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The role of ganglioside GM_3 in the modulation of conformation and activity of sarcoplasmic reticulum Ca^{2+}-ATPase

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Abstract

Rabbit sarcoplasmic reticulum does contain trace amounts of gangliosides, and the main species is GM_3. Incorporation of GM_3 into the SR vesicles or addition of it to the soybean phospholipid used for reconstitution of proteoliposomes obviously increased ATP hydrolysis, as well as, Ca^{2+} uptake activity of sarcoplasmic reticulum Ca^{2+}-ATPase. Conformation changes of Ca^{2+}-ATPase induced by GM_3 were also observed by circular dichroism, intrinsic fluorescence and fluorescence quenching measurements. © 1997 Elsevier Science B.V.

Keywords: Ganglioside GM_3; Sarcoplasmic reticulum Ca^{2+}-ATPase; Proteoliposomes; Conformation

1. Introduction

In the recent years, study on the lipid–protein interaction of SR Ca^{2+}-ATPase is being carried out in our laboratory. We found that a proper transmembrane Ca^{2+} gradient may play an important role in modulating the conformation and activity of SR Ca^{2+}-ATPase by mediating the physical state change of the phospholipids [1–5]. It may be deduced that during the contraction–relaxation cycle of muscle cells, in consequence of release of stored Ca^{2+} through the channel, a decrease in transmembrane Ca^{2+} gradient will be occurred, resulting in an increase of membrane fluidity, followed by activating of Ca^{2+}-ATPase, which will uptake Ca^{2+} back into SR and reestablish a Ca^{2+} gradient. This leads to a decrease in lipid fluidity, and hence, a conformation change of Ca^{2+}-ATPase with concomitant inhibition of its activity. It seems that in addition to the direct effect of Ca^{2+} on SR Ca^{2+}-ATPase [6,7], the transmembrane Ca^{2+} gradient-mediated change in fluidity of phospholipids (mainly the inner layer of membrane) may also be involved in such modulation

Abbreviations: *n*-AF, *n*-(9-anthroyloxy) fatty acids; CD, circular dichroism; Cer, ceramide; Gal, galactose; GM_3, NeuNAcα 2 → 3Galβ 1 → 4Glcβ 1 → β 1'Cer; Gls, ganglioside; HB, Hypocrellin B; HPTLC, high performance thin layer chromatography; LBSA, lipid bound sialic acid; NADH, reduced form of nicotinamide adenine dinucleotide; NeuNAc, *N*-acetylneuraminic acid; PM, plasma membrane; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPL, soybean phospholipids; SR, sarcoplasmic reticulum; GT1b, NeuNAcα 2 → 3Galβ 1 → 3GalNAcβ 1 → 4(NeuNAcα 2 → 8NeuNAcα 2 → 3)Galβ 1 → 4Glcβ 1 → 1'Cer; GDla, NeuNAcα 2 → 3Galβ 1 → 3GalNAcβ 1 → 4(NeuNAcα 2 → 3)Galβ 1 → 4Glcβ 1 → 1'Cer; GDlb, Galβ 1 → 3GalNAcβ 1 → 4(NeuNAcα 2 → 8NeuNAcα 2 → 3)Galβ 1 → 4Glcβ 1 → 1'Cer; Glc, glucose; Gls, ganglioside; GM1(= GM1a), Galβ 1 → 3GalNAcβ 1 → 4(NeuNAcα 2 → 3)Galβ 1 → 4Glcβ 1 → 1'Cer

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process. And, PC, mainly located in the inner layer of SR membrane, plays an important role in the modulation of SR Ca^{2+}-ATPase by the transmembrane Ca^{2+} gradient.

The another approach is focusing on the effect of ganglioside GM_3 on the conformation and activity of SR Ca^{2+}-ATPase. This article will present some results of our research on this aspect. Gangliosides have generally been known to be localized primarily in the plasma membrane of most vertebrate cells. Even though the effects of gangliosides on plasma membrane proteins, e.g., Na^+, K^+-ATPase [8], Ca^{2+}-ATPase [9], phospholipase C [10], Ca^{2+} channels [11], protein kinase [12] and epidermal growth factor receptor [13,14], have been well documented in recent years, the distribution and role of gangliosides in the intracellular membranes are still poorly understood. Subcellular contents and distribution of gangliosides have been reported in hepatocytes by Matyas and Moore [15], but their localization and role in the skeletal muscle SR membranes has not been documented so far. Owing to the vital importance of Ca^{2+} as a second messenger, and the pivotal position of SR Ca^{2+}-ATPase in maintaining intracellular calcium homeostasis, here SR Ca^{2+}-ATPase is chosen as the target membrane protein to study its interaction with gangliosides.

