J. Biochem. 120, 66-73 (1996)

Interaction of Ganglioside with Specific Peptide Sequences as a Mechanism for the Modulation of Calmodulin-Dependent Enzymes[1]

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Received for publication, January 11, 1996

We examined the interaction between gangliosides and synthetic peptides of calmodulin (CaM)-dependent enzymes to confirm the hypothesis that interaction between gangliosides and the CaM-like site (CLS) of the enzyme is a mechanism for the modulation of the enzyme activity by gangliosides. Gangliosides, GD1b, GT1b, and GD1a, inhibited the activity of CaM-independently activated cAMP-phosphodiesterase and their inhibition was cancelled by a peptide consisting of 17 amino acid residues of a plasma membrane Ca^{2+}-pump CLS, suggesting the involvement of the interaction between the peptide and the gangliosides. The peptide of an assumed CLS of phosphodiesterase also cancelled the inhibition. On the other hand, the gangliosides interacted with synthetic CaM-binding site (CBS) peptides of phosphodiesterase, calcineurin, Ca^{2+}-pump, and Ca^{2+}/calmodulin-dependent protein kinase II. Moreover, gangliosides GM3 and LM1, that activate but do not inhibit phosphodiesterase, interacted with the CBS peptides, whereas they did not bind to CLS peptides. On the basis of these new findings, we propose a revised model for the ganglioside-mediated modulation of CaM-dependent enzymes, *i.e.* without CaM, gangliosides bind to CBS and thus stimulate the enzyme activity, acting like CaM. At higher concentrations, they bind to CLS of the enzymes as well and inhibit the activity, acting like the CBS of the enzyme.

Key words: calcineurin, calcium pump, calmodulin, ganglioside, phosphodiesterase.

Gangliosides modulate intercellular recognition and transmembrane signal transduction (*2*). While gangliosides are mainly distributed in the outer leaflet of the plasma membrane, some are distributed in the cytosol (*3-9*) in association with specific proteins (*10-15*). This indicates that gangliosides interact with cytosolic enzymes as well as membrane proteins.

Gangliosides inhibit the activity of CaM-stimulated enzymes *in vitro* (*16, 17*). Without CaM, gangliosides stimulate CaM-dependent enzyme activity at low concentrations and inhibit the activity at higher concentrations. According to the flip-flop model proposed by Jarrett and Madhavan (*18*), in the absence of CaM, CaM-binding sites (CBS) of CaM-dependent enzymes are covered by their own CaM-like sites (CLS) and as a result, the enzymes are not activated, while in the presence of CaM, CBS are occupied by CaM and the enzymes are activated. We found that particular gangliosides bound to CaM and modulated the enzyme activities (*16*). On the basis of these lines of evidence, we proposed a working hypothesis for the molecular mechanism, *i.e.* gangliosides modulate CaM-dependent enzymes through their binding to CaM and the CLS of the enzymes (*17*).

We have examined the interaction between gangliosides and synthetic peptides of CaM-dependent enzymes to confirm our hypothesis. The interaction was analyzed by means of competition with cAMP-phosphodiesterase and by ganglioside titration of tyrosine or tryptophan fluorescence spectroscopy of the peptides. The results confirmed our idea that gangliosides directly inhibit the enzyme activity through binding to the CLS. Further, the results using CBS peptides of the enzymes demonstrated interaction between gangliosides and the CBS peptides, indicating that direct activation of the enzyme by gangliosides occurs through their binding to a CBS. Thus, we propose a revised model for the ganglioside-mediated bidirectional modulation of CaM-dependent enzymes, *i.e.*, not only the interaction between gangliosides and CLS, but also that between gangliosides and CBS is a mechanism for the modulation.

EXPERIMENTAL PROCEDURES

Materials—Boc-amino acid derivatives and resins were obtained from the Peptide Institute, Osaka. Other reagents for peptide synthesis were of peptide synthesis grade obtained from Kokusan Chemical Works, Tokyo, or Watanabe Chemical Industries, Hiroshima. Cyclic AMP, snake venom from *Crotalus atrox*, and bovine brain CaM were purchased from Sigma, St. Louis, MO. GM3 and LM1

[1] This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (No. 02259103) from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: CaM, calmodulin; CBS, calmodulin-binding site; CLS, calmodulin-like site. Abbreviations by Svennerholm (*1*) for gangliosides and IUPAC-IUB Recommendations for lipids used are: GA1, GgOse$_4$Cer; GM3, II^{3}NeuGc-LacCer; GM1, II^{3}NeuAc-GgOse$_4$Cer; GD1a, II^{3}NeuAc, IV^{2}NeuAc-GgOse$_4$Cer; GD1b, II^3(NeuAca2-8NeuAc)-GgOse$_4$Cer; GT1b, II^3(NeuAca2-8NeuAc), IV^{2}NeuAc-GgOse$_4$Cer; LM1, IV^{3}NeuGc-nLcOse$_4$Cer; and sulfatide, I^{3}HSO$_3$-GalCer.

were prepared as described (*19*). Sulfatide was prepared from bovine brain as described (*17*). GD1a, GD1b, and GT1b were purchased from Iatron Lab., Tokyo.

