Characteristics of the Cyclic Nucleotide Phosphodiesterases in a Transplantable Pheochromocytoma and Adrenal Medulla of the Rat

Robert M. Levin and Benjamin Weiss

ABSTRACT

A pheochromocytoma was maintained in rats from the New England Deaconess Hospital by giving the rats s.c. injections of isolated tumor cells. The animals were sacrificed 3 to 4 weeks after transplantation, the tumors were excised, and purified tumor cells were prepared. Cyclic nucleotide phosphodiesterase of the purified tumor cells was characterized and compared with that of the adrenal medulla.

At high concentrations of cyclic adenosine 3':5'-monophosphate (cyclic AMP), the activity of phosphodiesterase from the adrenal medulla was twice that of the pheochromocytoma; at low substrate concentrations, the cyclic AMP phosphodiesterase activity of the pheochromocytoma was more than 3 times that of the adrenal medulla. By contrast, the rate of hydrolysis of cyclic guanosine 3':5'-monophosphate (cyclic GMP) of the adrenal medulla was approximately 2 times that of the pheochromocytoma at all substrate concentrations studied.

The adrenal medulla and pheochromocytoma displayed biphasic kinetics for cyclic AMP hydrolysis; the apparent Michaelis constants (Km) for the adrenal medulla (8 and 130 μM) were significantly higher than the Km's for the pheochromocytoma (0.9 and 18 μM). Kinetic analysis of phosphodiesterase activity in the subcellular components demonstrated that in the pheochromocytoma all fractions contained the high-affinity form of phosphodiesterase. However, in the adrenal medulla only the nuclear fraction contained a high-affinity form of phosphodiesterase.

Theophylline, papaverine, cyclic GMP, trifluoperazine, and dipyridamole were relatively ineffective in inhibiting the cyclic AMP phosphodiesterase from either the adrenal medulla or pheochromocytoma.

Our findings that the pheochromocytoma has a greater activity of a high-affinity cyclic AMP phosphodiesterase but a lesser activity of cyclic GMP phosphodiesterase when compared with that of the normal adrenal medulla suggest that pheochromocytoma cells may have a relatively low ratio of cyclic AMP to cyclic GMP. This is consistent with the proposition that low intracellular concentrations of cyclic AMP or a low ratio of cyclic AMP to cyclic GMP may be associated with neoplastic activity.

INTRODUCTION

Alterations in the cyclic nucleotide system have been implicated in the development and progression of a variety of tumors and transformed cell lines. In general, neoplastic activity has been associated with low intracellular concentrations of cyclic AMP (1, 9, 17, 24) and in some cases with high intracellular concentrations of cyclic GMP (12, 27). Abnormally low concentrations of cyclic AMP may be caused either by a reduced activity of the enzyme that catalyzes its biosynthesis (adenylate cyclase) or by an increased activity of the enzyme that catalyzes its hydrolysis (phosphodiesterase). There is evidence that both of these defects exist in certain types of cells. For example, low activities of adenylyl cyclase have been reported in astrocytoma cells (38), thyroid tumors (23), and hepatomas (16), and abnormally high activities of phosphodiesterase have been found in certain leukemic lymphocytes (13, 14).

Tumors of the adrenal medulla (pheochromocytomas) provide a particularly interesting neoplastic model for testing the hypothesis that abnormal growth and function may be associated with an abnormal metabolism of cyclic nucleotides. These tumors are both neoplastic and functional; they produce and release into the circulation large quantities of the catecholamines epinephrine and norepinephrine. Accordingly, the clinical manifestations are similar to those seen with high doses of catecholamines, including vascular accidents, myocardial infarction, congestive heart failure, and malignant hypertension (15).

A rat model for pheochromocytoma has recently been developed by Warren et al. (34, 35) through irradiation of a strain of rats developed at the New England Deaconess Hospital. When transplanted s.c., this tumor grows rapidly to approximately 1 cm in 30 days. The tumor-bearing animals live between 30 and 60 days following transplantation and are characterized at the time of death by a loss of body weight, high blood pressure, proteinuria, and renal and cardiac lesions (34).

