersed extract as previously described [24,48]. Membranes from OB1771 or F442A cells were prepared as described above under “Cell homogenates and membrane preparations”. Activity of adenylyl cyclase from rat brain was determined with a reaction mixture containing 50 mM triethanolamine•HCl, pH 7.5, 5 mM MnCl₂, 100 µM forskolin, 1 mM 3-isobutyl-1-methylxanthine, 0.1% (w/v) Lubrol-PX, 1 mg bovine serum albumin per ml, 2 mM creatine phosphate, 100 µg creatine kinase per ml, and 100 µM 5′ATP with [α-32P]-5′-ATP (10⁵ to 10⁶ cpm) as tracer, in a volume of 100 µl, for 15 min at 30°C. Reactions with membrane preparations from OB1771 cells also included 100 µg myokinase per ml to aid in maintaining ATP concentrations constant. Reactions were started by the addition of [α-32P]-5′ATP and were ended by the addition of zinc acetate and sodium carbonate [48]. [32P]-cAMP was purified by sequential chromatography on Dowex-50 and alumina and was quantified in a scintillation spectrometer by Čerenkov radiation.

Inhibition kinetics were evaluated as previously described [49] with variable concentrations of Mn•5′-ATP and free cation held fixed in excess of the 5′-ATP concentration.

**Cardiac ventricular myocytes and Cl current measurements.** Adult male guinea pigs, weighing 300-500 g, were sacrificed by peritoneal injection of sodium pentobarbitone solution (1 ml of 390 mg/ml). Hearts were removed and cardiac myocytes were enzymatically isolated as described by Gao et al. [50]. Isolated cells were maintained in a buffer containing 83 mM KCl, 30 mM K₂HPO₄, 5 mM MgSO₄, 5 mM sodium pyruvate, 5 mM β-OH-butyrate 5 mM creatine, 20 mM taurine, 10 mM glucose, 0.5 mM EGTA, 2 mM KOH, 5 mM Na₂-ATP, and pH 7.2.

Cell membrane Cl-current (I_{Cl}) was measured by the whole cell patch clamp technique, with an Axopatch 1A amplifier (Axon Instruments, Inc.). Patch-pipette resistances were 2 MΩ to 3 MΩ prior to sealing. The pipette solution contained 92 mM potassium aspartate, 13 mM KCl, 45 mM KOH, 1 mM MgCl₂, 11 mM
EGTA, 1 mM CaCl₂, 5 mM MgSO₄, 10 mM glucose, 5 mM Na₂-ATP, 5 mM HEPES, and pH 7.2. The external Tyrode solution contained 137.7 mM NaCl, 2.3 mM NaOH, 5.4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, 1 mM CdCl₂, 2 mM BaCl₂, 5 mM HEPES, and pH 7.4. With these conditions, L-type Ca²⁺-current, Na⁺/Ca²⁺ exchange current, and K⁺-conductance were eliminated.

Cells, held at 0 mV, were pretreated with 2',5'-dd-3'-AMP-bis(t-Bu-SATE) for 15 min before ICl was activated by the application of 0.1 μM isoproterenol to the external solution. Cell membrane capacitance was measured in the current clamp mode by applying a -50 pA current and observing the membrane potential change. The density of ICl was obtained by normalizing the current to the cell’s membrane capacitance (pA/pF). All experiments were carried out at room temperature (24 ± 0.5°C). Patch clamp data, with a sampling rate of 50 μs/point with a 10 K Hz low pass filter, were acquired with pClamp7 software (Axon Instruments, Inc.) for later analysis.

RESULTS

**Synthesis of 9-substituted adenine derivatives** - The family of 9-substituted adenine derivatives reported here include three principal groups of protected nucleotides with two types of phosphate protection (Figure 1). Protected nucleotide groups included monophosphate precursors of two of the most potent inhibitors of adenylyl cyclases, the competitive pre-transition-state inhibitor, β-L-2',3'-dd-5'-ATP (compounds 7 - 9), and the non-competitive post-transition-state, P-site ligand, β-D-2',5'-dd-3'-ATP (compounds 1 - 5) [2-4,6]. The derivative of β-L-2',5'-dd-3'-AMP (compound 6) is a stereo isomer of compound 4. The third group included acyclic phosphonate derivatives of adenine (compounds 10 - 15) that form precursors for known inhibitors of viral reverse transcriptase, PMPApp and PMEApp [e.g. 31,39,42,51]. These two agents also inhibit adenylyl cyclase in vitro [7]. Compounds 16 and 17 are enantiomeric derivatives of 2'-phosphoryl-9-(cyclopentyl)-adenine, for which the corresponding 2'-triphosphate forms are also competitive inhibitors of adenylyl cyclase. Phosphate groups were protected by either cyclo-saligenyl-derivatives (compounds 1 and 2) [29,34-36] or S-acyl-2-thioethyl- (SATE-) derivatives [33,39] (compounds 3 - 17). The comparison of these several
ligands allows a distinction to be made in the efficacy of pre- and post-transition-state inhibitors of adenylyl cyclases for use in intact cell systems and a distinction to be made between pro-nucleotides that are spontaneously deprotected (cyclo-saligenyl-derivatives) and those that require deprotection by cellular enzymes (SATE-derivatives).

**Inhibition of adenylyl cyclase and of $[^3H]cAMP$ formation in preadipocytes** - The adenylyl cyclase in membranes isolated from OB1771 preadipocytes was inhibited by adenine nucleosides with a rank-order of potency similar to that previously reported for inhibition of the enzyme isolated from rat brain (Table 1). Inhibitory potency increased significantly as the number of 3'-phosphates was increased, but potency was somewhat less in the preadipocytes. These data suggest a probable range within which pro-nucleotide potency should fall. Potency of inhibition of cAMP formation in intact cells will depend upon the efficacy of pro-nucleotide uptake by cells and the extent of its subsequent deprotection and phosphorylation within cells.

To evaluate effects of agents to inhibit cAMP formation in intact cells, responses were tested on cells that had been pre-labeled with $[^3H]$adenine. This technique yields a very sensitive measure of agents that modulate adenylyl cyclase activity in intact cells and is especially useful for inhibitors of this enzyme. When preadipocytes were challenged with forskolin the amount of newly-formed $[^3H]cAMP$ increased markedly (Figure 2). Accumulation of $[^3H]cAMP$ showed dependence on time and forskolin concentration; it was nearly maximum after 15 min and was nearly maximally stimulated with 30 µM forskolin. Consequently, these conditions were used in subsequent experiments.