Peptide Synthesis—Peptides were constructed by using solid-phase common *t*-butoxycarbonyl chemistry in a Biosearch model 9500 peptide synthesizer. After hydrogen fluoride cleavage, the crude peptides were successively purified by column chromatography with Sephadex G-25F and carboxymethylcellulose CM-52, preparative HPLC with ODS columns, and finally column chromatography with Sephadex G-25F. Preparative HPLC was performed with a Shimadzu LC-8A system with an ODS column (20× 250 mm). To purify CaP-CLS1 (Table I), which contains a free -SH group, the carboxymethylcellulose CM-52 chromatography was omitted. The structure and the purity of the peptide were confirmed by analytical HPLC, amino acid analysis, and FAB-mass spectrometry. Analytical HPLC was conducted on a Shimadzu LC-6A system with an ODS column (4.6×250 mm). Amino acids were analyzed on a Beckman System Gold instrument after hydrolysis in 6 M hydrochloric acid at 110°C for 24 h and derivatization by 4-dimethylaminoazobenzene-4′-sulfonyl chloride. Mass spectra were measured on a JEOL JMS-HX100 mass spectrometer.

Phosphodiesterase Assay—The method of Cheung (*20*) was followed with a slight modification. Activator-deficient 3′:5′-cyclic nucleotide phosphodiesterase from bovine brain (P-9529, Sigma) was used. Little, if any, CaM-independent phosphodiesterase activity was detectable in this enzyme preparation. In the first stage of the incubation, the reaction mixture (0.5 ml) contained 40 mM Tris-HCl buffer (pH 8.0), 5 mM $MgSO_4$, 50 μM $CaCl_2$, 2 mM cyclic AMP, 0.02 unit of unmodified or trypsinized enzyme and the concentrations of glycosphingolipids and peptides described below. The reaction was initiated by adding cyclic AMP. After 10 min at 30°C, the tubes were placed in a boiling water bath for 2 min to terminate the reaction. After thermal equilibration to 30°C, 0.05 ml of snake venom (1 mg/ml) was added followed by a 10 min incubation. The reaction was terminated by adding 0.05 ml of 55% trichloroacetic acid and 0.75 ml of water, then 0.15 ml of 2.5% ammonium molybdate in 5 N H_2SO_4 was added. The denatured proteins were removed by centrifugation. The clear supernatant was decanted into tubes containing 0.05 ml of Fiske-SubbaRow reagent. The blue color that developed due to released phosphate was measured at 660 nm. To assess the ganglioside-inhibited trypsinized enzyme activity restored by peptides, inhibitory concentration of gangliosides and an appropriate concentration of the peptide were mixed with trypsinized enzyme for the first reaction. To assess the inhibition of the glycosphingolipid-stimulated enzyme activity by the peptide, activatory glycosphingolipids or 200 ng of CaM and an appropriate concentration of the peptide were mixed with unmodified enzyme for the first reaction. Phosphodiesterase was digested with trypsin according to Davis and Daly (*21*) with a modification as described (*17*).

Analysis of Protein Sequences for Hydropathy and Secondary Structure—The hydrophobicity was plotted using the Kyte-Doolittle algorithm (*22*). Predicted secondary structures were assigned according to Gibrat *et al.* (*23*).

Scatchard Plot Analysis—Scatchard plot analysis for the estimation of dissociation constant and interacting molar ratio was performed as described previously (*17*).

Fluorescence Measurements—Tryptophan fluorescence measurements were made on a Hitachi fluorescence spectrophotometer, Model F-4010. Excitation wavelengths of 280 and 290 nm were used for tyrosine and tryptophan fluorescence, respectively, with a slit width of 5 nm. The resultant fluorescence intensity, F and concentrations of ligand [L] were fitted well by a Hanes-Woolf plot or a Lineweaver-Burk plot when F and [L] are taken as viscosity and substrate concentrations, respectively. Thus, maximum or minimum fluorescence was obtained by these methods and the concentrations of free and ligand-bound peptides were estimated. Concentrations of free and peptide-bound ligands were calculated by supposing the number of ligand-binding sites for peptide to be 1. Using these values, apparent affinities between peptides and ligands were obtained by Scatchard plot analysis as described above.

RESULTS AND DISCUSSION

Assumption of CaM-Like Site—To confirm interaction of gangliosides and CLS, we chose CLS of human erythrocyte plasma membrane Ca^{2+}-pump, since it is the only enzyme of which the CLS has been identified (*24, 25*). The enzyme has two CLSs. We synthesized the first CLS peptide consisting of 17 amino acid residues, CaP-CLS1 (Table I), which contains the CBS interacting sequence, 537-544 residues, of the Ca^{2+}-pump (*24*). The interaction between this peptide and CBS of the Ca^{2+}-pump was confirmed by the effects on the tryptophan fluorescence spectrum of CaP-CBS (Table I and Fig. 1B). CaP-CLS1 was predicted to contain 100% α-helical structure by the method of Gibrat *et al.* (*23*). We expected that the sequence would form an

TABLE I. **Synthetic peptides used in this study.**

Peptide	Source (numbers of amino acid residues)	Amino acid sequence[a]	References
CaP-CLS1	Plasma membrane Ca^{2+} pump (531-547)	VGNKTECALLGFVTDLK	(*24*)
CaP-CLS2	Ca^{2+} pump hPMCA1 isoform (250-267)	VKKSLDKDPLLLSGTHVM	(*25, 33*)
CaP-CLSS		VGNKTE<u>S</u>ALLGFVTDLK[b]	
PDE-CL1	61 kDa phosphodiesterase (212-229)	KYKNPYHNLIHAADVTQT	(*34*)
CaM-3	Mammalian and chicken calmodulin (92-109)	FDKDGNGYISAAELRHVM	(*35*)
CaP-CBS	Plasma membrane Ca^{2+}-pump	LRRGQILWFRGKNRIQTQIKVVNA	(*36*)
PDE-CBS	61 kDa phosphodiesterase (23-43)	TEKMWQRLKGILR<u>S</u>LVKQLEK[b]	(*34*)
CaN-CBS	Mouse brain calcineurin	ARKEVIRNKIRAIGKMARVFSVLR	(*37*)
CaMKII-CBS	CaM-kinase II-α (295-317)	ARRKLKGAILTTMLATRNFSGGK	(*38*)