The treatment of choice in pheochromocytoma is surgery, but where multiple and ectopic foci exist, or in cases of malignant pheochromocytoma, surgery often provides only temporary relief, and symptoms frequently reappear. Current pharmacological treatment also provides only a temporary reduction in the hypertensive symptoms and cannot permanently control the destructive nature of circulating catecholamines (26). The high level of catecholamines localized in the pheochromocytoma, moreover, can protect the tumor against radiation therapy (28). Thus, there exists a need for an effective therapeutic treatment in cases not amenable to surgery.

Since abnormal concentrations of cyclic AMP and cyclic GMP have been associated with various forms of malignant...
of the cyclic nucleotide phosphodiesterase activity of pheo
tumors, we have studied the characteristics of the cyclic
nucleotide phosphodiesterase activity of pheo-
cyclic nucleotides in the diseased tissue with specific phar-
macological agents. As a first step in determining whether
this tack may profitably be taken in reducing the growth of
pneumatocytomas, we have studied the characteristics
of normal adrenal medulla.

**MATERIALS AND METHODS**

**Preparation of Pheochromocytoma.** The pheochro-
mocytoma was maintained by injection of approximately 1 x
10⁶ tumor cells s.c. (in the intrascapular area) into 6- to 8-
week-old NEDH rats. The tumor-bearing animals were sac-
riﬁed by cervical dislocation 3 to 4 weeks after transplantation.
The tumors were excised, rinsed in Hanks’ balanced
salt medium, minced, and suspended in Hanks’ medium
(maintained at 4°). The suspension was ﬁltered twice
through glass wool to remove the debris and particulate
matter. The resulting suspension consisted of large num-
bers of tumor cells and erythrocytes. For puriﬁcation of the
tumor cells, the suspension was kept at 4° for 20 min,
during which time the heavier tumor cells settled to the
bottom of the tube. The supernatant ﬂuid containing the
erthrocytes was discarded, and the settled tumor cells were
resuspended in Hanks’ medium. The suspension was
centrifuged at 100 x g for 5 min, and the supernatant ﬂuid
was discarded. The cells were resuspended and centrifuged
as done previously. The ﬁnal preparation contained approx-
imately 95% tumor cells and 5% erythrocytes. The puriﬁed
tumor cells were then suspended in 50 mm Tris buffer, pH
7.0, containing 1 mm Mg²⁺; homogenized with the use of a
Polytron homogenizer; and sonically dispersed for 10 sec
at 200 watts with the use of a Branson cell sonifer. Each
experiment utilized enzymes derived from a separate tumor.

**Preparation of the Adrenal Medulla.** The adrenals from
both the control and tumor-bearing rats were surgically
exised and dissected free of fat and connective tissue under
a dissecting microscope as described by Guidotti and Costa
(11). A slit was made in the adrenal gland, and under mild
pressure applied to the side opposite the slit, the adrenal
medulla was separated from the cortex and capsule. The
adrenal medulla was dissected free of any cortex adhering
to the medulla; homogenized in 50 mm Tris buffer, pH 7.0,
containing 1 mm Mg²⁺; and sonically dispersed as de-
scribed above. Each experiment utilized enzymes derived
from 2 adrenal medullae.

**Phosphodiesterase Activity.** Cyclic AMP phosphodies-
terase activity was measured by the luciferin-luciferase method as previously described (37). Each reaction vessel
contained 50 mm glycyglycine buffer (pH 8.0), 25 mm
ammonium acetate, 3 mm MgCl₂, 2 μg myokinase, 1 μg
pyruvate kinase, 400 μM cyclic AMP, 100 μM CaCl₂, and the
phosphodiesterase preparation in a total volume of 180 μl.
5'-AMP standards were incubated under the same condi-
tions as the phosphodiesterase so that corrections could
be made for any influence the compounds under study
might have on the assay system.
EGTA from Eastman Organic Chemicals, Rochester, N. Y. Other reagents were obtained from general commercial sources.