In the present paper, the following three questions are answered: (1) Does SR have gangliosides as the topological basis of interaction with SR proteins? If so, what is the main ganglioside species? (2) Has any effect on the activity of SR Ca^{2+}-ATPase following the incorporation of main ganglioside species? (3) Could the main ganglioside species affect the activity and conformation of SR Ca^{2+}-ATPase reconstituted into liposomes?

2. Materials and methods

2.1. Materials

HPTLC plates were from E. Merck, Darmstadt, Gls standards (GM_3, GM_2, GM_1, GD1a, GD1b and GT1b) were from Fidia; octyl glucoside, reactive red-120 agarose, soybean phospholipids and ADP were from Sigma. Sephadex G-50 was from Pharmacia. GM_3 from canine erythrocytes was prepared by the method described by Tsui et al. [16], its purity was 90%. Hypocrellin B (HB) was provided by Prof. Jia-Chang Yue from the Institute of Biophysics, Academia Sinica. Other reagents were commercially available in China and were of AR grade.

2.2. Isolation, purification and HPTLC analysis of SR Gls

This was performed according to the method based on Ladisch and Gillard [17] and modified by Zhang and Tang [18].

2.3. Preparation of SR Ca^{2+}-ATPase

Rabbit SR was prepared according to MacLennan [19], and SR Ca^{2+}-ATPase according to Coll and Murphy [20] with slight modification. The enzyme was purified to homogeneity on SDS-PAGE.

2.4. Preparation of proteoliposome containing SR Ca^{2+}-ATPase

Preparation of SPL proteoliposomes was based on the methods described by Gould et al. [21] and Tu and Yang [1]. The lipid–protein ratio was 100:1 (μmol/μmol). In the preparation of GM_3 containing proteoliposomes, the GM_3/SPL ratio (μmol/μmol) was 0.25:40, 0.5:40, 1.5:40 and 2:40, respectively. Electron microscope examination of the negative stained proteoliposomes revealed well-sealed intact vesicles, indicating successful preparation.

2.5. ATP hydrolysis activity and Ca^{2+} uptake of Ca^{2+}-ATPase

The ATP hydrolysis activity was monitored at 30°C by continuous spectrophotometry following the oxidation of NADH as described by Carafoli et al. [22]. Ca^{2+} uptake activity was measured at 30°C by following the decrease in the absorbance of arsenano III used as Ca^{2+} indicator (675–685 nm) in a Hitachi model 557 spectrophotometer as described by Gould et al. [21].

2.6. Fluorescence measurement

The steady-state intrinsic fluorescence of Ca^{2+}-ATPase was measured using a Hitachi F4010 spectrophotometer equipped with temperature control. Measurement was made in the ratio mode with excitation and emission band widths of 5 nm. Enzyme concentration was 200 μg/ml. Samples were excited at 285 nm, and the emission light monitored at 300–400 nm [23].

2.7. Circular dichroism

CD spectropolarimeter of JASCO-J-500C was used to monitor the CD spectra of Ca^{2+}-ATPase with or without GM_3. CD measurements were carried out in Hepes/KOH buffer at 25°C with path length 0.1 mm. The final spectra were the average of eight scans with time constant, 1 s and scan speed, 100 nm/mm. After correction for appropriate background, the spectra were smoothed by the fast Fourier transform algorithm supplied with the machine. The unknown spectra were matched with the four standard secondary structures by the computer to give their percentage contents.

2.8. Fluorescence decay lifetime determination by nanosecond time-resolved fluorescence technique

The fluorescence decay lifetime of the reconstituted Ca^{2+}-ATPase into liposomes was measured by the nanosecond time-resolved fluorescence system of Edinburgh M299T. The working conditions of the system were: the gas pressure of the hydrogen flash-lamp was 0.5 ps, repetition rate 40 kHz, resolving power was 0.1 ns/ch, range of measurement was 100 ns, Ex was 285 nm, and the emission light was monitored at 340 nm.

The measurement data were stored in a Multi-Channel Analyzer (MCA) to build up a histogram of the fluorescence decay, and analyzed by software FLA-900S which is based on a Marquardt minimization of nonlinear, least-squares fit. The fluorescence decay is given by:

$$I(t) = f_1 e^{-t/\tau_1} + f_2 e^{-t/\tau_2}$$

The fluorescence lifetimes referred to as τ_1 and τ_2 with their proportion f_1 and f_2 are yielded by the above equation.