9-(Cyclopentyl)adenine and 2',5'-dd-Ado are cell permeable P-site ligands that have been shown to inhibit cAMP formation and to accelerate differentiation in OB1771 preadipocytes [18]. With $[^3H]$adenine prelabeled preadipocytes 2',5'-dd-Ado inhibited the forskolin-induced increase in $[^3H]cAMP$ formation with an IC$_{50}$ ~13 µM (Figure 3, *Intact Cells curve*). This compares with the IC$_{50}$ ~21 µM for inhibition of adenylyl cyclase in membranes isolated from these same cells (Figure 3, *Cell Membranes curve*; cf. Table 1). For comparison, 9-(cyclopentyl)adenine inhibited forskolin-stimulated $[^3H]cAMP$ formation with an IC$_{50}$ between 10 µM and 30 µM (not shown).
**The prodrug effect** - The efficacy of 2',5'-dd-3'-AMP and a protected form, 2',5'-dd-3'-Ado-3'-O-bis(pivaloyl-2-thioethyl)-phosphate (2',5'-dd-3'-AMP-bis(t-Bu-SATE)), to inhibit adenylyl cyclase in membranes isolated from preadipocytes were compared with their efficacy to inhibit [3H]cAMP formation in cells prelabeled with [3H]adenine and stimulated with forskolin (Figure 4). Inhibition of adenylyl cyclase by 2',5'-dd-3'-AMP occurred with the expected potency (IC\textsubscript{50} ~3 to 10 \(\mu\)M), whereas the SATE-protected ligand was essentially without effect (20% inhibition at 100 \(\mu\)M). Not surprisingly, 2',5'-dd-3'-AMP showed a weaker potency towards inhibiting [3H]cAMP formation in intact cells (~40% inhibition at 30 \(\mu\)M) than it did on the isolated adenylyl cyclase. Presumably, 2',5'-dd-3'AMP does not enter cells intact and the inhibition noted here could be due to the partial extracellular hydrolysis of the 3'-phosphate and the formation of some 2',5'-dd-Ado. By contrast, 2',5'-dd-3'-AMP-bis(t-Bu-SATE) inhibited [3H]cAMP formation in intact cells with remarkable potency (IC\textsubscript{50} ~ 30 nM). This was in the same range as noted with 2',5'-dd-3'-ATP for inhibition of adenylyl cyclase in membranes isolated from the preadipocytes (Table 1). The data imply that intact cells are required for inhibition of cAMP formation by the protected nucleotide and suggest that inhibition occurs as a consequence of the nucleotide's being taken up and metabolized to a ligand more potent than the precursor nucleoside 3'-monophosphate.

**Pro-nucleotide comparison** - The efficacies of pro-nucleotides to inhibit the formation of [3H]cAMP in intact cells depended on characteristics of the 9-substituted adenine derivative and of the phosphate protecting groups. Protection of 2',5'-dd-3'-AMP by either H-saligenyl- or methyl-saligenyl-groups yielded 2',5'-dd-3'-AMP derivatives with potencies slightly greater than that of the precursor nucleoside (2',5'-dd-Ado; cf. Figure 3) and an order of magnitude greater than unprotected 2',5'-dd-3'-AMP (compare Figures 4 and 5 and Table 1). The H-Sal- derivative (1; IC\textsubscript{50} ~1.2 \(\mu\)M) was slightly more potent than the Me-Sal-derivative (2; IC\textsubscript{50} ~2.0 \(\mu\)M), consistent with its more rapid hydrolysis [29,34]. In fact, the general lack of efficacy of these ligands likely lies with their relative stability; they undergo spontaneous deprotection in aqueous solutions with relatively long half-lives. For related compounds the half-lives were: 2',3'-dd-5'-
AMP-(H-Sal), \( t_{1/2} \approx 8 \text{ hrs} \) and 2',3'-dd-5'-AMP-(Me-Sal), \( t_{1/2} \approx 35 \text{ hr} \) in phosphate buffer [29,35]. Consequently, the saligenyl-derivatives of 2',5'-dd-3'-AMP (compounds 1 and 2) were substantially less effective inhibitors of \([\text{H}]cAMP\) formation in intact cells than was 2',5'-dd-3'-AMP-bis(t-Bu-SATE) (4; Figure 5). The t-Bu-SATE-group \textit{per se} did not impart to ligands the capacity to inhibit \([\text{H}]cAMP\) in intact cells because bis(t-Bu-SATE)-PMEA was without effect (compound 14; Figure 5). Had this protected adenine derivative been taken up and processed by the OB1771 cells a greater effect would have been expected. PMEA and PMEApp inhibited adenylyl cyclase \textit{in vitro} with IC\(_{50}\) ~66 µM and 0.18 µM, respectively (Table 1).

\textbf{Effects on other cell types} - In experiments similar to those above, saligenyl- and SATE-protected 2',5'-dd-3'-AMP were compared with \([\text{H}]\)adenine prelabeled F442A cells. First, the rank-order of potency of the SATE- and saligenyl-protective groups was maintained, that is H-Sal > Me-Sal and bis(Me-SATE) > bis(t-Bu-SATE) > bis(Ph-SATE) (Figure 6, Table 2). The \textit{acyl} group clearly influenced potency. And second, these compounds exhibited slightly lower potencies from those seen with the OB1771 cells (cf. Figures 4 and 5, Table 2). Differences in efficacy of the pro-nucleotides were more pronounced when human macrophages were tested (Table 2). Although potency of the bis(t-Bu-SATE)-protected 2',5'-dd-3'-AMP (IC\(_{50}\) ~76 nM) was between those observed with OB1771 (IC\(_{50}\) ~51 nM) and F442A (IC\(_{50}\) ~146 nM) cells and the bis(Ph-SATE)-pro-nucleotide also exhibited a potency in this range (IC\(_{50}\) ~95 nM), the bis(Me-SATE)-pro-nucleotide, rather than being the more potent was actually somewhat less potent (IC\(_{50}\) ~247 nM). In each set of experiments cells were exposed to pro-nucleotide for 15 min before the challenge with forskolin, which again was for 15 min. These observations suggest that uptake and processing of pro-nucleotides differ between cell types and that this may be dependent on the protecting group. By accepted models, processing of these pro-nucleotides requires uptake, deprotection, and subsequent poly-phosphorylation. Therefore, variations in pro-nucleotide potency could be due to differences at any stage. For example, some differences in potency among the bis(S-\textit{acyl}-2-thioethyl)-phosphate derivatives would be expected to be due to differences in their processing rates. The half-lives for similar phosphotriester derivatives of 3'-azido-5'-TMP
in CEM-SS cell extracts varied considerably (t_{1/2} < 5 min for bis(Me-SATE)- and t_{1/2} ~ 63 min for bis(t-Bu-SATE)-derivatives) [33]. Because of the differences in uptake and/or processing rates, similar potencies among the SATE-protected compounds (bis(Me-, t-Bu-, and Ph-SATE)) might be expected if appropriately longer exposure times were considered.

**Comparisons of other pro-nucleotides** - Protected forms of the L-isomer of 2',5'-dd-3'-AMP (β-L-2',5'-dd-3'-AMP), β-L-2',3'-dd-5'-AMP, and, as noted above, acyclic 9-substituted adenine derivatives, PMEA and PMPA, and several other compounds were also synthesized and tested. It was not surprising that pro-nucleotide forms of β-L-2',5'-dd-3'-AMP were without effect on [3H]cAMP formation in intact cells, because the triphosphate form of this nucleoside was also without effect on adenylyl cyclase *in vitro* (Tables 1 and 2). The bis(t-Bu-SATE)- derivatives of (1R,2S)- and (1S,2R)-9-(cyclopentyl)-Ade-2'-phosphate were likely ineffective for similar reasons (Table 2). However, bis(t-Bu-SATE)- derivatives of the acyclic derivatives of adenine (PMEA and PMPA; Figures 5 and 6), were unexpectedly ineffective. This lack of effect was somewhat surprising because, first, the uptake and metabolic processing of the protected forms of these compounds have been established in other cell types. And second, the diphosphate forms of these agents (PMEApp and PMPApp) are fairly potent inhibitors of adenylyl cyclases in their own right (Table 1).