RESULTS

Functional Characteristics of Pheochromocytoma. The functional nature of the pheochromocytoma was determined by analyzing the total catecholamine content of the tumor, adrenal medulla, urine, and plasma. The concentration of catecholamines in the normal adrenal medulla was 95 nmol total catecholamine per mg protein, which is about 4 times greater than that of the pheochromocytoma (29 nmol/mg protein). This is not surprising when one considers the large amount of connective tissue in the whole tumor. The tumor was functional, however, since it released large quantities of catecholamines into the circulation as evidenced by the high concentrations of free catecholamines in the plasma (1.2 ± 0.2 nmol/ml) and urine (15 ± 6 nmol/24 hr) of tumor-bearing animals. By contrast, the concentration of catecholamines in plasma of control rats was less than 0.2 nmol/ml, and the quantity excreted in urine was 1.6 ± 0.4 nmol/24 hr. These results are consistent with the biochemical characterization of the tumor reported by Warren and Chute (34) and Chalfie and Perlman (2).

Kinetic Properties of Phosphodiesterase Isolated from the Adrenal Medulla and Pheochromocytoma. Chart 1 compares the activities of cyclic AMP phosphodiesterase in adrenal medulla and pheochromocytoma. At high concentrations of cyclic AMP, the phosphodiesterase activity of the adrenal medulla was twice that of the pheochromocytoma. However, as the concentration of cyclic AMP decreased, the ratio of phosphodiesterase activity of adrenal medulla to that of the pheochromocytoma also decreased until, at concentrations of cyclic AMP less than 50 μM, the phosphodiesterase activity of the adrenal medulla fell below that of the pheochromocytoma. At substrate concentrations of 1 μM, the pheochromocytoma had about 3 times greater phosphodiesterase activity than did the adrenal medulla.

By contrast, the rate of hydrolysis of cyclic GMP was approximately 2 times higher in the adrenal medulla than in the pheochromocytoma at all concentrations of cyclic GMP studied (Chart 2).

The phosphodiesterase activities of adrenal medullae from control and tumor-bearing rats were similar.

Kinetic analyses of the hydrolysis of cyclic AMP and cyclic GMP in homogenates of adrenal medulla and pheochromocytoma are shown in Charts 3 and 4. The adrenal medulla and pheochromocytoma both displayed biphasic kinetic properties for cyclic AMP hydrolysis, resulting in 2 apparent Michaelis constants (Kₘ's), each having a different maximum velocity (Vₘₜₐₓ). However, the Kₘ’s for the phosphodiesterase of the pheochromocytoma were substantially lower than those for the adrenal medulla. This would account for the relatively high activity of the enzyme isolated from the pheochromocytoma when low concentrations of cyclic AMP were used. The hydrolysis of cyclic GMP by both the adrenal medulla and pheochromocytoma displayed linear kinetics and had approximately the same affinity constants.

Effect of Phosphodiesterase Activator and EGTA on Phosphodiesterases in Pheochromocytoma

Phosphodiesterase Activity of Adrenal Medulla and Pheochromocytoma. Table 1 shows that neither the purified phosphodiesterase activator nor EGTA had any influence on cyclic AMP phosphodiesterase activity of adrenal medulla or pheochromocytoma over a wide range of substrate concentrations. These treatments also failed to influence the hydrolysis of cyclic GMP in adrenal medulla and pheochromocytoma (data not shown).

Subcellular Distribution of Cyclic AMP Phosphodiesterase. The subcellular distribution of cyclic AMP phosphodiesterase is shown in Table 2. There were no marked differences between the adrenal medulla and pheochromocytoma in the distribution of phosphodiesterase activity in any of the subcellular fractions. At 400 μM substrate concentration, the adrenal medulla had about twice the specific activity of the pheochromocytoma. In the adrenal medulla, the nuclear fraction displayed biphasic kinetics similar to that of the crude homogenate. All other fractions of the adrenal medulla displayed linear kinetics with Kₘ's ranging from 50 to 240 μM. In the pheochromocytoma, all subcellu-
lar fractions displayed biphasic kinetics similar to that seen in the crude homogenates.

Effect of Inhibitors on Phosphodiesterase Activity. Several pharmacological agents were examined for their ability to inhibit cyclic AMP phosphodiesterase prepared from adrenal medulla and pheochromocytoma (Table 3). Theophylline, papaverine, and cyclic GMP were competitive inhibitors and showed no pronounced differences in their ability to inhibit either phosphodiesterase preparation. Tri- fluoperazine, a potent inhibitor of phosphodiesterase from brain (20, 32, 36), and dipyridamole, an agent effective against phosphodiesterase of platelets (25) and lymphocytes (W. N. Hait and B. Weiss, unpublished observations), showed a mixed type of inhibition of the phosphodiesterase of both adrenal medulla and pheochromocytoma.