2.9. Quenching of intrinsic fluorescence of reconstituted Ca^{2+}-ATPase by KI

To 1 ml-reaction mixture containing 200 μg reconstituted Ca^{2+}-ATPase, add KI to a final concentration of 0.5, 5, 50 or 100 mM, and the fluorescence intensities were measured. The Ex and Em were 285 nm and 340 nm, respectively. The buffer was Hepes/KOH buffer containing KCl and $Na_2S_2O_3$. KCl was used to maintain the ionic strength at a constant value in each of the iodide quenched solutions. Trace $Na_2S_2O_3$ was added to the KI stock solution to retard I_3^- formation.

2.10. Quenching of intrinsic fluorescence of reconstituted Ca^{2+}-ATPase by HB [24]

To 1 ml-reaction mixture containing 200 μg reconstituted Ca^{2+}-ATPase, add HB to a final concentration of 1, 2, 4, 8 or 10 μM respectively, and the fluorescence intensities were measured. The Ex and Em were 285 nm and 340 nm, and the measuring temperature was 30°C.

After incubation for 20 min, the measurements were carried out by taking the average reading of the first 20 s. The data were fitted with Stern–Volmer equation [25]:

$$F_0/\Delta F = 1/E + 1/E \cdot K_{sv}[Q] \quad (1)$$

F and F_0 are the intrinsic fluorescence values with and without quenching agent, respectively. $\Delta F = F_0 - F$, $[Q]$ is the concentration of the quenching agent, and E is the quenching efficiency. K_{sv} is the Stern–Volmer quenching constant. K_{sv} and E could be obtained by plotting by $F_0/\Delta F$ against $1/[Q]$.

The dynamic quenching of different fluorescence lifetime components could be calculated by Eq. (2):

$$\tau_{oi}/\tau_i = 1 + K_{svi}[Q] \quad (2)$$

τ_{oi} and τ_i are the fluorescence lifetimes with and without quenching agent, respectively. K_{svi} is the quenching constant of different fluorescence lifetime components, $[Q]$ is the concentration of the quenching agent.

2.11. Measurement of lipid fluidity by n-AF probes

The membrane labeling and polarization measurement of *n*-AF(2-AS, 9-AS, 12-AS, 16-AP) were referred to the previous paper [3].

2.12. Other methods

Lipid bound sialic acid (LBSA) was determined according to Aminoff's method [26]. Protein was determined by Lowry's method [27].

3. Results and discussions

3.1. Rabbit SR does contain gangliosides and the main species is GM_3

Rabbit SR was prepared, purified (purity = 96%) and subjected to total ganglioside extraction and separation. The total ganglioside content, in terms of lipid-bound sialic acid (LBSA), was 0.36 ± 0.02 nmol/mg protein, which was much less than that of the plasma membrane (4.91 ± 0.25 nmol/mg protein). The HPTLC pattern of SR gangliosides revealed that the main ganglioside species was GM_3 [28]. Densitometric scanning of the chromatogram after developing the plate by a resorcinol–HCl spray reagent gave the percentage contents of GM_3 (86%) and GM_1 (8%). Besides, there were traces of unknown components. The HPTLC pattern of plasma membrane glangliosides was different from that of SR and showed that the ganglioside species was GM_3, GM_1, GDla and GDlb (Table 1).

Generally, gangliosides have been known to the localized primarily in the plasma membrane. The above results showed that rabbit skeletal muscle sarcoplasmic reticulum does contain trace amounts of gangliosides (0.36 nmol/mg protein) and the main species is GM_3. Thus, it is reasonable to choose GM_3 as the exogenous ganglioside used in the present study.

Table 1
Contents of gangliosides of plasma membrane and SR vesicles from rabbit skeletal muscle cells

	GM_3	GM_1	GDla	GDlb
	Contents %[a]			
Plasma membrane	52 ± 3.2	20 ± 2.4	26 ± 3.1	3 ± 0.7
SR	86 ± 12	8 ± 1.6	–	–

[a]The percentage contents of individual Gls in total Gls of PM or SR membrane from rabbit skeletal muscle calculated from the results of HPTLC measured by scanning densitometry.

Table 2
Activation of SR Ca^{2+}-ATPase by incorporation of GM_3 into SR vesicles

	Control	$+GM_3$	Increase (%)
ATP hydrolysis (μmol/min mg protein)	0.42	1.11	164
Ca^{2+} uptake (μmol/min mg protein)	0.05[a]	0.11	120

[a]Initial rate.

3.2. Increase in enzyme activity of rabbit SR Ca^{2+}-ATPase following incorporation of exogenous ganglioside GM_3

It was found that if ganglioside GM_3 from canine erythrocytes was incubated with rabbit SR vesicles, a significant incorporation could be observed. The amounts of incorporation were determined both by HPTLC and lipid bound sialic acid (LBSA) analysis. The optimum incorporation was observed when 8 μM GM_3 was incubated with SR vesicles for 90 min at 35°C.