A similar conundrum occurred with S-acyl-2-thioethyl-derivatives of β-L-2',3'-dd-5'-AMP. None of these pro-nucleotides inhibited the forskolin-stimulated formation of [3H]cAMP in isolated cells, yet β-L-2',3'-dd-5'-ATP is one of the most potent known inhibitors of adenylyl cyclases (IC_{50} ~ 24 nM; Table 1). That is, the expectation was that pro-nucleotide forms of either 2',5'-dd-3'-AMP or β-L-2',3'-dd-5'-AMP would have behaved similarly because the triphosphate forms of these nucleotides were comparably potent inhibitors of adenylyl cyclases. However, only protected derivatives of 2',5'-dd-3'-AMP yielded effective inhibitors of [3H]cAMP formation in intact cells and the most potent of these were the 2',5'-dd-Ado-3'-O-bis(S-acyl-2-thioethyl)-phosphates.

**Effects on cardiac tissue and cells** - Pro-nucleotides also inhibited formation of cAMP in cardiac tissue
and cells, but with reduced potency. In these experiments two approaches were taken. In rat atria, the end-point was forskolin-stimulated formation of \(^{[3]H}\)cAMP from tissue pre-labeled with \(^{[3]H}\)adenine. Atria were excised from male albino rats, were minced, and then were suspended in a Krebs-Henseleit buffer supplemented with 2 mM pyruvate. Minced atria were prelabeled with \(^{[3]H}\)adenine, then treated either with 2',5'-dd-3'-AMP-bis(t-Bu-SATE) or vehicle (dimethylsulfoxide) for 15 min in the presence of 100 µM IBMX, and then stimulated with 30 µM forskolin as had been done with isolated cells. Under these conditions the increase in the formation of \(^{[3]H}\)cAMP induced by forskolin (6.5 ± 1.1-fold; n=12) was more modest than had been observed with the isolated cells described above. In addition, the sensitivity to inhibition by 2',5'-dd-3'-AMP-bis(t-Bu-SATE) was also muted (1 µM caused a reduction of 54 ± 8 %; n = 3). The reduced sensitivity to forskolin and to the pro-nucleotide may have been due to characteristics of cardiac tissue per se or that this was with minced tissue, into which agents might diffuse poorly, or because of which the preparation contained a lot of damaged tissue. To circumvent these problems and to establish that the efficacy of these pro-nucleotides was not, in fact, a consequence of the tritium-labeling assay method, a different approach was used with guinea-pig cardiac ventricular myocytes. These cells express a cAMP-dependent Cl\(^-\)-channel; Cl\(^-\)-current is increased with increasing cellular levels of cAMP [52,53]. When isolated cells were treated for 15 min with varying concentrations of 2',5'-dd-3'-AMP-bis(t-Bu-SATE), isoproterenol-activated Cl\(^-\)-current was suppressed with an IC\(_{50}\) ~800 nM (Figure 7). This concentration was in the range of that noted above for inhibition of forskolin-stimulated formation of \(^{[3]H}\)cAMP in atrial tissue, suggesting that the less potent inhibition by this pro-nucleotide was a characteristic of cardiac tissue and was not a consequence of the assay method. When compared with other cell types, sensitivity to inhibition by 2',5'-dd-3'-AMP-bis(t-Bu-SATE) followed the order macrophages \(\cong\) OB1771 cells \(>\) 3T3 F442A \(>\) cardiomyocytes (Figure 7). Because the bis(SATE)-pro-nucleotides inhibited either cAMP formation or a cAMP-mediated process in four different cell types as measured with two different end-points, the differences in sensitivity likely lie with tissue-dependent characteristics of pro-nucleotide processing, with differences in sensitivities of the adenyl cyclase isozymes present in these several cell types, or with
differences imposed by the techniques and conditions we used.

**Pro-nucleotide metabolism and the effects of metabolites** - Deprotection of bis(SATE)-protected nucleotides is thought to occur through a carboxyesterase catalyzed elimination of one SATE-group, followed by spontaneous intramolecular decomposition and elimination of episulfide, yielding the nucleotide-SATE-diester (Scheme 3) [33]. The removal of the second SATE-group by this mechanism occurs more slowly and may in fact be rate-limiting. An alternative cleavage by the action of a phosphodiesterase is also possible. Several experiments were conducted to determine whether this process occurred with the SATE-protected pro-nucleotides used in this study and to ascertain whether such metabolites might affect cAMP formation in intact cells or by adenylyl cyclase activity in isolated membranes. Because of the small amount of tissue and the HPLC-UV analytical techniques we used, it was not possible to measure the formation of the exceedingly small amounts of nucleotide metabolites that actually appear in the amounts of tissue or cell extracts we used. Consequently, and to allow visualization of products, these experiments typically involved the incubation of 30 µM to 100 µM 2',5'-dd-3'-AMP-bis(t-Bu-SATE) with culture medium, cells, or cell homogenates, and the identification and quantifying of products by HPLC-UV techniques.

With whole homogenates of OB1771 preadipocytes, a time- and tissue-dependent appearance of a major product (retention time ~110 min) was noted (Figure 8, lower panel). It was also evident upon exposure of 2',5'-dd-3'-AMP-bis(t-Bu-SATE) to minced rat atrial tissue (not shown). As expected, formation of the ~110 min peak material was much less with buffer alone. This material exhibited elution and spectral characteristics consistent with its being the expected mono-SATE-derivative (Scheme 3) and it was easily distinguished from the initial 2',5'-dd-3'-AMP-bis(t-Bu-SATE) (retention time ~118 min) by reverse phase HPLC techniques. Neither the starting bis(t-Bu-SATE)-derivative (~118 min peak) nor the metabolite (~110 min peak) had any effect on adenylyl cyclase activity in membranes isolated from preadipocytes (Figure 8, upper panel).

Because metabolism of 2',5'-dd-3'-AMP-bis(t-Bu-SATE) occurred with cell homogenates (above), it was
of interest to determine if this also occurred with intact cells. Incubation of preadipocytes or macrophages
with the pro-nucleotide resulted in its substantial breakdown (Figure 9). This was more apparent with
macrophages (Figure 9, middle panel) than with the preadipocytes (Figure 9, lower panel), though with the
preadipocytes disappearance of the pro-nucleotide was complete after 24 hr (not shown). Breakdown of
2',5'-dd-3'-AMP-bis(t-Bu-SATE) was slight in the DMEM medium used with the preadipocytes (lower
panel), in PFHM-II medium (protein free hybridoma medium from Gibco # 12040-077; middle panel), and in
RPMI 1640 medium (Gibco #1875-093), whether supplemented with L-glutamine or with β-mercapto-
ethanol and fetal bovine serum (not shown). In these experiments samples were taken either from medium
above the preadipocytes or from suspensions containing comparatively small volumes of macrophages, the
metabolism of 2',5'-dd-3'-AMP-bis(t-Bu-SATE) is presumed to have occurred extracellularly. Because of the
possible cell-surface formation of degradation products, it was possible that the effects of the pro-nucleotides
on formation of [3H]cAMP in intact cells might have been due to such a metabolite, with a plausible
candidate being the material exhibiting the ~110 min retention time. However, when this material was tested
for its ability to block formation of [3H]cAMP in preadipocytes, it was ineffective (Figure 9, top panel);
2',5'-dd-3'-AMP-bis(t-Bu-SATE) exhibited an IC₅₀ ~8.1 nM, whereas the metabolite was not inhibitory at
concentrations to 1 µM. Taken together the data are consistent with the predicted deprotection pattern for
this class of pro-nucleotide (Scheme 3) and suggest that the inhibition of cAMP formation in intact cells and
tissues requires the further breakdown of the pro-nucleotide to product nucleoside(s)/nucleotide(s). Although
some extracellular breakdown of the nucleotide can occur, it is likely that effective inhibition of adenylyl
cyclase occurs via intracellular processing of the pro-nucleotide.

