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Gangliosides modulate the activity of the plasma membrane Ca^{2+}-ATPase from porcine brain synaptosomes

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Abstract

We systematically examined the effects of gangliosides on the plasma membrane Ca^{2+}-ATPase (PMCA) from porcine brain synaptosomes. Our results showed that GD1b (two sialic acid residues) stimulated the activity, GM1 (one sialic acid residue) slightly reduced the activity, while asialo-GM1 (no sialic acid residue) markedly inhibited it, suggesting that sialic acid residues of gangliosides are important in the modulation of the PMCA. We also examined the oligosaccharide effects by using GM1, GM2, and GM3 whose only difference was in the length of their oligosaccharide chain. GM1, GM2, and GM3 reduced the enzyme activities, whereas GM2 and GM3 were potent inhibitors. Gangliosides affect both affinity for Ca^{2+} and the V_{max} of enzyme. It was observed that GD1b and GM2 increased the affinity of the enzyme for Ca^{2+}. GD1b, GM2 affected the V_{max} with an increase of GD1b, but decreases of GM2. The study of the affinity for ATP and the V_{max} of enzyme in the presence of gangliosides showed that GD1b and GM2 had little effect on the ATP binding to the enzyme, but the V_{max} was apparently changed. Moreover, the effects of gangliosides are additive to that of calmodulin, suggesting that the modulation of PMCA by gangliosides should be through a different mechanism. The conformational changes induced by gangliosides were probed by fluorescence quenching. We found that fluorescent quenchers (I^- and Cs^+) with opposite charges had different accessibility to the IAEDANS binding to the PMCA in the presence of gangliosides. An apparent red shift (25 nm) with increased maximum of fluorescence spectrum was also observed in the presence of GD1b.
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Keywords: Plasma membrane Ca^{2+}-ATPase, gangliosides; Monosialoganglioside-G_{M1}; Monosialogangliosides-G_{M2}; Monosialogangliosides-G_{M3}; Disialogangliosides-G_{D1b}

The plasma membrane Ca^{2+}-ATPase (PMCA) is a P-type ATPase that plays a crucial role in regulation of cell calcium homeostasis [1,2]. Its function is to extrude Ca^{2+} from the cytosol to the extracellular space to maintain the resting low intracellular calcium concentration and to prevent cells from a lethal overload of calcium. The pump shares the catalytic properties of ion-motive ATPases of the P-type family, but has distinctive regulation properties due to its long (~150 amino acids) C-terminal intracellular tail, which contains calmodulin binding domain and some other regulatory sites [3]. Thus, the PMCA is modulated by (CaM)[1] [4,5], protein kinases A [6] and C [7], acidic phospholipids [8], controlled proteolysis [9,10], autoaggregation [11], organic solvents [12–14], phosphatidylethanol [15], and ceramide and sphingosine [16].

Gangliosides (sialic acid-containing glycosphingolipids) are the ubiquitous vertebrate glycolipids, and are especially abundant in the plasma membrane of neurons [17–19], where they play different roles in

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[1] *Abbreviations used:* GM1, monosialoganglioside-G_{M1}; GM2 monosialogangliosides-G_{M2}; GM3, monosialogangliosides-G_{M3}; GD1b, disialogangliosides-G_{D1b}; asialo-GM1, asialoganglioside-G_{M1}; CaM, calmodulin; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; FITC, fluorescein 5-isothiocyanate; IAEDANS, 5-((2-((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid.

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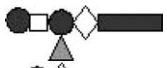
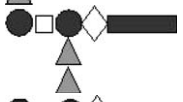
Table 1
Ganglioside used in this study

Gangliosides	Structure
GM1	
GM2	
GM3	
GD1b	
Asialo-GM1	
Cerebroside	
Lactocerebrosides	

Neuraminic acid, ▲; *N*-acetyl-galactosamine, □; galactose, ●; glucose, ◇; and ceramide, ▬.

controlling cell growth, cell adhesion, and cell–cell interaction [20,21]. Of great interest has recently been Ca^{2+}-homeostasis regulated by gangliosides [22]: gangliosides, such as GM1, can potentiate the activity of a nuclear envelope Na^{+}–Ca^{2+}-exchanger [23]. A Ca^{2+} channel is gated by interaction with GM1, promoting Ca^{2+} influx across the plasma membrane in neuroblastoma cells [24]. The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) can be modulated by gangliosides GM1, GM2, and GM3 [25–27]. The simple glycosphingolipids have also been implicated in regulating Ca^{2+}-homeostasis, e.g., glucosylceramide, one of the simplest glycolsphingolipids, increases Ca^{2+} mobilization from intracellular stores, i.e., endoplasmic reticulum (ER), via the activation of the ryanodine receptor (RyaR), the major Ca^{2+}-release channel of the ER [28,29].

We have previously shown that GM1 and GM3 have an antagonistic effect on the SERCA [25,26]. However, few study the possible effect of gangliosides on the PMCA, which shares essential basic properties with the SERCA, but differs in structural and functional aspects, pertaining particularly to its regulation [30]. In the present study, we systematically examine the effect of gangliosides, i.e., GD1b, GM1, GM2, GM3, and asialo-GM1 (Table 1), on the PMCA.

Materials and methods

Materials

Calmodulin–Sephorose CL-4B was from Pharmacia. Phosphatidylcholine and phosphatidylethalamine were from Avanti Polar Lipids GD1b, GM1, GM2, GM3, asilo-GM1, fluorescein 5-isothiocyanate (FITC), ATP, NADH, pyruvate kinase, lactate dehydrogenase, phosphor(enol)pyruvate, A23187, and leupeptin were from Sigma. 5-((2-((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS) was from Molecular Probes. Bio-beads were from Bio-Rad. Other reagents were commercially available in China and were of AR grade.