It is interesting to observe that in consequence of incorporation, the ATP hydrolysis and Ca^{2+} uptake activity of SR Ca^{2+}-ATPase were markedly increased (Table 2). Such an activation was also dependent on GM_3 concentration, incubation time and temperature. The optimum activation was observed when 8 μM GM_3 was incubated with SR vesicles for 120 min at 30°C.

3.3. GM_3 markedly increases the activity of reconstituted Ca^{2+}-ATPase into soybean phospholipid (SPL) liposomes

Rabbit SR Ca^{2+}-ATPase was purified and reconstituted into soybean phospholipid liposomes in the presence and absence of GM_3. Electron microscopic examination of the negatively stained proteoliposomes showed the reconstituted vesicles were

well-sealed and intact. Then, ATP hydrolysis activities of reconstituted Ca^{2+}-ATPase in these two kinds of vesicles were determined and compared. From the results shown in Table 3, it can be seen that GM_3 increased ATP hydrolysis in a concentration-dependent manner. The highest enzyme activity was observed in proteoliposomes with a GM_3/SPL ratio of 1:40 (μmol/μmol) [28].

Furthermore, Ca^{2+} uptake of Ca^{2+}-ATPase containing proteoliposomes with or without GM_3 were determined. As shown in Fig. 1, similar to the ATP hydrolysis activity, the Ca^{2+} transport rate of reconstituted Ca^{2+}-ATPase enhanced drastically with increasing in GM_3 concentration. At a GM_3/SPL ratio higher than 1:40 neither Ca^{2+} uptake nor ATP hydrolysis activity of reconstituted Ca^{2+}-ATPase was further increased significantly. The results obtained in the present study clearly showed that addition of GM_3 to the reconstitution system could also markedly increase the ATP hydrolysis as well as Ca^{2+} uptake activity of SR Ca^{2+}-ATPase. This may deduce that GM_3 present in SR is not just acting as a 'passerby' in its transport within the cell, but is involved in the modulation of SR Ca^{2+}-ATPase activity.

Aiming at elucidating the molecular mechanism of GM_3 activation of SR Ca^{2+}-ATPase its conformations in reconstituted proteoliposomes with and without GM_3 have been compared by using CD spectroscopy, steady state fluorescence, nanosecond time-resolved fluorescence and quenching techniques.

Table 3
Effect of GM_3 on ATP hydrolysis activity of reconstituted Ca^{2+}-ATPase into soybean phospholipid (SPL) liposomes

GM_3/SPL (μmol/μmol)	Activity of ATP hydrolysis [a] (μmol/min mg protein)	Increase (%)
0:40	2.20±0.01	
0.5:40	3.12±0.03	41
1:40	7.41±0.02	232
2:40	7.20±0.05	222

[a] Ca^{2+}-ATPase activity was determined by using a coupled enzyme assay in a medium containing 40 mM Hepes/KOH (pH 7.2), 5 mM $MgSO_4$, 2.1 mM ATP, 0.42 mM phosphoenolpyruvate, 0.15 M NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume 2.5 ml. The reaction was initiated by addition of an aliquot of a 25 mM $CaCl_2$ solution.

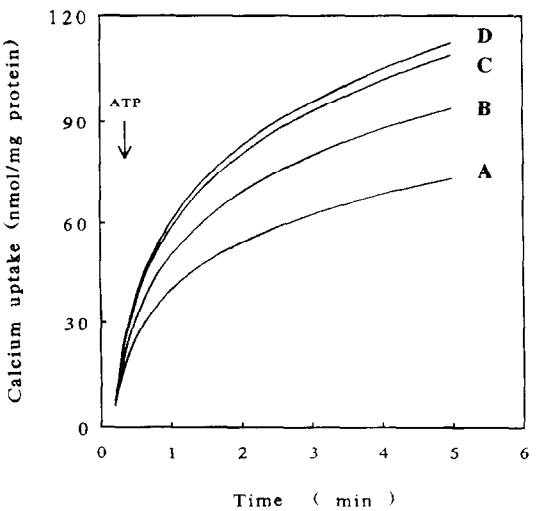

Fig. 1. Effect of GM_3 on Ca^{2+} uptake of reconstituted Ca^{2+}-ATPase into soybean phospholipid (SPL) liposomes. The measurements were carried out at 30°C as described by Gould et al. [21]. The medium contained 40 mM Hepes/KOH, 100 mM KOH, 5 mM $MgSO_4$, 70 μM arsenano III, pH 7.2. Uptaking of Ca^{2+} was initiated by addition of 0.4 mM ATP. A: GM_3/SPL = 0:40, B: GM_3/SPL = 1:80. C: GM_3/SPL = 1:40, D: GM_3/SPL = 1:20.