**DISCUSSION**

Many adenine nucleosides or nucleoside derivatives have been identified as cell permeable, but not
particularly potent, inhibitors of adenylyl cyclases [11-24]. Substantially more potent nucleotides have been
identified that exhibit IC₅₀s in the low nanomolar range, especially 2',5'-dd-3'-ATP and β-L-2',3'-dd-5'-ATP
[2-4,6], but these are not cell permeable. To take advantage of the potency of these nucleotides, the concept
of phosphate protective groups to neutralize charge to allow entry into cells, effective for other nucleotides, was adapted for these studies. Examples of derivatives that have been used in studies by others include (pivaloyloxy)methyl- [30,39], dithioethyl- [32,33,39], aryl phosphoramidate- [37,38], alkylamino- [54,55], cyclosaligenyl- [29,45], S-acyl-2-thioethyl- (SATE) [32,33], and combinations of these groups [54-56]. For the studies reported here SATE- and cyclosaligenyl- protective groups were used. These were coupled to four types of nucleoside phosphate, β-D- and β-L-isomers of 2',5'-dd-3'-AMP, β-L-2',3'-dd-5'-AMP, the acyclic phosphonate derivatives of adenine, PMEA and PMPA, and two enantiomers of 9-(cyclopentyl)-adenine-2'-phosphate. The resulting pro-nucleotides were tested on four cell types with two different end-points. In three cell types, OB1771 and 3T3 F442A preadipocytes, and human macrophages, the end-point was the reduction in forskolin-stimulated formation of [3H]cAMP in cells that had been pre-labeled with [3H]adenine. In ventricular cardiomyocytes the end-point was a reduction in catecholamine-stimulated current of a cAMP-sensitive Cl⁻-channel.

There were substantial differences in the responses of the cell lines to these agents. The acyclic phosphonate derivatives and the β-L-2',3'-dd-5'-AMP derivatives were surprisingly ineffective. Neither class of compound, regardless of protective group, attenuated forskolin-stimulated formation of [3H]cAMP in cells. This was unexpected for several reasons. PMEApp and PMPApp inhibit adenyl cyclase in vitro (IC₅₀ ~ 175 nM and 500 nM, respectively; Table 1). Because PMEApp and PMPApp inhibit viral reverse transcriptase in vitro, and protected precursor forms of PMEA and PMPA inhibit viral reverse transcriptase activity in intact cells [25-29], it was expected that protected form of PMEA and PMPA would also have inhibited adenyl cyclase in intact cells. But they did not. It was similarly expected that protected derivatives of β-L-2',3'-dd-5'-AMP would reduce adenyl cyclase in intact cells. These were unexpected observations in part because, from established behavior of other SATE-protected nucleotides, the uptake and deprotection of each of these ligands would have been expected to be comparable. It is known that the SATE-protected forms of β-L-2',3'-dd-5'-AMP are taken up by cells [57,58] and the triphosphate form,
β-L-2',3'-dd-5'-ATP, is one of the most potent inhibitors of adenylyl cyclase in vitro (Table 1; IC$_{50}$ ~ 24 nM [6]). The lack of effectiveness of protected forms of these compounds to block adenylyl cyclase in intact cells contrasted sharply with the behavior of the pro-nucleotide forms of 2',5'-dd-3'-AMP. In each instance, protected forms of this 3'-nucleotide elicited a reduction in the formation of [3H]cAMP, with potencies reflecting and being dependent on the protecting group. Least effective were the 2',5'-dd-3'-AMP-cyclosaligenyl derivatives, with IC$_{50}$s in the micromolar range. Even though these compounds would be expected to enter cells, the half-life for their presumed spontaneous deprotection to 2',5'-dd-3'-AMP (t$_{1/2}$ ~ 8 hr to ~35 hr [29,35]) might have been too long for the experiments conducted here, thereby not yielding sufficiently high intracellular concentrations of the corresponding 3'-polyphosphates to inhibit adenylyl cyclase.

Protection of 2',5'-dd-3'-AMP with S-acyl-2-thioethyl-groups, where acyl was either a methyl-, tert-butyl-, or phenyl- group, yielded a group of compounds exhibiting all the hallmarks of prodrugs. These compounds did not inhibit adenylyl cyclase in vitro (Figures 4 and 8), but were strikingly potent inhibitors of forskolin-stimulated formation of [3H]cAMP in intact cells, with IC$_{50}$ values in the low nanomolar range (Table 2). The data are fully consistent with the concept that the protected ligand must be taken up, be deprotected, and be polyphosphorylated to be active (cf. Scheme 3). The overall elimination of the bis(SATE)-protective groups would be expected to follow the order Me-SATE- > t-Bu-SATE- > Ph-SATE- [cf. 33], and this was reflected in the potencies noted here (Table 2). The differences in rates and mechanism of deprotection appeared not to hinder the efficacy of 2',5'-dd-3'-AMP protected with bis(Me-SATE)- and possibly bis(t-Bu-SATE)-groups, but could account for the reduced apparent potency of the bis(Ph-SATE)-protected ligand, resulting in the rank-order of potency of the 2',5'-dd-3'-AMP-bis(SATE) derivatives tested (Figure 6 and Table 2). That is, longer exposure of cells to bis(t-Bu-SATE)- and bis(Ph-SATE)-protected ligand might have resulted in potencies similar to that noted with the bis(Me-SATE)-nucleotide.

Attempts were made to verify the sequence of uptake and deprotection events, but the HPLC-UV isolation and detection techniques available to us were not sufficiently sensitive for the purpose. One
predominant metabolic product was noted. On this chromatography system it exhibited a slightly shorter retention time (~110 min peak) than the initial pro-nucleotide (~118 min peak) and it conformed spectrally to the mono-(t-Bu-SATE)-diester of 2',5'-dd-3'-AMP. The possibility was further examined that this compound was, in fact, the active agent, but it neither inhibited adenylyl cyclase *per se* (Figure 8) nor blocked the formation of [H]cAMP in prelabeled cells (Figure 9). This suggests that the active agent for inhibiting [H]cAMP formation in intact cells was, in fact, 2',5'-dd-3'-AMP-bis(t-Bu-SATE).

