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Activation of Rat Cerebral Cortical 3′,5′-Cyclic Nucleotide Phosphodiesterase Activity by Gangliosides

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SUMMARY

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Mixed brain gangliosides stimulated the activity of rat cerebral cortical calcium-dependent and calcium-independent cyclic nucleotide phosphodiesterases in a concentration-dependent manner. The extent of stimulation was similar with both enzymes, but calcium-dependent enzyme was at least 10 times more sensitive to gangliosides. Activation occurred rapidly and was readily reversible. The V_{max} of the calcium-dependent enzyme was increased, while the K_m was unaffected. Maximal activation of the calcium-dependent enzyme by gangliosides was only 50% of that elicited by lysolecithin or calcium-dependent activator protein. Activation by lysolecithin and gangliosides was apparently additive, while the activation by activator protein was slightly diminished in the presence of gangliosides. Trypsin pretreatment of the calcium-dependent enzyme resulted in a loss of sensitivity to subsequent activation by gangliosides or activator protein without altering the activation by lysolecithin. Potassium ions inhibited basal activity of the calcium-dependent phosphodiesterase but had no effect on enzyme activated by gangliosides, activator protein, or lysolecithin. Phosphodiesterase in the membrane-bound state did not appear to be affected by gangliosides.

INTRODUCTION

Intracellular concentrations of cyclic AMP and cyclic GMP are regulated, in part, by hydrolysis to 5′-nucleotides by 3′:5′-cyclic nucleotide phosphodiesterases. Multiple forms of the enzyme have been shown to exist in brain (1–6) as well as other tissues (1, 6–9). Both calcium-dependent and calcium-independent enzymes are present in rat brain (6, 10). The calcium-dependent phosphodiesterases are stimulated by calcium ions only in the presence of a thermostable, low molecular weight protein termed the calcium-dependent activator protein[1] (11–14). The calcium-dependent phosphodiesterase can be activated not only by calcium-dependent activator protein but also phospholipids (15, 16), fatty acids (15, 16), and proteolytic enzymes (11, 17, 18). In contrast to the calcium-dependent enzyme, the calcium-dependent phosphodiesterase is insensitive to these activators. The present investigation describes the activation by gangliosides of the soluble calcium-dependent and calcium-independent enzymes from rat cerebral cortex.

MATERIALS AND METHODS

Materials. Cyclic [8-^{3}H]GMP (21 Ci/mmol) and cyclic [8-^{3}H]AMP were from Amersham; anion exchange resin AG1-X8 (Cl^- form, 100–200 mesh) and 2-mercaptoethanol were from Bio-Rad; snake venom (*Crotalus adamanteus*), trypsin (bovine pancreas), trypsin inhibitor (soybean), and mixed brain gangliosides (Type III) were from Sigma Chemical Company; potassium chloride and sodium chloride were from Baker Chemical Company; lysolecithin (egg) was from Applied Science Laboratories; Sephadex G-200 was from Pharmacia; various nucleosides, nucleotides, and cyclic nucleotides were from Sigma, Boehringer-Mannheim, or Plenum Scientific Research.

Preparation of phosphodiesterases. The procedure for separation of phosphodiesterase from rat cerebrum was essentially that described by Kakiuchi *et al.* (6, 11). Briefly, mature, female Sprague–Dawley rats (Taconic Farms, Germantown, N. Y.) were sacrificed by decapitation and the brains were immediately excised and rinsed in ice-cold 20 mM Tris/Cl buffer, pH 7.5, containing 1 mM $MgCl_2$, 3 mM 2-mercaptoethanol and 0.1 mM EGTA[2] (extraction buffer). The tissue was homogenized in 2 vol of the same solution using a motor-driven glass–Teflon homogenizer. The homogenate was centrifuged at 105,000*g* for 60 min and the supernatant fluid (crude

[1] This protein has also been referred to as a calcium-dependent regulator protein, modulator protein, and calmodulin.

[2] The abbreviations used are: EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*′,*N*′-tetraacetic acid.

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extract) was filtered through glass wool to remove the fat. The 105,000*g* pellet was used for the source of membrane-bound phosphodiesterase (see below).