[a]Amino acids are expressed as one-letter abbreviations. The sequences of CBS are aligned according to Ikura *et al.* (*39*). [b]The original cysteine residue was replaced with serine in the synthetic peptide. The substituted serine is underlined.

amphiphilic helix like that of CaM. The hydrophobicity profile of the peptide was similar to that of 4 homologous portions of the CaM sequence, residues 19-36, 55-72, 92-109, and 128-145 (Fig. 1A). Thus, we supposed that CLS would take a similar structure to these sequences and we searched the sequences in the second CLS of Ca^{2+}-pump, residues 206-271 (*25*). We found a single sequence, residues 251-267 (Table I) with predicted 88% helical conformation and similar hydropathy (Fig. 1A). Interaction between the peptide, CaP-CLS2 (Table I), and CBS of the Ca^{2+}-pump was demonstrated by the tryptophan fluorescence spectrum of CaP-CBS (Fig. 1B). Apparent affinities of CaP-CLS1 and CaP-CLS2 to CaP-CBS obtained from the spectrofluorimetric titration were similar (Table II). The phosphodiesterase is a typical CaM-dependent enzyme and the modulation by CaM and gangliosides is relatively simple, compared with other CaM-dependent enzymes. Therefore, we selected the phosphodiesterase and picked up its CLS from the whole sequence of the enzyme in the manner as described above. We picked up a single sequence, residues 212-229, with predicted 94% helical conformation and the characteristic hydropathy (Fig. 1A). Interaction between the peptide, PDE-CL1 (Table I), and CBS of phosphodiesterase was demonstrated by the tryptophan fluorescence spectrum of PDE-CBS (Fig. 1C). Apparent affinity between them was similar to that between CaP-CLS1 and CaP-CBS (Table II).

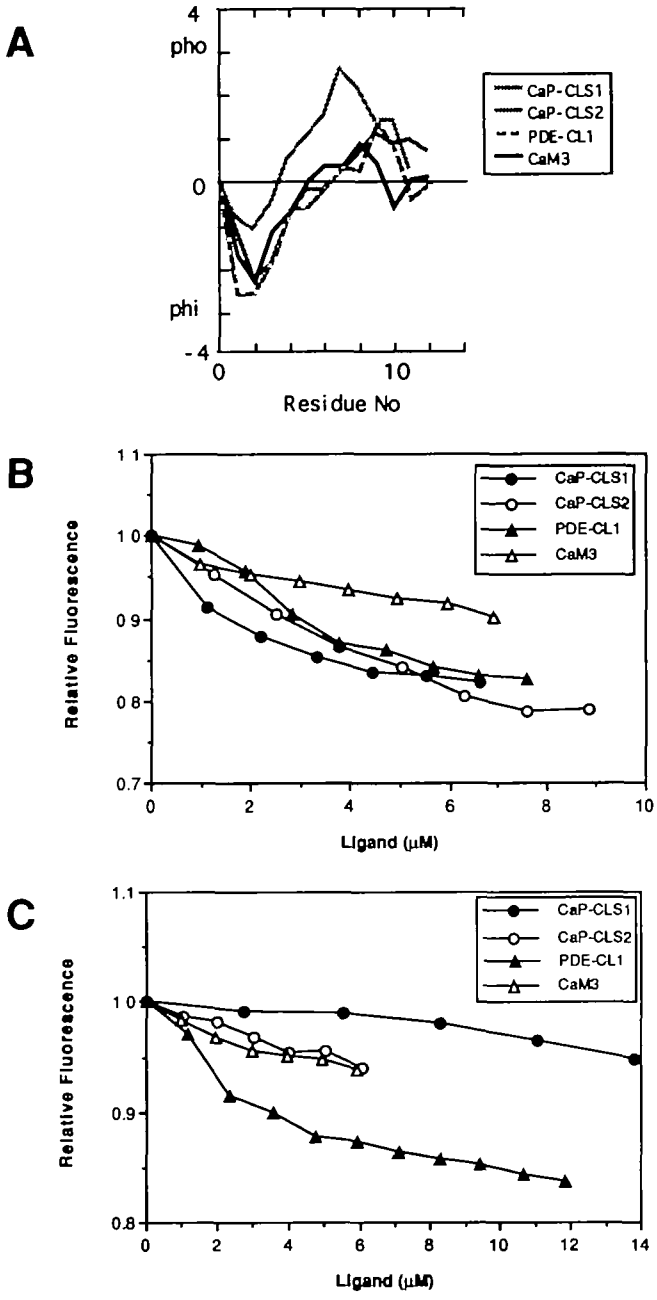

Fig. 1. **Properties of CLS sequences.** (A) Hydrophobicity profiles of CLS peptides and the peptide of residues 92-109 of CaM (CaM3) are shown. (B, C) Effects of CLS peptides and CaM3 on tryptophan fluorescence of CaP-CBS (B) and PDE-CBS (C). Ratio of emission florescence intensity at peak wavelength in the presence of the titrated ligands to that in their absence is plotted. Fluorescence was measured in 50 mM Hepes, pH 7.5, 1 mM EGTA at 25°C. In the case of CaM3, 2 mM $CaCl_2$ was added to the same solution. Concentrations of 3.04 μM CaP-CBS and 3.87 μM PDE-CBS were used. Abbreviations and peptide sequences are listed in Table I.