Purification of plasma membrane (Ca^{2+} + Mg^{2+})—ATPase from pig brain

The PMCA was purified from porcine brain by calmodulin affinity column chromatography in the presence of Triton X-100 by the method of Salvador and Mata [31]. The purified ATPase was divided into appropriate aliquots, quick-frozen in liquid nitrogen, and stored at −80 °C in the elution buffer containing 20 mM Hepes/KOH, pH 7.4, 130 mM KCl, 2 mM EDTA, 0.06% Triton X-100, and 2 mM of 2-mercaptoethanol. The protein concentration was measured by using the slight modification of the Lowry's procedure [31,32] to avoid interference by Triton X-100 and 2-mercaptoethanol, using bovine serum albumin as a standard.

Reconstitution of the purified ATPase by the Bio-Beads

The reconstitution protocol was that described by Niggli et al. [8], except that 80 mg/ml Bio-Beads were added to the mixture of phospholipid solution and ATPase every hour for three times and the mixture was agitated slowly at room temperature.

Determination of Ca^{2+}-ATPase activity

Gangliosides were added to a medium (100 μl) containing 130 mM KCl, 20 mM Hepes/KOH, pH 7.4, 1 mM $MgCl_2$, 2 mM ATP, 1 mM EGTA, and the appropriate quantity of $CaCl_2$, to obtain the desired free calcium concentrations. Aliquots of reconstituted PMCA (about 10 μg of protein/ml) were added to this medium to start the reaction. The reaction was carried out for 30 min at 37 °C and was stopped by addition of SDS at 0.5%. The phosphate produced by ATP hydrolysis was determined according to the method of Hergenrother and Martin [33]. Appropriate blanks were included to correct any interference with the colorimetric method.

When measuring Ca^{2+}-ATPase's affinity for ATP, the enzyme activity was measured spectrophotometrically at 340 nm with the aid of a coupled enzyme assay [34]. Briefly, microsomes plus a certain concentration of gangliosides were added to the reaction mixture that contained, in a final volume of 1 ml, 40 mM Hepes/KOH, pH 7.4, 120 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, 0.15 mM NADH, 0.42 mM phospho(enol)pyruvate, 10 IU pyruvate kinase, and 28 IU lactate dehydrogenase. After 2 min incubation at 37 °C, the reaction was started by the addition of 1 mM $CaCl_2$ (10 μM free $CaCl_2$).

The Ca^{2+}-ATPase activity was obtained after subtraction of the Mg^{2+}-dependent activity, measured in the presence of 1 mM EGTA. Defined concentrations of free Ca^{2+} were established with the aid of $CaCl_2$ and EGTA solutions (determined using an algorithm [35] and software available at http://www.standford.edu/~cpatton/maxc.html).

Measurement of calcium uptake by the proteoliposomes

Ca^{2+} uptake was measured at 37 °C by dual wavelength spectrophotometer using arsenazo III as Ca^{2+} indicator as described by Gould et al. [36]. Ca^{2+} uptake was followed by monitoring the differential (675 versus 685 nm) absorption changes which were linearly proportional to changes of the Ca^{2+} concentration in the external medium. The reaction mixture contained 100 mM KCl, 40 mM Hepes–K^+, pH 7.4, 5 mM $MgCl_2$, 20 μM arsenazo III, and 25–30 μg of proteoliposomal protein per ml. Additional $CaCl_2$ (40 μM) was added after obtaining the absorption base line to standardize the absorption changes. Then the reaction was started by the addition of 0.5 mM ATP. When the absorption had no changes, 10 μM A23187 was added to the mixture.

FITC and IAEDANS labeling and fluorescence measurement

Labeling by FITC was essentially as described by Nakamura et al. [37]. FITC was added to the medium of 50 mM Tris–HCl, pH 8.0, 2 mM EGTA, 5 mM $MgCl_2$, and 0.1 M KCl with protein concentration of 0.1 mg/ml. The reaction was kept in dark at 30 °C for 1 h. Unreacted label was removed by dialysis thoroughly.

The Ca^{2+}-ATPase was labeled with IAEDANS in a medium of 10 mM Tris–HCl, pH 8.0, 0.1 M KCl, 1 mM $CaCl_2$, and 5 mM $MgCl_2$ with the protein concentration of 0.1 mg/ml in dark for 30 min at 25 °C essentially as described by Coan and Inesi [38]. IAEDANS was added 100-fold excess. Unreacted label was removed by dialysis.

Fluorescence measurement was carried on Hitachi 4500-fluorescence spectrophotometer, with an excitation bandpass of 5 nm and emission bandpass of 5 nm. For FITC label, $\lambda_{ex} = 480$ nm, $\lambda_{em} = 520$ nm, the temperature of measurement is 25 °C. For quenching experiments of FITC–PMCA, standard suppression medium contained 50 μg/ml FITC–PMCA, 5 mM $MgCl_2$, 40 mM Tris–HCl, pH 8.0, 0.1 M KCl, and 2 mM EGTA in a final volume of 0.3 ml. The spectrum and yield of labeled PMCA with IAEDANS were determined with excitation at 340 nm and emission scanning from 480 to 550 nm. For quenching experiments of IAEDANS–PMCA, the emission was recorded at 460 nm. The concentrated quenchers (KI, CsCl) were sequentially added and the fluorescence was recorded for 3 min. Twenty micromolar gangliosides were added to the medium to get the changes of fluorescence quenching.