To be clear, it remains a slight possibility that this pro-nucleotide need not be taken up by cells to exert its effects and that it might act indirectly, for example, through a cell-surface receptor-mediated mechanism. There is no evidence to support this alternative possibility. Although these compounds are adenine nucleotides, they do not fit the structure profile for purinergic receptor agonists. Taken together, the data fit best the model of prodrugs that are deprotected within cells to agents that are then further metabolized to active inhibitors of adenylyl cyclases. Once deprotected, 2',5'-dd-3'-AMP presumably undergoes sequential phosphorylation to the intracellularly active and most potent form, 2',5'-dd-3'-ATP (Scheme 3). Nucleoside kinases are numerous and are relatively promiscuous and the intracellular phosphorylation of 2',5'-dd-3'-AMP may be expected. Although phosphorylation of 2',5'-dd-3'-AMP to the corresponding diphosphate has not been described, the subsequent phosphorylation to 2',5'-dd-3'-ATP can be catalyzed by several common enzymes, for example, creatine kinase or 3-phosphoglycerate kinase, but not pyruvate kinase [59].

Several considerations are important in understanding why pro-nucleotide forms only of 2',5'-dd-3'-AMP inhibited adenylyl cyclase in intact cells, whereas those of PMEA, PMPA, or β-L-2',3'-dd-5'-AMP did not. The efficacy of pro-nucleotides to block cAMP-mediated events in intact cells will depend not only on their structures, but also on the mechanisms of uptake, deprotection, phosphorylation, and subsequent breakdown. Uptake and deprotection can be influenced if not controlled by the nature of the protective group. Simple changes in the structures of the pro-nucleotide protecting groups can have significant effects on the rates of deprotection. For example, the addition of a hydroxyl group to the t-Bu-SATE-group, increases the half-life of a protected 3'-azido-5'-TMP in CEM-SS cell extracts from 1.2 to 7.5 hr [54]. Behavior of pro-nucleotides
can be modified significantly by the use of mixed phosphotriester derivatives, e.g. with one SATE-group and a second group susceptible to hydrolysis by an enzyme other than carboxyesterases. Examples include thioesterases for dithioethyl-groups, or phosphoramidases for amidate moieties (glycine, [54]; tyrosine, [56]) or alkylamines (isopropyl-amine [54]). These substituents also influence solubility, effecting a balance between aqueous solubility and lipophilicity. But perhaps more important, because the uptake mechanisms and the deprotecting enzymes expressed in different cell types will differ, it may be possible that modifications to the protective groups could afford some degree of tissue selectivity for prodrug inhibitors of adenylyl cyclases in intact cells and tissues. Additional targeting specificity can be developed through modifications to the nucleotide precursor itself.

Intracellular metabolic stability of deprotected ligands can be important and these processes could be differentially affected by experimental conditions. For example, enzymes capable of cleaving 3’-nucleotides are less abundant than those that cleave 5’-phosphates, suggesting that 3’-phosphates might be longer lived in vivo than are mono-, di-, or triphosphate-forms of β-L-2’,3’-dd-Ado or PMEA, for example. Second, the unexpected lack of effect of the bis(t-Bu-SATE) derivatives of PMEA, PMPA, or β-L-2’,3’-dd-5’-AMP, could have been due to impaired processing within cells because of conditions used in the experiments conducted here. For example, although IBMX greatly facilitates the amount of [3H]cAMP measured upon stimulation of cells with forskolin due to its inhibition of cyclic nucleotide phosphodiesterases, the xanthine might have adversely affected processing of one pro-nucleotide relative to another by effects on other enzymes involved in their processing. There is no evidence for this, but a full understanding of pro-nucleotide processing must consider such experimental variations. Also, deamination or depurination of these latter two compounds may occur at greater rates than for the corresponding derivatives of 2’,5’-dd-Ado. For example, whereas β-Ado and 2’-d-Ado were excellent substrates for adenosine deaminase, 2’-d-3’-AMP, 2’-d-5’-AMP, β-L-2’,3’-dd-Ado, 2’,5’-dd-3’-AMP, PMEA, PMPA, and PMPApp were not (not shown).

Although the considerations regarding variations in processing efficacy or mechanism could explain differences in responsiveness of the several cell types to the pro-nucleotides, the differences might also
reflect in part the different structures and hence susceptibilities of the adenylyl cyclase isozymes being expressed. For example, the SATE-protected forms of 2',5'-dd-3'-AMP reduced [3H]cAMP formation in OB1771 cells in the low nanomolar range but a comparable response in cardiac ventricular myocytes required high nanomolar concentrations (Table 2). If these pro-nucleotide were effectively converted to the suggested 2',5'-dd-3'-ATP, then the number, amounts, and sensitivity of the different isozymes expressed in the respective tissues could influence the response seen. From past experience, however, the differences in selectivity for adenine nucleoside 3'-polyphosphates among the isozymes we have tested (I, II, VI, VII, and VIII) was not great, with IC₅₀s varying from 90 nM (type VII) to 280 nM (type II) (5). The other isozymes have not yet been tested nor have we explored which isozymes are expressed in the cells studied here.

And lastly, the mechanisms of inhibition of adenylyl cyclase differ considerably for the agents tested here. β-L-2',3'-dd-5'-ATP is a competitive inhibitor of adenylyl cyclase [6], inhibition by PMEApp or PMPApp is mixed [7], whereas 2',5'-dd-3'-ATP elicits non-competitive or un-competitive inhibition, depending on reaction conditions [2]. This and the observation that 2'-d-3'-AMP is competitive with cAMP in inhibiting the reverse reaction of a chimeric construct of adenylyl cyclase [60] suggest that 2',5'-dd-3'-ATP is a post-transition-state inhibitor, binding to adenylyl cyclase only after products cAMP and metal-pyrophosphate have left the catalytic cleft. Consequently, this class of pro-nucleotide would not be susceptible to the competitive pressures characteristic of the mechanism for β-L-2',3'-dd-5'-ATP and that pro-nucleotides leading to the formation of 2',5'-dd-3'-ATP should be very specific inhibitors of adenylyl cyclases in intact cells.

The expectation is that the number and characteristics of prodrug inhibitors of adenylyl cyclases can be expanded and that tissue and possibly isozyme selective ligands will result. Much can be learned about cell and tissue functions through the inhibition of adenylyl cyclases by ligands such as these. Inhibition of a signaling pathway can be as informative as its stimulation. Potent and specific ligands provide a chemical means for reducing the influence of an enzyme cascade that might otherwise be achieved only through knockout techniques. Moreover, chemical inhibition implies the capacity to cause graded responses.
Cellular responses depend on the degree of change in cAMP levels. If a stimulatory ligand elicits changes in the expression of a group of genes, it is possible that quite different information becomes available if an inhibitory ligand is used and different questions can be asked. For example, if the level of cAMP must be low for cells to passage mitosis [61], can the inhibition of adenylyl cyclase during G2 provide information regarding changes in gene expression necessary for mitosis per se? If the inhibition of adenylyl cyclase causes an increased rate of differentiation of preadipocytes [18], are the changes in gene expression similar to those induced by insulin and triiodothyronine or by bromo-palmitate? That is, is the cassette of genes whose expression is altered by reduced cAMP similar to or different from those associated with differentiation per se? By use of selective adenylyl cyclase inhibitors, one can gain insights into the roles of the adenylyl cyclase-cAMP signaling cascade in cell and tissue function that are not otherwise possible. Moreover, this concept may well be expanded easily to guanylyl cyclases and to other enzymes in signaling cascades with the attendant additional insights that can be gained from blockade of the respective pathways.
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Table 1

