Increased Cyclic Nucleotide Phosphodiesterase Activity in a Mutant S49 Lymphoma Cell

CHARACTERIZATION AND COMPARISON WITH WILD TYPE ENZYME ACTIVITY*

Virginia M. Brothers†, Naomi Walker, and Henry R. Bourne‡

From the Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, and the Cardiovascular Research Institute, University of California Medical Center, San Francisco, California 94143

(Received for publication, January 17, 1981)

We have characterized the markedly elevated cyclic AMP and cyclic GMP phosphodiesterase activities of a recently isolated S49 mouse lymphoma mutant, termed K30a, and compared both activities to enzyme activities in the parental wild type S49 cell. cAMP phosphodiesterase activity in K30a appears to be slightly larger than the major wild type cAMP-hydrolyzing enzyme, in sucrose gradient sedimentation and gel filtration. Both cAMP phosphodiesterase activities elute from DEAE-cellulose columns at a 0.4 mM salt concentration. cAMP phosphodiesterase in K30a, as compared to wild type cells, exhibits a component of activity with higher affinity for substrate ($K_{m} = 0.15 \pm 0.02 \text{ versus } 0.53 \pm 0.05 \mu M$), much greater sensitivity to competitive inhibition by cGMP ($K_{i} = 0.04 \mu M \text{ versus greater than } 100 \mu M$ in wild type) and cyclic IMP, and greatly reduced sensitivity to inhibition by the synthetic inhibitor, RO 20-1724. By the same criteria, cGMP-hydrolyzing activities in the two cell types appeared similar or identical, although K30a cells contain much more high affinity cGMP-hydrolyzing activity than do wild type cells. Both cGMP-hydrolyzing enzymes exhibited a high affinity component with a $K_{m}$ of 0.04 $\mu M$ for cGMP as substrate. The cAMP- and cGMP-hydrolyzing activities of K30a probably are catalyzed by the same enzyme, because the two activities co-migrate exactly on DEAE-cellulose, sucrose gradients, and gel filtration, and because hydrolysis with either substrate is inhibited by low concentrations of the other substrate. From these results, we propose that the K30a mutation caused increased expression of a cyclic nucleotide phosphodiesterase different from the predominant cAMP-hydrolyzing activity of wild type S49 cells. Wild type S49 cells express, in addition to the predominant cAMP-hydrolyzing activity, a phosphodiesterase similar to or identical with the enzyme whose expression is increased in K30a.

Most mammalian tissues contain multiple forms of cyclic nucleotide phosphodiesterase (EC 3.1.4.17), which differ in kinetic and other properties, including substrate specificity and susceptibility to stimulation by calcium and calmodulin (1-4). Biochemical investigations are just beginning to elucidate the regulation and physiological roles of these phosphodiesterases as modulators and mediators of hormone action (5-10). To complement these approaches, it should be useful to examine the phenotypes of mutant cells bearing genetic defects that alter phosphodiesterase activities.

By virtue of its susceptibility to killing by cyclic AMP, the S49 mouse lymphoma cell has provided a useful model for genetic analysis of the actions of hormones that utilize cAMP as a second messenger (11). Most of the previously reported S49 mutants bore lesions affecting either cAMP-dependent protein kinase or the guanine nucleotide-binding regulatory component (the N (11) or G/F (12) protein) of hormone-sensitive adenylate cyclase. S49 mutants resistant to killing by synthetic cAMP analogs proved useful in elucidating the role of cAMP-dependent protein kinase in mediating cAMP's action (13). An S49 mutant resistant to actions of agents that stimulate cAMP synthesis (14) led to the discovery, characterization, and eventual purification of the N protein (12, 15).

Both of these protein components of the cAMP second messenger system appeared functionally intact in a recently isolated S49 mutant, termed K30a (16). In comparison to the wild type S49 parental cell line, K30a cells exhibited unchanged adenylate cyclase and N activities and similar sensitivity to killing by N6-@-dibutyryl-cAMP, but were almost completely resistant to the cAMP-elevating and cytotoxic effects of cholera toxin. This resistance was associated with increased capacity of K30a extracts to degrade cAMP (16).

Despite the usefulness of S49 cells as models for studying cyclic nucleotides as second messengers, the enzymes responsible for degrading cAMP and cGMP in S49 cells have received little attention. Here we report the initial biochemical characterization of cAMP and cGMP phosphodiesterase activities in the K30a mutant and in the parental wild type S49 cell line. Comparison of the two phenotypes suggests that genetic analysis may help to unravel complexities in the regulation and function of cyclic nucleotide phosphodiesterases. Although heritable alterations in cAMP-degrading activity are known in Drosophila (17), the K30a cell is the first mammalian example of a mutation that primarily affects cyclic nucleotide phosphodiesterase activities.