The quenching constants (K_q) were calculated using the Stern–Volmer equation. $F_0/F = 1 + K_q[Q]$, where K_q is the Stern–Volmer quenching constant, F_0 is the fluorescence in the absence of quencher, F is the fluorescence in the presence of quencher, and [Q] is the molar concentration of quencher. The data presented were averages of three different experiments.

Fluorescence lifetime measurement

Fluorescence lifetimes of FITC–PMCA in the presence or absence of gangliosides were measured on an Edinburg 299T time resolved fluorescence spectrophotometer [39]. The medium contained 50 μg/ml FITC–PMCA, 40 mM Tris–HCl, pH 8.0, 0.1 M KCl, 0.5 mM $MgCl_2$, and 2 mM EGTA. The kinetics decay was fitted with a single exponential process: $F(t) = Ae^{-t/\tau}$, τ represents the lifetime of FITC.

Results

Antagonistic effect of GD1b and GM2 on the PMCA in the synaptosomal plasma membranes

GD1b is one of most abundant gangliosides in the synaptosomal plasma membranes [40]. The effects of GD1b and GM2 on the PMCA in porcine brain synaptosomes were tested by adding GD1b or GM2 into the plasma membrane vesicles. As shown in Fig. 1, the GD1b significantly enhanced the Ca^{2+}-dependent ATPase activity while GM2 decreased, indicating that gangliosides could modulate the PMCA from synaptosomes. This result prompted us to purify the PMCA from porcine cerebella and study the modulation of the enzyme by gangliosides.

Dependence of the reconstituted PMCA activities upon sialic acid residues of gangliosides

The purified PMCA was inactive due to the delipidation in the course of calmodulin affinity chromatography, but could be fully reactivated by reconstituting into liposomes containing phosphatidylcholine. Furthermore, the activity of the reconstituted enzyme was enhanced upon CaM by ~1.5-fold (data not shown), indicating that the reconstituted enzyme in our experiments was fully functional.

Consistent with the observation that GD1b enhanced the PMCA activity in synaptosomal plasma membranes (Fig. 1), the reconstituted PMCA could also be activated by GD1b (Fig. 2). To study the mechanism by which gangliosides modulate the PMCA, a series of ganglio-

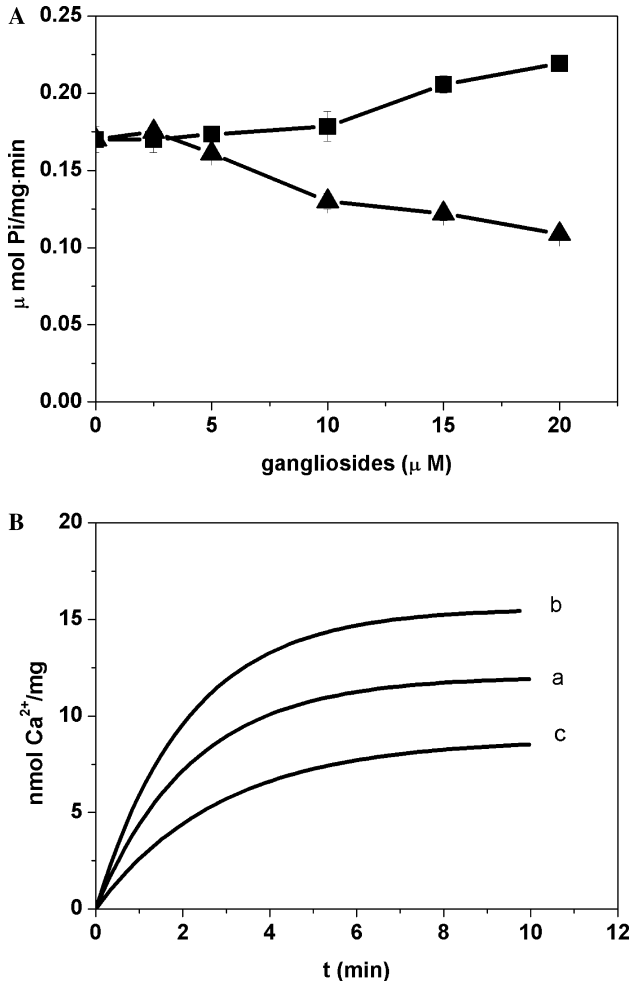


Fig. 1. Effects of GD1b, GM2 on the activity of PMCA in porcine brain synaptosomes (A), ATPase activity. The reaction medium contained 130 mM KCl, 20 mM HEPES/KOH, (pH 7.4), 2 mM ATP, 1 mM MgCl, 1 mM EGTA, and the amount of $CaCl_2$ to give a final Ca^{2+} concentration of 10 μM and the indicated concentrations of gangliosides. The reaction was started by the addition of 25 μg/ml synaptosomes in a final volume of 0.1 ml. After 30 min incubation, the reaction was stopped by the addition of 0.5% SDS. ■, GD1b; ▲, GM2. (B) Ca^{2+} uptake. The reaction mixture contained 100 mM KCl, 40 mM Hepes–K^+, pH 7.4, 5 mM $MgCl_2$, 20 μM arsenazo III, and 500 μg of synaptosomal protein per ml. Additional $CaCl_2$ (40 μM) was added after obtaining the absorption base line to standardize the absorption changes. Then the reaction was started by the addition of 0.5 mM ATP. Experiments were carried out at 37 °C in the absence (a) or in the presence of 20 μM GD1b (b) or GM2 (c).

