MOLECULAR PHARMACOLOGY, 16, 851-864

Cyclic Nucleotide Phosphodiesterase of Normal and Leukemic Lymphocytes

Kinetic Properties and Selective Alteration of the Activity of the Multiple Molecular Forms

WILLIAM N. HAIT AND BENJAMIN WEISS

Department of Pharmacology, Medical College of Pennsylvania, 3300 Henry Avenue, Philadelphia, Pennsylvania 19129

(Received November 13, 1978)
(Accepted June 13, 1979)

SUMMARY

HAIT, WILLIAM N., AND BENJAMIN WEISS. Cyclic nucleotide phosphodiesterase of normal and leukemic lymphocytes: Kinetic properties and selective alteration of the activity of the multiple molecular forms. *Mol. Pharmacol.* **16**, 851-864 (1979).

The multiple forms of cyclic AMP and cyclic GMP phosphodiesterase of normal and leukemic lymphocytes were separated by gel electrophoresis and characterized by measuring their kinetic properties and their response to activators and inhibitors of phosphodiesterase. The pattern of the cyclic AMP phosphodiesterases of normal and leukemic cells was similar: both evidenced 4 forms of the enzyme, designated as I, IA, III and IV. Peaks I, IA and IV had classic Michaelis-Menten kinetics with apparent K_m values of 10, 10 and 70 μM respectively. Peak III, a major form of phosphodiesterase activity, had anomalous kinetic behavior, suggestive of an allosteric enzyme displaying positive cooperativity. By contrast, the pattern of the cyclic GMP phosphodiesterases of leukemic lymphocytes was different from that of the normal lymphocytes. Normal lymphocytes had 3 forms of cyclic GMP phosphodiesterase corresponding to peaks IA, III, and IV of cyclic AMP phosphodiesterase. However, leukemic lymphocytes showed only 2 forms of cyclic GMP phosphodiesterase; these corresponded to peaks III and IV of cyclic AMP phosphodiesterase. The apparent K_m values for peak III (the major peak) was similar in the two types of cells (100 μM). Peak IV showed K_m values of 10 μM for normal lymphocytes and 40 μM for leukemic lymphocytes. Peak IA was present only in normal lymphocytes and had an apparent K_m of 16 μM. A study of the cyclic AMP phosphodiesterase activities of mouse cerebrum and salivary gland showed that the major form of phosphodiesterase in these tissues did not correspond to that found in leukemic lymphocytes. Calmodulin increased the activity of the major form of phosphodiesterase isolated from cerebrum but had no effect on the forms of phosphodiesterase isolated from lymphocytes or several other tissues. Cyclic GMP (5 μM) increased the activity of the major form of cyclic AMP phosphodiesterase from normal and leukemic lymphocytes but had no effect on other forms of phosphodiesterase of lymphocytes. This selective activation by cyclic GMP of the phosphodiesterase of lymphocytes was found to normalize the anomalous kinetic behavior of this form of the enzyme. The various forms of phosphodiesterase from leukemic lymphocytes and normal tissues of the mouse could be selectively inhibited by

This work was supported in part by Grant #CA15883 awarded by the National Cancer Institute and Grant #MH30096 awarded by the National Institute of Mental Health, DHEW.

0026-895X/79/060851-14$02.00/0

drugs. Thus, isobutylmethylxanthine, a competitive antagonist, was a more potent inhibitor of the form of the enzyme with high Michaelis constants (K_m) than those with low K_m values. Chlorpromazine, a drug that interferes with the calmodulin-induced activation of phosphodiesterase, was a potent inhibitor of the activable form of phosphodiesterase from cerebrum but a weak inhibitor of the other forms of the enzyme. Papaverine, which has a mixed mechanism of action, was an effective inhibitor of all forms of phosphodiesterase examined. Dipyridamole was a potent inhibitor of the major form of phosphodiesterase from normal and leukemic lymphocytes but was less effective in inhibiting the major form of phosphodiesterase from cerebrum. When these drugs were tested on the phosphodiesterase activity of crude homogenates, their order of potency reflected their potency as inhibitors of the major form of the enzyme isolated by gel electrophoresis. These studies demonstrate that the phosphodiesterases isolated from normal and malignant tissues of the mouse could be selectively activated and inhibited. Since an abnormal metabolism of cyclic nucleotides may be associated with malignancy, the data suggest the possibility of developing chemotherapeutic agents that act by selectively altering the metabolism of cyclic nucleotides in malignant tissue.

INTRODUCTION

The control of cellular proliferation and neoplastic cell growth by the cyclic nucleotides, adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP) has recently received much attention (1–3). The evidence, in general, supports the concept that low concentrations of cyclic AMP permit rapid cellular proliferation and prevent cellular differentiation. Low concentrations of cyclic AMP are found during mitosis (4, 5), in rapidly proliferating cells (6), and in cells grown at low cell-density (7, 8). Cyclic GMP, as in other systems (9), appears to antagonize the action of cyclic AMP on cell growth. The concentration of cyclic GMP is increased in rapidly proliferating cells (10), and an increased concentration of cyclic GMP can counteract the growth inhibitory effects of cyclic AMP (11).

The role of cyclic AMP in the growth of malignant cells is more controversial. Whereas most malignant tissues grown *in vitro* have lower concentrations of cyclic AMP than their normal counterparts (7, 8, 12) only certain malignant tissues *in vivo* have lower levels of cyclic AMP (13–17). This may be due to the presence of mixed cell populations in *in vivo* preparations (18).

Studies of the role of cyclic nucleotides in hematological malignancies showed that the concentration of cyclic AMP is lower in leukemic lymphocytes than in normal lymphocytes in both experimental animals (15, 19) and man (16). This decreased concentration of cyclic AMP is probably not due to an increased activity of adenylate cyclase but may be due to an increased activity of phosphodiesterase in leukemic cells (14, 16, 20–22).

Previous studies showed that the activity of the various molecular forms of phosphodiesterase can be selectively inhibited and activated by drugs (23–25). The demonstration of multiple forms of phosphodiesterase in leukemic lymphocytes (22) suggests the possibility of selectively altering the activity of the dominant form of phosphodiesterase in these cells and, thereby, selectively altering their intracellular concentration of cyclic nucleotides. Accordingly, we have isolated and characterized the forms of phosphodiesterase from normal and leukemic lymphocytes and other normal tissues of the mouse and determined if the activity of these forms of the enzyme could be selectively modified. The results show that the forms of phosphodiesterase in leukemic lymphocytes can be altered differentially and that the major forms of phosphodiesterase in leukemic lymphocytes can be inhibited by dipyridamole at concentrations having relatively little effect on other major forms of phosphodiesterase.

MATERIALS

Pyruvate kinase, myokinase and phosphoenol pyruvate were obtained from Boehringer/Mannheim; firefly luciferin-lu-

ciferase from Dupont de Nemours; acrylamide, N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethyl ethylenediamine from Eastman Organic Chemicals, Rochester, New York; Ficoll-Hypaque from Pharmacia; and the L1210 cell line from A. D. Little and Co. The preparative polyacrylamide electrophoresis apparatus was obtained from Shandon Scientific Co.