We assumed that the structure of the CLS of the enzymes would contain an arrangement of acidic and hydrophobic amino acids in an α-helical structure and we gave priority to the hydrophobic profile and the α-helical structure over other criteria. CaP-CLS1 was predicted to make an acidic amphiphilic helix. This strategy utilizes different assumptions from those proposed by Jarrett and Madhavan (*18*), who used the criteria that CLS should have more than two clusters of acidic amino acids adjacent to hydrophobic residues and aromatic residues should be present among the hydrophobic residues.

The Interaction of Gangliosides with CLS Peptides of CaM-Dependent Enzymes—The activity of phosphodiesterase, which was activated in a CaM-independent manner by trypsin digestion, is inhibited by gangliosides such as GD1b, GT1b, and GD1a (*17*). We examined whether or not the synthetic peptides could cancel this inhibition. As shown in Fig. 2, A and B, using GD1b as an inhibitor ganglioside, CaP-CLS1, but not CaP-CLS2, could cancel

TABLE II. **Apparent dissociation constants of interaction of CLS peptides for CBS peptides and gangliosides for CLS or CBS peptides.** Values were estimated as equimolar interactions from the results of Figs. 1, B and C, and 3 by the method described in "EXPERIMENTAL PROCEDURES."

Peptide	Ligand	Apparent dissociation constant (μM)
CaP-CBS	CaP-CLS1	0.52
	CaP-CLS2	0.97
	GD1a	2.17
	GD1b	2.89
	GT1b	0.84
	GM3	5.90
	CaM	1.85
PDE-CBS	PDE-CL1	0.70
	GD1a	1.10
	GD1b	1.91
	GT1b	0.47
	GM3	5.00
	CaM	0.37
PDE-CL1	GD1a	11.5
	GD1b	9.90
	GT1b	6.60
	GM3	33.0

the inhibition in a concentration-dependent manner. This suggests that the ganglioside-binding sequence is located in the first, but not in the second, CLS of the Ca^{2+}-pump. Similar results were obtained when GD1a or GT1b was substituted for GD1b (data not shown). CaP-CLSS, a peptide having a substitution of cysteine of CaP-CLS1 with serine, was still effective even though the substitution reduced the effect to less than half that of the unsubstituted peptide, suggesting that disulfide bonding is not sufficient for binding. The peptides had no effect on the activity of the trypsinized phosphodiesterase itself at the concentrations used (Fig. 2, A and B). The results indicate that the gangliosides, which inhibit phosphodiesterase activity in the absence of CaM, directly interact with the CLS of the

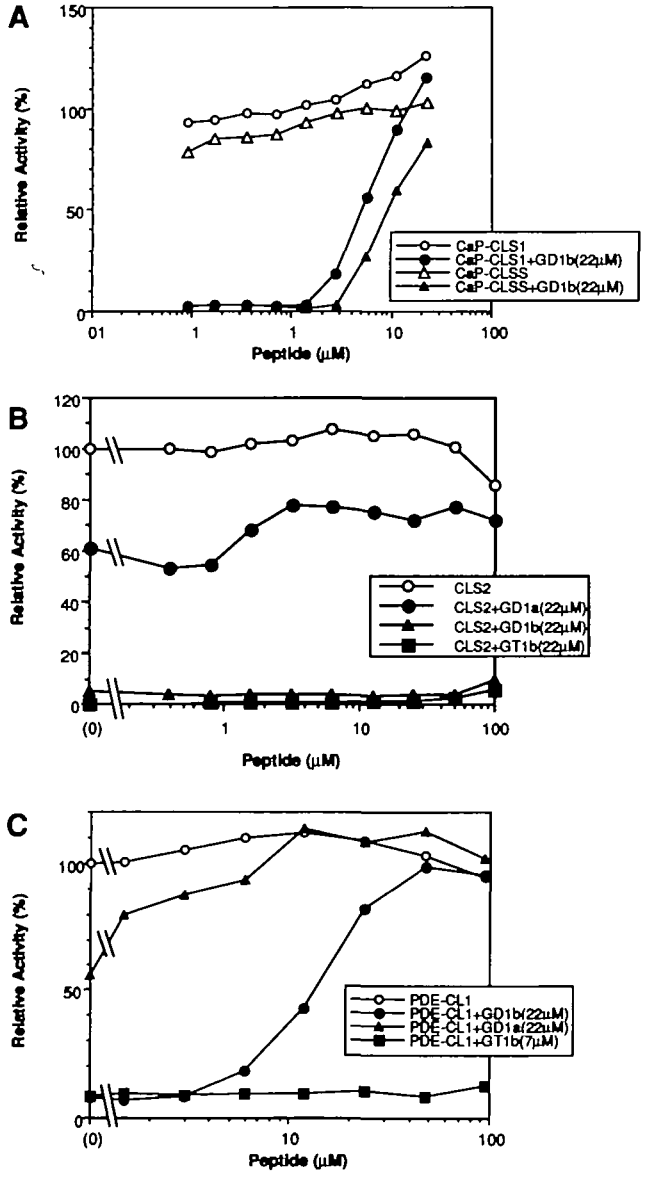

Fig. 2. **Interaction of CLS and CaM-derived peptides with gangliosides.** Peptide and ganglioside binding was determined as recovery by the peptides of the ganglioside-inhibited activity of CaM-independently activated phosphodiesterase. Synthetic peptides at various concentrations were added to reaction mixtures of phosphodiesterase CaM-independently activated by trypsin digestion, with or without the indicated concentrations of gangliosides. Activity is expressed as relative to that without peptides and gangliosides. Abbreviations and the peptide sequences are listed in Table I.