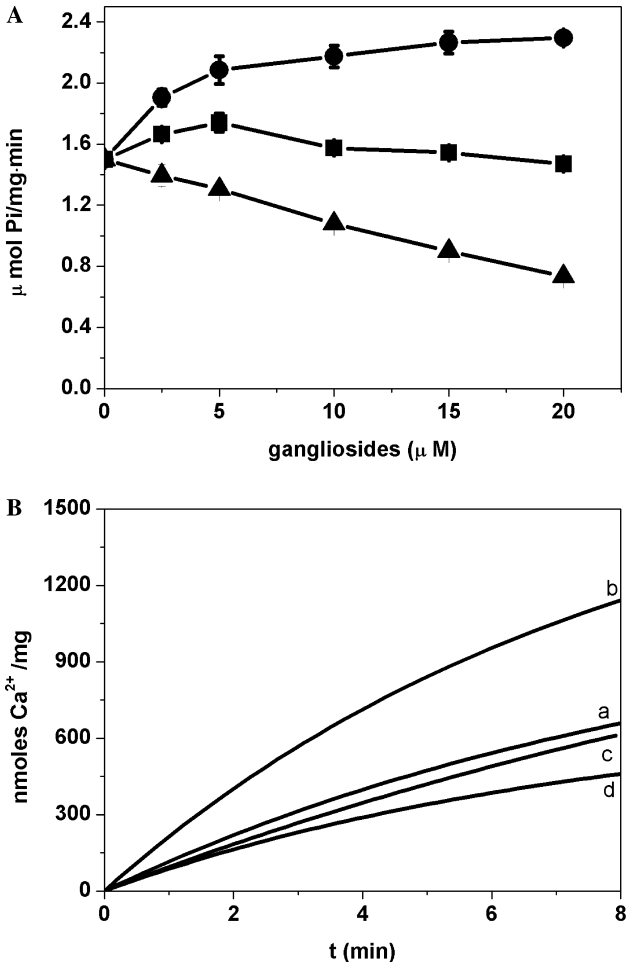


Fig. 2. Effects of GD1b, GM1, or asialo-GM1 on the activity of reconstituted PMCA (A), ATP hydrolysis. Experimental conditions were as in Fig. 1A, except that 5 μg/ml reconstituted PMCA was added to the reaction. ●, GD1b; ■, GM1; and ▲, asialo-GM1. (B) Ca^{2+} uptake. Experimental conditions were as in Fig. 1B, except that 25–30 μg of proteoliposomal protein was added to the reaction. Experiments were carried out in the absence (a) or in the presence of 20 μM GD1b (b), GM1 (c) or asialo-GM1 (d).

sides were used to examine their effects on the PMCA. Among GD1b (two sialic acid residues), GM1 (one sialic acid residue), and asialo-GM1 (no sialic acid residue), GD1b enhanced the activity, while asialo-GM1 inhibited it. In addition, GM1 at relatively low concentrations stimulated the activity, whereas slightly decreased at relatively high concentrations (Fig. 2A). Similar effects on the Ca^{2+} uptake were also observed (Fig. 2B). This result suggested that sialic acid residue(s) of gangliosides be important in the modulation of the PMCA.

Dependence of the reconstituted PMCA activities upon oligosaccharide of gangliosides

We next examined the oligosaccharide effect by using GM1, GM2, and GM3 whose only difference was in the length of their oligosaccharide chain. As shown in Fig. 3A, after slightly activated at relatively low concentrations of gangliosides, the enzyme activity was then reduced over the experimental concentrations, whereas GM2 and GM3 were potent inhibitors. In an agreement with the ATPase activity, GM1, GM2, and GM3 also inhibited the Ca^{2+} uptake (Fig. 3B). The results indicated that the oligosaccharide was also involved in the modulation of the PMCA by gangliosides. Moreover, to explore whether other ganglioside structural determinants might contribute to the modulation of the PMCA, similar experiments with lactocerebrosides, cerebrosides,

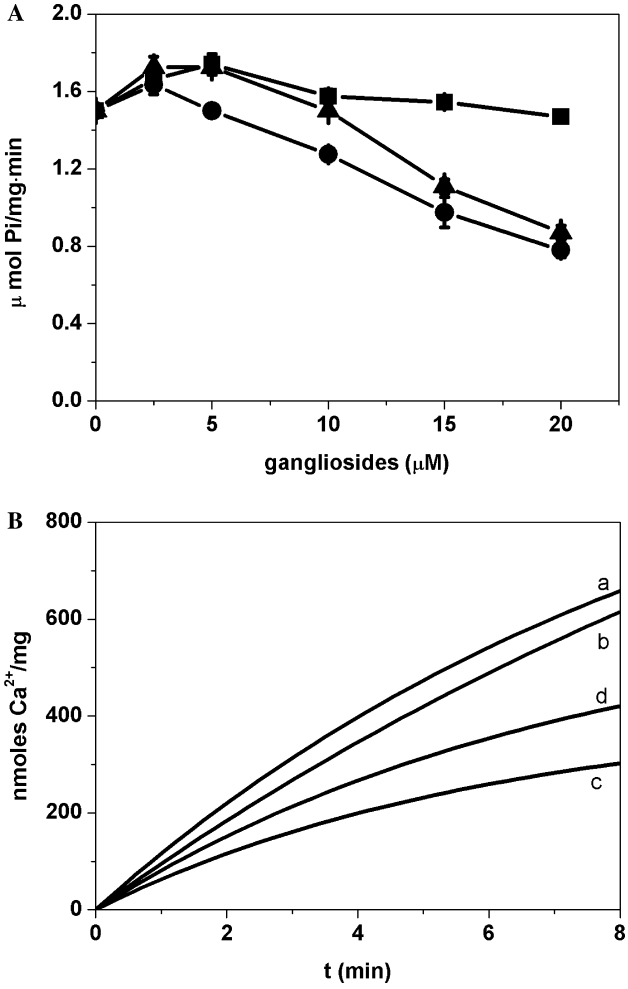


Fig. 3. Effects of GM1, GM2, or GM3 on the activity of reconstituted PMCA. Experiments conditions were as in Fig. 2. (A) ATP hydrolysis. ■, GM1; ▲, GM3; ●, and GM2. (B) Ca^{2+} uptake. Experiments were carried out in the absence (a) or in the presence of 20 μM GM1 (b), GM2 (c) or GM3 (d).

ceramide, and sialic acid were also performed. None of them apparently exerted effects on the PMCA (data not shown).