IC$_{50}$ values for inhibition of the adenylyl cyclase of preadipocytes by adenine nucleoside 3’-phosphates

Adenylyl cyclase activities were measured as described under “Experimental Procedures”. IC$_{50}$ values were determined from graphical analyses.

| Compound                  | OB1771-cell membranes (µM) | Rat Brain-detergent extract (µM) |
|---------------------------|----------------------------|---------------------------------|
| 2′,5′-dd-Ado               | 21.3                       | 2.7                             |
| 2′,5′-dd-3′-AMP            | 5.71                       | 0.46                            |
| 2′,5′-dd-3′-ADP            | 0.39                       | 0.1                             |
| 2′,5′-dd-3′-ATP            | 0.12                       | 0.04                            |
| 2′,5′-dd-3′-A4P            | 0.007                      |                                 |
| 2′,5′-dd-3′-AMP-HSal       | 29                         | 8.4                             |
| 2′,5′-dd-3′-AMP-MeSal      | 50                         | 6.9                             |
| β-L-2′,3′-dd-5′-AMP        | 62                         |                                 |
| β-L-2′,3′-dd-5′-ATP        | 0.024                      |                                 |
| PMEA                      | 66                         |                                 |
| PMEApp                     | 0.175                      |                                 |
| PMPA                       | 6.3                        |                                 |
| PMPApp                     | 0.50                       |                                 |

a) Values for the rat brain adenylyl cyclase are from Désaubry et al. [2-4].

b) Values are from Shoshani et al. [6].

c) Values are from Shoshani et al. [7].
Table 2

IC$_{50}$ values for various pro-nucleotides to inhibit forskolin-stimulated formation of [$^3$H]cAMP from cells prelabeled with [$^3$H]adenine.

| Pro-Nucleotide | OB1771 Preadipocytes | F442A Preadipocytes | Human Macrophages |
|----------------|----------------------|---------------------|------------------|
|                | IC$_{50}$ (nM)       | IC$_{50}$ (nM)      | IC$_{50}$ (nM)   |
| 2',5'-dd-3'-AMP-bis(Me-SATE) (3) | 18 | 29 | 247 |
| 2',5'-dd-3'-AMP-bis(t-Bu-SATE) (4) | 51 | 146 | 76 |
| 2',5'-dd-3'-AMP-bis(Ph-SATE) (5) | 324 | 740 | 95 |
| β-L-2',5'-dd-3'-AMP-bis(t-Bu-SATE) (6) | ~2000 | | |
| β-L-2',3'-dd-5'-AMP-bis(t-Bu-SATE) (8) | >30000 | | >30000 |
| t-Bu-SATE-PMPA (11) | | >30000 | |
| t-Bu-SATE-PMEA (14) | >30000 | | |
| 2',5'-dd-3'-AMP-HSal (1) | ~1220 | | ~1030 |
| 2',5'-dd-3'-AMP-MeSal (2) | ~2020 | | |
| (1R,2S)-9-(cyclopentyl)-Ade-2'-phosphate-bis(t-Bu-SATE) (16) | >30000 | | |
| (1S,2R)-9-(cyclopentyl)-Ade-2'-phosphate-bis(t-Bu-SATE) (17) | >30000 | | |

Bold numbers in parentheses refer to structures shown in Figure 1. IC$_{50}$ values for the 2'-triphosphate forms of (1R,2S)- and (1S,2R)- enantiomers of 9-(cyclopentyl)-adenine for inhibition of the rat brain adenylyl cyclase were 450 nM and 9860 nM, respectively; these values can be compared with those shown in Table 1.
FIGURE LEGENDS

Figure 1. Structures of 9-substituted adenine derivatives. Structures are referred to by number in the text.

Figure 2. [3H]cAMP formation in OB1771 preadipocytes. OB1771 cells were incubated with [3H]adenine for 2 hr. Cells were washed with serum-free medium and equilibrated for 15 min. Forskolin at the indicated concentrations was then added. At the indicated times medium was aspirated from the cells and 1 ml of ice-cold 0.3 M HClO₄ and 0.5 mM cAMP was added. The solution was neutralized with ice-cold KOH, cell debris and KClO₄ were removed by centrifugation, and [3H]cAMP was purified and quantified, as described under “Experimental Procedures”. Upper panel: Time course for [3H]cAMP formation at various forskolin concentrations. Lower panel: Effect of varying forskolin concentration after a 15 min incubation.

Figure 3. Inhibition of cAMP formation in OB1771 preadipocytes by 2',5'-dd-Ado: Intact cells versus a cell membrane preparation. OB1771 cells were grown, prelabeled with [3H]adenine, and the formation of [3H]cAMP (■) was determined as described under “Experimental Procedures”. 2',5'-dd-Ado was added 15 min before the addition of 30 µM forskolin. Adenylyl cyclase activities (●) in membranes isolated from OB1771 cells were determined as described under “Experimental Procedures”.

Figure 4. The prodrug effect. A comparison was made of the effects of 2',5'-dd-3'-AMP (square symbols) and 2',5'-dd-3'-AMP-bis(t-Bu-SATE) (round symbols) on adenylyl cyclase activity in membranes isolated from OB 1771 preadipocytes (grey symbols & dashed lines) and on the rate of the forskolin-stimulated formation of [3H]cAMP in preadipocytes prelabeled with [3H]adenine (solid symbols & solid lines). The determination of forskolin-stimulated formation of [3H]cAMP in cells prelabeled with [3H]adenine follows the procedures depicted in Figure 2 and described under “Experimental Procedures”. 2',5'-dd-3'-AMP-bis(t-Bu-SATE) was added as solutions in dimethylsulfoxide with dilutions of 100-fold into assays to give the indicated concentrations. For intact cells, compounds were added 15 min before the addition of 30 µM forskolin. Adenylyl cyclase activities in membranes isolated from OB1771 cells
were determined as described under “Experimental Procedures”.

Figure 5. The effects of 2',5'-dd-3'-AMP protected with saligenyl- and bis(t-Bu-SATE)-groups on forskolin-stimulated formation of [3H]cAMP in OB1771 preadipocytes. OB1771 cells were grown, prelabeled with [3H]adenine, and the formation of [3H]cAMP was determined as described for Figure 2 and under “Experimental Procedures”. Compounds were added as solutions in dimethylsulfoxide with dilutions into cell medium of 100-fold to give the indicated concentrations. Compounds were added 15 min before the addition of 30 µM forskolin. The lack of effect of (t-Bu-SATE)-PMEA is shown for comparison.

Figure 6. The importance of the acyl-group of 2',5'-dd-3'-AMP-bis(S-acyl-2-thioethyl)-derivatives to inhibit [3H]cAMP formation in 3T3 F442A preadipocytes. The 3T3-F442A cells were grown, prelabeled with [3H]adenine, and the formation of [3H]cAMP was determined as described under “Experimental Procedures”. Compounds were added as solutions in dimethylsulfoxide and dilutions into cell medium were 100-fold. Compounds were added 15 min before the addition of 30 µM forskolin. The lack of effect of bis(t-Bu-SATE)-PMPA is shown for comparison.