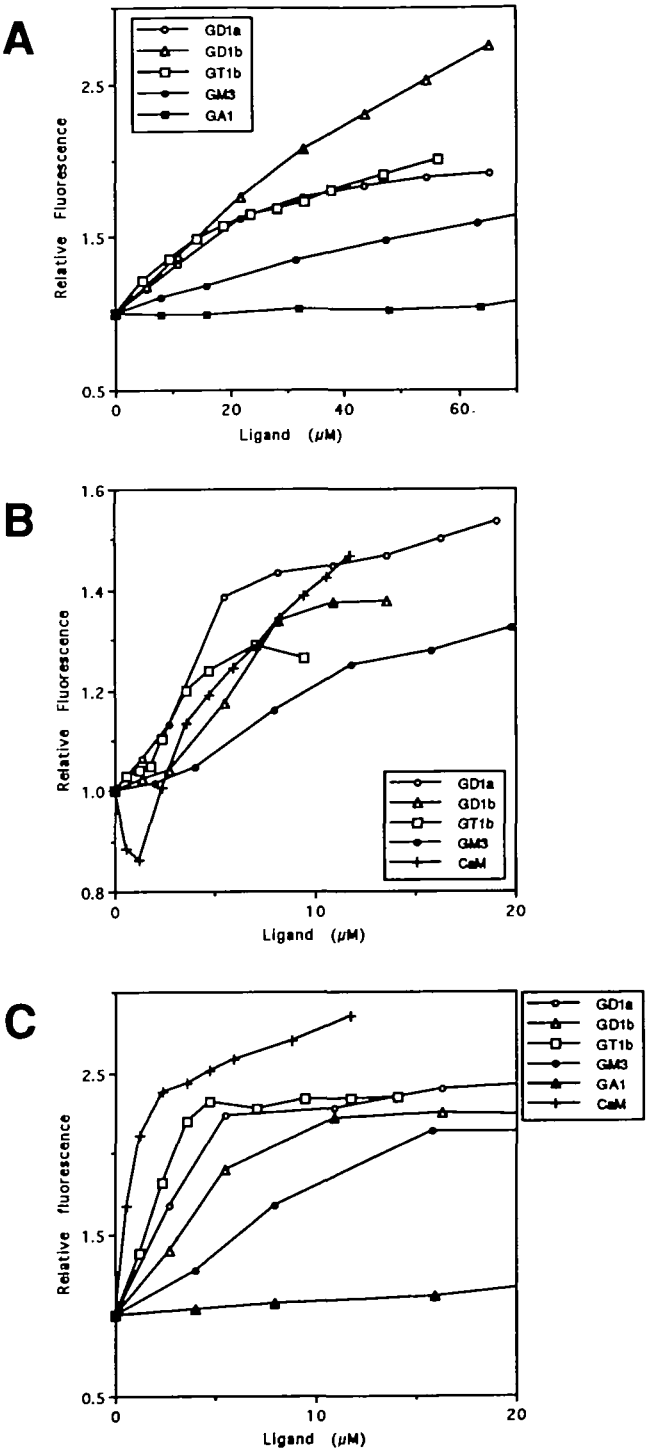

Fig. 3. **Effects of gangliosides and CaM on tyrosine fluorescence of PDE-CL1 (A), and tryptophan fluorescence of CaP-CBS (B) and PDE-CBS (C).** The ratio of emission fluorescence intensity at peak wavelength in the presence of the titrated ligands to that in their absence is plotted. Fluorescence was measured in 50 mM Hepes, pH 7.5, 1 mM EGTA at 25°C. In the case of CaM, 2 mM $CaCl_2$ was added to the same solution. Concentrations of 4.73 μM PDE-CL1, 3.04 μM CaP-CBS, and 3.87 μM PDE-CBS were used.

Ca^{2+}-pump.

The gangliosides, LM1 and GM3 do not inhibit but activate phosphodiesterase (*26*). We examined the effect of the peptide on this activation. The peptides did not have any effect on the activation by LM1 or GM3 (data not shown), suggesting that these gangliosides do not interact with the CLS.

The interaction of the assumed CLS peptide of phosphodiesterase, PDE-CL1, with gangliosides was assayed as described above. The peptides cancelled the inhibitory effects of GD1b and GD1a on the trypsinized phosphodiesterase (Fig. 2C), indicating that these gangliosides bind to the assumed CLS of phosphodiesterase. Effects of GD1b and GD1a on the tyrosine fluorescence of PDE-CL1 showed direct interaction between the gangliosides and the peptide (Fig. 3A). However, PDE-CL1 failed to cancel the inhibition by GT1b (Fig. 2C). It is possible that the affinity of GT1b for trypsinized phosphodiesterase was too high for competition with the peptide to occur under our conditions, since interaction between PDE-CL1 and GT1b was observed by tyrosine fluorescence measurement (Fig. 3A). The peptide had no effect on the phosphodiesterase activity stimulated by LM1 or GM3 (data not shown), suggesting that the gangliosides, which directly activate but do not inhibit phosphodiesterase, do not interact with the assumed CLS peptide. Tyrosine fluorescence measurement also showed low affinity of GM3 to PDE-CL1 (Fig. 3A and Table II). The ganglioside binding specificity of the peptide was similar to that of CaP-CLS1. These results indicate that phosphodiesterase has CaM-like sites and that the gangliosides directly interact with the sites and the interaction leads to the inhibition of the enzyme activity.