Drugs were obtained from the following sources; papaverine, isobutylmethylxanthine from Sigma Chem. Co.; 1-ethyl-4(isopropylidene-hydrazino)-1H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid (SQ20009), from Squibb Pharmaceutical Co.; dipyridamole, from Boehringer-Ingelheim; and chlorpromazine from Smith Kline and French.

METHODS

Preparation and tissue. Normal lymphocytes were isolated from the spleen of DBA/2 mice, the strain in which the leukemic cell line originated, by a modification (21) of the method of Boyum (26). Spleens were minced in Hank's solution, and particles were dispersed by aspirating them through an 18 gauge needle. Cell suspensions were further purified by passage through a plastic column fitted with 40 × 7 mm of loosely-packed glass wool. The cells were washed twice in Hank's solution and collected by centrifuging at 480 × *g* for 10 minutes. The pellet was resuspended in 4 ml of buffer and layered on 3 ml of Ficoll-Hypaque solution and centrifuged at 400 × *g* for 35 minutes at 23°. The lymphocyte band was removed and washed as above in Hank's solution.

The lymphocytic leukemia cell line, L1210, was grown in the peritoneal cavity of $C_3D_2F_1$ mice for 6 days by transplanting 1×10^5 cells. The cells were purified by the procedure used for preparing normal lymphocytes.

Using these techniques, both normal and leukemic lymphocyte preparations contained more than 95% lymphocytes, less than 5% red blood cells and less than 1% platelets; 99% of the lymphocytes were viable as evidenced by their ability to exclude Trypan Blue. Cells were resuspended in 50 mM Tris buffer, pH 8.0, containing 0.3 M sucrose and 3 mM magnesium chloride and were disrupted by sonication for 20 seconds at 200 Watts using a Branson Cell Sonifier. Phosphodiesterase activity of sonicated cells was similar to that of cells disrupted by a Polytron or Duall homogenizer.

The cerebrum and salivary gland from DBA/2 mice were prepared by homogenizing the tissue in 50 mM Tris buffer, pH 8.0, containing 0.3 M sucrose and 3 mM magnesium chloride, in a glass homogenizer with a tightly fitting Teflon pestle and sonicated for 15 seconds at 200 Watts.

Separation of the multiple forms of phosphodiesterase. The multiple forms of phosphodiesterase were separated by preparative polyacrylamide gel electrophoresis according to procedures described previously (27). Briefly, 1.5 ml of the 100,000 × *g* soluble supernatant were placed on the water-cooled column beneath an electrolyte buffer (Tris-glycine, 0.08 M, pH 8.3). A constant current of 25 mAmp was maintained for the first hour. The current was then increased to 50 mAmp and this amperage was maintained for the remainder of the separation. The voltage varied between 240–280 volts. The electrophoresis was allowed to proceed for 33 hours. Two hundred fractions were collected, accounting for about 30% of the initial phosphodiesterase activity. Peaks of phosphodiesterase activity were pooled and stored at −70° for up to two weeks (with no loss of activity) before being assayed. Each figure shown is one representative of at least 3 similar experiments.

Assay of cyclic nucleotide phosphodiesterase. The activity of cyclic AMP phosphodiesterase was determined by a modification (21) of the firefly luciferin-luciferase procedure (28), and the activity of cyclic GMP phosphodiesterase was measured as described previously (29). Enzyme activity is expressed as substrate hydrolyzed/ml/min rather than on a protein basis because the purified enzymes were collected in 0.1% bovine serum albumin to increase their stability (30). In all assays, the effect of tissue and added drugs on the standard curves was determined and appropriate corrections made. Drugs were dissolved in distilled water, adjusting the pH to insure their

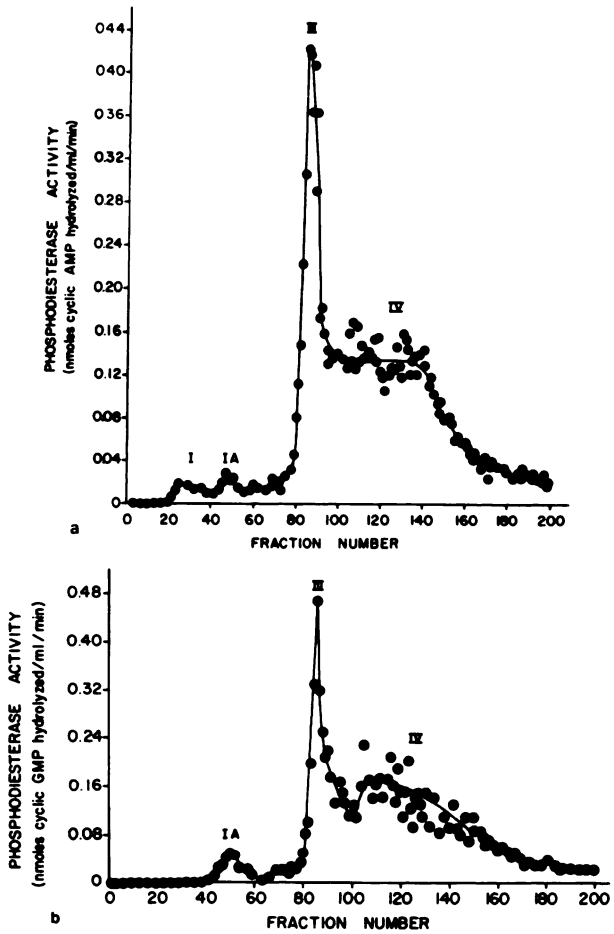

FIG. 1. *Multiple molecular forms of cyclic AMP and cyclic GMP phosphodiesterase of normal lymphocytes (DBA)*

The soluble 100,000 × *g* supernatant fraction of the sonicates from 1 × 10^8 cells was fractionated by preparative polyacrylamide gel electrophoresis as described in METHODS. The activity of phosphodiesterase was determined in each of 200 two-ml fractions using either 200 μM cyclic AMP (Fig. 1a) or 100 μM cyclic GMP (Fig. 1b) as substrate. The figure represents the data from 1 of 4 similar experiments.

complete solubility. The potency of each drug is expressed as the concentration required to obtain 50% inhibition of enzyme activity (I_{50}). K_i values were not calculated because of the anomalous kinetics of the major form of phosphodiesterase of leukemic lymphocytes.

Other procedures. Calmodulin[1] was prepared from bovine brain by the method of Teo and Wang (31) and from murine brain and lymphocytes by a modification of the method of Cheung (32). Protein was assayed according to the method of Lowry *et al.* (33) using bovine serum albumin as standards. Cells were counted using an Improved Neubauer Hemocytometer.

[1] Calmodulin is the calcium-dependent protein modulator of cyclic nucleotide phosphodiesterase which has also been referred to as CDR, activator protein, phosphodiesterase activator, calcium dependent modulator, and others.

