Characterization of the Yeast Low $K_m$ cAMP-Phosphodiesterase with cAMP Analogues

APPLICATIONS IN MAMMALIAN CELLS THAT EXPRESS THE YEAST PDE2 GENE*

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The essential interactions between cAMP and the yeast low $K_m$ cAMP-phosphodiesterase have been analyzed using cAMP analogues and phosphodiesterase inhibitors. cAMP specificity is conferred by hydrogen bonding at the N-6 and N-7 positions. In contrast to the other yeast phosphodiesterase, $(R,)-$adenosine 3',5'-monophosphorothioate is not hydrolyzed. Eleven standard phosphodiesterase inhibitors were not highly effective.

In Chinese hamster ovary (CHO) cells that express the yeast cAMP-phosphodiesterase (PDE2) gene, cAMP levels cannot be raised by cholera toxin. cAMP analogues that are efficiently hydrolyzed by the yeast cAMP-phosphodiesterase had no effect on the growth of CHO cells that express the PDE2 gene, even though they block the growth and alter the morphology of control cells. cAMP analogues that are not hydrolyzed by the yeast enzyme inhibited the growth and changed the morphology of both control and PDE2 expressing CHO cells. We have developed a method for creating cell lines in which cAMP levels can be reduced by expression of an exogenous cAMP-phosphodiesterase gene. By employing cAMP analogues that are not hydrolyzed by this phosphodiesterase, the inhibitory effects of the enzyme can be bypassed.

One of the problems in the study of signal transduction has been to determine to what extent the cAMP second messenger system is responsible for the effects of a hormone or growth factor. For example, a hormone receptor may be coupled to more than one second messenger system, only one of which may be generated by adenylate cyclase. One experimental approach to determine which physiological responses depend on cAMP and which do not is to introduce a gene whose product degrades cAMP and to determine the effect that this has on cells stimulated with specific agonists. We have introduced an expression vector carrying the yeast PDE2 (=SRA5) gene, coding for the low $K_m$ cAMP-phosphodiesterase, into Chinese hamster ovary (CHO) cells, and we have shown that the gene is transcribed and codes for an active enzyme which vitiated the growth inhibitory effects of cholera toxin on CHO cells (1).

The yeast low $K_m$ cAMP-phosphodiesterase has been purified to homogeneity and shown to be a zinc-binding enzyme that requires Mg$^{2+}$ for activity (2). It has a molecular mass of 61,000 daltons and an apparent $K_m$ of 170 nM. The enzyme is not stimulated or inhibited by cAMP and falls into the class IV designation of phosphodiesterases (3). The pharmacological properties of the yeast cAMP-specific phosphodiesterase were largely unknown.

We followed two approaches for creating conditions in which the effects of the yeast cAMP-phosphodiesterase can be overcome. First, we searched for specific inhibitors of the yeast cAMP-phosphodiesterase. Second, we searched for non-hydrolyzable, membrane-permeable cAMP analogues, which can activate the cAMP-dependent protein kinase in vivo. None of 11 cyclic nucleotide phosphodiesterase inhibitors that we assessed were active and therefore we focused on non-hydrolyzable cAMP analogues.

To be able to predict the structure-activity relationship of such cAMP analogues, we mapped the essential molecular interactions between substrate and the yeast cAMP-phosphodiesterase responsible for both binding and hydrolysis by using a "test kit" of systematically modified analogues (4, 5). This constellation of cAMP analogues has been successfully used to characterize a variety of cyclic nucleotide-binding proteins. These include cAMP-binding protein from Escherichia coli (6, 7), both binding sites from different cAMP-dependent protein kinases (8-12), diverse cyclic nucleotide phosphodiesterases (13-19), and Dictostelium discoideum cell surface receptors (20-22). Comparison of the binding data for the yeast cAMP-phosphodiesterase with those of the cAMP-dependent protein kinases led to the identification of several cAMP analogues which can bypass the block imposed on the cAMP-dependent protein kinase by the expression of the yeast cAMP-phosphodiesterase gene in mammalian cells. Thus, cells that carry the yeast cAMP-phosphodiesterase gene can be prevented from carrying out cAMP-dependent functions, but in the manner of a conditional mutant, this ability can be restored by application of analogues.