EXPERIMENTAL PROCEDURES

Cells—S49 mouse lymphoma cells of the wild type and K30a phenotype were grown in Dulbecco's modified Eagle's medium with 3 g/liter of glucose, supplemented with 10% heat-inactivated horse serum as previously described (18). All cell extracts were made from cells in the logarithmic phase of growth (less than 10^6 cells/ml).

The clonedly selected K30a cell population (16) was subcloned in soft agar without selective pressure, and 15 of 15 randomly selected colonies exhibited the K30a phenotype. One of these subclones was used in all the experiments presented here. Its phenotype has re-
mained stable in the absence of selective pressure after more than 250 generations in culture.

Preparation of Cell Extracts—Cell suspensions were centrifuged at 1000 rpm for 5 min and the medium was removed. The sedimented cells were washed in cold 250 mM sucrose, 50 mM Tris-HCl, pH 6.5, 4 mM β-mercaptoethanol and centrifuged at 4 °C at 1500 rpm for 10 min. The supernatant fluid was removed and the cells were resuspended in 50 mM Tris-HCl, pH 6.5, 1 mM phenylmethylsulfonyl fluoride, and 4 mM β-mercaptoethanol, at a concentration of 10^6 cells/ml. After incubation on ice for 15 min, the cells were homogenized in a Dounce homogenizer. Each 10-ml portion of the homogenate was sonicated three times for 30 s, each time using a Biosonik IV sonicator at a setting of 50. The sonicated homogenate was centrifuged at 1000 rpm for 5 min and the medium was removed. The sedimented material was frozen in aliquots in liquid nitrogen for further use. This procedure routinely released 50–70% of the phosphodiesterase activity into the supernatant fraction, from both wild type and K30a cells.

Protein was determined by a modification of the Lowry procedure (19), using bovine serum albumin as a standard.

Phosphodiesterase Assay—cAMP and cGMP phosphodiesterase activities were measured by the two-step isotopic method of Thompson and Appleman described in Ref. 20. cAMP phosphodiesterase activity was measured in 300 µl of a reaction mixture containing (final concentrations) 40 mM Tris-HCl, pH 8.5, 5 mM MgCl₂, 4 mM β-mercaptoethanol (assay buffer), [³H]cAMP (300–600,000 cpm), [¹⁴C]β-cGMP (10,000–20,000 cpm), and an appropriate amount of enzyme. Substrate concentration was 0.2 µM unless stated otherwise. Incubations were carried out at 30 °C for an appropriate time and terminated by boiling for 1 min. After cooling on ice, 0.2 unit of 5'-nucleotidase was added. A second incubation at 30 °C was carried out for 25 min. At the end of the second incubation, 1 ml of AG 1-X8 resin slurry (1:4 settled resin volume in deionized water) was added. After 20 min at room temperature, samples were centrifuged at 1000 rpm for 5 min and 0.5 ml of the supernatant was added to 6 ml of Aquasure for counting. Enzyme activities were corrected for binding of adenosine to the resin and for blanke containing heat-inactivated enzyme.

For determination of cGMP phosphodiesterase activity, [³H]cGMP (400,000–80,000 cpm) and [¹⁴C]β-cGMP (10,000–20,000 cpm) were used and incubations were carried out as described above. Substrate concentration was 0.1 µM unless indicated otherwise.

Linearity of enzyme activity with enzyme concentration and time was studied in the 100,000 × g supernatant with both cAMP and cGMP as substrate. All assays were carried out with dilutions of enzyme that were within the linear range. All fractionated samples were assayed within this range for 5–60 min so that substrate consumption did not exceed 30%.

DEAE-cellulose Chromatography—Frozen 100,000 × g supernatants (12 mg of protein) were applied to a DEAE-cellulose column (1.2 × 10.6 cm) which had been equilibrated with 50 mM sodium acetate, pH 6.5, containing 4 mM β-mercaptoethanol (20). After sample application, the column was washed with 2 column volumes of 70 mM NaAc, pH 6.5, containing 4 mM β-mercaptoethanol and then eluted with 120 ml of a 70–1000 mM NaAc gradient, pH 6.5, containing β-mercaptoethanol. Fractions (1.5 ml) were collected and assayed for phosphodiesterase activity. Peak fractions were pooled, bovine serum albumin was added to a final concentration of 1 mg/ml, and the pooled peak was frozen at −70 °C for subsequent use.

Determination of Kinetic Constants—Values of kinetic constants were obtained by fitting the data with the aid of a nonlinear least squares curve-fitting computer program obtained from Dr. Vojtech Licko of the Cardiovascular Research Institute, University of California, San Francisco.