RESULTS

Isolation of multiple forms of phosphodiesterase from normal and leukemic lymphocytes. Figure 1 shows the multiple forms of cyclic AMP and cyclic GMP phosphodiesterase activities from the soluble supernatant fraction of homogenates of normal lymphocytes prepared from spleens of DBA

mice. Three distinct peaks of cyclic AMP phosphodiesterase activity were found (Fig. 1a). These peaks are designated I, IA, and III in reference to the migration of phosphodiesterase similarly prepared from cerebrum (30). An additional area of activity emerged from the column after Peak III. The activity of this peak, designated Peak IV, was more variable than the others. Peaks IA, III and IV also had appreciable cyclic GMP phosphodiesterase activity (Fig. 1b). However, no cyclic GMP phosphodiesterase activity was detectable in Peak I.

Figure 2 shows the multiple forms of phosphodiesterase of leukemic lymphocytes. As in normal lymphocytes, there were 3 distinct peaks of cyclic AMP phosphodiesterase activity as well as an additional area of activity (labeled Peak IV) Fig. 2a). The pattern of cyclic GMP phosphodiesterase in the leukemic lymphocytes was different from that of the normal lymphocytes (Fig. 2b). In leukemic lymphocytes cyclic GMP phosphodiesterase activity was seen only in Peaks III and IV; no cyclic GMP phosphodiesterase activity was detectable in Peak IA.

Figures 1 and 2 also show that in the leukemic lymphocyte preparations the total

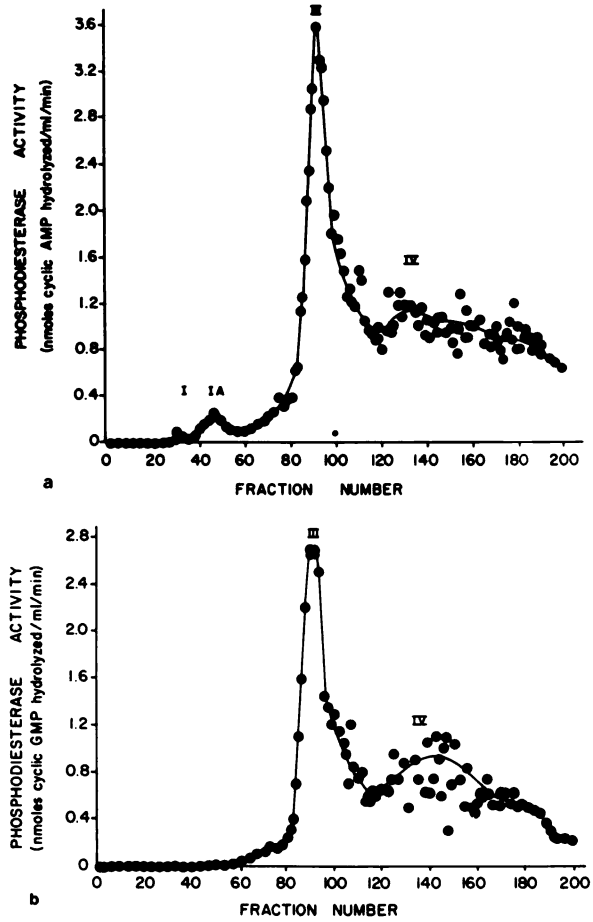

FIG. 2. *Multiple molecular forms of cyclic AMP and cyclic GMP phosphodiesterase of leukemic lymphocytes (L1210)*

The soluble 100,000 × *g* supernatant fraction of the sonicates from 1 × 10^8 cells was fractionated by preparative polyacrylamide gel electrophoresis as described in METHODS. The activity of phosphodiesterase was determined in each of 200 two-ml fractions using either 200 μM cyclic AMP (Fig. 2a) or 100 μM cyclic GMP (Fig. 2b) as substrate. The figure represents the data from 1 of 3 similar experiments.

cyclic AMP phosphodiesterase activity was approximately 9 times greater and the cyclic GMP phosphodiesterase about 6 times greater than in the normal lymphocytes.

Kinetic analyses of the phosphodiesterase from normal and leukemic lymphocytes. Kinetic analysis of the isolated forms of cyclic AMP phosphodiesterase from normal and leukemic lymphocytes demonstrated that the analogous peaks from both cell types had similar kinetics. In both the leukemic (Fig. 3) and normal lymphocytes, Peaks I, IA and IV demonstrated linear kinetics whereas Peak III demonstrated non-linear kinetics. Moreover, in both preparations the K_m values for cyclic AMP were lower in Peaks I and IA (10–40 μM) than in Peaks III and IV (approximately 100 μM) (Table 1).

The kinetic analysis of the cyclic GMP phosphodiesterases, also summarized in Table 1, shows that Peak IA, which was present only in the normal cells, had a K_m of about 16 μM; Peak III, which was present in both cell types, had a K_m of approximately 100 μM; Peak IV, which was also

TABLE 1

Apparent Michaelis constants (K_m) for the multiple forms of phosphodiesterase of normal and leukemic lymphocytes

The multiple forms of phosphodiesterase were prepared as described in Methods. Fractions under each peak were pooled and stored at −70°. The activity of each form was determined at concentrations of substrate ranging from 1–250 μM. The concentration of enzyme and the time of incubation was adjusted so that less than 10% of the substrate was hydrolyzed. In each analysis, the line of best fit was determined by linear regression analysis, the K_m values were estimated by the method of Lineweaver and Burk (34). Each value represents the mean of 4 determinations from one of 2 separate experiments. There was less than 10% experimental variation between them.

Peak	K_m Values (μM)			
	Cyclic AMP phosphodiesterase		Cyclic GMP phosphodiesterase	
	Normal lymphocytes	Leukemic lymphocytes	Normal lymphocytes	Leukemic lymphocytes
I	10	10	—	—
IA	40	10	16	—
III	100	100	150	100
IV	100	70	10	40

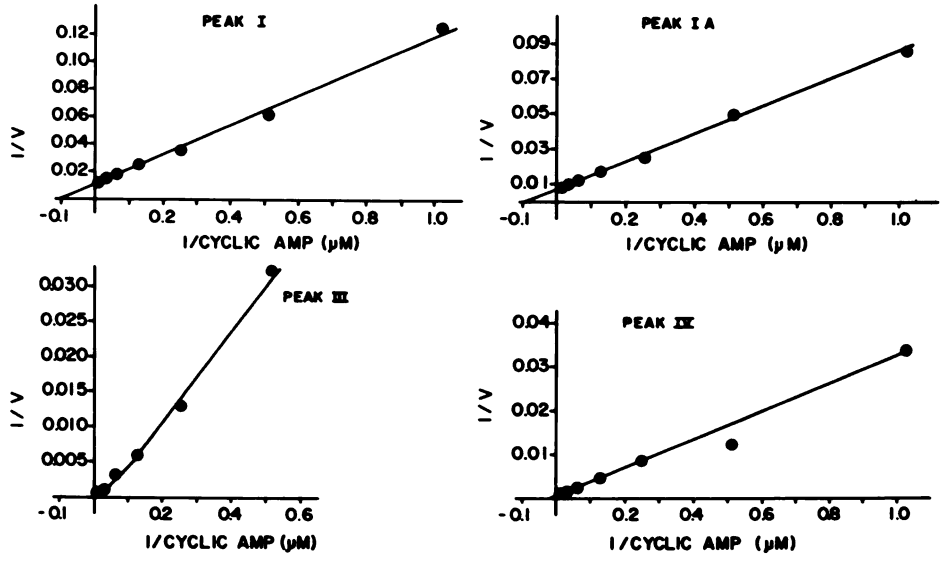

FIG. 3. *Kinetic analysis of the multiple forms of cyclic and phosphodiesterase of leukemic lymphocytes*