Figure 7. Sensitivity to inhibition of [3H]cAMP formation by 2',5'-dd-3'-AMP-bis(t-Bu-SATE) varies with cell type. For human macrophages, OB1771 preadipocytes, and 3T3 F442A preadipocytes, cells were grown, prelabeled with [3H]adenine, and the formation of [3H]cAMP was determined as described for Figure 2 and under “Experimental Procedures”. Compounds were added as solutions in dimethylsulfoxide with dilutions into cell medium of 100-fold to give the indicated concentrations. Compounds were added 15 min before the addition of 30 µM forskolin. For ventricular cardiomyocytes, cells exhibiting channel activity were identified, pro-nucleotide was added, and after 15 min cells were challenged with 0.1 µM isoproterenol. The response after a 15 min exposure to catecholamine is shown.

Figure 8. Initial metabolism of 2',5'-dd-3'-AMP-bis(t-Bu-SATE) by homogenates of OB1771 preadipocytes and the lack of effect of the diester derivative on adenylyl cyclase activity. Lower
Homogenates were prepared from OB1771 preadipocytes as described under “Experimental Procedures”. 2’,5’-dd-3’-AMP-bis(t-Bu-SATE) (100 μM) was incubated at 30°C either with homogenizing buffer (250 mM sucrose, 3 mM dithiothreitol, and 20 mM glycylglycine, pH 7.5) or with a 965 μl sample of the preadipocyte homogenate in the presence of 5 mM MgCl₂ and a final volume of 1 ml. A 500 μl sample was removed immediately after mixing for the zero time value and was placed on ice. The remainder was incubated for 60 min. Samples with homogenizing medium were filtered through Spartan syringe filters (0.22 μM), whereas homogenates were filtered through Spartan 3 mm filters with 0.45 μM pores. Approximately 195 μl was recovered from these filtrates, of which 130 μl was injected onto a reverse-phase HPLC column (Ultrasphere, 4.6 x 300 mm). The column was developed with a complex elution scheme that first eluted nucleosides and nucleoside phosphates by varying ionic strength, followed by a washing period, and the very hydrophobic protected nucleotides were eluted by varying solvent polarity. Peaks were identified by a Waters 996 photodiode array detector and were quantified by Waters Millennium32 software. Upper panel - Washed membranes were prepared from OB1771 preadipocytes and assayed for adenylyl cyclase activity as described under “Experimental Procedures”, in a reaction mixture containing 50 mM triethanolamine! HCl, pH 7.5, 100 μM ATP, 5 mM MnCl₂, 100 μM IBMX, 1 mM dithiothreitol, 2 mM creatine phosphate, 100 μg creatine kinase per ml, 100 μg myokinase per ml, and 100 μM forskolin. HPLC peaks eluting from the reverse phase column at ~118 min and ~110 min (see text) were pooled and the concentration of nucleotide was estimated from the absorbance at 259 nm with an assumed ε = 15400 M⁻¹. The effect of 3 μM 2’,5’-dd-3’-AMP is shown for comparison.

Figure 9. Extracellular breakdown of 2’,5’-dd-3’-AMP-bis(t-Bu-SATE) and the effect of product on formation of [3H]cAMP in prelabeled OB1771 preadipocytes.

Lower panel - To a 35 mm culture dish of confluent OB1771 preadipocytes, grown as described under “Experimental Procedures”, was added 2 ml of DMEM medium and 2’,5’-dd-3’-AMP-bis(t-Bu-
SATE) to a final concentration of 50 µM. A second dish without cells was used as the ‘medium’ control. From each was immediately removed a 200 µl aliquot as a zero time point sample. Thereafter, the dishes were incubated at 37°C under CO₂ and 200 ml samples were taken at the indicated time points and placed on ice. Samples were then subjected to HPLC on a C18-reverse phase column. Nucleotides were quantified from UV peaks detected with a Waters 996 photodiode array detector and analyzed with Waters Millennium32 software.

**Middle panel** - Macrophages were grown as described under “Experimental Procedures”.

Medium (PFHM-II; serum-free; protein-free hybridoma medium) alone or cells and medium (6 ml) were incubated in glass tubes at 37°C with shaking, but without CO₂. 2',5'-dd-3'-AMP-bis(t-Bu-SATE) was added to a final concentration of 100 µM. At the indicated times 500 µl portions were removed to which 500 µl of ice-cold 0.6 M HClO₄ was added and then samples were placed on ice. Triethanolamine-HCl (116 µl of 0.5 M) and ice-cold KOH (43 µl of 8.4 N) were added to precipitate protein and KClO₄.

Samples were clarified by centrifugation and then by filtration through syringe filters (Spartan 3, 0.45 µm pore) before being subjected to reverse phase HPLC as described for Figure 8.

**Upper panel** - OB1771 preadipocytes were prelabeled with [³H]adenine and forskolin-stimulated formation of [³H]cAMP was measured as described under “Experimental Procedures”. Samples of the material eluting at ~110 min (see legend to Figure 8 and text) and 2',5'-dd-3'-AMP-bis(t-Bu-SATE) were added in dimethylsulfoxide at 100-fold dilutions to give the indicated concentrations. Concentration of nucleotide was estimated as described for Figure 8.
Scheme 1. Synthesis of 2',5'-dd-3'-AMP-cyclosaligenyl derivatives.
Scheme 2. Synthesis of β-L-2',5'-dd-3'-AMP-bis(t-Bu-SATE).

Reagents and conditions: a) Ph-S-S-Ph, Bu₃P/pyridine; b) Raney Ni/MeOH-iPrOH

Reagents and conditions: c) 1H-tetrazole/THF-DMF then tBuOOH/toluene
Scheme 3. Processing of the pro-nucleotide 2',5'-dd-3'-AMP-bis(t-Bu-SATE). Adapted from Lefebvre et al. [33].
Figure 1. Structures of 9-substituted adenine derivatives.
Figure 2. \[^{[H]}\text{cAMP}\] formation in OB1771 preadipocytes.
Figure 3. Inhibition of cAMP formation in OB1771 preadipocytes by 2',5'-dd-Ado:

Intact cells versus a cell membrane preparation.
Figure 4. The prodrug effect.
Figure 5. The effects of 2',5'-dd-3'-AMP protected with saligenyl- and bis(t-Bu-SATE)-groups on forskolin-stimulated formation of [3H]cAMP in OB1771 preadipocytes.
Figure 6. The importance of the acyl-group of 2',5'-dd-3'-AMP-bis(S-acyl-2-thioethyl)-derivatives to inhibit $[^3]$HcAMP formation in 3T3 F442A preadipocytes.
Figure 7. Sensitivity to inhibition of $[^3H]$cAMP formation by 2',5'-dd-3'-AMP-bis(t-Bu-SATE) varies with cell type.
Figure 8. Initial metabolism of 2',5'-dd-3'-AMP-bis(t-Bu-SATE) by homogenates of OB1771 preadipocytes and the lack of effect of the diester derivative on adenylyl cyclase activity.
Figure 9. Extracellular breakdown of 2',5'-dd-3'-AMP-bis(t-Bu-SATE) by THP1 macrophages and the effect of product on forskolin-stimulated formation of [3H]cAMP in prelabeled cells.
1. **Abbreviations used:**

