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Mechanistic study of modulation of SR Ca^{2+}-ATPase activity by gangliosides GM1 and GM3 through some biophysical measurements

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On the basis of confirming the antagonistic effects of GM1 and GM3 on the activity of Ca^{2+}-ATPase, we further demonstrated that some of the components of these two gangliosides, including sialic acid (NeuNAc), asialo-GM1, asialo-GM3 and ceramide, failed to show any effects on the activity of Ca^{2+}-ATPase. Thus it is apparent that the intact molecules of these two gangliosides with their specific conformations were needed to perform their effects on Ca^{2+}-ATPase. From the fluorescence resonance energy transfer measurements, the energy transfer between Cys 670/674 and Lys 515 was decreased by GM1 and increased by GM3, indicating GM1 induced the conformation of the hydrophilic region of Ca^{2+}-ATPase to be less compact, while GM3 induced it to be more compact. From the CD spectra measurements, GM1 and GM3 both reduced the content of α-helical structures of Ca^{2+}-ATPase, but GM1 caused a stronger decrease than that of GM3. Using DPH as the probe, we found that the membrane lipid fluidity of the proteoliposomes containing Ca^{2+}-ATPase was decreased by GM1 and tend to increase by GM3.

Keywords: **Ca^{2+}-ATPase, circular dichroism, fluorescence resonance energy transfer, ganglioside GM1 and GM3, membrane lipid fluidity, protein conformation, proteoliposomes, sarcoplasmic reticulum**

Abbreviations: **CD, Circular dichroism; DPH, Diphenylhexatriene. FITC, Fluorescein 5′-isothiocyanate; FRET: fluorescence resonance energy transfer; GM1, Galβ1-3 GalNAcβ1-4 (NeuNAcα2-3) Galβ1-4Glcβ1-1′ Cer; GM3, NeuNAcα2-3Galβ1-4Glcβ1-1′ Cer; IAEDANS, 5-[2-((Iodoacetyl)-amino) ethyl] amino-naphthalene-1-sulfonic acid: NeuNAc, N-acetylneuraminic acid; SR, sarcoplasmic reticulum; TLC, thin-layer chromatography**

Introduction

Gangliosides are acidic glycosphingolipids with sialic acid residues covalently attached to the sugar chain. They are implicated in a series of biological processes, such as mediators of cell-cell recognition and adhesion, receptors of some bacterial toxins, modulators and transducers of signal transduction pathways [1–3]. The above functions of gangliosides are mainly derived from the studies on plasma membrane, however, it has been reported that gangliosides are also distributed on subcellular membranes [4,5], and we believe that the subcellular gangliosides are not just act as passers-by on their trafficking route to their plasma membrane destination, they should have their *in situ* functions.

Our group has reported that GM3 and GM1 are the two main gangliosides in the sarcoplasmic reticulum (SR) of rabbit skeletal muscle [4]. Furthermore GM3 was found to activate Ca^{2+}-ATPase both in natural SR vesicles [4,6]. and in proteoliposomes, and GM1 to inhibit it [7]. Since it is known that the ganglioside modulation of protein functions is mediated by their interactions, thus changing the conformation of the protein, and the conformational changes would account for the changes of their functions. Based on this generalization, using some biophysical approaches, including FITC labeling, FRET, CD, and membrane lipid fluidity measurements, we investigated the effects of GM1 and GM3 on the conformation of Ca^{2+}-ATPase. The findings are reported here.

Materials and Methods

Chemicals

GM1 (from bovine brain), ceramide, N-acetylneuraminic acid, NADH, Na_2ATP and soybean phospholipids were purchased from Sigma (St. Louis, MO, USA). Sephadex G-50 was from

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Pharmacia (Uppsala, Sweden). Sodium cholate and deoxycholate were from Fluka (Buchs, Switzerland). High performance TLC plates were from E. Merck (Darmstadt, Germany). GM3 was prepared in this laboratory from canine erythrocytes by the method described by Tsui, et al. [8], its TLC purity was no less than 95%. Asialo-GM1 and asialo-GM3 were obtained by hydrolysis of GM1 and GM3 in 1 M formic acid and identified by TLC. FITC, IAEDANS and DPH were from Molecular Probes (Eugene, USA). Other reagents were commercially available in China and were of AR grade.

Isolation of SR and purification of Ca^{2+}-ATPase

Rabbit SR was prepared according to MacLennan [9], and Ca^{2+}-ATPase was purified according to Banerjee, et al. [10]. The purity of the enzyme was 90–95% as assayed by SDS-PAGE and stained with Coomassie brilliant blue.