Fractions under each peak of phosphodiesterase activity were pooled and stored in 2 ml portions at −70°. The activity of phosphodiesterase was determined using 1–250 μM cyclic AMP. The concentration of enzyme and time of incubation were adjusted so that the reaction was linear and less than 10% of the substrate was hydrolyzed. The line of best fit was determined, where possible, by linear regression analysis. Each point represents the mean of quadruplicate determinations.

present in both cell types, had K_m values of approximately 40 μM and 10 μM in leukemic and normal lymphocytes, respectively.

Isolation of multiple forms of phosphodiesterase from other tissues of the mouse. The soluble supernatant fractions of cerebrum and submaxillary gland homogenates were subjected to polyacrylamide gel electrophoresis and the phosphodiesterase analyzed. The pattern of phosphodiesterase activities of these tissues was markedly different from that found in lymphocytes. The submaxillary gland had 2 major forms of phosphodiesterase; the first peak, which appeared to coincide with Peak IA of lymphocytes, accounted for about 60% of the activity and the second peak, which didn't coincide with any of the phosphodiesterase peaks isolated from lymphocytes, accounted for about 40% of the activity. In mouse cerebrum, the major form of phosphodiesterse (designated as Peak II accounted for about 85% of the total recovered activity, and did not coincide with any of the peaks of activity isolated from lymphocytes or submaxillary gland.

Effect of calmodulin on phosphodiesterase activity. Figure 4 demonstrates that calmodulin (activator) prepared from bovine brain selectively increased the activity of the major form of phosphodiesterase of cerebrum (Peak II). The other forms of phosphodiesterase (i.e., Peaks I, IA, III and IV) were not activated, even when assayed over a concentration range of calmodulin from 0.05 to 12 μg and substrate concentrations from 1 to 250 μM. Identical results were obtained with calmodulin prepared from cerebrum, normal or leukemic lymphocytes or mouse brain. Furthermore, these non-activable forms of phosphodiesterase were not inhibited by the calcium chelating agent, EGTA, suggesting that these enzymes are not already maximally activated.

Effect of cyclic GMP on phosphodiesterase activity. The major form of cyclic AMP phosphodiesterase (Peak III) from normal and leukemic lymphocytes displayed anomalous kinetics reflected in a non-linear Lineweaver Burk plot (Figs. 3, 5). The addition of 5 μM cyclic GMP increased the activity of the phosphodiesterase when the enzyme was measured at concentrations of cyclic AMP less than 80 μM, resulting in a linear Lineweaver Burk plot (Fig. 5). By contrast,

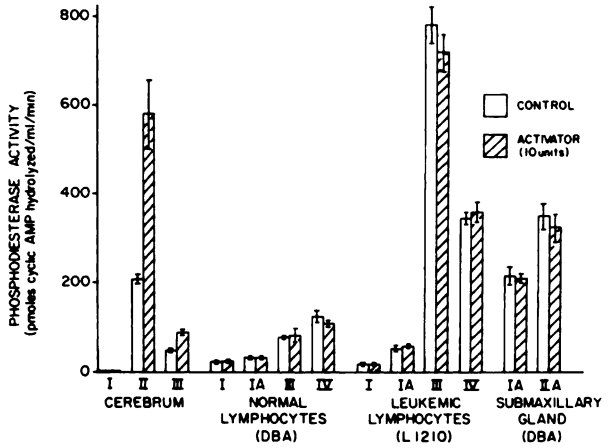

FIG. 4. *Effect of the endogenous calcium-dependent protein activator (calmodulin) on the multiple forms of cyclic AMP phosphodiesterase from various tissues of the mouse*

The molecular forms of phosphodiesterase from various tissues were prepared by polyacrylamide gel electrophoresis as described in METHODS. Cerebrum exhibited 3 peaks of activity (labeled I, II and III according to the order in which they emerged from the column), lymphocytes 4 peaks, and submaxillary gland 2 peaks of activity. Fractions under each peak of phosphodiesterase activity were pooled and stored in 2 ml portions at −70°. Phosphodiesterase activity was determined in the presence or absence of 10 units of bovine brain activator (calmodulin) using a substrate concentration of 200 μM cyclic AMP. Each value represents the mean ± SEM of 5 determinations.

5 μM cyclic GMP failed to alter the activity of any of the other forms of phosphodiesterase isolated from lymphocytes at concentrations of cyclic AMP of 2, 5 and 200 μM (Fig. 6).

Effect of phosphodiesterase inhibitors on the multiple forms of phosphodiesterase. Table 2 shows the effect of several drugs on the activity of the multiple forms of phosphodiesterase of normal and leukemic lymphocytes. Chlorpromazine was a weak inhibitor of all forms of phosphodiesterase of lymphocytes. Isobutylmethylxanthine was most effective against Peak IV, a form of the enzyme having a relatively high K_m. Papaverine was a potent inhibitor of most of the forms of phosphodiesterase of normal and leukemic lymphocytes although Peak I of leukemic lymphocytes was less sensitive to inhibition (I_{50} = 160 μM) than was Peak I of normal lymphocytes (I_{50} = 25 μM). Dipyridamole was also a potent inhibitor of most forms of phosphodiesterase prepared from lymphocytes.

The effect of these compounds on the multiple forms of cyclic GMP phosphodiesterase is also summarized in Table 2. As with the cyclic AMP phosphodiesterases, there was some selectivity with which the drugs inhibited the various forms of cyclic GMP phosphodiesterase: papaverine and dipyridamole were particularly effective against Peak III. Moreover, isobutylmethylxanthine and papaverine were more potent inhibitors of Peak III cyclic GMP phosphodiesterase than Peak III cyclic AMP phosphodiesterase.

Comparison of the effect of drugs on the major forms of phosphodiesterase from various tissues. Table 3 shows that dipyridamole was 4 times more potent in inhibiting the major form of phosphodiesterase of leukemic lymphocytes (Peak III; I_{50} = 20 μM) than that of cerebral cortex (Peak II; I_{50} = 80 μM). This was true whether the activity of phosphodiesterase was measured in the presence or absence of calmodulin. By contrast, chlorpromazine was about 4 times more potent in inhibiting the major form of phosphodiesterase of cerebral cortex (Peak II; I_{50} = 45 μM) that that of leukemic lymphocytes (Peak III; 200 μM) when enzyme activity was measured in the presence of calmodulin.