**EXPERIMENTAL PROCEDURES**

**Methods**

*Source of the Enzyme—Saccharomyces cerevisiae* contains only two phosphodiesterases capable of hydrolyzing cAMP (23, 24), one with a $K_m$ of about 170 nM which is specific for cAMP encoded by the PDE2 (=SRA5) gene (2, 25, 26) and another with a $K_m$ of about 0.1...
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mM, which degrades both cAMP and cGMP and is encoded by the PDEI gene (2, 23). The strain J105 (23) was used to produce the enzyme. This strain carries a disruption of the yeast PDEI gene, and, therefore, all of its cAMP-phosphodiesterase activity is due to the PDEZ (=SRA5) gene product. Following the procedure of Londesborough and Suoranta (24), 1 liter of cells was grown in YEP medium to stationary phase, harvested by centrifugation at 12,000 \( \times g \) for 5 min, resuspended in homogenization buffer (10 mM potassium phosphate, pH 7.5, 1 mM MgCl\(_2\), 0.1 mM EDTA, 7 mM \( \beta \)-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.6 M mannitol, 0.45 M KCl). The cells were broken by vortexing with glass beads and centrifuged at 12,000 \( \times g \) for 30 min. The supernatants were centrifuged again for 1 h at 48,000 \( \times g \). All operations were at 4 °C. The supernatant was brought to 1 mM EDTA and ammonium sulfate was added to 25 g/100 ml over the course of 20 min. After 30 min on ice, the preparation was centrifuged for 15 min at 12,000 \( \times g \). An additional 25 g/100 ml of ammonium sulfate was added to the supernatant. After 30 min the preparation was centrifuged at 12,000 \( \times g \), and the pellet was resuspended in 10 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 1 mM MgCl\(_2\), and 1.4 mM \( \beta \)-mercaptoethanol, and 0.5 M KCl. Four ml of a resuspended pellet were applied to a Sephacryl S200 column (bed volume 82 ml) and eluted with the same buffer. The peak active fractions were pooled and stored in 40% glycerol at -20 °C.

**cAMP-Phosphodiesterase Assays for Measurement of Apparent \( V_{\text{max}} \)**—The assay buffer contained 10 mM Tris-HCl, pH 8.0, 5 mM MgCl\(_2\), 25 nM of [2,8-\( ^3 \)H]cAMP (80 nCi; 32.9 Ci/mmol) and 20 \( \mu l \) of a 10-fold dilution of the partially purified enzyme and varying concentrations of cAMP analogues or phosphodiesterase inhibitors in a final volume of 100 \( \mu l \). Incubations were carried out for 30 min at 30 °C and stopped by boiling for 2 min. Reaction mixtures were then treated with snake venom (Ophiophagus hannah), and the reaction products were separated and measured as described by Bulgakov and Van Haastert (27).

**cAMP-Phosphodiesterase Assays for Measurement of \( K_{\text{m}} \)**—The buffer described above was used, omitting radioactive cAMP. Assays contained 10 \( \mu l \) of the undiluted partially purified enzyme in a final volume of 25 \( \mu l \). The reactions were stopped by the addition of 1 M phosphoric acid and reduced to pH 3.0 with 100 mM phosphoric acid. The hydrolysis of the various cAMP analogues was monitored by high pressure liquid chromatography using a \( \mu \)Bondapak C18 column. The mobile phase was 10 mM phosphate, pH 3.0, 5 mM triethylamine, 0.5 mM tributylamine, and varying concentrations of methanol (2-35%) which depended on the hydrophobicity of the analogue. The flow rate was 1 ml/min and absorbance was monitored at 254 nm. Percentage hydrolysis was determined by integration of the peaks. Substrate concentrations at least 10-fold greater than the \( K_{\text{m}} \) of the analogue for the enzyme were employed.

**Growth of Chinese Hamster Ovary Cells—**CHO cells lines (derived from strain 10001) were grown in a modified minimal essential medium supplemented with 10% fetal calf serum (GIBCO), 50 units/ml penicillin, 50 \( \mu \)g/ml streptomycin, and 1 mg/ml G418 (GIBCO) as described by Gottesman et al. (28). The creation of the CHO-C3 control vector-transfected cell line and of the CHO-PDE12 cell line that expresses the yeast PDE2 (=SRA5) gene transcribed from a murine sarcoma virus long terminal repeat has been described previously (1). The CHO-PDE12 cell line was maintained in 100 ng/ml cholera toxin to select against loss of the phosphodiesterase gene.