The binding affinity of the native type of CaP-CLS1 was about twice as high as that of CaP-CLSS, in which cysteine is substituted by serine (Table I and Fig. 2A). Furthermore, the native type of peptide stimulates phosphodiesterase activity by functioning as CaM, but serine substitution decreased this potential by less than 30% (Higashi, H. and Yoshida, S., unpublished observation). The effects of this substitution were probably caused by the loss of the cysteine side chain, which may be important for the binding to CBS and gangliosides. PDE-CL1 failed to activate phosphodiesterase by substituting for CaM at concentrations between 0.4 and 100 μM, in contrast to CaP-CLS1 (Higashi, H. and Yoshida, S., unpublished observation). A peptide with the sequences of a limited portion of the

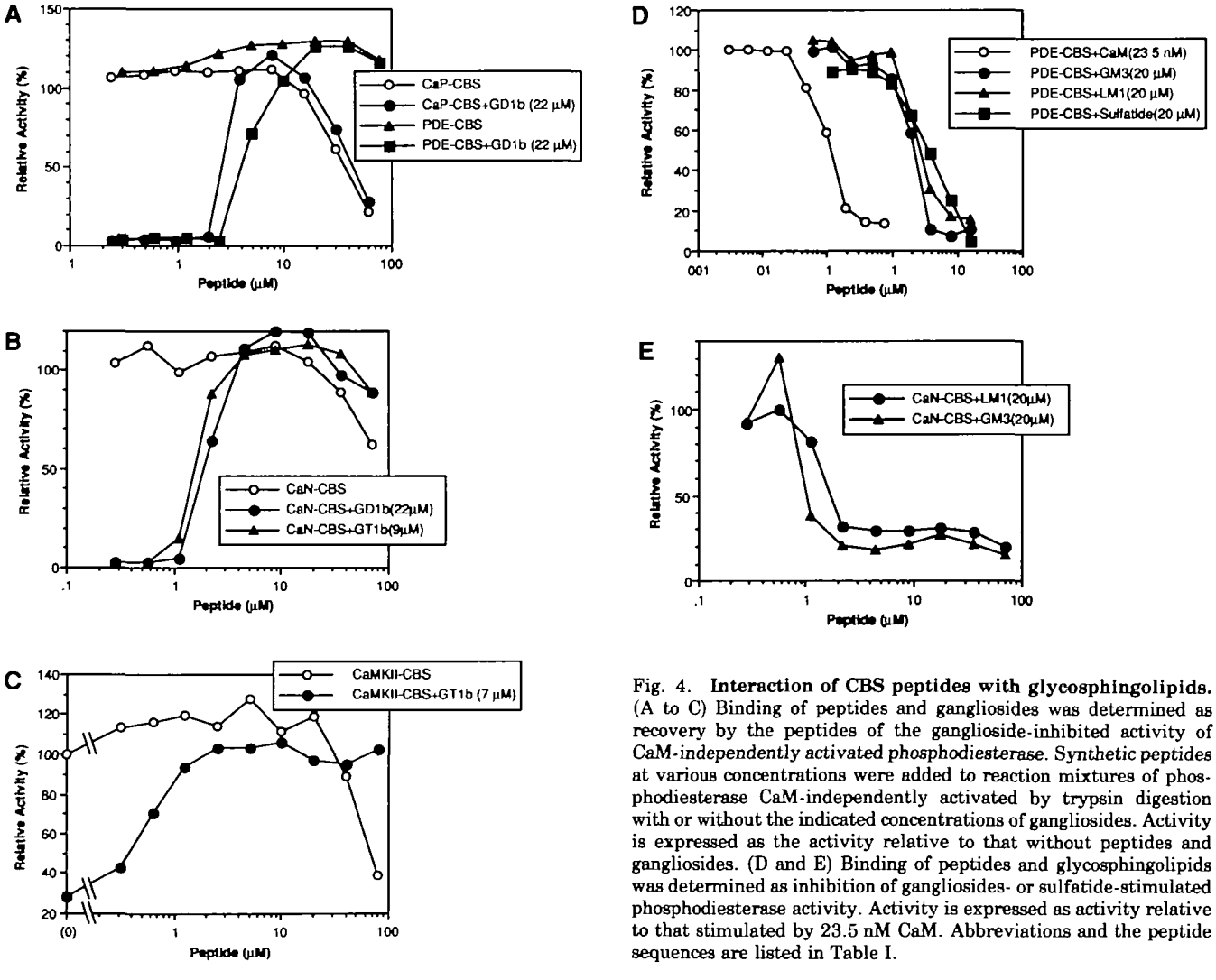

Fig. 4. **Interaction of CBS peptides with glycosphingolipids.** (A to C) Binding of peptides and gangliosides was determined as recovery by the peptides of the ganglioside-inhibited activity of CaM-independently activated phosphodiesterase. Synthetic peptides at various concentrations were added to reaction mixtures of phosphodiesterase CaM-independently activated by trypsin digestion with or without the indicated concentrations of gangliosides. Activity is expressed as the activity relative to that without peptides and gangliosides. (D and E) Binding of peptides and glycosphingolipids was determined as inhibition of gangliosides- or sulfatide-stimulated phosphodiesterase activity. Activity is expressed as activity relative to that stimulated by 23.5 nM CaM. Abbreviations and the peptide sequences are listed in Table I.

enzyme should have reduced the affinity to the "original" target molecules, but they retained some affinity for CBS peptides and gangliosides (Figs. 1and 3 and Table II). The predicted conformation of this peptide was not a typical acidic amphiphilic helix, in contrast to CaP-CLS1. This may explain the weak effects upon the phosphodiesterase.

The results using CLS peptides indicated that the enzyme was directly inhibited by gangliosides through interaction between ganglioside and CLS, but the interaction may not be the mechanism of the direct activation, since GM3 and LM1, that fully activate the phosphodiesterase, did not interact with the peptides (or their affinity for the peptides is very low).