Other compounds studied also showed preferential inhibition of the major form of phosphodiesterase of certain tissues. For example, SQ20009 was 10 times more potent in inhibiting the major form of phosphodiesterase of salivary gland (I_{50} = 12 μM) than in inhibiting the major form of phosphodiesterase of the leukemic lymphocytes (I_{50} = 120 μM).

Effect of drugs on phosphodiesterase activity of whole homogenates of various tissues. Figure 7a demonstrates that dipyri-

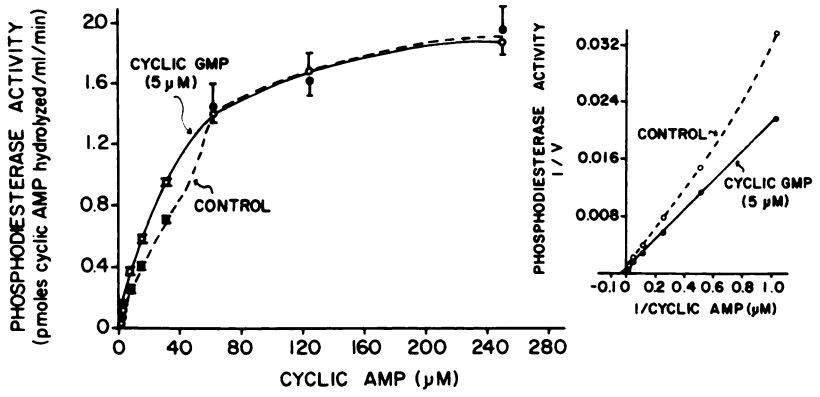

FIG. 5. *Effect of cyclic GMP on the kinetics of Peak III cyclic AMP phosphodiesterase of leukemic lymphocytes*

Peak III phosphodiesterase was prepared from leukemic lymphocytes (L1210) as described in METHODS. Cyclic AMP phosphodiesterase activity was determined in the presence of 5 μM cyclic GMP at concentrations of up to 250 μM cyclic AMP. Each point represents the mean ± SEM of quadruplicate samples. The insert is a Lineweaver-Burk analysis of these data.

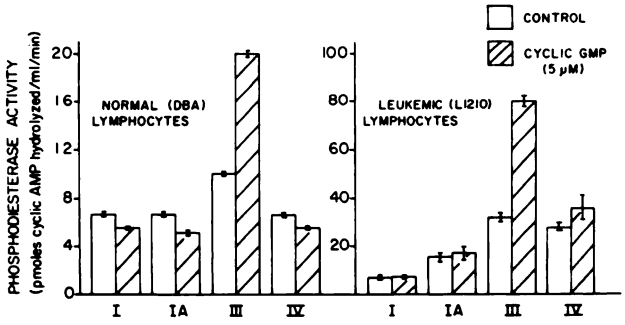

FIG. 6. *Effect of cyclic GMP on the multiple forms of cyclic AMP phosphodiesterase of normal and leukemic lymphocytes*

The multiple forms of phosphodiesterase were prepared and stored as described in METHODS. Cyclic AMP phosphodiesterase activity was determined in the presence or absence of 5 μM cyclic GMP using 2 μM cyclic AMP as substrate. Each bar represents the mean ± SEM of 4 determinations.

damole was about 3 times more potent in inhibiting the activity of phosphodiesterase of crude homogenates of leukemic lymphocytes (I_{50} = 20 μM) than that of crude homogenates of cerebral cortex (I_{50} = 64 μM). Conversely, chlorpromazine was a more potent inhibitor of the phosphodiesterase of cerebral homogenates than that of lymphocyte homogenates (Fig. 7b).

DISCUSSION

The present results confirm previous findings (19–21) that there is a several-fold greater activity of cyclic nucleotide phosphodiesterase in leukemic lymphocytes than in normal lymphocytes. The phosphodiesterase activity in the soluble supernatant fraction of sonicates prepared from normal lymphocytes isolated from spleen was of the same order of magnitude as that found previously in B cells, T cells, whole thymus, whole spleen or in mixed populations of cells puified from lymph nodes (20, 21, 35).

Other reports using polyacrylamide gel electrophoresis to separate the phosphodiesterases revealed three forms of cyclic AMP and cyclic GMP phosphodiesterase in purified T and B cells prepared from homozygous nude mouse spleen and heterozygous mouse spleen, respectively (35). Studies of normal and leukemic cell lines, in which whole cell sonicates were fractionated on a linear sucrose gradient, showed two forms of cyclic AMP phosphodiesterase and either one or two forms of cyclic GMP phosphodiesterase, depending on the cell type (19).

The present studies of the electrophoretic pattern of the soluble fraction of normal and leukemic lymphocytes (L1210) revealed the presence of 3 distinct forms of cyclic nucleotide phosphodiesterase. An analysis of these peaks of activity showed that whereas the pattern of cyclic AMP phosphodiesterase from the normal and leukemic lymphocytes was similar, there was a marked difference in the pattern of cyclic GMP phosphodiesterase. Three forms of cyclic GMP phosphodiesterase were detected in normal cells (IA, III, IV), but only 2 forms were seen in the leukemic cells (III and IV). Since the total cyclic GMP phosphodiesterase activity emerging from the electrophoretic column was 5–10 fold greater for leukemic lymphocyte preparations, this lack of cyclic GMP phosphodiesterase activity for Peak IA in leukemic cells cannot be attributed to a lack of sensitivity of the assay, to differences in the recovery of the enzymes (approximately 30% of the phosphodiesterase activity was recovered from the gel electrophoresis columns in each preparation) or to differences in contaminants of the preparations (21). Moreover, the consistency with which the electrophoretic patterns were seen suggests that these forms were not caused by the interconversion of one form of the enzyme to another as has been shown in other systems (36, 37).

Analysis of the isolated forms of phos-

TABLE 2

Effect of phosphodiesterase inhibitors on the multiple molecular forms of cyclic AMP and cyclic GMP phosphodiesterase isolated from normal and leukemic lymphocytes

The multiple forms of phosphodiesterase were prepared by polyacrylamide gel electrophoresis. Fractions under each peak of phosphodiesterase activity were pooled and stored in 2 ml portions at −70°. Drugs were dissolved in distilled water and tested over a concentration range of 4–250 μM at a substrate concentration of 200 μM cyclic AMP or cyclic GMP. The effect of each drug on the assay was determined and where necessary corrected by adding the drug to the 5'AMP or 5'GMP standard curves. Each value represents the concentration of drug required to produce 50% inhibition of enzyme activity and was calculated from 4 dose-response curves for each form of the enzyme. The I_{50} values are the mean of 3 experiments with less than 10% variability among them.