3.4. CD spectra of reconstituted SR Ca^{2+}-ATPase in the presence and absence of GM_3

CD spectra of reconstituted SR Ca^{2+}-ATPase into SPL liposomes with and without GM_3 in the wavelength range of 200–250 nm were measured. The spectra were matched with four standard secondary structures by computer to estimate their percentage contents. Results showed that GM_3 could significantly increase the content of α-helix, and decrease that of β-turn and random coils.

3.5. Fluorescence study of SR Ca^{2+}-ATPase-containing proteoliposomes with or without GM_3

The fluorescence emission spectra of reconstituted Ca^{2+}-ATPase in proteoliposomes with and without GM_3 were measured with excitation at 285 nm, and difference in emission intensity was recorded at 300–400 nm. The results showed that the intrinsic fluorescence intensity of Ca^{2+}-ATPase increased when GM_3 was added to the system and was GM_3

concentration-depended, but the emission maximum at 340 nm did not shift significantly [28]. Obtained results implied that the microenvironment of Trp residues of Ca^{2+}-ATPase has been altered due to the conformation changes induced by GM_3. Within one molecule of SR Ca^{2+}-ATPase, there are 13 tryptophan residues which produce the intrinsic fluorescence. Results of various experiments have showed that the fluorescence changes of Trp residues are associated with conformational changes of SR Ca^{2+}-ATPase during Ca^{2+} transport [29] and transmembrane Ca^{2+} gradient formation [4].

Time-resolved emission measurement were also carried out in attempt to determine the behavior of tryptophan residues of SR Ca^{2+}-ATPase into SPL liposomes with GM_3. The time-resolved decays of Trps intrinsic fluorescence intensity of the reconstituted Ca^{2+}-ATPase were fit by a doubly exponential lifetime decay curve (Fig. 2). The lifetime shown in Table 4 suggest that SR Ca^{2+}-ATPase contains at least two populations of tryptophan residues, which are characterized by different fluorescence lifetimes, i.e., longer time τ_1 (5.08 ns) and the shorter lifetime τ_2 (1.77 ns). According to the three-dimensional model of Ca^{2+}-ATPase, 10 out of 13 Trps are located in the intramembraneous hydrophobic portion of the protein [30,31]. Various experiments have indicated that these membrane-embedded Trps fall into two categories, residues close to or at the lipid–protein interface (with longer fluorescence lifetime), others are away from the lipid–protein junctions (with shorter lifetime) [32]. From Table 4, it can be seen that GM_3 could significantly prolong both τ_1 (from 5.08 to 8.03 ns) and τ_2 (from 1.77 to 2.64 ns). The change of protein intrinsic fluorescence lifetime is influenced by the polarity of the lipid microenvironment surrounding the tryptophan residue [29]. So, according to the time-resolved fluorescence results it

Table 4
Effect of GM_3 on the time-resolved fluorescence lifetime of Trps of reconstituted Ca^{2+}-ATPase

	Fluorescence lifetime (ns)		
	τ	$\tau_1(f_1)$	$\tau_2(f_2)$
$-GM_3$	3.95	5.08(65.9)	1.77(34.1)
$+GM_3^a$	4.46	8.03(33.8)	2.64(66.2)

(f): Percentage of different fluorescence lifetime components τ_1 and τ_2 of reconstituted Ca^{2+}-ATPase.
[a]Ratio of GM_3/SPL in reconstitution of Ca^{2+}-ATPase was 1:40.