Table 1
Effect of phosphodiesterase activator and EGTA on cyclic AMP phosphodiesterase activity of rat adrenal medulla and pheochromocytoma

<table>
<thead>
<tr>
<th>Cyclic AMP (μM)</th>
<th>Adrenal medulla</th>
<th>Pheochromocytoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Activator</td>
</tr>
<tr>
<td>1</td>
<td>0.16 ± 0.02a</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.43 ± 0.02</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>100</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>400</td>
<td>4.4 ± 0.8</td>
<td>4.4 ± 0.8</td>
</tr>
</tbody>
</table>

a Mean ± S.E. of 4 determinations.
Subcellular distribution of cyclic AMP phosphodiesterase in adrenal medulla and pheochromocytoma

Each homogenate was centrifuged successively at 900 × g for 10 min, 10,500 × g for 20 min, and 100,000 × g for 60 min. Each particulate fraction was washed by resuspending in Tris-HCl buffer and recentrifuging. The resulting washed particulate fractions were resuspended in Tris-HCl buffer and assayed for protein content and cyclic AMP phosphodiesterase activity with the use of 400 μM cyclic AMP as substrate for determination of the total phosphodiesterase activity and the specific activity. Each value represents the mean of 3 separate experiments.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% of total recovered phosphodiesterase activity</th>
<th>Specific activity (nmol cyclic AMP hydrolyzed/mg protein/min)</th>
<th>Michaelis constants³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adrenal medulla</td>
<td>Pheochromocytoma</td>
<td>Adrenal medulla</td>
</tr>
<tr>
<td>Homogenate</td>
<td>100</td>
<td>100</td>
<td>3.9 ± 0.2b</td>
</tr>
<tr>
<td>900 × g (nuclear)</td>
<td>35</td>
<td>41</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>10,500 × g</td>
<td>6</td>
<td>12</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>(mitochondrial)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100,000 × g</td>
<td>3</td>
<td>5</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>(microsomal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant fluid</td>
<td>56</td>
<td>42</td>
<td>3.9 ± 0.3</td>
</tr>
</tbody>
</table>

³ The Kₘ's were determined by kinetic analysis with the use of substrate concentrations ranging from 0.5 to 500 μM.

b Mean ± S.E.

Effect of several phosphodiesterase inhibitors on phosphodiesterase activity of adrenal medulla and pheochromocytoma

Cyclic AMP phosphodiesterase activity of homogenates of adrenal medulla and purified pheochromocytoma was measured in the presence of various phosphodiesterase inhibitors. Enzyme activity was determined in triplicate at 7 different inhibitor concentrations for each of 4 substrate concentrations. The data were plotted according to the method of Dixon (5), and the Kₘ's were determined from the median point at which the lines intersected.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Adrenal medulla</th>
<th>Pheochromocytoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trifluoperazine</td>
<td>60</td>
<td>175</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>Cyclic GMP</td>
<td>150</td>
<td>270</td>
</tr>
<tr>
<td>Papaverine</td>
<td>260</td>
<td>270</td>
</tr>
<tr>
<td>Theophylline</td>
<td>650</td>
<td>850</td>
</tr>
</tbody>
</table>

DISCUSSION

The major points made in this study are: (a) normal adrenal medulla had a higher maximum rate of hydrolysis (Vₘₐₓ) of cyclic AMP phosphodiesterase than the pheochromocytoma cells; and (b) the pheochromocytoma cells can hydrolyze low (and perhaps physiological) concentrations of cyclic AMP at a far greater rate than can the normal adrenal medulla; and (c) this apparently is due to the presence in the pheochromocytoma cells of a low-Kₘ (high-affinity) form of cyclic AMP phosphodiesterase not found in the normal adrenal medulla. Thus, although both the pheochromocytoma and adrenal medulla displayed biphasic kinetics for cyclic AMP phosphodiesterase activity, the apparent Kₘ's for cyclic AMP hydrolysis in the pheochromocytoma were significantly lower (between 5- and 10-fold) than those of the normal adrenal medulla.