Sucrose Density Gradient Centrifugation—Two hundred- to 250-µl samples of fresh or frozen 100,000 × g supernatant fractions or DEAE-cellulose peaks were applied to 5-ml, 5–20% linear sucrose gradients. The gradients were centrifuged for 14–16 h at 40,000 rpm in a Beckman SW 28.1 rotor. The assay buffer containing sucrose. Marker proteins (fumarase, lactase dehydrogenase, malic dehydrogenase, and cytochrome c) were run on each gradient. The hydrodynamic properties of these proteins have been summarized (21). Phosphodiesterase activity was measured using 0.2 µM cAMP and 0.1 µM cGMP.

Gel Filtration—One-ml samples of 100,000 × g supernatant fractions from wild type and K30a (2.8 mg of protein) were applied to a Sepharose 6B column (0.9 × 60 cm) in assay buffer. The column was run at 5.5 ml/h and 0.75-ml fractions were collected. Columns were calibrated with blue dextran, H₂O, and protein standards (see figure legends) (21).

Materials—H₂O, [2,8-³H]cAMP (36.4 Ci/mmol), [8,5'-³H]cGMP (33.0 Ci/mmol), [U-¹⁴C]β-cAMP, and Aquasure were purchased from New England Nuclear. [U-¹⁴C]β-cGMP was obtained from ICN. cAMP, cGMP, and 5'-nucleotidase purified from Crotalus atrox venom (Grade IV) and marker proteins were from Sigma. AG 1-X8 was from Bio-Rad, DEAE-cellulose DE52 from Whatman, and Sephrose 6B from Pharmacia. Ultrapure sucrose was purchased from Schwart/Mann. RO 20-1724 was obtained from Hoffman-LaRoche. Purified bovine testis calmodulin was obtained from Dr. Joan Egrie, University of California, Berkeley. All other reagents were obtained from Sigma.

RESULTS

Crude Extracts and Ion Exchange Chromatography—Compared to the 100,000 × g supernatant fraction of wild type cells, the corresponding extract of K30a cells contained 5- to 10-fold more cAMP phosphodiesterase activity (measured at 0.2 µM cAMP) and 50- to 100-fold more cGMP phosphodiesterase activity (measured at 0.1 µM cGMP). Phosphodiesterase activities in mixtures of wild type and K30a supernatant extracts were completely additive (not shown) This suggests that the differences in enzyme activities were not caused by endogenous inhibitors in the wild type extracts or by activators in the K30a extracts.

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** DEAE-cellulose fractionation of phosphodiesterase activity (PDE) from wild type (A) and K30a (B). The 100,000 × g supernatant fractions (4.3 ml containing 2.8 mg of protein/ml) were applied to a DEAE-cellulose column (1.2 × 10.6 cm) and eluted as described in under “Experimental Procedures.” Fractions (1.5 ml) were collected and assayed with 100 µM cAMP (●), 0.2 µM cAMP (○), and 0.1 µM cGMP (×). The sodium acetate gradient (■—■) was determined by conductivity measurements. A line was drawn to indicate the range of enzyme activity in peak fractions (3–10%).
revealed phosphodiesters of either cell type. To remove hypothetical modulators of cyclic nucleotide hydrolysis from or cGMP from 0.01-100 μM, hydrolysis; hydrolysis estimated from four separate experiments on phosphodiesterase activity eluted from DEAE-cellulose were identical, whether column fractions were assayed at 0.2 μM or 100 μM cAMP.

cAMP and cGMP phosphodiesterase activities in supernatant extracts of either wild type or K30a cells co-migrated in a single peak on DEAE-cellulose chromatography, eluting at a salt concentration of approximately 0.4 M (Fig. 1). In both wild type and K30a extracts, the activity profiles of cAMP phosphodiesterase activity eluted from DEAE-cellulose were identical, whether column fractions were assayed at 0.2 μM or 100 μM cAMP.

The peaks of cAMP- and cGMP-hydrolyzing activity in both wild type and K30a extracts were neither stimulated by calmodulin and Ca2+, nor inhibited by a calcium chelator, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid. In addition, no other peaks of enzyme activity were detected when column fractions were assayed in the presence of calmodulin and Ca2+, and cGMP did not activate hydrolysis of cAMP (results not shown).

Enzyme Kinetics and Inhibitors—DEAE-column fractions corresponding to peak phosphodiesterase activities in wild type and K30a were pooled for measurements of cAMP and cGMP hydrolysis at substrate concentrations ranging from 0.01-100 μM. Eadie-Hofstee plots of the data, with both substrates and in both cell types (Fig. 2), were nonlinear and concave-upward. This indicates either that more than one enzyme hydrolyzed each substrate or that the enzymes exhibited negative cooperativity with both substrates. A computer curve-fitting procedure separated the enzyme activities into two hypothetical components (indicated by dashed lines in Fig. 2), one of high affinity (characterized by Km and Vmax), and another of lower affinity (characterized by Km2 and Vmax2). Table 1 lists the mean apparent Km and Vmax values for the high and low affinity components of cAMP and cGMP hydrolysis estimated from four separate experiments on DEAE-cellulose eluates of each cell type.