Influence of gangliosides on the Ca^{2+} and ATP dependencies of ATPase activity

The effect of gangliosides on the affinity of the PMCA for Ca^{2+} was determined by incubating the enzyme with different concentrations of free Ca^{2+} in the presence of 15 μM GD1b, GM2, and asialo-GM1, by which the enzyme activity was significantly affected (Fig. 4). It may be observed that GD1b and GM2 increased the affinity of the enzyme for Ca^{2+} ($K_m = 121 \pm 9$ nM for GD1b, $K_m = 141 \pm 11$ nM for GM2) with respect to the control ($K_m = 200 \pm 8.6$ nM), while asialo-GM1 had no apparent effect ($K_m = 200 \pm 15$ nM). Moreover, GD1b, GM2, and asialo-GM1 affected the V_{max} with an increase of GD1b ($V_{max} = 2.2$ μmol Pi/mg min), but decreases of

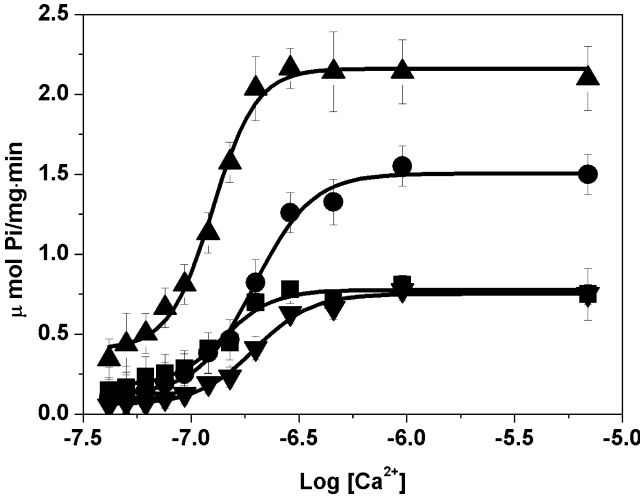


Fig. 4. Effects of gangliosides on Ca^{2+}-ATPase's affinity for Ca^{2+}. Experimental conditions were as in Fig. 2A in the presence of 15 μM gangliosides, except that the final free Ca^{2+} concentration was obtained upon addition of 1 mM EGTA and the calculated quantity of $CaCl_2$. ●, control; ▲, GD1b; ■, GM2; ▼, and asialo-GM1.

GM2 ($V_{max} = 0.7$ μmol Pi/mg min) and asialo-GM1 ($V_{max} = 0.7$ μmol Pi/mg min) with respect to the control ($V_{max} = 1.5$ μmol Pi/mg min).

The ATP dependence of the PMCA in the presence of gangliosides was shown in Fig. 5. In contrast to increasing the affinity of the PMCA for Ca^{2+}, GD1b, and GM2 at a concentration of 10 μM had little effect on the ATP binding to the enzyme in view of $K_m = 6.7 \pm 0.33$ μM for GD1b and $K_m = 6.7 \pm 0.83$ μM for GM2

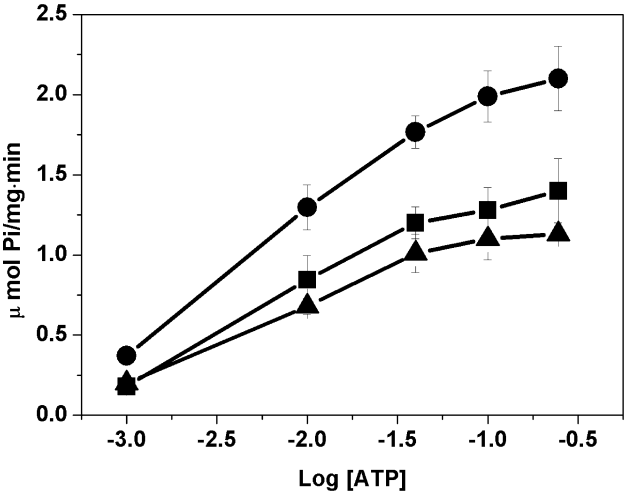


Fig. 5. Effects of gangliosides on Ca^{2+}-ATPase's affinity for ATP. The reaction mixture contained, in a final volume of 1 ml, 40 mM Hepes/KOH, pH 7.4, 120 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, the indicated concentrations of ATP, 0.15 mM NADH, 0.42 mM phospho(enol)pyruvate, 10 IU pyruvate kinase, and 28 IU lactate dehydrogenase. After 2 min incubation at 37 °C, the reaction was started by the addition of 1 mM $CaCl_2$ (10 μM free $CaCl_2$). Experiments were carried out in the absence of gangliosides (■), in the presence of 10 μM GD1b (●) or GM2 (▲).

with respect to the control ($K_m = 6.8 \pm 0.65\,\mu M$). However, the V_{max} were apparently changed with GD1b and GM2. GD1b increased the V_{max} ($V_{max} = 2.2\,\mu mol\,Pi/mg\,min$), while GM2 reduced the V_{max} to $1.12\,\mu mol\,Pi/mg\,min$ with respect to the control ($V_{max} = 1.4\,\mu mol\,Pi/mg\,min$).