An aliquot (3–6 ml) of the crude extract was applied to a Sephadex G-200 column (2.5 × 40 cm) previously equilibrated with extraction buffer containing 0.1 M NaCl. The column was eluted with the same buffer. The enzyme activity eluting at V_e/V_o of 1.0 represented a high-K_m calcium-independent phosphodiesterase. Peak enzyme activity eluting at a V_e/V_o of 1.5 was used as the source of calcium-dependent phosphodiesterase. The enzyme was not inhibited by EGTA indicating the absence of significant amounts of activator protein.

Further purification of the calcium-dependent enzyme was achieved using activator protein–Sepharose affinity chromatography as described by Miyake *et al.* (19). The calcium-dependent enzyme was applied to the affinity column (1.5 × 10 cm) previously equilibrated with 20 mM Tris/Cl (pH 7.5) containing 1 mM $MgCl_2$, 3 mM 2-mercaptoethanol and 1 mM $CaCl_2$. The column was washed with 2.5 to 3 column vol of the same buffer and then eluted with 3 column vol of 20 mM Tris/Cl (pH 7.5) containing 1 mM $MgCl_2$, 3 mM 2-mercaptoethanol and 0.1 M EGTA. An aliquot of each fraction was assayed for phosphodiesterase activity as described below. The calcium-dependent enzyme so obtained showed two distinct protein bands following sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[3]

Particulate phosphodiesterase (membrane) was prepared by two methods using the 105,000*g* pellet. In method 1 the pellet was resuspended in 2 vol of extraction buffer by gentle vortexing and was centrifuged at 105,000*g* for 10 min. This procedure was repeated five times and the pellet was finally resuspended in the same volume of extraction buffer using a glass–Teflon homogenizer. Method 2 was essentially the same but instead of resuspending the pellet by gentle vortexing, the pellet was resuspended after each wash by glass–Teflon homogenization. Both qualitative and quantitative differences in resulting membrane preparations were apparent (see Results).

Assay of phosphodiesterase. The assay method for phosphodiesterase was a modification (9, 20, 21) of that originally described by Thompson and Appleman (8). The standard reaction mixture contained in a final volume of 0.1 ml: Tris/Cl buffer (pH 7.5) 5 μmol; $MgCl_2$ 0.5 μmol; and enzyme as indicated. In most instances, 0.1 nmol cyclic [8-^{3}H]GMP, containing about 1.2×10^5 cpm, was used as substrate. In experiments in which activator protein was used, calcium ions (0.4 μmol) were included in the assay mixture unless otherwise noted. The reaction was initiated by the addition of either substrate or enzyme and was carried out at 37° for 4–10 min. The reaction was terminated by heating the mixture at 98–100° for 1 min. A second incubation was then carried out in the presence of 5′-nucleotidase (*C. adamanteus* snake venom, 75–100 μg) for 30 min at 37° and the reaction was terminated by heating. Unreacted nucleotides were separated from dephosphorylated products using anion exchange chromatography (AG1-X8) as described elsewhere (9, 20, 21). Appropriate amounts of enzyme and appropriate incubation times were used to limit hydrolysis of substrate to less than 25% under the assay conditions. All assays were performed in triplicate with a variation of less than 8%. The values were adjusted for blank values obtained in the absence of added phosphodiesterase and corrected for 60–70% recovery of the nucleoside.

Other methods. In some experiments the calcium-dependent phosphodiesterase was pretreated with trypsin. Under these conditions the enzyme was preincubated with trypsin (30 ng/μg enzyme protein) for 5 min at 0°, after which soybean trypsin inhibitor (240 ng/μg enzyme protein) was added. Control preparations contained either soybean trypsin inhibitor alone or trypsin added together with an eightfold excess of trypsin inhibitor.

Mixed brain gangliosides and lysolecithin were exhaustively dialyzed against 10 mM Tris/Cl, pH 7.5 containing 1 mM EGTA prior to use. This procedure effectively removed calcium ions contained in the commercial preparation of lysolecithin but was ineffective in removing the calcium in mixed brain gangliosides. The latter still contained 3 ng calcium/μg gangliosides as determined by atomic adsorption spectrometry.

Activator protein was prepared from rat cortex through the DEAE-cellulose step by the method of Lin *et al.* (22). Protein was determined by the method of Lowry *et al.* (23) using bovine serum albumin as standard.