**Fig. 1. Kinetics of the yeast low \( K_{\text{m}} \) cAMP-phosphodiesterase.** Time course of hydrolysis of 25 nM [2,8-\( ^3 \)H]cAMP (■) or 82 nM [2,8-\( ^3 \)H]cAMP (○). Inset, Eadie-Hofstee plot of [2,8-\( ^3 \)H]cAMP hydrolysis at different substrate concentrations. \( v \) is the rate of hydrolysis in nmol/min/mg protein. \( v/[S] \) is expressed as nmol/min/mg protein/PM. Assays were performed as described under "Experimental Procedures." Results shown are the means of three experiments in which each point was assayed in triplicate.

**Fig. 2. Structures of cAMP analogues and inhibitors in Table 1.**
Kinetic Properties of the Partially Purified Yeast Low $K_m$ cAMP-Phosphodiesterase—We have taken advantage of the demonstration of Nikawa et al. (29) that yeast contains only two phosphodiesterase activities capable of hydrolyzing cAMP and that the genes which code for these enzymes can be separately mutated. By using strain J105, with a disrupted high $K_m$ cyclic nucleotide phosphodiesterase gene (PDE1), we were able to prepare an extract which contained only one activity, that due to PDE2 (=SRAS), the gene that codes for the low $K_m$ cAMP-phosphodiesterase. To confirm that the enzyme in the partially purified J105 extract is exclusively the low $K_m$ PDE2-cAMP-specific phosphodiesterase, we measured hydrolysis of cAMP and cGMP at low substrate concentrations. The results in Fig. 1 demonstrate that the enzyme degrades CAMP. There is little hydrolysis of cGMP (<2% h$^{-1}$) at low substrate concentrations (82 nM), which is the expected result if the strain of yeast used to produce the enzyme lacks the nonspecific cyclic nucleotide phosphodiesterase. Kinetic analysis of the partially purified yeast cAMP-phosphodiesterase with varying substrate concentrations shows nonlinearity in an Eadie-Hofstee plot (inset, Fig. 1) as described previously for the enzyme purified to homogeneity (2). These authors concluded that the nonlinearity was caused by the presence of both intact and nicked forms of the enzyme with $K_m$ values of 115 nM and 1.63 µM, respectively (2), which agrees well with the results shown in Fig. 1.

Mapping of cAMP-cAMP-Phosphodiesterase Interactions

Rationale for Selection of cAMP Analogues—Compounds 1–10 were selected according to the test kit concept (4, 5) to map the essential molecular interactions formed between cAMP and the yeast cAMP-phosphodiesterase and to compare them with results obtained with the same analogues for other cAMP-binding proteins (6–22). Compounds 12 and 13 were chosen to check the steric influences at the C-8 position and because these analogues are known to be good activators of the cAMP-dependent protein kinases (39, 40). Compounds 11, 15, 16, 17, and 18 were selected to prove the proposed hydrogen bonding at the N-6 position. Compounds 16, 17, and 18 also provided information on hydrogen bonding toward the N-3 position and hydrophobic interactions between the enzyme and the purine moiety. The double-modified compound 14 was designed to combine a good binding property to the cAMP-dependent protein kinase (data not shown) with resistance to hydrolisis by the yeast cAMP-phosphodiesterase. This compound has the further advantage that it is relatively lipophilic (data not shown) and will therefore cross the plasma membrane with greater ease. Compound 10 is a cAMP-dependent protein kinase antagonist (41). The structure of these analogues is shown in Fig. 2.