Effects of gangliosides on the PMCA in the presence of calmodulin

It is well known that the calmodulin stimulates the PMCA through its calmodulin binding domain in the C-terminal intracellular tail. We examined the effects of GD1b and GM2 on the ATPase activity in the presence of calmodulin. As shown in Fig. 6, after addition of calsmodulin to the same preparation as described in Fig. 2, a more pronounced increase in ATPase activity was observed. Moreover, an additive effect was obtained upon addition of both effectors added simultaneously, suggesting the modulation of gangliosides and calmodulin through different mechanisms.

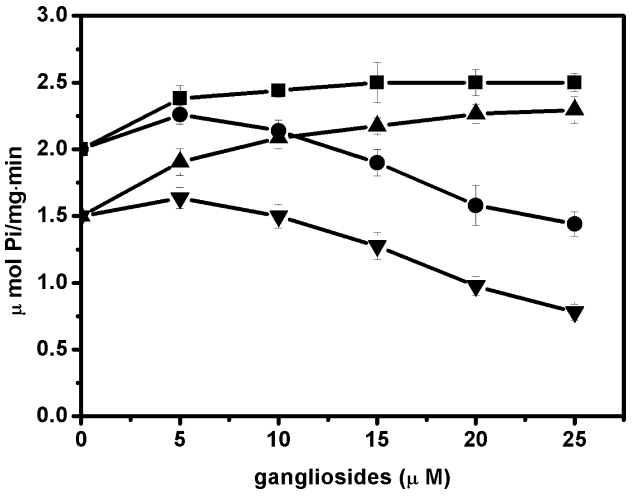


Fig. 6. Effects of gangliosides on the PMCA in the absence or in the presence of calmodulin. Different gangliosides at various concentrations were added to the reaction mixture of PMCA stimulated by 2 μg/ml calmodulin. Experiments conditions were as in Fig. 2A. ▲, GD1b; ▼, GM2 in the absence of calmodulin. ■, GD1b; and ●, GM2 in the presence of calmodulin.

Fluorescence study of conformational changes of the PMCA in the presence of gangliosides

The conformational changes of the PMCA were probed by fluorescence quenching. FITC was used to covalently label on a Lys591 located within the nucleotide domain of the catalytic site of the PMCA [41]. Dynamic quenching by a fluorescent quencher (KI) was studied in the presence of 20 μM gangliosides (Table 2). No apparent differences of the quenching constants (K_q) were obtained in the presence of GD1b, GM2, and asialo-GM1 compared with free gangliosides. The GD1b, GM2, and asialo-GM1 did not affect the accessibility of KI to the FITC, suggesting that the conformation near the Lys591 was not altered. The measurements of lifetimes of the FITC labeled PMCA in the presence of gangliosides supported this finding (Table 3). The invariance of the lifetimes meant that the behavior of FITC fluorescence followed the first order exponential process showing no changes in the presence of gangliosides, reflecting little effects of gangliosides on the conformation near the Lys591.

A cysteine/cysteines can be specifically labeled by IAEDANS [42,43]. An apparent red shift (25 nm) with increased maximum of fluorescence spectrum was observed in the presence of 20 μM GD1b with respect to free gangliosides, although fluorescence spectrum behaved similarly in the presence of GM2 or asialo-GM1 (Fig. 7). Obviously, GD1b induced conformational changes of the enzyme. The effects of fluorescent quenchers (I^- and Cs^+) with opposite charges were also tested in the presence of gangliosides (Fig. 8). The K_q values were significantly increased in the presence of

Table 3
Fluorescence lifetimes of FITC–PMCA in the presence or absence of gangliosides

Gangliosides	τ (ns)
None	4.24 ± 0.01
GD1b	4.25 ± 0.0073
GM2	4.25 ± 0.01

Lifetimes were measured in the same experimental conditions used for Table 1.Values were calculated from the experimental data by fitting with a single exponential decay.

Table 2
K_q values for KI or CsCl as quenchers of the fluorescence of FITC–PMCA or IAEDANS–PMCA in the presence or absence of gangliosides[a]

	None gangliosides		GD1b		GM2		Asialo-GM1	
	FITC labeled	IAEDANS labeled	FITC labeled	IAEDANS labeled	FITC labeled	IAEDANS labeled	FITC labeled	IAEDANS labeled
KI	2.5 ± 0.22	0.39 ± 0.04	2.33 ± 0.23	1.36 ± 0.05	2.22 ± 0.22	0.62 ± 0.05	2.56 ± 0.22	0.57 ± 0.04
CsCl		0.27 ± 0.04		1.11 ± 0.06		0.15 ± 0.04		0.24 ± 0.03
R_q		1.44		1.23		4.13		2.38

[a] Values for K_q are expressed as M^{-1}. Data are presented as means ± SD of three normalized experiments with different preparations. R_q is the ratio K_q (I^-)/K_q (Cs^+).