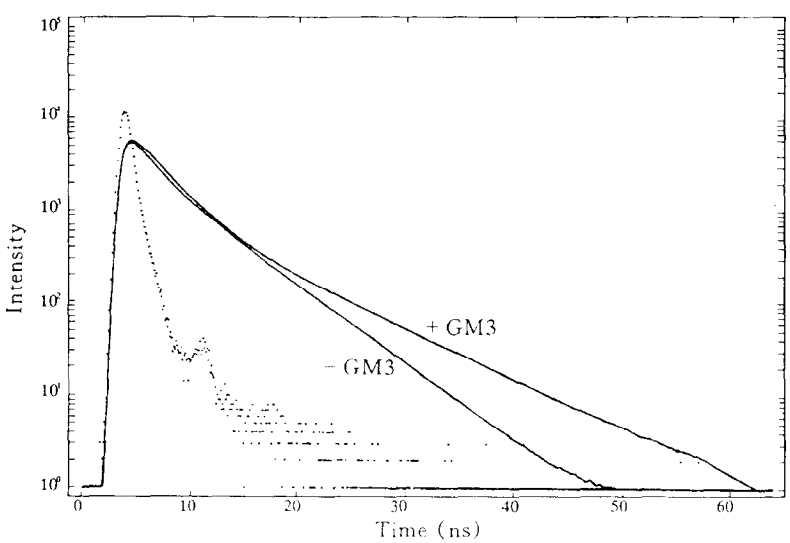

Fig. 2. Effect of GM_3 on the nanosecond-time resolved fluorescence lifetime of Trps of reconstituted Ca^{2+}-ATPase. Continuous curves refer to the best fit of the data with two exponential decays. The sample was excited at 285 nm and a microcuvette was used to minimize scattering and inner filter effects. The membrane protein concentration was 200 μg/ml in the assay medium (40 mM Hepes/KOH, 100 mM KOH, 5 mM $MgSO_4$, 1.01 mM EGTA, pH 7.2). $-GM_3$:(GM_3/SPL: 0:40, μmol/μmol); $+GM_3$:(GM_3/SPL: 1:40, μmol/μmol).

may be deduced that in consequence of the interaction of the hydrophilic oligosaccharide chain and the hydrophobic ceramide moiety of GM_3 with their counterparts in the Ca^{2+}-ATPase the conformation of the membrane domain of the enzyme molecule has been changed. In the following quenchers which selectively quench Trps belonging to different groups were further used to explore the molecular mechanism of GM_3-mediated modulation of SR Ca^{2+}-ATPase.

3.6. Fluorescence quenching study of SR Ca^{2+}-ATPase incorporation vesicles with and without GM_3

Since iodide is an ionic quencher, it normally quenches the fluorescence of surface Trps. The quenching efficiency of KI is proportional to the degree of exposure of the tryptophan residues to the hydrophilic surface. So, the change of quenching efficiency of the Trps intrinsic fluorescence by KI could reflect, to some extent, the conformational change of the hydrophilic domain of the membrane protein. For SR Ca^{2+}-ATPase, only 3 Trps residues located in the hydrophilic region which are considered to be easily accessed by KI [29]. It can be seen in Fig. 3 the Stern–Volmer plot for iodide shows downward curvature. This is commonly seen in proteins containing more than one class of Trps and suggests that a fraction of total emission is not accessible to the quencher. But an obvious difference could be observed between the proteoliposomes with and without GM_3. By fitting up the data with Eq. (1), the quenching efficiency E and the quenching constant K_{sv} obtained are given in Table 5. It can be seen that K_{sv} decreases from 3.34 M^{-1} in SPL proteoliposomes to 2.21 M^{-1} if GM_3 is also present in the vesicles. It may suggest that in consequence of interaction of GM_3 with SR Ca^{2+}-ATPase, the Trp residues in the hydrophilic domain undergo conformational changes, which would lead them being less accessible to the KI. Possibly, this may facilitate ATP to bind to nucleotide binding domain of SR Ca^{2+}-ATPase, resulting in the increase in enzymatic activity. Furthermore, the effect of GM_3 on the quenching of Trp residues in the hydrophobic domain of reconstituted SR Ca^{2+}-ATPase was studied in the following.

Hypocrellin B (HB) is a photosensitive pigment

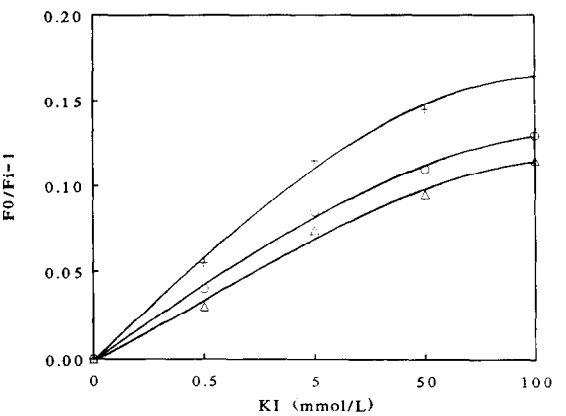

Fig. 3. Stern–Volmer quenching curve of intrinsic fluorescence by KI in Ca^{2+}-ATPase-containing proteoliposomes with or without GM_3. The membrane protein concentration was 200 μg/ml in the Hepes/KOH buffer containing NaCl and $Na_2S_2O_3$. [KCl]+[KI]=0.50 M. The fluorescence intensity was measured at 30°C, the wavelengths of excitation and emission were 285 nm and 340 nm, respectively. The Stern–Volmer plots were drawn by applying the Stern–Volmer equation to the quenching data. + = 0:40; O = 0.5:40: Δ = 1:40 (GM_3/SPL, μmol/μmol).