Binding of cAMP Analogues—To determine the ability of a variety of cAMP analogues to compete for the cAMP-binding site, we measured the hydrolysis of 25 nM [2,8-$^3$H]cAMP in the presence of varying concentrations of analogue. Fig. 3 shows the results of experiments with seven of a series of 24 analogues. The concentration of the cAMP analogue which reduces the hydrolysis of low concentrations of [2,8-$^3$H]cAMP by 50% (IC$_{50}$) is equal to the $K_m$ of the cAMP derivative, assuming Michaelis-Menten kinetics and provided that [S] $\ll$ $K_m$. Michaelis-Menten kinetics cannot be assumed as our preparation shows nonlinearity in Eadie-Hofstee plots (inset, Fig. 1). Therefore, the point of 50% inhibition of hydrolysis of [2,8-$^3$H]cAMP represents an apparent $K_m$ ($K_{app}$), as defined by Van Haastert et al. (15). The $K_{app}$ values of all the analogues tested relative to the $K_m$ for cAMP are shown in Table I. Data are also expressed as $\delta AG$ values, which represent a reduction of free energy of binding (in kJ/mol) of the cAMP analogue compared with cAMP, where $\delta AG = RT \ln (K_m$ analogue/$K_m$ cAMP) (42). This allows assessment of the type of interaction that has been interfered with (43) and comparison with other cAMP-binding proteins assayed under different conditions (42).

Making use of the rationale for selection of cAMP analogues described above, we have mapped the essential chemical interactions of cAMP with the active site. cAMP is recognized and bound by the very specific hydrogen bond between one of the hydrogens at the N-6 position and the enzyme. This is inferred by the strongly reduced $K_a$ values of compounds like 6-Cl-PUMP (compound 3) and 4-cNBIMP (compound 16). A second hydrogen bond is probable between the enzyme and the N-7 position. The removal of this nitrogen, as in 7-deaza-cAMP (compound 4), yields a very strong reduction of affinity. All analogues modified at the N-6 or N-7 position (compounds 3, 4, 11, 16, 17, 18, 23, and 24) have a $\delta AG$ of between 10 and 25 kJ/mol, which is the range of the binding energy of a hydrogen bond (43). No hydrogen bond is formed at the 2'-OH, 3'-O or at the 5'-O positions because 2'-deoxy-cAMP (compound 6), 3'-amino-3'-deoxy-cAMP (compound 7) and 5'-amino-5'-deoxy-cAMP (compound 8) bind relatively well to the enzyme. The results with benzim-
dazole derivatives (compounds 15, 16, and 17) and the N'-oxide-cAMP (compound 2) suggest that no hydrogen bonding is likely at the N-1 and N-3 positions. The high $\Delta G$ of N'-oxide-cAMP (compound 2) is probably due to its high polarity (see below). cAMP probably binds to the enzyme in the syn conformation because the introduction of a bulky group at the C-8 position (compounds 5, 13, and 14), which shifts the natural syn-anti-equilibrium of 1:1 for cAMP to the syn conformation does not interfere with binding. Decreased hydrophobicity of the purine moiety (compounds 2, 23, and 24) reduces the affinity for the enzyme substantially, whereas increased hydrophobicity, as in the benzimidazole derivatives (compounds 17, 18, and 19) bind relatively well to the enzyme when taking into consideration the increase in $\Delta G$ due to the disruption of the hydrogen bond at the N-6 position. The introduction of a sulfur atom in an equatorial (comp-
Correlation of \( \delta G \) values of systematically modified cAMP analogues for the yeast cAMP-phosphodiesterase with those of other cAMP-binding proteins

\( \delta G \) values for the yeast low \( K_m \) cAMP-phosphodiesterase (YPDE) are taken from Table I; for the beef heart type I calcium-calmodulin-stimulated cyclic nucleotide phosphodiesterase (PDEI) and the \( D. discoideum \) cyclic nucleotide phosphodiesterase (DdPDE) from Van Haastert et al. (15); for the type II cGMP-stimulated cyclic nucleotide phosphodiesterase (PDEII) from Coughie et al. (14); for the \( D. discoideum \) cell-surface cAMP receptor (DdR) from Van Haastert and Kien (20); for the \( E. coli \) cAMP-binding protein (CAP) from Scholubbers (7); and for the A- and B-binding sites of the regulatory subunit of the type I CAMP-dependent protein kinase (RIA and RIB) from De Wit et al. (11). \( \delta G \) values represent the reduction of binding energy (in kJ/mol) of a cAMP analogue compared with cAMP. RO 20-1724 inhibits members of one class of mammalian low \( K_m \), cAMP (compound 13).