RESULTS

The calcium-independent and calcium-dependent phosphodiesterases were stimulated in a dose-dependent manner by mixed brain gangliosides (Fig. 1). Both enzymes exhibited the same maximal degree of stimulation (100–130%) by gangliosides. However, maximal activation of the calcium-dependent enzyme occurred with 1 μg gangliosides while for the calcium-independent phosphodiesterase 10 μg gangliosides were required. The stimulation of the calcium-dependent enzyme rapidly decreased to the control values at higher concentrations of nondialyzed gangliosides (data not shown). With dialyzed

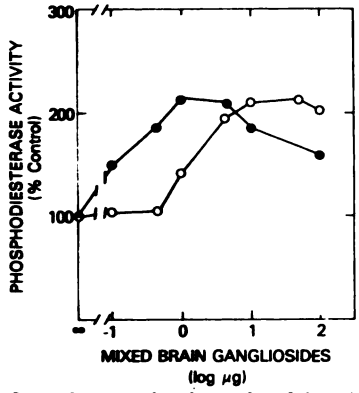

FIG. 1. *Dose-dependent activation of calcium-independent and calcium-dependent phosphodiesterases by gangliosides*

The enzymes were assayed as described under Materials and Methods using 1 μM cyclic GMP as substrate. Control activities for the calcium-independent (○, 1.4 μg) and calcium-dependent (●, 0.05 μg) enzymes were 1.24 and 1.50 pmol/min, respectively.

[3] C. R. Creveling, unpublished observation.

gangliosides the inhibition of higher concentrations was less pronounced (Fig. 1). Quantitatively identical stimulation of cyclic AMP hydrolysis by the enzymes was also observed (data not shown). Activation of both enzymes was readily reversible, i.e., enzyme preincubated with 5 μg of gangliosides and then diluted prior to assay exhibited no change in activity. A subsequent activation of both enzymes to mixed brain gangliosides was unaltered (data not shown).

The activation of the calcium-dependent phosphodiesterase was confirmed using a highly purified enzyme preparation obtained following activator protein–Sepharose chromatography (Fig. 2). The enzyme was eluted by 0.1 mM EGTA as a sharp peak. Gangliosides in amounts of 0.1 and 1 μg stimulated the activity of these fractions 55 and 120%, respectively.

Reaction kinetics as a function of enzyme protein amount and incubation time are shown in Fig. 3. A twofold activation of the calcium-dependent phosphodiesterase occurred irrespective of enzyme amount up to 100 ng protein but decreased above this amount as the reaction in the presence of gangliosides became nonlinear. The activation was rapid, occurring within 45 sec, and remaining constant for incubation times up to 5 min. Kinetic analysis revealed that the stimulation of the enzyme occurred through an increase in V_{max} without significantly affecting the K_m for cyclic GMP (Fig. 4).

The effects of calcium-dependent activator protein, mixed brain gangliosides, and lysolecithin on the activity of the calcium-dependent enzyme were examined. Activator protein (1.3 μg), gangliosides (0.5 μg), and lysolecithin (100 μM) stimulated the enzyme activity 200, 95, and 220%, respectively (Fig. 5). The stimulation by activator protein was slightly decreased in the presence of gangliosides. In contrast, activation by a combination of lysolecithin and gangliosides was additive, attaining a value 300% above control. There was no evidence for a direct interaction of gangliosides and activator protein. Thus,

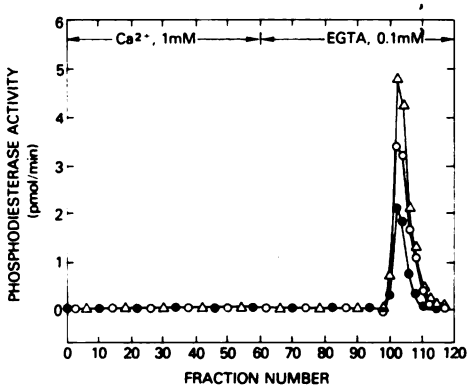

FIG. 2. *Affinity chromatography of the calcium-dependent phosphodiesterase*

Chromatography was on activator protein–Sepharose 4B (1.5 × 10 cm) with a flow rate of 0.3 ml/min and fraction size of 0.8 ml. An aliquot (2.5 μl) from each fraction was assayed for phosphodiesterase activity in either the absence (●) or presence of 0.1 μg (○) or 1 μg (△) gangliosides as described under Materials and Methods using 1 μM cyclic GMP as substrate. Fractions 1–60 were eluted with extraction buffer containing 1 mM calcium with subsequent fractions eluted with extraction buffer containing 0.1 mM EGTA.