Kinetic experiments assessing cAMP and cGMP hydrolysis in 100,000 × g supernatant extracts of wild type and K30a (not shown) revealed Km values virtually identical with those estimated in DEAE-cellulose eluates (Table I). Although estimates of Vmax were lower (per mg of protein) in the supernatant fractions than in DEAE-extracts, relative differences between K30a and wild type were similar to those in Table I. Thus, fractionation on DEAE-cellulose did not remove hypothetical modulators of cyclic nucleotide hydrolysis from phosphodiesterases of either cell type.

For each of the four enzymatic activities (cAMP and cGMP hydrolysis in both wild type and mutant extracts), the computer curve-fitting procedure was used to fit the data to models containing one, two, or three saturable Michaelis-Menten components. A two-component model provided the best fit for each of the four activities. Enzyme assays were performed as described in the legend of Fig. 2. Values reported are the mean ± S.E. from four determinations.

Estimates of kinetic parameters for cAMP hydrolysis (Table I) indicate that the largest relative differences between wild type and K30a extracts were observed at low substrate concentrations. As compared to wild type, cAMP phosphodiesterase activity in K30a exhibited a lower Km (p < 0.001) and a higher Vmax (p < 0.001) in K30a than in wild type extracts (p < 0.05). Similarly, the largest differences in rates of cGMP hydrolysis between wild type and K30a were observed at low substrate concentrations. With cGMP, however, the differences at low substrate reflect an apparent 50- to 100-fold greater Vmax in K30a, while the estimated Km for cGMP hydrolysis is identical in wild type and K30a. At high concentrations of substrate (approaching 100 μM), rates of cGMP hydrolysis were similar in wild type and K30a extracts.

In addition to differences in kinetic properties, cAMP phosphodiesterase activities in wild type and K30a extracts behaved differently in the presence of other cyclic 3',5'-purine nucleotides or synthetic inhibitors of phosphodiesterase (Table II). In K30a, very low concentrations of cGMP inhibited cAMP hydrolysis (0.05 μM cGMP inhibited hydrolysis of 0.2 μM cAMP by 50%). Similarly, cyclic IMP (2 μM) inhibited hydrolysis of 0.2 μM cAMP by 80% in K30a. In contrast, cGMP and cIMP almost completely failed to inhibit cAMP hydrolysis in wild type extracts, even when the cyclic nucleotides were present in 500-fold excess over substrate. A variety of

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Km (μM)</th>
<th>Vmax (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>Wild type</td>
<td>K30a</td>
</tr>
<tr>
<td></td>
<td>0.05 ± 0.05</td>
<td>1140 ± 120</td>
</tr>
<tr>
<td></td>
<td>0.15 ± 0.02</td>
<td>4110 ± 440</td>
</tr>
<tr>
<td>cGMP</td>
<td>Wild type</td>
<td>K30a</td>
</tr>
<tr>
<td></td>
<td>0.04 ± 0.01</td>
<td>4.22 ± 1.02</td>
</tr>
<tr>
<td></td>
<td>0.04 ± 0.01</td>
<td>355 ± 54</td>
</tr>
</tbody>
</table>

Note: Km and Vmax values for cAMP and cGMP are given in Table I.

It should be stressed that the computer-fitted curves and the Km and Vmax parameters that define them do not define the number or molecular nature of the enzymes in the cell extracts we tested. This qualification should be borne in mind in relation to our interpretation of the difference between wild type and K30a (see "Discussion"), which suggests that K30a expresses an increased amount of a cAMP- and cGMP-hydrolyzing enzyme that is present in low abundance in wild type extracts; we suggest that this enzyme's cAMP-hydrolyzing activity in wild type is overshadowed by the much greater activity of a phosphodiesterase that hydrolyzes cAMP, but not cGMP. Thus, fitting the substrate-velocity relation for cAMP hydrolysis in either extract to a two-component model could not assign the kinetic components to specific enzymes; this could only be accomplished by separating and purifying the enzyme(s) involved. Accordingly, the two kinetic components of cAMP hydrolysis (Table I) are not to be attributed to the two postulated cAMP phosphodiesterase activities in wild type extracts. Similarly, optimal fit of the curves to a two-component model (see Table I) did not rule out the presence of one or more additional kinetic components (or additional enzymes); such an optimal fit merely indicated that it was not possible to detect an additional component, within the limits of precision of the available data.
other nucleotides failed to inhibit cAMP hydrolysis in either wild type or K30a extracts.