which derives its name from *Hypocrella banbusae* (B. and Br.) *Sacc.*, a parasitic fungus of *Sinarundinaria* sp. growing in the northwestern region of Yunnan Province in China [33]. It is a peryloquinone derivative (Fig. 4) with excitation wavelength of 490 nm and maximum emission wavelength at 630 nm. It has been reported that HB is mainly incorporated in the lipid bilayer of membrane and could act as a very efficient collisional quencher of Trp residues embedded in the hydrophobic domain of membrane proteins [34]. Besides, no change in enzyme activity of SR Ca^{2+}-ATPase was observed in the presence of HB. Thus it was used in the present study to unravel the effect of GM_3 on the conformation of the hydrophobic domain of reconstituted SR Ca^{2+}-ATPase.

Table 5
Effect of GM_3 on the quenching of intrinsic fluorescence of reconstituted Ca^{2+}-ATPase by KI

	K_{sv} (M^{-1})	E (%)
$-GM_3$	3.34	14.8
$+GM_3$[a]	2.21	10.1

[a]Ratio of GM_3/SPL in reconstitution of Ca^{2+}-ATPase was 1:40.

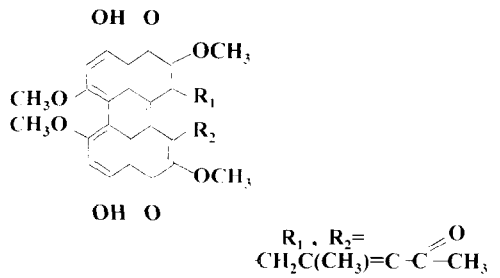

Fig. 4. Structure of hypocrellin B (HB).

Results shown in Fig. 5 demonstrate that the HB quenching of intrinsic fluorescence of reconstituted SR Ca^{2+}-ATPase is concentration-dependent. In the case of the Ca^{2+}-ATPase-containing proteoliposomes reconstituted with SPL alone, the Stern–Volmer quenching constant was 2.05×10^5 M^{-1} and the quenching efficiency might reach 62%. This may deduce that compared to KI, HB is more easily getting accessed to the Trp residues of the hydrophobic domain of reconstituted Ca^{2+}-ATPase. It can clearly be seen from the Table 6 that both quenching constant and quenching efficiency of the reconstituted Ca^{2+}-ATPase-incorporating proteoliposomes in the presence of GM_3 were significantly lower than that in the absence of GM_3. This result may indicate a decrease in accessibility of Trp residues in the presence of GM_3. As the Trps in membrane domain of SR Ca^{2+}-ATPase could be divided into two categories, close to the lipid–protein interface and away from lipid–protein junctions [32]. So in the following, the quenching of fluorescence of the reconstituted Ca^{2+}-ATPase by HB was further studied by time-resolved fluorescence technique. As could be seen from Table 7, the K_{sv} for both τ_1 and τ_2 were significantly reduced in the reconstituted Ca^{2+}-ATPase in the presence of GM_3. This result give us another indication that in consequence of conformation change induced by GM_3, the environment of Trps with longer lifetime (τ_1) and shorter lifetime (τ_2) are both altered. If groups of Trp residues with different lifetimes are thought to be distributed at different location in membrane domain of SR Ca^{2+}-ATPase [29], this may suggest that the Trp residues that undergo conformational changes induced by GM_3 are probably not only these located at the lipid–aqueous environment but also those deeply embedded in the membrane.

Based on the results obtained from the steady-state fluorescence and nanosecond time-resolved fluorescence experiments, it is clearly indicate that GM_3 could induce the conformation changes of both hy-

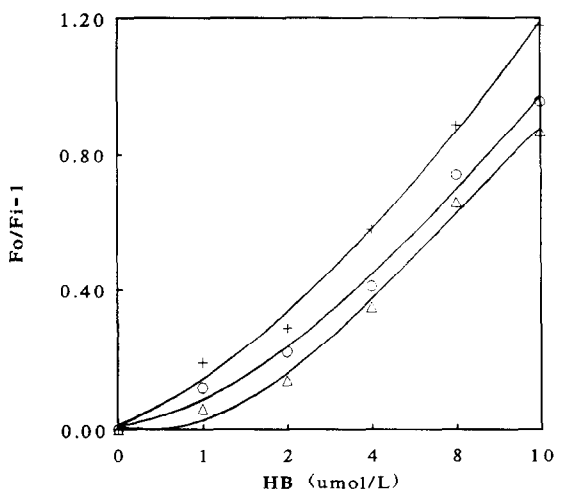

Fig. 5. Stern–Volmer quenching curve of intrinsic fluorescence by HB in Ca^{2+}-ATPase-containing proteoliposomes with and without GM_3. The membrane protein concentration was 200 μg/ml in the Hepes/KOH buffer. The fluorescence intensity was measured at 30°C, the wavelengths of excitation and emission were 285 nm and 340 nm, respectively. The Stern–Volmer plots were drawn by applying the Stern–Volmer equation to the quenching data. + = 0:40; O = 0.5:40; Δ = 1:40 (GM_3/SPL, μmol/μmol).