Kinetic analysis of the cyclic AMP phosphodiesterase activity of the subcellular fractions demonstrated a difference in the distribution of the high- and low-affinity forms of phosphodiesterase. In the adrenal medulla, the high-affinity form of phosphodiesterase was localized in the nuclear fraction, whereas all fractions of the pheochromocytoma contained the low-Kₘ form of phosphodiesterase.

This difference in the high- and low-Kₘ phosphodiesterases between adrenal medulla and pheochromocytoma cells may explain our observation that the rate of hydrolysis of cyclic AMP in the tumor is greater than that of the adrenal medulla when measured at low substrate concentrations but lower at high substrate concentrations. Similar findings have been reported by Clark et al. (4) and Hickie et al. (18) for rat hepatomas. Both groups report an increased activity of a low-Kₘ form of cyclic AMP phosphodiesterase in the neoplasm compared to that of normal liver. These studies show again the complexity of the cyclic nucleotide phosphodiesterase system and emphasize the need for studying phosphodiesterase activity over a wide range of substrate concentrations when different tissues are being compared.

Phosphodiesterase exists in multiple forms with varying physiological and pharmacological characteristics, such as substrate specificity (19, 30), stability (32, 33), and response to activator and inhibitors (8, 20, 32, 33, 36). One form of phosphodiesterase is particularly sensitive to an endogenous calcium-dependent activator of phosphodiesterase (33, 36). Uzunov et al. (31) demonstrated the presence of this activator-sensitive phosphodiesterase in adrenal medulla after separating the phosphodiesterase isozymes by polyacrylamide gel electrophoresis. In our experiments, the addition of activator or EGTA (which would inhibit the activity of the calcium-dependent activatable phosphodiesterase) failed to affect the phosphodiesterase activity of homogenates of adrenal medulla or pheochromocytoma at any concentration of cyclic AMP. This suggests that the major form of phosphodiesterase present in the adrenal medulla or pheochromocytoma is not activated by this calcium-dependent activator nor is the enzyme already activated. Our results, which are consistent with the observations of Egrie and Siegel (6), who also failed to demonstrate an activable form of phosphodiesterase in adrenal medulla, suggest that the activator-sensitive form of phosphodiesterase does not contribute significantly to the total phosphodiesterase activity observed at any concentration of substrate.

Since the multiple forms of phosphodiesterase can be...
differentially inhibited by drugs (8, 36), it may be possible to find a pharmacological agent specific for the high-affinity form of phosphodiesterase which is present in the pheochromocytoma but not in the adrenal medulla. In a preliminary attempt to find such a drug, we chose compounds known to be effective inhibitors of phosphodiesterase in other tissues and which may have different mechanisms of action: papaverine and theophylline, which are competitive inhibitors of phosphodiesterase (36); trifluoperazine, which is a potent inhibitor of phosphodiesterase of brain (36) and which acts by preventing the activation of phosphodiesterase (20); and dipyridamole, which has been shown to be a particularly effective inhibitor of phosphodiesterase of lymphocytes (W. N. Hain and B. Weiss, unpublished observation). Unfortunately, none of these pharmacological agents examined so far were particularly effective inhibitors of the phosphodiesterase isolated from either the adrenal medulla or pheochromocytoma, although the enzyme from the adrenal medulla was somewhat more sensitive to inhibition by trifluoperazine and dipyridamole.

In summary, we have confirmed the growth pattern and functional activity of a transplantable rat pheochromocytoma and characterized the activity of cyclic nucleotide phosphodiesterase isolated from purified pheochromocytoma cells and compared it with the phosphodiesterase activity of the adrenal medulla. Our demonstrations that pheochromocytoma cells have a lower activity of cyclic GMP phosphodiesterase and a higher activity of the high-affinity cyclic AMP phosphodiesterase when compared with that of the adrenal medulla are consistent with the proposition that tumor growth may be associated with either a reduced intracellular concentration of cyclic AMP or a decreased ratio of cyclic AMP to cyclic GMP.

ACKNOWLEDGMENTS

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REFERENCES

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