CAMP hydrolysis in K30a, as compared to wild type, was 100-fold less sensitive to inhibition by the phosphodiesterase inhibitor RO 20-1724 and 4- to 5-fold more sensitive to inhibition by methylisobutylxanthine and theophylline (Table II).

In contrast to cAMP phosphodiesterase activity, cGMP phosphodiesterase activity in wild type and K30a extracts behaved almost identically in the presence of cAMP, cIMP, and phosphodiesterase inhibitors (Table II). Low concentrations of cAMP inhibited cGMP hydrolysis in both wild type and K30a. High concentrations of RO 20-1724, similar to those required to inhibit cAMP hydrolysis in K30a, were required to inhibit cGMP hydrolysis in both wild type and K30a extracts. cIMP (2 μM) inhibited hydrolysis of 0.1 μM cGMP by 72% in K30a and 63% in wild type.

cGMP competitively inhibited cAMP hydrolysis in K30a extracts (Fig. 3), with an apparent K, of 0.04 μM, which was identical with the K, for cGMP hydrolysis in K30a (Table I). Although low concentrations of cAMP inhibited cGMP hydrolysis in extracts of both cell types (Table II), the much more rapid hydrolysis of cAMP prevented accurate determination of K, values for inhibition of cGMP hydrolysis.

Hydrodynamic Properties—To determine the hydrodynamic properties of cAMP and cGMP phosphodiesterases, soluble extracts of both cell types were subjected to gel filtration on Sepharose 6B and to centrifugation through sucrose gradients. As assessed by gel filtration, the Stokes radius of cAMP phosphodiesterase activity was slightly larger in K30a than in wild type (Fig. 4, Table III). cGMP phosphodiesterase activity co-migrated precisely with cAMP phosphodiesterase in K30a, with a Stokes radius of 6.5 nm (Fig. 4B). In wild type extracts, the major peak of cGMP phosphodiesterase activity migrated close to the position of cAMP phosphodiesterase, corresponding to a Stokes radius of 6.2 nm.

3 cGMP (2 μM) inhibited cAMP hydrolysis in K30 extracts by more than 90%; the same concentration of cGMP inhibited hydrolysis of 0.2 μM cAMP by 5–8% in wild type extracts; higher concentrations of cGMP (up to 100 μM) produced less than 20% inhibition of cAMP phosphodiesterase in wild type extracts. Other nucleotides failed to inhibit cAMP phosphodiesterase in either wild type or K30a extracts. These nucleotides, including ATP, ADP, AMP, GTP, GDP, GMP, cUMP, and cCMP, were tested at 2 μM and 100 μM in the presence of 0.2 μM cAMP as substrate.

FIG. 3. Lineweaver-Burk plot of cAMP hydrolysis in K30a in the presence of cGMP. cAMP phosphodiesterase activity was measured using the DEAE-cellulose enzyme peak in the presence of either 0 (●), 0.1 μM (○), 0.25 μM (×), 0.5 μM (□), or 1.0 μM (△) cGMP. The incubations were performed at 30°C for 5–30 min so that substrate consumption did not exceed 20%. Lines drawn represent the least squares line of the data.

FIG. 4. Sepharose 6B gel filtration of phosphodiesterase activity (PDE) from wild type (A) and K30a (B). The 100,000 × g supernatant fractions (1 ml containing 2.8 mg of protein) were applied to a Sepharose 6B column as described under "Experimental Procedures." Fractions were assayed for phosphodiesterase activity using 0.2 μM cAMP (●—●) or 0.1 μM cGMP (○—○) and for the marker proteins. Recovery of enzyme activity was 90–70%. Arrows indicate peaks of markers, including blue dextran (BD), β-galactosidase (B), fumarase (F), lactic dehydrogenase (L), malic dehydrogenase (M), and 3H2O (HI).

The behavior of wild type cGMP phosphodiesterase activity on Sepharose 6B was somewhat variable, sometimes showing one or more minor components that eluted earlier than the major peak (Fig. 4A).

On sucrose gradients (Fig. 5A, Table III), the major peak of

TABLE II
Effect of nucleotides and inhibitors on phosphodiesterase activity

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>100</td>
</tr>
<tr>
<td>cGMP</td>
<td>0.1</td>
</tr>
<tr>
<td>ATP</td>
<td>10</td>
</tr>
<tr>
<td>ADP</td>
<td>10</td>
</tr>
<tr>
<td>AMP</td>
<td>10</td>
</tr>
<tr>
<td>GTP</td>
<td>10</td>
</tr>
<tr>
<td>GDP</td>
<td>10</td>
</tr>
<tr>
<td>GMP</td>
<td>10</td>
</tr>
<tr>
<td>cUMP</td>
<td>10</td>
</tr>
<tr>
<td>cCMP</td>
<td>10</td>
</tr>
</tbody>
</table>