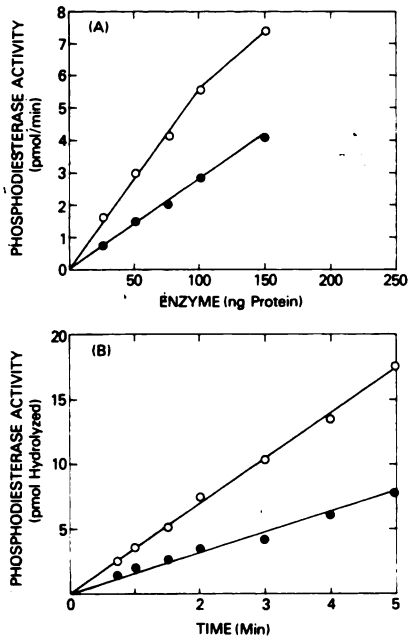

FIG. 3. *Dependency of phosphodiesterase activity on protein concentration and time in the presence and absence of mixed brain gangliosides*

(A) Dose-dependent hydrolysis of cyclic GMP (1 μM) by calcium-dependent phosphodiesterase. The enzyme was assayed as described under Materials and Methods in the absence (●) or presence of 1 μg gangliosides (○). Incubation time was 3 to 5 min. (B) Time course of the hydrolysis of cyclic GMP (1 μM) by calcium-dependent phosphodiesterase in the absence (●) or presence (○) of 1 μg gangliosides. The enzyme (50 ng) was assayed as described under Materials and Methods.

activation by gangliosides was unaffected by the presence of activator protein in absence of calcium ions (data not shown). Furthermore, gangliosides do not cause a change in the potency of the activator protein (data not shown).

Limited proteolysis of calcium-dependent phosphodiesterase from rat brain has been shown to concomitantly

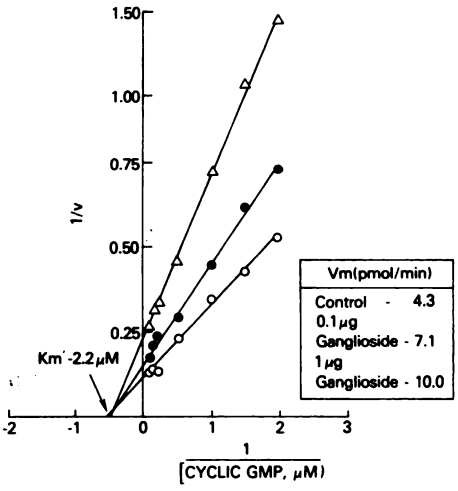

FIG. 4. *Double reciprocal plot of the hydrolysis of cyclic GMP by the calcium-dependent phosphodiesterase from rat cerebral cortex*

The enzyme (45 ng) was assayed in the absence (△) or presence of 0.1 μg (●) or 1 μg (○) gangliosides as described under Materials and Methods. Substrate concentration ranged from 0.5 to 10 μM.

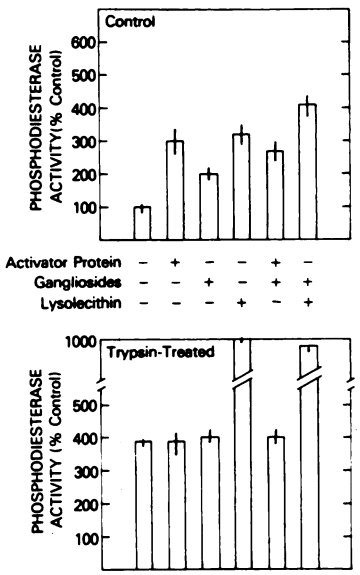

FIG. 5. *Effect of various agents on the activity of the calcium-dependent phosphodiesterase preincubated in the absence or presence of trypsin*