The results in the lower panel of Table I show that a number of frequently used cyclic nucleotide phosphodiesterase inhibitors are inactive or less active than they are with certain low \( K_m \) forms of (type IV) mammalian enzymes. Isobutylmethylxanthine has a \( K_m \) for mammalian enzymes that is in the range of 1–5 \( \mu M \). To inhibit the yeast enzyme, 100-fold higher concentrations must be used to achieve half-maximal inhibition (IC\(_{50}\)). The compound RO 20–1724 had no discernible inhibitory effect on the yeast enzyme. RO 20–1724 inhibits members of one class of mammalian low \( K_m \) cAMP-phosphodiesterase that, like the yeast enzyme, are insensitive to cGMP (45). Substrate concentrations below the \( K_m \) were used to approximate \( K_m \).

Table II uses values taken from the literature to compare the binding of cAMP analogues to the yeast low \( K_m \) cAMP-phosphodiesterase with the binding of the same analogues to three other cAMP and cyclic nucleotide phosphodiesterases, the cell surface cAMP receptor from \( D. discoideum \), \( A \) and \( B \) sites on the regulatory subunits of the cAMP-dependent protein kinase, and the CAP protein from \( E. coli \). The relative binding energies (\( \delta G \)) for the twelve test kit analogues (compounds 1–12) are compared through calculation of correlation coefficients. Although in some cases not all the analogues have been tested for binding to each of the proteins, in no case could a significant correlation coefficient be found.

The Effects of cAMP Analogues on CHO Cells That Express Yeast Low \( K_m \), cAMP-Phosphodiesterase

Normal CHO cells respond to agents that increase cAMP content in two ways: growth is slowed, and the cells become spindle shaped and clustered. Cholera toxin or 8-Br-cAMP have these effects on control CHO cells. When the yeast cAMP-phosphodiesterase gene (\( PDE2 \)) is introduced into these cells, the yeast enzyme is synthesized and can be distin-

\[
\begin{array}{cccccccc}
\text{No.} & \text{Analogue} & \text{YPDE} & \text{PDEI} & \text{PDEII} & \text{DdPDE} & \text{DdR} & \text{CAP} & \text{RIB} \\
1. & cAMP & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
2. & N'-Oxide-cAMP & 12.4 & 10.4 & 1.2 & 2.2 & 14.6 & 1.6 & -0.1 \\
3. & 6'-Cl-PuMP & 22.0 & 5.0 & 2.1 & 4.6 & 15.1 & 1.1 & -0.8 \\
4. & 7'-Deaza-cAMP & 19.4 & 4.4 & -0.5 & 13.3 & 10.2 & 17.1 & 18.1 \\
5. & 8'-Br-cAMP & 3.4 & 3.7 & -6.5 & 4.5 & 17.2 & 16.8 \\
6. & 2'-Deoxy-cAMP & 5.3 & 2.2 & 3.6 & 5.6 & 15.0 & 15.2 \\
7. & 3'-Amine-3'-deoxy-cAMP & 9.1 & 2.9 & 3.6 & 5.6 & 15.0 & 17.1 & 18.1 \\
8. & 5'-Amine-5'-deoxy-cAMP & 17.0 & 6.0 & 13.1 & 10.7 & 5.7 & 9.2 & 9.4 \\
9. & S,,-cAMPs & 5.2 & 4.4 & 13.1 & 10.7 & 5.7 & 9.2 & 9.4 \\
10. & R,,-cAMPs & 18.0 & 6.4 & 2.5 & 9.2 & 6.3 & 5.6 \\
\end{array}
\]

\[
\begin{array}{cccccccc}
\text{Correlation with YPDE} & 1.00 & 0.56 (9) & 0.24 (4) & -0.15 (7) & 0.38 (8) & -0.50 (6) & -0.40 (9) & -0.39 (9) \\
\end{array}
\]

Hydrolysis of cAMP Analogues

Competition with [2,\( \delta \)-\( ^{14} \)N]cAMP hydrolysis does not reveal whether the enzyme can hydrolyze the analogue. Hydrolysis of the cAMP analogues was examined by comparing the rate of hydrolysis of cAMP, at [S] >> \( K_m \), with that of an analogue. Analysis of the products was by high performance liquid chromatography using a reverse-phase C18 column. Table I (last column) presents relative \( V_{\text{max}} = V'_{\text{max}} \) analogue/\( V_{\text{max}} \) cAMP) values for 14 compounds. Many of these bind to the enzyme, as reflected in the relative \( K_m \) values but are not efficiently hydrolyzed, as indicated by the very low relative \( V_{\text{max}} \) values. All monophosphorothioate derivatives (compounds 9, 10, and 14) fall into this category. The only other tested compound that binds well but is hydrolyzed slowly is 8-(4-CPT)-cAMP (compound 13).