The cyclic nucleotides were used as inhibitors at concentrations of 0.01-100 μM cGMP or 0.05-5 μM cAMP. Other inhibitors were tested over a concentration range of 1 μM-1 mM. Each value represents the concentration of the compound which produced 50% inhibition of enzyme activity, calculated from two separate experiments. The IC50 values did not vary more than 2-fold between experiments and the -fold differences between K30a and wild type were the same in both experiments.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>100</td>
</tr>
<tr>
<td>cGMP</td>
<td>0.1</td>
</tr>
<tr>
<td>ATP</td>
<td>10</td>
</tr>
<tr>
<td>ADP</td>
<td>10</td>
</tr>
<tr>
<td>AMP</td>
<td>10</td>
</tr>
<tr>
<td>GTP</td>
<td>10</td>
</tr>
<tr>
<td>GDP</td>
<td>10</td>
</tr>
<tr>
<td>GMP</td>
<td>10</td>
</tr>
<tr>
<td>cUMP</td>
<td>10</td>
</tr>
<tr>
<td>cCMP</td>
<td>10</td>
</tr>
</tbody>
</table>

The cyclic nucleotides were used as inhibitors at concentrations of 0.01-100 μM cGMP or 0.05-5 μM cAMP. Other inhibitors were tested over a concentration range of 1 μM-1 mM. Each value represents the concentration of the compound which produced 50% inhibition of enzyme activity, calculated from two separate experiments. The IC50 values did not vary more than 2-fold between experiments and the -fold differences between K30a and wild type were the same in both experiments.
Hydrodynamic properties of phosphodiesterase

Values represent mean ± 1 S.E. The number of determinations is shown in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>K30a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cAMP</td>
<td>cGMP</td>
</tr>
<tr>
<td>Stokes radius, a (nm)</td>
<td>6.2 ± 0.17 (3)*</td>
<td>6.2 ± 0.17 (3)*</td>
</tr>
<tr>
<td>Sedimentation coefficient, s_{20w} (S)</td>
<td>3.8 ± 0.15 (4)*</td>
<td>4.1 ± 0.11 (4)*</td>
</tr>
<tr>
<td>Molecular weight, M_r</td>
<td>104,000 ± 5000*</td>
<td>112,000 ± 4100*</td>
</tr>
</tbody>
</table>

* Difference not significant.
\( P < 0.005 \) using a paired t test.
\( ^{d} \) Difference not significant.
\( ^{d} \) Calculated from the following equation:

\[
M_r = \frac{6\pi N_t \mu_{20w}}{1 - f_{20w} \cdot a - \rho_{20w}}
\]

where \( N \) is Avogadro’s number, \( \nu_{20w} \) is the viscosity of water at 20 °C, and \( \rho_{20w} \) is the density of water at 20 °C. The value used for \( \pi \) is the mean of values reported in the literature for the protein standards (21).

\( ^{d} \) Difference not significant.

The insets show the migration of phosphodiesterase activity (indicated by arrows) relative to the marker proteins fumarase (F), lactic dehydrogenase (L), malic dehydrogenase (M), and cytochrome c (C).

**Fig. 5.** Sedimentation profile of phosphodiesterase activity (PDE) in wild type (A) and K30a (B). The 100,000 × g supernatant fractions (700 µg of protein) were sedimented through 5-20% sucrose gradients as described under “Experimental Procedures.” Phosphodiesterase activity was measured using 0.2 µM cAMP (●) or 0.1 µM cGMP (○). The recovery of enzyme activity was 65-75%. The inset shows the migration of phosphodiesterase activity (indicated by arrows) relative to the marker proteins fumarase (F), lactic dehydrogenase (L), malic dehydrogenase (M), and cytochrome c (C).

Cyclic GMP phosphodiesterase in wild type extracts (4.1 S) consistently sedimented slightly faster than did wild type cAMP phosphodiesterase activity (3.8 S). cAMP and cGMP phosphodiesterase activities in K30a extracts co-sedimented at 4.6 S (Fig. 5B). Thus, cAMP phosphodiesterase appeared to sediment more rapidly in K30a than in wild type extracts, whereas the cGMP phosphodiesterase exhibited similar values in both cell types. The presence of 0.4 M NaCl in samples and gradients did not affect sedimentation rates of phosphodiesterase activities of either wild type or K30a extracts. In addition, in some preparations of both wild type and K30a, more rapidly sedimenting forms (7.2-7.4 S) of both cAMP and cGMP phosphodiesterase activities were seen (e.g. in Fig. 4A for cGMP hydrolysis). The amount of these rapidly sedimenting forms was quite variable, especially in K30a, for reasons we have not been able to determine.