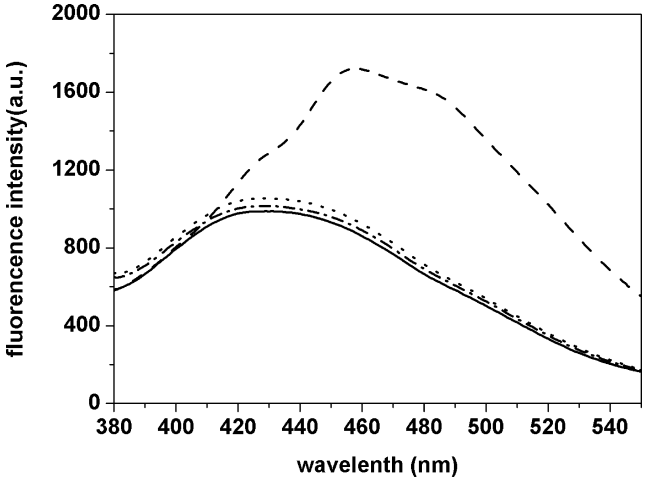


Fig. 7. The fluorescence emission spectra of IAEDANS–PMCA and IAEDANS–PMCA in the presence of gangliosides. The media contained 50 mM Tris, pH 8.0, 100 mM KCl, and 5 mM $MgCl_2$. Excitation was performed at 340 nm. IAEDANS–PMCA was measured in the absence of gangliosides (——) or in the presence of 20 μM GD1b (– –), asialo-GM1 (– · – · –) or GM2 (· · · ·).

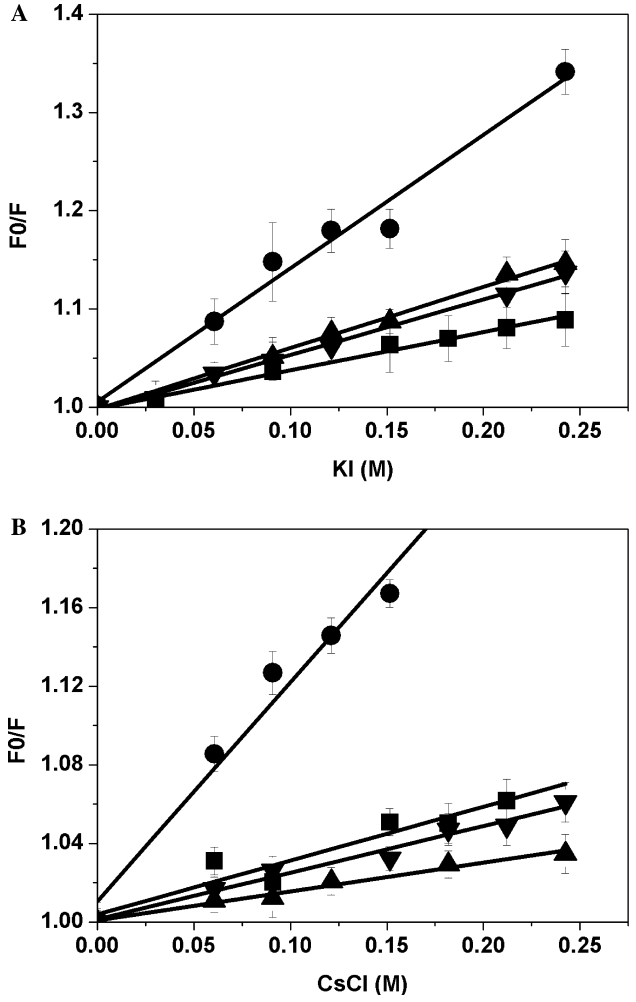


Fig. 8. Fluorescence quenching of IAEDANS–PMCA by iodide (A) or cesium (B). The media contained 50 mM Tris, pH 8.0, 100 mM KCl, and 5 mM $MgCl_2$. The curves represent the relative quenching obtained for PMCA–IAEDANS (■), or IAEDANS–PMCA in the presence of 20 μM GD1b (●), GM2 (▲), and asialo-GM1 (▼).

20 μM GD1b, indicating that the enzyme was more accessible to I^- or Cs^+. The ratio, R_q, defined as $K_q(I^-)/K_q(Cs)^+$ had little changes in the presence of GD1b (1.22) with respect to in the absence of GD1b (1.44). Since I^- and Cs^+ have opposite charges, the closer R_q values implied that GD1b induced conformational changes had little influences on exposures of the charged amino acids located in the vicinity of the bound IAEDANS. Fig. 8 also shows that the K_q value for KI in the presence of GM2 or asialo-GM1 slightly increases, but that for CsCl in the presence of GM2 in contrast slightly decreases. The K_q value for CsCl in the presence of asialo-GM1 has no significant changes. The results suggested that the quenchers (I^- and Cs^+) had less accessibility caused by conformational changes induced by GM2 or asialo-GM1 compared with GD1b. Meanwhile, the R_q values in the presence of GM2 or asialo-GM1 are also increased (Table 2), implying that a cluster of positive charges could be exposed, therefore enhancing quenching by I^- through electrostatic attraction.

Discussion

The impetus for the current study was our earlier observation of an antagonistic effect of GM1 and GM3 on the SERCA [25,26]. Since the PMCA is the homologue of SERCA, but functions to pump Ca^{2+} from the cytosol to the extracellular space, here we carry on the systematic study to determine the mechanism by which gangliosides regulate the PMCA.

The regulation of the PMCA by gangliosides depends upon the defined structural features of the gangliosides. A ganglioside consists of sialic acid residue(s), oligosaccharide and ceramide. The observation in Fig. 2 points to the importance of sialic acid residue(s) of the gangliosides in this regulation. The GD1b with two sialic acid residues significantly stimulates the PMCA, while the GM1 with one sialic acid residue has a small effect. Moreover, the asialo-GM1 without sialic acid residues strongly inhibits the PMCA. The remarkable different effects of GD1b, GM1, and asialo-GM1 on the PMCA, thus, appear to be related to the number of sialic acid residues presented on the gangliosides. However, sialic acid alone fails to have any influence on the PMCA. Taken together, the sialic acid residue must be associated with the defined glycosphingolipidic structure to exert its effect on the PMCA.