Interaction of Gangliosides and Sulfatide with CBS Peptides of CaM-Dependent Enzymes—These results rather conflicted with our notion that ganglioside-binding to CLS is also a mechanism for activation of CaM-dependent enzymes. Moreover, in contrast to our notion, Fukunaga *et al.* (*27*) have reported that gangliosides activate CaM-kinase II through binding their CBS. Thus, we examined the interaction of gangliosides with the CBS of several CaM-dependent enzymes. CBS peptides of phosphodiesterase, calcineurin, CaM-kinase II, and the Ca^{2+}-pump listed in Table I were synthesized. As the CBS of phosphodiesterase contains a cysteine residue, it was substituted by serine to avoid effects caused by disulfide bonding. Inhibition by GD1b, GD1a, and GT1b of the trypsinized phosphodiesterase activity was prevented by all CBS peptides tested, as shown in Fig. 4, A to C, and Fig. 5A. Moreover, the peptides prevented LM1, GM3, and sulfatide from activating phosphodiesterase, indicating the direct interaction of the peptides with these glycosphingolipids that activate, but do not inhibit phosphodiesterase (Fig. 4, -D and -E). Furthermore, the effects of these gangliosides on the tryptophan fluorescence of PDE-CBS or CaP-CBS demonstrated a direct interaction between the gangliosides and the peptides (Fig. 3, B and C). These results indicated that the gangliosides and sulfatide interact with CBS of the enzymes. This explained the mechanism of the CaM-independent activation of CaM-dependent enzymes by gangliosides at low concentrations (*17, 21, 27-30*). In particular, the interaction of GM3, LM1, and sulfatide with CBS can explain the CaM-independent and maximal activation of phosphodiesterase by these glycosphingolipids (*26*). Thus, we concluded that the two groups of glycosphingolipids activate the enzyme by the same mechanism as CaM, namely by binding to the CBS of the enzyme.

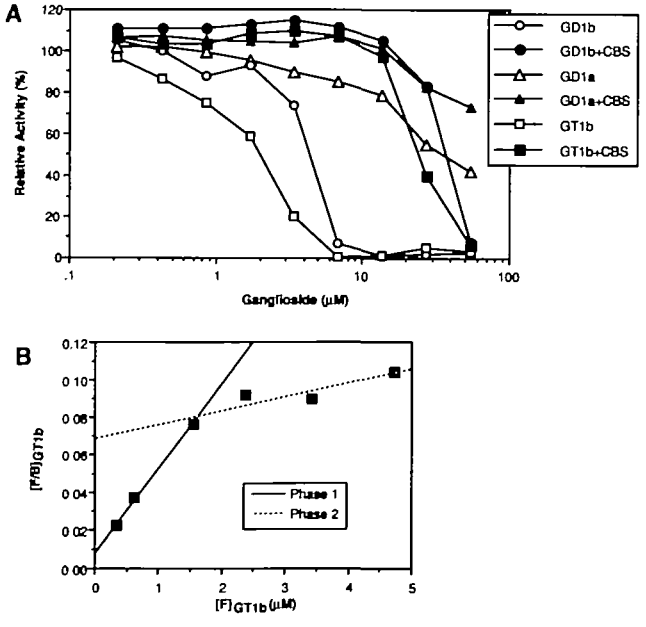

Fig. 5. **Effects of CBS peptides on inhibition of CaM-independently activated phosphodiesterase by gangliosides.** (A) The inhibition by gangliosides of trypsinized phosphodiesterase was assessed in the presence of 7.7 μM of PDE-CBS peptide. Activity is expressed as the relative activity to that without the peptide and the gangliosides. (B) Scatchard plots of the binding of the peptide and GT1b obtained in Fig. A. $[F]_{GT1b}$, concentration of free GT1b; $[F/B]_{GT1b}$, ratio of free *versus* bound GT1b. Phases 1 and 2 correspond to those in Table III.

Mechanisms for Modulation of CaM-Dependent Enzyme Activity by Gangliosides—The results presented here using synthetic peptides of CBS and CLS demonstrated that gangliosides and sulfatide interact with the CBS and activate the enzyme, and that gangliosides interact with the CLS and directly inhibit the enzyme activity. Thus, we revised the previous hypothesis. Gangliosides and sulfatide activate the enzyme through binding to the CBS as a CaM agonist, and inhibit the CaM-dependent activity through binding to CaM and by directly binding to the CLS of the enzyme, acting as its CBS (Fig. 6). Some *in vivo* interactions between gangliosides and cytosolic proteins (*10-15*) may occur by this mechanism.

Kinetics of Binding between Gangliosides and CBS—The affinity and molar ratio of the interaction between the gangliosides and CBS peptides of phosphodiesterase were estimated by Scatchard plots of the binding ratios obtained from Fig. 5A. Scatchard plots revealed the biphasic kinetics of the peptide binding to GD1b and GT1b, as shown in Fig. 5B. Table III summarizes the results. The affinity of the CBS peptide of phosphodiesterase to gangliosides was slightly lower than that of CaM to ganglioside GD1b. The K_d value of the peptide for GD1b was one order higher than that of CaM for GD1b (*17*). The affinity of the assumed CLS peptide of phosphodiesterase to gangliosides seemed weaker than that of CBS to gangliosides. Actually the apparent affinity, which was estimated from tyrosine and tryptophan fluorescence measurement, of ganglioside and PDE-CL1 was lower than that of gangliosides and PDE-CBS (Table II). Thus, gangliosides should have higher affinity to the CBS than to the CLS of phosphodiesterase. As described above, LM1, GM3, and sulfatide, which fully activate phosphodiesterase, have no or very low affinity for CLS peptides. Gangliosides such as GD1a inhibit, but do not activate, the activity of calcineurin (Higashi, H. and Yoshida, S., unpublished observation). The affinity of GD1a for the CLS should be higher than that for the CBS of calcineurin. The results presented here indicated that the activation and inhibition of the phosphodiesterase by gangliosides are mediated through their binding to the CBS and CLS, respectively, of the enzyme (Fig. 6). The affinity of gangliosides and the ganglioside-binding sites, CBS and CLS, should differ among gangliosides and enzymes. Thus, modulation of the enzyme activity by gangliosides should differ among enzymes.