The results in the lower panel of Table I show that a number of frequently used cyclic nucleotide phosphodiesterase inhibitors are inactive or less active than they are with certain low \( K_m \) forms of (type IV) mammalian enzymes. Isobutylmethylxanthine has a \( K_m \) for mammalian enzymes that is in the range of 1–5 \( \mu M \). To inhibit the yeast enzyme, 100-fold higher concentrations must be used to achieve half-maximal inhibition (IC\(_{50}\)). The compound RO 20–1724 had no discernible inhibitory effect on the yeast enzyme. RO 20–1724 inhibits members of one class of mammalian low \( K_m \) cAMP-phosphodiesterase that, like the yeast enzyme, are insensitive to cGMP (45). Substrate concentrations below the \( K_m \) were used to approximate \( K_m \).
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**FIG. 5. Morphology of control CHO-C3 cells and yeast cAMP-phosphodiesterase expressing CHO-PDE12 cells treated with cAMP analogues.** Cells from the experiment shown in Fig. 3 were photographed before exposure to trypsin. A–C, CHO-PDE12 cells expressing the yeast cAMP-phosphodiesterase gene; D–F, CHO-C3 cells containing the control plasmid. A and D, no additions; B and E, 1 mM 8-Br-cAMP; C and F, 1 mM S$_p$-8-Br-cAMPS.

**TABLE III**
cAMP analogues that inhibit growth of CHO-C3 control cells and yeast cAMP-phosphodiesterase expressing CHO-PDE12 cells equally well

CHO-C3 and CHO-PDE12 cells were seeded at 10$^4$ cells/well of a 12-well tissue culture dish in the presence of varying concentrations of the cAMP analogues. After 3 days cells were trypsin treated and counted. Data are presented as IC$_{50}$ values, which is the concentration of cAMP analogue which inhibits growth by 50% of both the CHO-C3 control cell line and of the CHO-PDE12 yeast cAMP-phosphodiesterase expressing cell line. The numbers in the first column refer to the compounds listed in Table I.

<table>
<thead>
<tr>
<th>No.</th>
<th>Analogue</th>
<th>IC$_{50}$ for growth μM</th>
</tr>
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<tbody>
<tr>
<td>14</td>
<td>S$_p$-8-Br-cAMPS</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>S$_p$-cAMPS</td>
<td>200</td>
</tr>
<tr>
<td>13</td>
<td>8-(4-CPT)-cAMP</td>
<td>200</td>
</tr>
<tr>
<td>11</td>
<td>N$^\alpha$-Benzoyl-cAMP</td>
<td>400</td>
</tr>
<tr>
<td>15</td>
<td>N$^\alpha$,O$^\beta$-Dibutyryl-cAMP</td>
<td>1000</td>
</tr>
</tbody>
</table>

guished from the endogenous enzymes of the host cell because of its low $K_m$ and insensitivity to the inhibitors described above. The cell line CHO-PDE12 expresses the yeast PDE2 (SRA5) gene (1). When cholera toxin is applied to this cell line there is little or no increase in intracellular cAMP, whereas there is about a 35-fold increase in CHO-C3 cells, which carry the control vector without the PDE2 insert (1).

Using the data presented in Fig. 3 and Table I, we can predict which cAMP analogues will cause both the control (CHO-C3) and yeast cAMP-phosphodiesterase containing cells (CHO-PDE12) to stop growing and change their morphology. 8-Br-cAMP (compound 5) is a good substrate for the yeast cAMP-phosphodiesterase and as the results in Fig. 4A demonstrate, CHO-PDE12 cells are relatively resistant to the growth inhibitory effects of 8-Br-cAMP when compared with CHO-C3 cells. S$_p$-8-Br-cAMPS (compound 14) binds to the yeast cAMP-phosphodiesterase but is not hydrolyzed (Table I). The results in Fig. 4B show that the growth of CHO-PDE12 and CHO-C3 cell lines are equally sensitive to S$_p$-8-Br-cAMPS (compound 14), as predicted. As is shown in Fig. 4, CHO cells are more sensitive to S$_p$-8-Br-cAMPS (IC$_{50}$
about 20 μM) than to 8-Br-cAMP (IC₅₀ about 100 μM), despite the fact that 8-Br-cAMP is a better activator of the cAMP-dependent protein kinase than S₂-8-Br-cAMPS (data not shown). The results indicate that S₂-8-Br-cAMPS enters the cells more readily, or that 8-Br-cAMP is hydrolyzed substantially by endogenous phosphodiesterases. If the latter is the case, one should be cautious of artifacts that may result from its metabolites.