Assay conditions and trypsin pretreatment were as described under Materials and Methods. Cyclic GMP (1 μM) was used as substrate. Where indicated the amounts of the agents were 1.3 μg, 1 μg, and 100 μM for activator protein, gangliosides, and lysolecithin, respectively. Values represent the mean ± SEM from at least three separate experiments. Control activity for the untreated phosphodiesterase (45 ng) was 1.37 pmol/min.

activate the enzyme, while desensitizing the enzyme to further stimulation by activator protein (11). Similarly, the calcium-dependent phosphodiesterase, after treatment with trypsin, was no longer sensitive to activation by mixed brain gangliosides (Fig. 5). In contrast, activation of the trypsin-pretreated enzyme by lysolecithin was not affected.

Potassium ions have recently been observed to cause profound inhibition of basal calcium-dependent enzyme activity without affecting activator protein-stimulated activity (24). Mixed brain gangliosides and lysolecithin were able to overcome the inhibition of enzyme activity produced by potassium (Table 1). Potassium had no effect on the activity of trypsin-treated enzyme under any condition.

Activator protein–Sepharose affinity chromatography of the trypsin-treated enzyme revealed qualitative changes in the enzyme (data not shown). The enzyme was no longer bound to the column but eluted as a sharp peak in the presence of 1 mM calcium. No activity was observed in the EGTA-eluted fractions. A similar observation has been reported by Sakai *et al.* (25). Enzyme activity eluted in the calcium wash was unresponsive to activator protein and mixed brain gangliosides (data not shown).

Activation of the calcium-dependent enzyme by gangliosides was independent of calcium (Fig. 6). At higher concentrations of calcium the activity of the enzyme in the presence of gangliosides was slightly diminished. In marked contrast the activator protein showed a marked dependence on calcium for activation, which is consistent with previously reported data (11–14).

TABLE 1

Effect of various agents on the activity of the calcium-dependent phosphodiesterase

Assay conditions and trypsin-pretreatment were as described under Materials and Methods. Cyclic GMP (1 μM) was used as substrate. Values presented are the means of triplicate assays from at least two separate experiments.

	Calcium-dependent phosphodiesterase activity (pmol/min)	
	No pretreatment	Trypsin pretreatment
Control	1.06	3.28
Potassium, 55 mM	0.20	3.08
Activator protein, 1.3 μg	3.18	3.41
Gangliosides, 1 μg	2.31	3.26
Lysolecithin, 100 μM	3.36	9.13
Activator protein + potassium	2.99	3.36
Gangliosides + potassium	2.19	3.24
Lysolecithin + potassium	3.25	8.94

The effect of mixed brain gangliosides on membrane-bound phosphodiesterase[4] activity was found to vary according to the method of preparation of the membrane fraction (Fig. 7). Enzyme activity in membranes prepared by method 1 (see Materials and Methods) was stimulated in a dose-dependent manner by gangliosides with near maximal stimulation (100%) at 10 μg. In marked contrast the phosphodiesterase activity in membranes prepared by method 2 showed very minimal effects of gangliosides in amounts up to 100 μg. When the final washed membrane homogenates from method 1 were centrifuged at 105,000*g* and enzyme activity of the recovered supernatant was measured in the presence of mixed brain gangliosides, results virtually identical to those of the homogenate were obtained (data not shown). The results suggest that phosphodiesterase activity, loosely bound to the membrane, must be "released" during homogenization before activation by gangliosides can be observed.

DISCUSSION

Relatively low concentrations of mixed brain gangliosides stimulated the activity of both the calcium-dependent and calcium-independent phosphodiesterases from rat cerebrum (Fig. 1). At higher concentrations, the mixed gangliosides inhibited the calcium-dependent enzyme. The inhibition was much more profound prior to dialysis of gangliosides, suggestive of the presence of dialyzable inhibitory components or the loss of stimulatory components.[5] Phosphodiesterases in the membrane-

[4] Sonication of initial membrane preparations yielded two enzymes with chromatographic properties corresponding to calcium-independent and calcium-dependent enzymes of soluble preparations. The enzyme eluting at the void volume was the major enzyme, i.e., the enzyme corresponding to the calcium-independent enzyme.