Cell type	Peak	I_{50} Values (μM)							
		Chlorpromazine		Isobutylmethylxanthine		Papaverine		Dipyridamole	
		Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
Normal lymphocytes (DBA)	I	>250	—	250	—	25	—	17	—
	IA	>250	100	130	>250	50	30	50	>250
	III	180	150	100	30	30	4	20	4
	IV	200	150	5	180	5	8	15	20
Leukemic lymphocytes (L1210)	I	>250	—	>250	—	160	—	40	—
	IA	>250	—	>250	—	70	—	50	—
	III	>250	150	100	50	30	25	15	16
	IV	180	150	60	200	15	25	15	16

TABLE 3

Comparison of the effects of dipyridamole and chlorpromazine on the major form of phosphodiesterase isolated from mouse leukemic lymphocytes and cerebral cortex

The phosphodiesterases of the 100,000 × *g* supernatant fraction of cerebrum and leukemic lymphocytes were separated by preparative polyacrylamide gel electrophoresis as described in METHODS. Fractions under each peak of phosphodiesterase activity were pooled and stored in 2 ml portions at −70°. Cyclic AMP phosphodiesterase was determined on the major form of phosphodiesterase of each tissue using 200 μM cyclic AMP in the presence of 10 units of calmodulin and varying concentrations of drug.

Tissue	Major form of phosphodiesterase	I_{50} Values (μM)	
		Dipyridamole	Chlorpromazine
Leukemic lymphocytes	Peak III	20	200
Cerebral cortex	Peak II	80	45

phodiesterase demonstrated that the peaks from normal and leukemic lymphocytes that had similar electrophoretic mobilities also had similar kinetic properties. It may be noted that Peak IA of cyclic GMP phosphodiesterase, present in normal lymphocytes, had a relatively low K_m value of 16 μM. Since it has been shown that the concentration of cyclic GMP is increased in lymphocytes that are rapidly proliferating (10) and that the concentration of cyclic GMP is increased in rapidly growing L1210 cells (38), the apparent loss of Peak IA cyclic GMP phosphodiesterase in these leukemic cells may have functional significance.

In addition to having different kinetic properties, the various forms of phosphodiesterase in normal and leukemic lymphocytes have different responses to endogenous and exogenous substances. For example, calmodulin, the heat-stable, protein activator of phosphodiesterase, had no effect on any form of phosphodiesterase of lymphocytes. This finding can be explained by the absence in lymphocytes of Peak II phosphodiesterase, the only major form of the enzyme that has been shown to be activated by calmodulin (23, 30). The presence of calmodulin in lymphocytes, albeit in lower concentrations than that found in cerebrum (21) or in other tissues devoid of an activable phosphodiesterase (25), may be explained by the observation that this cal-

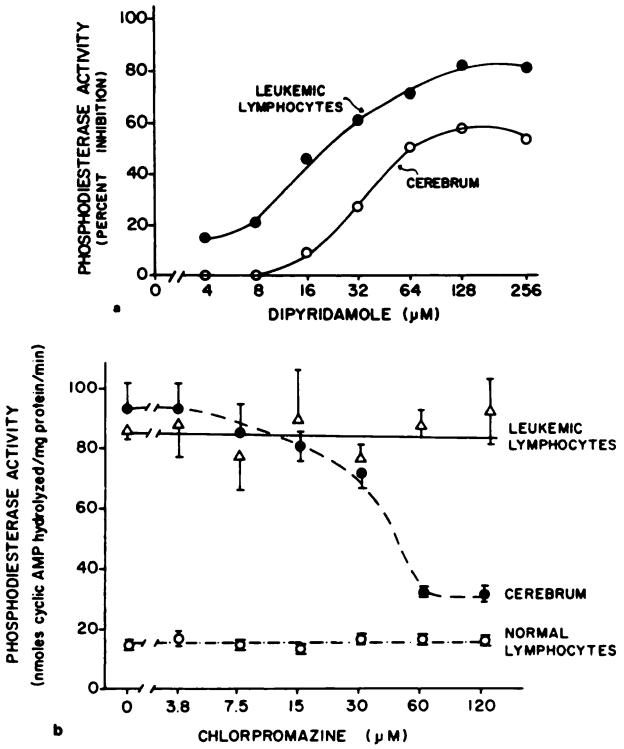

FIG. 7. *Effect of dipyridamole and chlorpromazine on phosphodiesterase activity of whole homogenates of leukemic lymphocytes and cerebral cortex*

Tissue was prepared from the cerebral cortex of DBA mice and from L1210 leukemic lymphocytes as described in METHODS. The activity of phosphodiesterase was determined in whole tissue homogenates in the presence of 10 units of calmodulin and either dipyridamole (Fig. 7a) or chlorpromazine (Fig. 7b) (up to 250 μM). The substrate concentration was 250 μM cyclic AMP. Each point represents the mean of 4 determinations. Brackets indicate the standard error.

cium-dependent protein also activates other enzyme systems, such as adenylate cyclase (39, 40) and ATPase (41).

Another endogenous material that has been reported to influence cyclic AMP phosphodiesterase activity is cyclic GMP; low concentrations have been shown to increase the activity of cyclic AMP phosphodiesterase whereas high concentrations inhibit it (42). The anomalous kinetic behavior of Peak III phosphodiesterase, which suggested allosteric sites on the enzyme, was normalized when 5 μM cyclic GMP was added to the preparation. Other cyclic nucleotides and purine derivatives failed to have any effect.[2] These data suggest that low concentrations of cyclic GMP may activate Peak III phosphodiesterase by occupying an allosteric site on the enzyme. Therefore, forms of the enzyme that do not appear to have an allosteric site should not be activated by cyclic GMP. The data (Fig. 6) demonstrated that only Peak III phosphodiesterase, the form of the enzyme that displayed anomalous kinetics, was activated by cyclic GMP.

The distinctive characteristic of Peak III phosphodiesterase suggests that drugs may be developed that selectively inhibit the major form of phosphodiesterase of leukemic lymphocytes by interacting at the

[2] Compounds tested include: 8-bromo cyclic AMP; 8-bromo cyclic GMP; N^2-monobutyl cyclic GMP; 8-p-chlorophenyl-thio-cyclic AMP; isobutylmethylxanthine; adenosine-5′-O-monothiophosphate; 6-thioguanine; 6-histoaminopurine; and 8-bromo acetyl-2′-3′5′-triacetyl guanosine; each over a concentration range of 1–500 μM using cyclic AMP concentrations of 2.5 and 200 μM.

allosteric site.

The differential inhibition of the various forms of phosphodiesterase by drugs is due to the different mechanisms by which drugs exert their inhibitory actions on the phosphodiesterase system. Thus, phenothiazine antipsychotics selectively inhibit the calmodulin-activable form of phosphodiesterase (Peak II) by binding to calmodulin (43). Since cerebrum has a relatively high amount of Peak II, its phosphodiesterase is very sensitive to the effects of chlorpromazine. By contrast, no Peak II phosphodiesterase was detectable in the lymphocytes, and predictably the phosphodiesterase of lymphocytes was relatively resistant to chlorpromazine inhibition.