The effects of 8-Br-cAMP and S₂-8-Br-cAMPS on cell growth are paralleled by changes in morphology as shown in Fig. 5. 8-Br-cAMP causes cell shape changes only in the control cells while S₂-8-Br-cAMPS causes both cell lines to grow with a clustered spindle shape. Thus, the properties of the enzyme observed in vitro are reflected in the behavior of cells exposed to the analogues. The IC₅₀ values for inhibition of growth by a number of CAMP analogues that are known activators of the cAMP-dependent protein kinase both in vitro and in vivo and that are poorly or not hydrolyzed by the yeast cAMP-phosphodiesterase are summarized in Table III. All these analogues are equally potent inhibitors of growth for CHO cells expressing the yeast cAMP-phosphodiesterase (CHO-PDE12) and for the control transfected cell line (CHO-C3). Note the high potency of 8-Br-cAMPS compared with the other compounds listed in Table III. These data are consistent with the idea that the cAMP-dependent protein kinase of control and transfected CHO cells is functional.

**DISCUSSION**

The cyclic nucleotide phosphodiesterases have been arranged into several classes (3) The type I group consists of calmodulin-sensitive cyclic nucleotide phosphodiesterases and type II of the cGMP-sensitive cyclic nucleotide phosphodiesterases. Type III is the rhodopsin-sensitive cGMP-phosphodiesterase. The yeast enzyme described in this study belongs to class IV, the low Kₘ cAMP-specific phosphodiesterases.

The interaction of cAMP with the binding sites of cAMP-binding proteins can be systematically studied using a constellation of cAMP analogues (test kit) that has been described previously (4, 5). Each of these analogues has a modification at a different position. A variety of cAMP-binding proteins have been analyzed in this way (see Table II), but of the cyclic nucleotide phosphodiesterases, only members of classes I and II have been studied with the systematic use of these analogues. In this work we show that the yeast low Kₘ cAMP-phosphodiesterase does not closely resemble any of these previously studied proteins (Table II).

The yeast enzyme is highly specific for cAMP. cAMP specificity as compared with cGMP is exhibited by the 6-amino moiety, a potential hydrogen bond donor. As can be seen in Table I and Fig. 3, modifications at this position severely reduce binding to the enzyme. Therefore, we postulate a hydrogen bond to the enzyme via the 6-NH₂ moiety. cGMP, which has an oxygen atom with non-bonded orbitals, will be repelled by a hydrogen bond acceptor on the protein, rationalizing the inability of cGMP to bind to the yeast cAMP-phosphodiesterase. The reduced binding by substitution of the hydrogen bond acceptor N-7 through a non-bonding CH-group implies an additional hydrogen bond.

Other class IV phosphodiesterases have only been studied with analogues with alterations in the C-2, N-6, and C-8 positions (46). The binding characteristics of these analogues is compatible with those shown here for the yeast low Kₘ cAMP-phosphodiesterase. However, the yeast enzyme is unique because of its relative insensitivity to 11 standard phosphodiesterase inhibitors. Some of these inhibit certain forms of the type IV low Kₘ cAMP-specific phosphodiesterases with high affinity.

By analogy with other enzymes, we propose a hydrophobic interaction between the π-electrons of the purine ring structure and the enzyme. This conclusion is consistent with the disruptive effects of the very polar N'-oxide-cAMP (compound 2, (18)), and the unexpected affinity of the benzimidazole derivatives (compounds 16, 17, and 18), which are relatively non-polar and exhibit a high potential for π-electron interactions.