[5] Structure–activity relationships for the activation of various gangliosides present in brain require investigation. Commercial preparations of purified gangliosides afforded marked differences in terms of potency for activation of calcium-dependent phosphodiesterase. GM_1 was relatively pure and was about 50-fold more potent than the mixed brain gangliosides. GD_1 and GT_1 were relatively impure: the former activated the enzyme, while the latter profoundly inhibited the enzyme.

bound state appeared to be unaffected by gangliosides, although after release of phosphodiesterase from membranes a ganglioside activation was clearly manifest (Fig. 7). The activation of phosphodiesterases by gangliosides was immediate and readily reversible. In contrast, activation of brain adenylate cyclase by gangliosides was irreversible and required 40- to 50-fold higher concentrations of gangliosides (26).

Activation of the calcium-dependent phosphodiesterase by gangliosides appeared in some respects similar to activation by calcium-dependent activator protein. For example, pretreatment of the calcium-dependent phosphodiesterase with trypsin resulted in a loss of subsequent sensitivity to activation by either gangliosides or activator protein. Furthermore, both gangliosides and activator protein were effective in restoring the fully activated state to potassium-inhibited enzyme activity. Finally, kinetic analysis revealed that the primary effect of ganglioside activation results from an increase in V_{max} of the enzyme. Similar effects on the enzyme using saturating amounts of activator protein have been reported (27). Differences between activation of phosphodiesterase by gangliosides or activator protein did pertain. Thus, the maximal stimulation of the calcium-dependent enzyme by gangliosides was only 50% of that by activator protein. However, combinations of activator protein and ganglioside had effects on enzyme activity less than activator protein alone (Fig. 5), suggesting a common site at which the ganglioside had partial agonist activity. Activation of the enzyme by activator protein was strictly dependent on calcium ions, while the activation by gangliosides was unaffected or slightly diminished by calcium in concentrations up to 100 μM (Fig. 7). It should be noted that the ganglioside preparation contained small amounts of calcium (3 ng calcium/μg ganglioside), following extensive dialysis against EGTA which may have been sufficient for activation. The most striking difference between the activation of phosphodiesterase activity by these two agents is the relative specificity. Activator protein had little or no effect on the activity of the calcium-dependent enzyme while gangliosides were effective in stimulating either the calcium-independent or

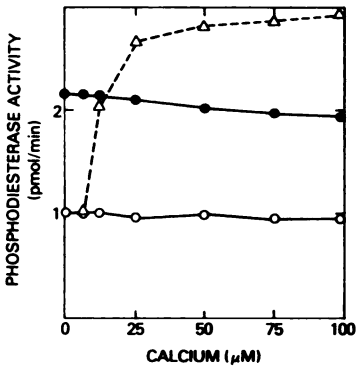

FIG. 6. *Effect of calcium on the activity of the calcium-dependent phosphodiesterase*

The enzyme (40 ng) was assayed in either the absence (○) or presence of 1 μg gangliosides (●) or 1.3 μg activator protein (△) as described under Materials and Methods. The concentration of cyclic GMP was 1 μM.

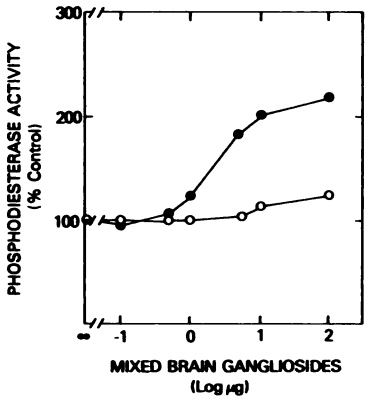

FIG. 7. *Effect of gangliosides on particulate (membrane) phosphodiesterase activity*

Particulate phosphodiesterases were prepared by either method 1 (●) or method 2 (○) as described under Materials and Methods. The enzymes prepared by method 1 (13.2 μg) and method 2 (12.9 μg) were assayed using 1 μM cyclic GMP and had control activities of 1.91 and 1.15 pmol/min, respectively.