Cross Reaction of CaM-Binding Sites among Different Enzymes—We found that excess CBS peptides of the

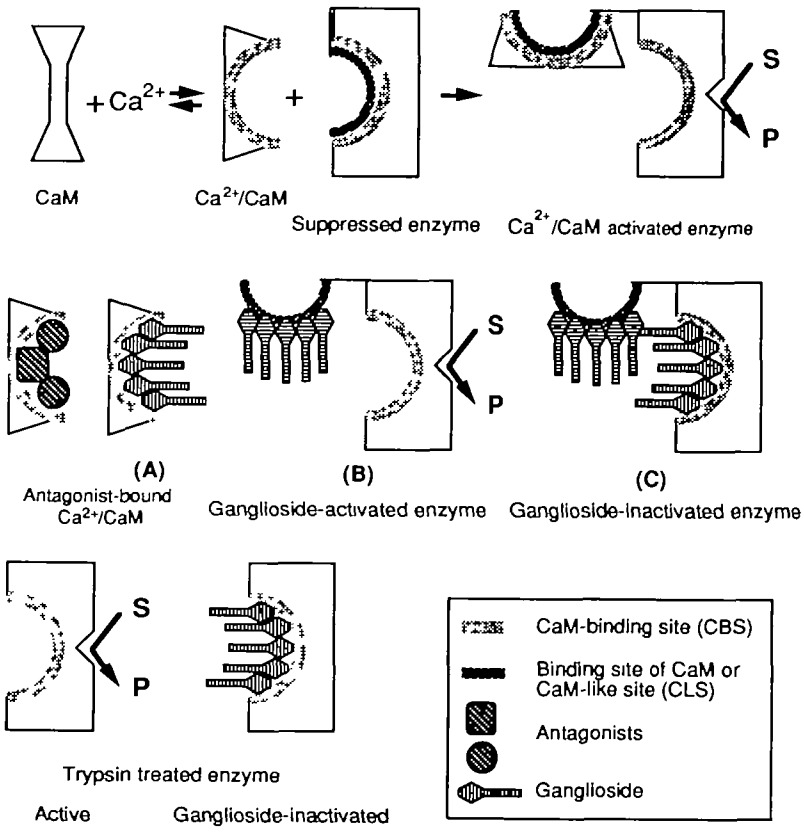

Fig. 6. **Proposed mechanism for the bimodal regulation of CaM-dependent enzymes by gangliosides.** The proposed model (*17*) was revised based upon this study. The flip-flop model proposed by Jarrett and Madhavan (*18*) was used with slight modifications. Gangliosides act as either CaM antagonists or agonists. They inhibit enzyme activity by competing with the CaM binding site for CaM (A). Gangliosides activate the enzyme by directly binding to the CaM-binding site to release it from the CaM-like site (B). In the meantime, gangliosides bind to the CaM-like site and enzyme inhibition occurs (C). CaM reverses this inhibition by competing with the CaM-like site for ganglioside-binding (*17*). Reaction (C) hypothesized previously was confirmed in this study and reaction (B), that had been proposed as binding of gangliosides to CLS, was revised as binding of gangliosides to CBS based on the present study. S, substrate; P, product.

TABLE III. **Dissociation constants and molar ratio of binding of gangliosides for CBS peptide of phosphodiesterase.** Values were obtained from Scatchard plots of the binding of the peptide and individual ganglioside shown in Fig. 5B for the case of GT1b.

Ganglioside	Phase	Dissociation constant, K_d (nM)	Ganglioside bound/ peptide (mol/mol)
GD1b	1	450	7.0
GD1b	2	960	42.0
GT1b	1	56	2.9
GT1b	2	540	5.3
GD1a		190	2.6

Ca^{2+}-pump, calcineurin, and CaM-kinase II inhibited the activity of trypsinized phosphodiesterase (Fig. 4, A to C). This indicated that the CBS of these enzymes cross-reacted with the CaM-independently activated phosphodiesterase. Enyedi and Penniston (*31*) have demonstrated the cross-reaction of CaM-binding domains of various Ca^{2+} transporters on a CaM-independently activated Ca^{2+}-pump. These results implied similar features of the CBS among different enzymes. Furthermore, we found that CaP-CLS1 activated phosphodiesterase by functioning as a CaM-like molecule (Higashi, H. and Yoshida, S., unpublished observation). These results suggest general roles for the CBS and the complementary CLS for autoregulation of enzyme activity, confirming the general applicability of the flip-flop model. Our revised hypothesis explains the *in vitro* nature of ganglioside modulation of phosphodiesterase activity. This and the flip-flop model may be easily applied to explain the mechanisms of the regulation of typical CaM-dependent enzymes that have rather simple autoregulatory structures, such as phosphodiesterase. For relatively complex enzymes, there are additional automodulation mechanisms such as a pseudosubstrate site located near or overlapping with CBS, as in the case of Ca^{2+}/CaM-dependent protein kinases (*32*). Myosin light chain kinase has 8 inhibitory domain-binding acidic residues within the catalytic core. Gallagher *et al.* demonstrated that these residues interact with substrate and/or CBS, indicating some of them construct CLS of the enzyme. Moreover, 6 of them are conserved among Ca^{2+}/CaM-dependent protein kinases (*32*). Thus the CBS-CLS interaction is present in such complex enzymes.

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