Differential binding of the two stereoisomers of the monophosphorothioate derivatives indicates that a regioselective charge-charge interaction between the equatorial exocyclic oxygen atom and the enzyme may exist. This regioselectivity can be obtained by additional hydrogen bonding toward the equatorial exocyclic oxygen. cAMP is presumably bound in the syn conformation because introduction of bulky groups at the C-8 position, which shifts the syn-anti equilibrium to the syn conformation, does not interfere with binding. A model of binding is presented in Fig. 6. Hydrolysis of cAMP probably takes place by a nucleophilic attack on the phosphate as described by Van Haastert et al. (15) for the beef heart type I cyclic nucleotide phosphodiesterase, as judged from the slow hydrolysis of S₂-cAMPS and the undetectable hydrolysis of R₃-cAMPS. This is in complete contrast to the yeast high Kₘ cyclic nucleotide phosphodiesterase. This enzyme hydrolyzes both R₃-cAMPS and S₂-cAMPS at reasonable rates (47) and does not require Mg²⁺ ions for activity.

The characterization of the yeast low Kₘ cAMP-phosphodiesterase is useful beyond allowing a comparison with other cAMP-binding proteins. Our previous experiments have shown that expression of the yeast cAMP-phosphodiesterase gene in CHO or other mammalian cells provides a way to block cAMP-mediated responses and to determine what effects of a hormone or growth factor require an elevation of cAMP levels for their action and which do not. Introduction of excess cAMP-phosphodiesterase into the cAMP cascade can also be useful in those situations, for example in differentiating cells, in which an agonist is not known, but a requirement for cAMP is suspected. Analogues that are not
hydrolyzed by the yeast cAMP-phosphodiesterase provide a way to bypass the effects of a high rate of degradation of intracellular cAMP. By comparing the ability of each analogue to be hydrolyzed by the enzyme with its ability to bind to the only confirmed intracellular target of cAMP, the regulatory subunit of the cAMP-dependent protein kinase, several compounds were found that were capable of activating the cAMP-dependent protein kinase but were only poorly or not at all hydrolyzed by the yeast enzyme. When applied to cells which did not produce the yeast cAMP-phosphodiesterase, the cell behavior in terms of growth and morphology became predictable. The high effectiveness of Z-8-Br-cAMPS (compound 14) at inhibiting the cell growth of CHO-C3 cells in comparison with 8-Br-cAMP (compound 5) demonstrates the usefulness in vivo of non-hydrolyzable, highly lipophilic cAMP analogues. The 8-halogen-modified monophosphorothioate derivatives of cAMP provide a new generation of cAMP-dependent protein kinase agonists and antagonists for use in vivo. We hope these compounds will overcome the artifactual effects of the metabolites of cAMP analogues that are resistant to CAMP-dependent protein kinase agonists and antagonists for use in vivo. In the initial experiments to select CHO cells which produced high amounts of the yeast low K_m, cAMP-phosphodiesterase, we observed that cells carrying the gene coding for this enzyme were resistant to both cholera toxin and to 8-Br-cAMP. Resistance to 8-Br-cAMP might have been due to the degradation of this compound by the yeast cAMP-phosphodiesterase or to a mutation that rendered the cells resistant to all CAMP analogues. The findings presented here show that the ability of membrane-permeable analogues of the cAMP dependent protein kinase to inhibit cell growth in CHO-PDE12 cells parallels the ability of the yeast cAMP-phosphodiesterase to hydrolyze the analogues. These results suggest that in CHO cells that express the yeast cAMP-phosphodiesterase, resistance to agents that elevate cAMP levels such as cholera toxin is caused by rapid hydrolysis of newly formed cAMP.

The mapping of the essential molecular interactions between cAMP and the yeast cAMP-phosphodiesterase, in combination with detailed knowledge of the interactions between cAMP and the cAMP-dependent protein kinase, allows us to predict the action of almost any cAMP analogue in cells expressing the yeast cAMP-phosphodiesterase gene, provided that the analogue is sufficiently lipophilic to cross the plasma membrane.

The expression vector containing the yeast cAMP-phosphodiesterase gene together with non-hydrolyzable, lipophilic cAMP-dependent protein kinase agonists, such as Z-8-Br-cAMPS, provides a tool to control CAMP-mediated events in mammalian cell lines in the manner of a conditional mutant.

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Characterization of the yeast low Km cAMP-phosphodiesterase with cAMP analogues. Applications in mammalian cells that express the yeast PDE2 gene.
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