Isobutylmethylxanthine, a structural analogue of the cyclic nucleotides, is a competitive inhibitor of phosphodiesterase (24). Thus, as one would anticipate, this drug was a more potent inhibitor of Peaks III and IV, which have high K_m values (about 100 μM), than of Peaks I and II which have K_m values of about 10 μM.

Papaverine, a drug that competitively inhibits phosphodiesterase at low concentrations and non-competitively inhibits it at high concentrations (44), is a relatively potent inhibitor of most forms of phosphodiesterase (23). This relative non-selectivity of inhibition was again seen in the present studies, although this drug was more effective against Peaks III and IV than against I and IA.

The mechanism by which dipyridamole inhibits phosphodiesterase has not been elucidated. However, it is a more potent inhibitor of the phosphodiesterase of platelets than of brain (26), and it has been shown to have differential actions on separated forms of phosphodiesterase isolated from human platelets (45). Our results showed that dipyridamole was the most potent inhibitor of the multiple forms of phosphodiesterase of normal and leukemic lymphocytes. Although there was no clear selectivity of this drug for the various forms of phosphodiesterase in lymphocytes, the drug was found to be a far more potent inhibitor of the major form of phosphodiesterase of leukemic lymphocytes than that of cerebrum (Table 3).

While examining other drugs as inhibitors of the isolated forms of phosphodiesterase, two additional observations of drug selectivity were made. SQ20009 was an extremely potent inhibitor of the second electrophoretic peak of phosphodiesterase of salivary gland, and isobutylmethylxanthine was a more selective inhibitor of the major form of phosphodiesterase of leukemic lymphocytes than that of salivary gland (3).

In considering the physiological relevance of the multiple forms of phosphodiesterase in different tissues and the pharmacological significance of their selective inhibition by drugs, one must address the question of whether these isolated forms of phosphodiesterase have similar properties as those found *in vivo*. Recent studies showed that the multiple forms of phosphodiesterase are interconvertible, i.e., that one form can give rise to others following appropriate manipulation, and that the pattern of phosphodiesterase can be effected by a variety of factors present during their preparation (36, 37). Evidence such as this may suggest that the forms identified by any one technique might not truly represent the forms of phosphodiesterase present in the intact cell. If, however, the purified forms were representative of the forms of phosphodiesterase in the intact cell, then the characteristics of the major form should reflect the characteristics of the enzyme before purification. To examine this, we compared the effect of various phosphodiesterase inhibitors on the major forms of phosphodiesterase isolated by gel electrophoresis and on the crude homogenates of various tissues. We found that those drugs that were most effective against the major form of phosphodiesterase isolated from a particular tissue were also the most potent inhibitors of the phosphodiesterase in crude homogenates of that tissue.

Most currently used cancer chemotherapeutic agents are non-selective since they interfere with the cellular components essential for proliferation of both normal and malignant cells. To date, few unique properties of malignant cells have been identified that are amenable to pharmacological intervention. It has now been shown that leukemic lymphocytes have markedly in-

creased activities of cyclic nucleotide phosphodiesterase and that these phosphodiesterases of leukemic lymphocytes can be selectively inhibited by drugs. These studies suggest the possibility of selectively altering cyclic nucleotide metabolism and perhaps the properties of malignant cells.

REFERENCES

1. Strada, S. J. and W. J. Pledger. The role of cyclic nucleotides in cell growth and development: Regulation and characterization of cyclic nucleotide phosphodiesterases in mammalian cells, In: *Cyclic Nucleotides in Disease* (ed. B. Weiss), University Park Press, Baltimore, Md., 1975, pp 3–34.
2. Ryan, W. L. and M. L. Heidrick. Role of cyclic nucleotides in cancer, In: *Advances in Cyclic Nucleotide Research* (eds. P. Greengard and G. A. Robison), Raven Press, N.Y., vol. 4, 1974, pp 81–116.
3. Weiss, B. and W. N. Hait. Selective cyclic nucleotide phosphodiesterase inhibitors as potential therepeutic agents. *Ann. Rev. Pharmacol. Toxicol.* **17:** 441–477, 1977.
4. Sheppard, J. R. and D. M. Prescott. Cyclic AMP levels in synchronized mammalian cells. *Expt. Cell Res.* **75:** 293–296, 1972.
5. Millis, A. J. T., G. Forrest and D. A. Pious. Cyclic AMP in cultured human lymphoid cells: Relationship to mitosis. *Biochem. Biophys. Res. Commun.* **49:** 1645–1649, 1972.
6. Otten, J., G. S. Johnson, and I. Pastan. Cyclic AMP levels in fibroblasts: Relationships to growth rate and contact inhibition of growth. *Biochem. Biophys. Res. Commun.* **44:** 1192–1198, 1971.
7. Otten, J., G. S. Johnson, and I. Pastan. Regulation of cell growth by cyclic adenosine 3′,5′-monophosphate. *Biochem. Biophys. Res. Commun.* **44:** 1192–1198, 1971.
8. Bannai, S. and J. R. Sheppard. Cyclic AMP, ATP and cell contact. *Nature* **250:** 62–64, 1974.
9. Goldberg, N. D., M. K. Haddox, S. E. Nicol., D. B. Glass, C. H. Sanford, F. A. Kuehl, Jr., and R. Estensen. Biological regulation through opposing influences of cyclic GMP and cyclic AMP: The Yin Yang hypothesis, In: *Adv. Cyclic Nucl. Res.* (ed. G. I. Drummond, P. Greengard and G. A. Robison). Raven Press, N.Y., pp 307–330, 1975.
10. Goldberg, N. D., M. K. Haddox, R. Estensen, J. G., White, C. Lopez, and J. W. Hadden. Evidence of a dualism between cyclic GMP and cyclic AMP in the regulation of cell proliferation and other cellular processes. In: *Cyclic AMP, Cell Growth and the Immune Response* (eds. W. Braun, L. M. Lichtenstein, C. W. Parker), Springer-Verlag, N.Y., pp 247–262, 1973.
11. Rudland, P. S., D. Gospodarowicz, and W. Seifert. Activation of guanyl cyclase and intracellular cyclic GMP by fibroblast growth factor. *Nature* **250:** 741–743, 1974.
12. Lincoln, T. M. and G. L. Vaughn. The role of adenosine 3′,5′-monophosphate in the transformation of Cloudman mouse melanoma cells. *J. Cell Physiol.* **86:** 543–552, 1975.
13. Goldberg, M. L., G. C. Burke, and H. P. Morris. Cyclic AMP and cyclic GMP content and binding in malignancy. *Biochem. Biophys. Res. Commun.* **62:** 320–327, 1975.
14. Hickie, R. A., C. M. Walker, and G. A. Croll. Decreased basal cyclic adenosine 3,5-monophosphate levels in Morris Hepatoma 5123 t.c. *Biochem. Biophys. Res. Commun.* **59:** 167–173, 1974.
15. Kemp, R. G., P-Y. Hsu, and R. J. Duquesnoy. Changes in lymphoid cyclic adenosine 3′:5′-monophosphate metabolism during murine leukemogenesis. *Cancer Res.* **35:** 2440–2445, 1975.
16. Monahan, T. M., N. W. Marchand, R. R. Fritz, and C. W. Abell. Cyclic adenosine 3′:5′-monophosphate levels and activities related enzymes in normal and leukemic lymphocytes. *Cancer Res.* **35:** 2540–2547, 1975.
17. Stevens, R. J., D. D. Smith, J. W. Osborne, and L. W. Oberley. Adenosine 3′,5′-cyclic monophosphate levels in X-ray induced small bowel adenocarcinoma in the rat. *J. Natl. Cancer Inst.* **57:** 43–45, 1976.
18. Cohen, L. and P-C. Chan. Intracellular cAMP levels in normal rat mammary gland and adenocarcinoma. In vivo *vs* in vitro. *Life Sci.* **16:** 107–115, 1975.
19. Epstein, P. M., J. S. Mills, C. P. Ross, S. J. Strada, E. M. Hersh, and W. J. Thompson. Increased cyclic nucleotide phosphodiesterase activity associated with proliferation and cancer in human and murine lymphoid cells. *Cancer Res.* **37:** 4016–4023, 1977.
20. Hait, W. N. and B. Weiss. Increased cyclic nucleotide phosphodiesterase activity in leukemic lymphocytes. *Nature* **259:** 321–323, 1976.
21. Hait, W. N. and B. Weiss. Characteristics of the cyclic nucleotide phosphodiesterases of normal and leukemic lymphocytes. *Biochim. Biophys. Acta* **497:** 86–100, 1977.
22. Weiss, B. and R. A. Winchurch. Analyses of cyclic nucleotide phosphodiesterases in lymphocytes from normal and aged leukemic mice. *Cancer Res.* **38:** 1274–1280, 1978.
23. Weiss, B., R. Fertel, R. Figlin, and P. Uzunov. Selective alteration of the activity of the multiple forms of cyclic 3′,5′-AMP phosphodiesterase of rat cerebrum. *Mol. Pharmacol.* **10:** 615–625,