Table 6
Effect of GM_3 on the quenching of intrinsic fluorescence of reconstituted Ca^{2+}-ATPase by HB

	K_{sv} (M^{-1})	E (%)
$-GM_3$	2.05×10^5	62.18
$+GM_3^a$	1.62×10^5	48.01

[a]Ratio of GM_3/SPL in reconstitution of Ca^{2+}-ATPase was 1:40.

Table 7
Effect of GM_3 on fluorescence quenching parameters of Trp residues in SR Ca^{2+}-ATPase by HB

	K_{sv} (app, M^{-1})	K_{sv} (τ_1, M^{-1})	K_{sv} (τ_2, M^{-1})
$-GM_3$	2.78×10^5	7.03×10^4	5.86×10^4
$+GM_3^a$	2.44×10^5	5.32×10^4	4.12×10^4

[a]Ratio of GM_3/SPL in reconstitution of Ca^{2+}-ATPase was 1:40.

Table 8
The fluorescence polarization measured with *n*-AF of reconstituted Ca^{2+}-ATPase with or without GM_3

Probe	*P*	
	Control	$+GM_3$
2AS	0.235 ± 0.005	0.224 ± 0.004
9AS	0.271 ± 0.004	0.277 ± 0.003
12AS	0.165 ± 0.002	0.186 ± 0.003
16AP	0.151 ± 0.001	0.176 ± 0.004

Fluorescence polarization was measured at 30°C, the excitation and emission wavelength were 365 and 440 nm, respectively. Each number represents an average of results from five experiments.

drophilic and hydrophobic domains of SR Ca^{2+}-ATPase.

3.7. Effect of GM_3 on the physical state of phospholipid of SR Ca^{2+}-ATPase-containing proteoliposomes

The difference in the physical state of phospholipids in the reconstituted SR Ca^{2+}-ATPase with and without GM_3 were measured by using probes of *n*-(9-anthroyloxy) fatty acids.

It is interesting to note from Table 8 that a significant difference can be observed in the case with 2AS, 12AS and 16AP as probe but not by using 9AS. As the activity of delipided SR Ca^{2+}-ATPase could be recovered by treatment with GM_3 (unpublished results). So, it is reasonable to suggest that in addition to the direct effect, GM_3-mediated change in physical state of phospholipids may also be involved in the modulation of reconstituted SR Ca^{2+}-ATPase.

4. Conclusions

To sum up, rabbit sarcoplasmic reticulum does contain trace amounts of gangliosides, and the main species is GM_3. GM_3 can significantly activate Ca^{2+}-ATPase by inducing an optimal conformation with higher activity to maintain the intracellular Ca^{2+} homeostasis.

Although glycosphinoglipids are small molecules compared to proteins and account mostly for only a few percent of the total cell lipid, they comprise 30–60% of the lipid molecules on the outer half of the plasma membrane, forming a sort of sugar lawn on the cell surface, and play important roles in cell differentiation, morphogenesis, and oncogenic transformation [35]. Formation of clusters or aggregation of trace amounts of glycolipids on the cell membrane surface are considered to be important in regulating the structure and function of many membrane proteins. Possibly, some gangliosides may also be highly concentrated in intracellular membranes under certain physiological conditions and modulate functions of subcellular organelles, similar to that in plasma membranes. The present paper clearly showed that incorporation of GM_3 into the SR vesicles or addition of it to the reconstituted system could markedly increase the ATP hydrolysis as well as Ca^{2+} uptake activity of SR Ca^{2+}-ATPase. The results suggest that GM_3 present in SR membranes is not just acting as a 'passerby' in its transport within the cell, but is involved in the modulation of SR Ca^{2+}-ATPase activity.

It is well known that GM_3 is an amphipathic molecule asymmetrically located in the membrane with its hydrophobic moiety, the ceramide, inserted into the lipid core and with hydrophilic oligosaccharide chain protruding into the surrounding medium [36]. It is possible that GM_3-mediated changes in conformation and activity of SR Ca^{2+}-ATPase are modulated either by direct interaction between ganglioside and enzyme molecules or by changes in physical state of lipid, or by both factors. Related further studies are still in progress.

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