calcium-dependent enzyme activities to the same maximal degree. Neither activator protein (28) nor gangliosides (Fig. 7) appeared to activate membrane-bound phosphodiesterase. The physiological significance of activation of membranal adenylate cyclase (26) and soluble phosphodiesterases by brain gangliosides is unknown. However, gangliosides might, like the calcium-dependent activator protein, be involved in a subtle control of cyclic AMP-generating systems. Both activator protein and ganglioside can, in brain membranes, activate adenylate cyclase and both could, if released into cytosol,[6] activate soluble phosphodiesterases. The release of activator protein has been proposed to be a cyclic AMP-dependent phenomenon (30), and might thus provide a control mechanism for regulating cyclic nucleotide levels. Factors that might affect release of gangliosides from neuronal membranes are unknown. However, in view of the differential effects of gangliosides—high concentrations required to activate adenylate cyclase and 40- to 50-fold lower concentrations required to activate phosphodiesterase—it is possible that release of only minor amounts of ganglioside might serve to activate the degradation of cyclic nucleotides.

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REFERENCES

1. Brooker, G., L. Thomas, Jr. and M. M. Appleman. The assay of adenosine 3',5'-cyclic monophosphate and guanosine 3',5'-cyclic monophosphate in biological materials by enzymatic radioisotopic displacement. *Biochemistry* 7: 4177–4181 (1968).
2. Cheung, W. Y. Cyclic nucleotide phosphodiesterases. *Adv. Biochem. Psychopharmacol.* 3: 51–64 (1970).
3. Kakiuchi, S., and R. Yamazaki. Calcium-dependent phosphodiesterase activity and its activating factor (PAF) from brain. *Biochem. Biophys. Res. Commun.* 41: 1104–1110 (1970).
4. Uzunov, P., and B. Weiss. Separation of multiple molecular forms of cyclic

[6] Gangliosides have recently been reported in cytosol fractions from calf brain (29).