1974.

24. Wells, J. N., Y. J. Wu, C. E. Baird, and J. G. Hardman. Phosphodiesterases from porcine coronary arteries: Inhibition of separated forms by xanthines, papaverine, and cyclic nucleotides. *Mol. Pharmacol.* **11:** 775–783, 1975.
25. Fertel, R. and B. Weiss. Properties and drug responsiveness of cyclic nucleotide phosphodiesterases of rat lung. *Mol. Pharmacol.* **12:** 678–687, 1976.
26. Boyum, A. Separation of leucocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21:** 79–90, 1968.
27. Uzunov, P. and B. Weiss. Separation of multiple forms of cyclic adenosine 3′,5′-monophosphate phosphodiesterase in rat cerebellum by polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* **284:** 220–226, 1972.
28. Weiss, B., R. Lehne, and S. J. Strada. Rapid microassay of adenosine 3′,5′-monophosphate phosphodiesterase activity. *Analyt. Biochem.* **45:** 222–235, 1972.
29. Fertel, R. and B. Weiss. A microassay for guanosine 3′,5′-monophosphate phosphodiesterase activity. *Analyt. Biochem.* **59:** 386–398. 1974.
30. Uzunov, P., H. M. Shein, and B. Weiss. Multiple forms of cyclic 3′,5′-AMP phosphodiesterase of rat cerebrum and cloned astrocytoma and neuroblastoma cells. *Neuropharmacol.* **13:** 377–391, 1974.
31. Teo, T. S. and J. H. Wang. Mechanism of activation of a cyclic adenosine 3′,5′-monophosphate phosphodiesterase from bovine heart by calcium ions. Identification of the protein activator as a Ca2+ binding protein. *J. Biol. Chem.* **248:** 5950–5955, 1973.
32. Cheung, W. Y. Cyclic 3′,5′-nucleotide phosphodiesterase. Demonstration of an activator. *Biochem. Biophys. Res. Commun.* **38:** 533–538, 1970.
33. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275, 1951.
34. Lineweaver, H. and D. Burk. Determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **56:** 658–666, 1934.
35. Winchurch, R. A., W. N. Hait, and B. Weiss. Cyclic AMP phosphodiesterase activity of Murine T and B lymphocytes. *Cell. Immunol.* **41:** 421–426, 1978.
36. Pichard, A. L. and W. Y. Cheung. Cyclic 3′:5′-nucleotide phosphodiesterase: Introconvertible multiple forms and their effects on enzyme activity and kinetics. *J. Biol. Chem.* **254:** 5726–5736, 1976.
37. Pledger, W. J., W. J. Thompson, and S. J. Strada. Serum modification of cyclic nucleotide phosphodiesterase forms independent of protein synthesis. *Biochim. Biophys. Res. Commun.* **70:** 58–65, 1976.
38. Coffey, R. G. Assays for cyclic nucleotides including clinical applications. In: *Immunopharmacology* (eds. J. W. Hadden, R. G. Coffey and F. Spreafico), Plenum Medical Book Co., N.Y., pp 389–412, 1977.
39. Brostrom, C. O., Y-C Huang, B. McL. Breckenridge, and D. J. Wolff. Identification of a calcium-binding protein as a calcium-dependent regulator of brain adenylate cyclase. *Proc. Nat. Acad. Sci. USA* **72:** 64–68, 1975.
40. Cheung, W. Y., L. S. Bradham, T. J. Lynch, Y. M. Lin, and E. A. Tallant. Protein activator of cyclic 3′:5′-nucleotide phosphodiesterase of bovine or rat brain also activates its adenylate cyclase. *Biochem. Biophys. Res. Commun.* **66:** 1055–1062, 1975.
41. Jarrett, H. W. and J. T. Penniston. Partial purification of the Ca2+ − Mg2+ ATPase activator from human erythrocytes. Its similarity to the activator of 3′,5′-cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* **77:** 1210–1223, 1977.
42. Beavo, J. A., J. G. Hardman, and E. W. Sutherland. Stimulation of adenosine 3′,5′-monophosphate hydrolysis by guanosine 3′,5′-monophosphate. *J. Biol. Chem.* **246:** 3841–3846, 1971.
43. Levin, R. M. and B. Weiss. Binding of trifluoperazine to the calcium dependent activator of cyclic nucleotide phosphodiesterase. *Mol. Pharmacol.* **13:** 690–697, 1977.
44. Weiss, B. Differential activation and inhibition of the multiple forms of cyclic nucleotide phosphodiesterase. *Adv. Cyclic Nucl. Res.* **5:** 195–211, 1975.
45. Pichard, A. L., J. Hanoune, and J. C. Kaplan. Human brain and platelet cyclic adenosine monophosphate phosphodiesterases: Different response to drugs. *Biochim. Biophys. Acta* **279:** 217–220, 1972.