Table III lists apparent molecular weights of cAMP and cGMP phosphodiesterase activities in wild type and K30a extracts, calculated from Stokes radii and sedimentation coefficients. Wild type cAMP phosphodiesterase (\( M_r = 104,000 \)) appears somewhat smaller than cAMP phosphodiesterase in K30a (\( M_r = 114,000 \)) and smaller than cGMP phosphodiesterase in both wild type and K30a (\( M_r = 112,000 \) and 114,000). The differences are not statistically significant, owing to variability in the parameters from which values for molecular weight were derived.

In extracts of both cell types, cAMP and cGMP phosphodiesterase activities appeared larger on gel filtration than would be expected from their sedimentation coefficients, relative to a series of globular marker proteins (Figs. 4 and 5). Thus, these phosphodiesterases may be elongated molecules.

**DISCUSSION**

K30a cells were isolated (16) by virtue of their ability to survive and proliferate in the presence of cholera toxin, a stimulator of adenylate cyclase, and RO 20-1724, a synthetic imidazolidinone that inhibits cyclic nucleotide phosphodiesterase (22). Evidently, the K30a mutation prevented cellular cAMP accumulation in the presence of these agents by providing the cell with a form of phosphodiesterase that is able to hydrolyze cAMP rapidly, even in the presence of RO 20-1724. How did this occur?

The present results are consistent with the following explanation. K30a cells express increased activity of a cyclic nucleotide phosphodiesterase subtly but definitely different from the predominant cAMP-hydrolyzing activity of wild type S49 cells. The wild type S49 line expresses, in addition to the predominant cAMP-hydrolyzing activity, a phosphodiesterase similar to or identical with that found in K30a; in wild type, this minor phosphodiesterase plays little or no role in regulating accumulation of cAMP. We propose, as a working hypothesis, that the K30a mutation has somehow altered the relative expression of two phosphodiesterase activities, both of which are present in wild type, thereby increasing expression of an enzyme that is resistant to inhibition by RO 20-1724.

The data strongly suggest that low concentrations of both cAMP and cGMP are predominantly hydrolyzed by the same enzyme in K30a extracts. Although further purification of the enzyme(s) will be required to establish this interpretation unequivocally, it is consistent with the following observations.

1. In K30a extracts, cAMP- and cGMP-hydrolyzing activities co-migrate precisely on DEAE-cellulose and gel filtration columns and upon centrifugation through sucrose gradients (Figs. 1, 4, and 5).
2. cGMP is a competitive inhibitor of cAMP hydrolysis in K30a, with a \( K_i \) equal to the \( K_m \) for cGMP hydrolysis, and low concentrations of cAMP inhibit hydrolysis of cGMP (Fig. 3, Table II).
3. Three phosphodiesterase inhibitors exhibited similar relative potencies in inhibiting hydrolysis of low concentrations of cAMP and cGMP in K30a extracts (Table II); hydrolysis of both cyclic nucleotides was
S49 Mouse Lymphoma Cyclic Nucleotide Phosphodiesterases

quite sensitive to inhibition by methylisobutylxanthine and quite resistant to inhibition by RO 20-1724.

In contrast, different enzymes appear to be responsible for cAMP and cGMP hydrolysis in wild type cells. cAMP hyd-
drolysis in wild type is not inhibited by cGMP, but is very
sensitive to inhibition by RO 20-1724, while cGMP hydrolysis
in extracts of these cells is inhibited by low concentrations of
cAMP and is insensitive to inhibition by RO 20-1724 (Table
II). In addition, wild type cGMP phosphodiesterase activity
consistently sedimented more rapidly in sucrose gradients
than did cAMP phosphodiesterase in the same extracts (Fig.
5A, Table III). The two enzyme activities exhibited similar
Stokes radii and behavior on DEAE-cellulose columns.

Several observations suggest that the small amount of
cGMP phosphodiesterase activity in wild type extracts is
similar to or identical with the predominant cyclic nucleotide
phosphodiesterase in K30a. The two activities exhibit similar
sedimentation coefficients and relative sensitivities to phos-
dodiesterase inhibitors. Both activities have the same K_m
for hydrolyzing cGMP, and both cAMP and cGMP inhibit
cGMP hydrolysis in both extracts. Activity of the high affin-
ity component of cGMP phosphodiesterase in wild type cells is
quite low (approximately 1% of the corresponding activity
observed in K30a (Table I)). Accordingly, rigorous tests of
the identity of the two activities are beyond the scope of the
present work. The cGMP phosphodiesterase in wild type is an
enzyme identical in properties with the K30a enzyme, this
enzyme would be responsible for less than 10% of the cAMP
hydrolysis observed in wild type extracts at low substrate
concentrations.4

The present data do not discriminate between the genetic
and biochemical mechanisms that could have resulted in
increased activity of a distinctive form of cyclic nucleotide
phosphodiesterase in K30a. Of the possible mechanisms, two
are particularly relevant in the context of current efforts to
eluicate regulation of cyclic nucleotide hydrolysis in mam-
nalian cells.