adenosine 3′,5′-monophosphate phosphodiesterase in rat cerebellum by polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* **284:** 220–226 (1972).
5. Pledger, W. J., G. M. Stangel, W. J. Thompson and S. J. Strada. Separation of multiple forms of cyclic nucleotide phosphodiesterases from rat brain by isoelectric focusing. *Biochim. Biophys. Acta* **370:** 242–248 (1974).
6. Kakiuchi, S., R. Yamazaki, Y. Teshima, K. Uenishi and E. Miyamoto. Multiple cyclic nucleotide phosphodiesterase activities from rat tissues and occurrence of a calcium-plus-magnesium-ion-dependent phosphodiesterase and its protein activator. *Biochem. J.* **146:** 109–120 (1975).
7. Beavo, J. A., J. G. Hardman and E. W. Sutherland. Hydrolysis of cyclic guanosine and adenosine 3′,5′-monophosphates by rat and bovine tissues. *J. Biol. Chem.* **245:** 5649–5655 (1970).
8. Thompson, W. J. and M. M. Appleman. Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry* **10:** 311–316 (1971).
9. Davis, C. W. and J. F. Kuo. Ontogenetic changes in levels of phosphodiesterases for adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate in the lung, liver, brain, and heart from guinea pigs. *Biochim. Biophys. Acta* **444:** 555–562 (1975).
10. Kakiuchi S., R. Yamazaki and Y. Teshima. Cyclic 3′,5′-nucleotide phosphodiesterase IV. Two enzymes with different properties from brain. *Biochem. Biophys. Res. Commun.* **42:** 968–974 (1971).
11. Cheung, W. Y. Cyclic 3′,5′-nucleotide phosphodiesterase: Evidence for and properties of a protein activator. *J. Biol. Chem.* **246:** 2859–2868 (1971).
12. Kakiuchi, S., R. Yamazuki and H. Nakajima. Properties of a heat-stable phosphodiesterase activating factor isolated from brain extract. II. Studies on cyclic 3′,5′-nucleotide phosphodiesterases. *Proc. Jap. Acad.* **46:** 587–592 (1970).
13. Teo, T. S. and J. H. Wang. Mechanism of activation of a cyclic adenosine 3′,5′-monophosphate phosphodiesterase from bovine heart by calcium ion: Identification of the protein activator as a Ca^{2+} binding protein. *J. Biol. Chem.* **248:** 5950–5955 (1973).
14. Wolff, D. J. and C. O. Brostrum. Calcium-binding phosphoprotein from pig brain: Identification as a calcium dependent regulator of brain cyclic nucleotide phosphodiesterase. *Arch. Biochem. Biophys.* **163:** 345–358 (1974).
15. Wolff, D. J. and C. O. Brostrum. Calcium-dependent cyclic nucleotide phosphodiesterases from brain. Identification of phospholipids as calcium-dependent activators. *Arch. Biochem. Biophys.* **173:** 720–731 (1976).
16. Pichard, A.-L. and W. Y. Cheung. Cyclic 3′,5′-nucleotide phosphodiesterase: Stimulation of bovine brain cytoplasmic enzyme by lysophosphotidylcholine. *J. Biol. Chem.* **252:** 4872–4875 (1977).
17. Epstein, P. M., W. J. Pledger, E. Gardner, G. M. Stancel, W. J. Thompson and S. J. Strada. Activation of mammalian cyclic AMP phosphodiesterases by trypsin. *Biochim. Biophys. Acta* **527:** 442–455 (1978).
18. Moss, J., V. C. Manganiello and M. Vaughan. Effects of chymotrypsin and a calcium-dependent protein activator on guanosine 3′,5′-monophosphate phosphodiesterase from rat liver. *Biochim. Biophys. Acta* **541:** 279–287 (1978).
19. Miyake, M., J. W. Daly and C. R. Creveling. Purification of calcium-dependent phosphodiesterases from rat cerebrum by affinity chromatography on activator protein-Sepharose. *Arch. Biochem. Biophys.* **181:** 39–45 (1977).
20. Davis, C. W., and J. F. Kuo. Purification and characterization of a guanosine 3′,5′-monophosphate-specific phosphodiesterase from guinea pig lung. *J. Biol. Chem.* **252:** 4078–4084 (1977).
21. Davis, C. W., and J. F. Kuo. Differential effects of cyclic nucleotides and their analogs and various agents on the activities of cyclic GMP-specific and cyclic AMP-specific phosphodiesterase purified from guinea pig lung. *Biochem. Pharmacol.* **27:** 89–99 (1978).
22. Lin, Y. M., Y. P. Liu and W. Y. Cheung. Cyclic 3′,5′-nucleotide phosphodiesterase. Purification, characterization, and active form of the protein activator from bovine brain. *J. Biol. Chem.* **249:** 4943–4954 (1974).
23. 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).
24. Davis, C. W. and J. W. Daly. Calcium-dependent 3′:5′-cyclic nucleotide phosphodiesterase: Inhibition of basal activity at physiological levels of potassium ions. *J. Biol. Chem.* **253:** 8683–8686 (1978).
25. Sakai, T., H. Yamanaka, R. Tanaka, H. Makino and H. Kasa. Stimulation of cyclic nucleotide phosphodiesterases from rat brain by activator protein, proteolytic enzymes, and a vitamin E derivative. *Biochim. Biophys. Acta* **483:** 121–134 (1977).
26. Partington, C. R. and J. W. Daly. Effect of gangliosides on adenylate cyclase activity in rat cerebral cortical membranes. *Mol. Pharmacol.* **15:** 484–491 (1979).
27. Brostrom, C. O. and D. J. Wolff. Calcium-dependent cyclic nucleotide phosphodiesterase from brain: Comparison of adenosine 3′,5′-monophosphate and guanosine 3′,5′-monophosphate as substrates. *Arch. Biochem. Biophys.* **172:** 301–311 (1976).
28. Lynch, T. J., E. A. Tallant and W. Y. Cheung. Rat brain adenylate cyclase: Further studies on its stimulation by a Ca^{2+}-binding protein. *Arch. Biochem. Biophys.* **182:** 124–133 (1977).
29. Sonnino, S., R. Ghidoni, S. Marchesini and G. Tettamanti. Cytosolic gangliosides: Occurrence in calf brain as ganglioside-protein complexes. *J. Neurochem.* **33:** 117–121 (1979).
30. Gnegy, M. E., J. P. Nathanson and P. Usunov. Release of the phosphodiesterase activator by cyclic AMP-dependent ATP: protein phosphotransferase from subcellular fractions of rat brain. *Biochim. Biophys. Acta* **497:** 75–85 (1977).

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