IBMX, 1-methyl-3-isobutylxanthine

\(2',5'\)-dd-Ado, 2',5'-dideoxyadenosine;

\(\beta-L-2',5'\)-dd-Ado, \(\beta-L-2',5'\)-dideoxyadenosine;

\(2',5'\)-dd-3'-AMP, 2',5'-dideoxyadenosine-3'-monophosphate;

\(2',5'\)-dd-3'-ADP, 2',5'-dideoxyadenosine-3'-diphosphate;

\(2',5'\)-dd-3'-ATP, 2',5'-dideoxyadenosine-3'-triphosphate;

\(2',5'\)-dd-3'-A4P, 2',5'-dideoxyadenosine-3'-tetraphosphate;

\(2',5'\)-dd-3'-AMP-(H-Sal), 2',5'-dideoxyadenosine-3'-O-cyclosaligenyl-phosphates;

\(2',5'\)-dd-3'-AMP-(Me-Sal), 2',5'-dideoxyadenosine-3'-O-cyclo-3-methyl-saligenyl phosphate;

\(2',5'\)-dd-3'-AMP-bis(Me-SATE), 2',5'-dideoxyadenosine-3'-O-bis(S-acetyl-2-thioethyl)-phosphate;

\(2',5'\)-dd-3'-AMP-bis(t-Bu-SATE), 2',5'-dideoxyadenosine-3'-O-bis(S-pivaloyl-2-thioethyl)-phosphate;

\(2',5'\)-dd-3'-AMP-bis(Ph-SATE), 2',5'-dideoxyadenosine-3'-O-bis(S-benzoyl-2-thioethyl)-phosphate;

\(\beta-L-2',5'\)-dd-3'-AMP-bis(Me-SATE), 2',5'-dideoxyadenosine-3'-O-bis(S-acetyl-2-thioethyl)-phosphate;

\(\beta-L-2',5'\)-dd-3'-AMP-bis(t-Bu-SATE), 2',5'-dideoxyadenosine-3'-O-bis(S-pivaloyl-2-thioethyl)-phosphate;

\(\beta-L-2',5'\)-dd-3'-AMP-bis(Ph-SATE), 2',5'-dideoxyadenosine-3'-O-bis(S-benzoyl-2-thioethyl)-phosphate;

\(2',3'\)-dd-Ado, 2',3'-dideoxyadenosine;

\(\beta-L-2',3'\)-dd-Ado, \(\beta-L-2',3'\)-dideoxyadenosine;

\(\beta-L-2',3'\)-dd-5'-AMP, \(\beta-L-2',3'\)-dideoxyadenosine-5'-monophosphate;

\(\beta-L-2',3'\)-dd-5'-ADP, \(\beta-L-2',3'\)-dideoxyadenosine-5'-diphosphate;

\(\beta-L-2',3'\)-dd-5'-ATP, \(\beta-L-2',3'\)-dideoxyadenosine-5'-triphosphate;

\(\beta-L-2',3'\)-dd-5'-AMP-bis(Me-SATE), 2',3'-dideoxyadenosine-5'-O-bis(S-acetyl-2-thioethyl)-phosphate;

\(\beta-L-2',3'\)-dd-5'-AMP-bis(t-Bu-SATE), 2',3'-dideoxyadenosine-5'-O-bis(S-pivaloyl-2-thioethyl)-phosphate;

\(\beta-L-2',3'\)-dd-5'-AMP-bis(Ph-SATE), 2',3'-dideoxyadenosine-5'-O-bis(S-benzoyl-2-thioethyl)-phosphate;

PMEA, 9-(2-phosphonomethoxyethyl)adenine;

PMEApp, (R)-9-(2-phosphonomethoxyethyl)adenine-diphosphate;

Me-SATE-PMEA, (R)-bis(S-acetyl-2-thioethyl)-9-(2-phosphonomethoxyethyl)-adenine;

t-Bu-SATE-PMEA, (R)-bis(S-pivaloyl-2-thioethyl)-9-(2-phosphonomethoxyethyl)-adenine;

Ph-SATE-PMEA, (R)-bis(S-benzoyl-2-thioethyl)-9-(2-phosphonomethoxyethyl)-adenine;

PMPA, 9-(2-phosphonomethoxypropyl)adenine;

PMPApp, (R)-9-(2-phosphonomethoxypropyl)adenine-diphosphate;

Me-SATE-PMPA, (R)-bis(S-acetyl-2-thioethyl)-9-(2-phosphonomethoxypropyl)-adenine;

t-Bu-SATE-PMPA, (R)-bis(S-pivaloyl-2-thioethyl)-9-(2-phosphonomethoxypropyl)-adenine;

Ph-SATE-PMPA, (R)-bis(S-benzoyl-2-thioethyl)-9-(2-phosphonomethoxypropyl)-adenine;
2. The forskolin-induced increase in the formation of $[^3]$HcAMP was diminished to only two-fold when longer periods of exposure to $[^3]$Hadenine were used, e.g. six hours, and when cells were equilibrated for longer periods after removal of the $[^3]$Hadenine (not shown). The reduced response to forskolin with the longer prelabeling times was probably due to the further distribution of $[^3]$Hadenine into other metabolic pools. Consequently, depicted experiments used the 2 hr prelabeling period.

3. After sample injection, the C18-column was washed for 5 min with the column equilibration buffer (A: 10 mM tetra-butylammonium hydroxide, 10 mM KH$_2$PO$_4$, 1% methanol, pH = 6.96). Nucleosides and nucleotides were eluted with a gradient that then changed over 40 min from Buffer A to Buffer B (2.8 mM tetra-butylammonium hydroxide, 100 mM KH$_2$PO$_4$, 30% methanol, pH = 5.5). At 55 min, Buffer B was exchanged via solvent valve to water with which the column was washed for 25 min. At 80 min a 30 min gradient from water to 100% methanol was introduced to elute the hydrophobic pro-nucleotides. The column was returned to water and then to Buffer A before the next sample was injected. These phosphate-containing buffers give excellent separations of ribo-, deoxyribo-, and dideoxyribo-nucleotides, but removal of the buffers is necessary before the introduction of high concentrations of methanol to prevent their precipitating within the HPLC system. With this protocol retention times for nucleotides were, in minutes: adenine, 8; 2',5'-dd-Ado, 15.7; 2',5'-dd-3'-AMP, ~24; 2',5'-dd-3'-ADP, ~33; 2',5'-dd-3'-ATP, 39; 2',5'-dd-3'-AMP-bis(t-Bu-SATE), 117-120.

4. For example, the bis(POM)-derivative of PMEA (adefovir dipivoxil; Hepsera, from Gilead Sciences) has recently been approved the treatment of chronic hepatitis B in adults.

5. Mixed inhibition in this context implies that plots of lines at increasing concentrations of inhibitor on a Lineweaver-Burke plot (1/velocity vs 1/[MnATP]) would intersect above the abscissa and to the left of the ordinate.
Pro-nucleotide inhibitors of adenylyl cyclases in intact cells
Wolfgang H. G. Laux, Praveen Pande, Ilana Shoshani, Junyuan Gao, Valérie Boudou-Vivet, Gilles Gosselin and Roger A. Johnson

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