1) The K30a phenotype could represent altered intercon-
version between different forms of phosphodiesterase (23, 24)
or activation of a latent enzyme, mediated by covalent modi-
fication, e.g. phosphorylation or proteolysis (5, 7). Hormones
and other environmental signals are known to increase activi-
ties of high affinity cAMP phosphodiesterase in many cell
types (5–10). For example, insulin increases cAMP phos-
dodiesterase activity in rat liver membranes through a mecha-
nism that appears to involve cAMP-dependent phosphoryla-
tion of the enzyme (5). Mixing experiments utilizing wild type
and K30a extracts provided no evidence for such a mechanism,
but certainly did not rule out enzyme interconversion or
covalent modification as causes of the K30a phenotype.

2) K30a could have resulted from a mutation that increased
transcription of a gene coding for a distinctive phosphodies-
terase (a gene that may be expressed at a low level in wild
type cells). Regulation of the synthesis of phosphodiesterases

4 More explicitly, the low K_m cGMP-hydrolyzing activity in wild
type is 1.2% of that observed in K30a (see Table I). If this en-
zyme in wild type hydrolyzes cAMP at 1.2% the rate observed in
K30a, it would account for 9.5% of the cAMP hydrolyzed by wild type extracts
at 0.2 μM substrate concentration (28 versus 312 pmol per mg per min,
calculated using the K_m and V_max, values in Table I. If so, the same propor-
tion of wild type cAMP-hydrolyzing activity should be sensitive
to inhibition by low concentrations of cGMP; this was the case, as
described in Footnote 3. Conversely, K30a extracts appear to contain a
small amount of "wild type" cAMP phosphodiesterase activity (2–
5% of the total activity at low substrate concentration), which is
resistant to inhibition by 100 μM GMP. Thus, the two cell lines may
express different amounts of the same two forms of cyclic nucleotide
phosphodiesterase.

is very poorly understood, although it is clear that hormones
can alter expression of phosphodiesterase activities in many
cell types (8, 9), including S49, by mechanisms that
require continued protein synthesis.

A third possibility is that the K30a phenotype resulted from
a point mutation producing substitution of an amino acid
in the predominant phosphodiesterase enzyme of S49 cells. Al-
though this interpretation would be consistent with the similar
hydrodynamic properties of the wild type and K30a enzyme
activities, it appears unlikely that such a small change could
have produced simultaneous (and opposite) changes in affinity
of the enzyme for cGMP and inhibitors of phosphodiesterase.

Finally, we should note certain similarities between phos-
dodiesterase activities in S49 cells and those studied in lym-
phocytes and leukemic cells (24–28). Like S49 cells, human
lymphocytes may contain a single peak of cAMP phospho-
diesterase activity that elutes at a high salt concentration from
DEAE-columns (25) and may lack enzyme forms, observed in
other tissues, that are regulated by calcium and calmodulin or
the cAMP phosphodiesterase that is stimulated by cGMP (25,
28). In addition, cGMP has been reported to inhibit hydrolysis
of low concentrations of cAMP in human lymphocyte extracts;
in leukemic lymphocytes or in mitogen-treated normal lym-
phocytes, inhibition by cGMP reportedly decreased (25, 26).

The increased inhibitory effect of cGMP was associated with
increased lymphocyte cyclic nucleotide phosphodiesterase activity, how-

REFERENCES
1. Strada, S., and Thompson, W. J. (1978) in Advances in Cyclic
Nucleotide Research (George, W. J., and Ignarro, L. J., eds)
Nucleotide Research (Greengard, P., and Robison, G. A., eds)
Vol. 8, pp. 119–143, Raven Press, New York
3. Vaughan, M., Danello, M. A., Manganiello, V. C., and Strewler,
G. J. (1981) in Advances in Cyclic Nucleotide Research (Du-
Press, New York
Biol. Chem. 246, 3841–3846
195, 633–660
Chem. 255, 7838–7844
U. S. A. 69, 269–273
181, 952–954
(1980) in Advances in Cyclic Nucleotide Research (Greengard, P.,
York
49, 533–564
(Tococelli, E., King, R. J. B., Landner, H. R., and Lippmann, M.
187, 750–752
15. Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S.,
S. A. 77, 6516–6520
19, 109–116
17. Kiger, J. A., Jr., Davis, R. L., Saltz, H., Fletcher, T., and Bowling,
M. (1981) in Advances in Cyclic Nucleotide Research (Du-