GM1, GM2, and GM3 decrease the PMCA activity in the order of GM2 = GM3 > GM1. Since these monosialo-gangliosides differ only in the length of their oligosaccharide chain, this suggests the importance of

the saccharide structure in the regulation of the PMCA. Moreover, the glycolipids lacking sialic acid residues, i.e., asialo-GM1 (four sugar groups), lactocerebrosides (two sugar groups), cerebroside (one sugar group), and ceramide (no sugar group) behave quite differently. With the exception of the asialo-GM1, neither of them tested has effect on the PMCA. This further supports the importance of oligosaccharide structure. Ceramide has been found to stimulate the PMCA from human erythrocytes [16]. It should be noted that four different PMCA isoforms known as PMCA1, PMCA2, PMCA3, and PMCA4 have been identified. The PMCA in human erythrocytes is a mixture of PMCA1 and PMCA4, while PMCA2 and PMCA3 are essentially restricted to the nervous cells with high concentrations of PMCA2 in the cerebellum [44]. It has been observed that the activation of the PMCA by ethanol is isoform-specific [14]. The different effect of ceramide on the PMCA from human erythrocytes [16] and synaptosomes, therefore, may imply the isoforms related. As a matter of fact, we tested the effects of GD1b and GM2 on the PMCA from human erythrocytes, the preliminary results showed that both GD1b and GM2 stimulated the enzyme (data not shown), suggesting that the effect of gangliosides on the PMCA was isoform dependence. This could be confirmed by studying the effect of gangliosides on each overexpressed isoform which is ongoing in our laboratory.

It should be noted that an uncoupling of Ca^{2+} uptake to ATP hydrolysis is observed (Figs. 1–3). Under our experimental conditions, this unfavorable ratio of Ca^{2+} uptake to ATPase activity could be due to the leakage in the course of our making reconstituted vesicles and/or a slippage pathway that involves the release of bound Ca^{2+} from the phosphorylated intermediate to the cytoplasmic side of the membrane before its transport into the lumen, resulting in ATP hydrolysis uncoupled from Ca^{2+} transport. Both the passive leak of transported Ca^{2+} out of the vesicle and the slippage have been observed for the PMCA [45].

Concerning the mechanism by which gangliosides modulate the PMCA activity, it is noteworthy that since the effects of gangliosides are additive to that of CaM, the modulation of PMCA by gangliosides should be through a different mechanism. The locus of interaction of PMCA with CaM is well documented [3]. Calpain attacks PMCA [9,46], removing portions of the calmodulin binding sequence and leading to their "irreversible" activation. We therefore tested the effects of gangliosides on the calpain digested enzyme. Of GD1b, GM2 and GM3 did not show significant effects on ATPase activity, suggesting that the portions of interaction of GD1b, GM2, and GM3 with the PMCA were removed by calpain (data not shown). A recent study, however, presented that the calpain digested the PMCA was the isoform dependence [47], i.e., the PMCA1 isoform was rapidly and completely degraded; a more complex pattern of degradation prevailed in the PMCA2 protein; only the calmodulin binding domain and the sequence downstream of PMCA4 were quantitatively removed. Since porcine brain synaptosomes used in the present study contain all the PMCA1–PMCA4, gangliosides' mechanism of action on this enzyme remains to be elucidated.

The interaction between gangliosides and the PMCA should lead to the conformational changes of the enzyme, thus affecting the activity. The fluorescence quenching of the IAEDANS labeled PMCA supports this idea. Our data indicate that the gangliosides, i.e., GD1b and GM2 exhibiting different effects on the PMCA activity have significantly induced the conformational changes, evidenced by their different accessibilities of the oppositely charged quenchers (I^- and Cs^+) to the IAEDANS. The apparent red shift of the fluorescence spectrum provides a further support for the conformational changes in the presence of GD1b.

Ganglioside modulation of calcium flux across the plasma membrane has been extensively investigated, but mainly focused on the influx of calcium through Ca^{2+} channels [22]. There is as yet only limited evidence indicating facilitation of Ca^{2+} efflux across the plasma membrane. It was suggested that this efflux of calcium may relate to various Ca^{2+}-dependent enzymes or Ca^{2+} exchange proteins in the plasma membrane. In the present study we, for the first, time show that gangliosides are able to affect both the membrane-bound (i.e., synaptosomal plasma membranes) and purified PMCA, indicating that their effects are not linked to the solubilization of the enzyme from the membrane environment. It is generally accepted that gangliosides are asymmetrically distributed on the outer leaflet of the plasma membrane, but some are found to exist in the cytosolic leaflet (e.g., 40% GM1, 30% GD1a, and 40% GD1b) [48] and cytosol (e.g., 47μM soluble forms of gangliosides in the brain) as well [49]. This indicates that gangliosides interact with cytosolic enzymes as well as membrane proteins. As shown in Figs. 1 and 2, gangliosides effectively modulate the activity of the PMCA at around these concentrations. Soluble cytosolic gangliosides or in cytosolic leaflet should thus be capable of modulating the PMCA, and the PMCA dependent cellular activities in vivo depending on their concentrations.

In the gangliosidoses, gangliosides accumulate due to defective activity of the lysosomal proteins responsible for their degradation, usually resulting in a rapidly progressive neurodegenerative disease. For instance, both Tay-Sachs and Sandhoff diseases are caused by the massive accumulation of ganglioside GM2 [50]. It was recently observed that GM2 accumulation in a mouse model of Sandhoff disease inhibited Ca^{2+}-uptake via SERCA, suggesting a direct physiological link between

GM2 accumulation and neuronal cell death mediated via SERCA [27]. Since gangliosides are able to modulate the PMCA as described in the current study, it would be interesting to study the effect of change of ganglioside content on the PMCA in vivo.

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