Preparation of proteoliposomal Ca^{2+}-ATPase and incorporation of gangliosides

Preparation of soybean phospholipid proteoliposomes was based on the method described by Gould et al [11]. The lipid/protein molar ratio was 1000/1. The incorporation of GM1 or GM3 into proteoliposomes was achieved by adding the desired amounts of gangliosides to the medium (GM1, 10 nmole/mg protein; GM3, 15 nmole/mg protein), followed by incubation at 30°C for 1 hr.

Measurement of Ca^{2+}-ATPase activity

The ATP hydrolysis activity was monitored by continuous spectrophotometry following the oxidation of NADH as described by Froud, et al. [12]. Assay mixture for hydrolysis activity contained 40 mM Hepes/KOH, 5 mM $MgSO_4$, 1.01 mM EGTA, 2.1 mM ATP, 0.47 mM phosphoenolpyruvate, 0.15 mM NADH (pH 7.2, total 2.5 ml). 7 U pyruvate kinase and 12 U LDH were added, the mixture was incubated at 30°C for 5 min, 30 µl 100 mM Ca^{2+} was added to trigger the reaction. The absorbance of 340 nm was measured by a Hitachi UVPC-2010 spectrophotometer. The Ca^{2+} uptake activity was measured by following the decrease in the absorbance of arsenazo III used as Ca^{2+} indicator as described by Gould, et al. [11]. Assay mixture for Ca^{2+} uptake activity contained 40 mM Hepes/KOH, 100 mM KCl, 5 mM $MgSO_4$, 70 mM arsenazo III (pH 7.2, total volume 1 ml). Suitable amounts of the sample and 5 µl 10 mM Ca^{2+} were added. After incubation at 30°C for 5 min, 20 µl 25 mM ATP was added to trigger the reaction, the dual wave-length absorbance of 675–685 nm was measured by a Shimadzu UV-3000 spectrophotometer.

Labeling of IAEDANS

The reconstituted Ca^{2+}-ATPase was labeled with IAEDANS in a medium of 40 mM Hepes, 5 mM $MgSO_4$, 1 mM EGTA, pH 7.4 and 1 mg protein/ml for 30 min at 25°C. IAEDANS was added to the medium at a molar ratio of protein/IAEDANS from 1 : 10 to 1 : 100. Un-reacted label molecules were removed by dialyzing the mixture against the 10 mM Na_2HPO_4 buffer (containing 0.85% NaCl, pH 7.4) for 24 hr at 4°C. The fluorescence emission intensity was measured with Hitachi F4010 fluorometer. Ex and Em wavelengths were 340 and 460 nm, respectively. Excitation and emission slits were of 5 nm and 10 nm, respectively.

Distance estimation using FRET:

FRET was performed according to the method described by Squier [13]. In our experiment, the FITC and IAEDANS were added to the samples, followed by incubation at 30°C for 20 min. IAEDANS was added at a molar concentration 80 times of the protein, and FITC was added from stock solution (2 mM) freshly prepared in the dimethyl formamide at the desired concentration. The reaction mixture was incubated for 30 min at 25°C. The measurement condition is the same as described in IAEDANS labeling experiment. The distance (R) between the donor and acceptor was estimated according to the formula [14]: $R = R_0[(1 - E)/E)]^{1/6}$, where R_0 is the distance giving 50% E (for Ca^{2+}-ATPase, R_0 is 49.0), E is the FRET between IAEDANS and FITC and is equal to $(1\text{-}F/F_0)$, F is the fluorescence yield of IAEDANS-Ca^{2+}-ATPase, and F_0 is the value in the absence of energy transfer acceptors.

Circular dichroism

Hitachi JASCO-J-720 spectrometer was used to record the CD spectra between 190 and 250 nm in a quartz cell 0.1 mm in width. The assay medium contained 20 mM Tris, 5 mM $MgCl_2$, pH 7.2, 0.5 mg protein/ml.

Membrane fluidity measurements

Fluorescence polarization is a parameter negatively correlated to membrane fluidity. The measurement of fluorescence polarization was performed by using Hitachi F4010 fluorometer equipped with a temperature controller. The membrane labeling and polarization measurements of DPH were performed according to Yang, et al. [15]. DPH was dissolved in tetrahydrofuran and added to the medium containing proteoliposome (50 µg protein/ml with and without GM1 or GM3) at a concentration of 1.3 µM. All the samples were incubated for 15 min at 30°C, and then 2 hr at room temperature. The samples were excited at 360 nm, and the emission intensities were measured at 430 nm parallel and perpendicular to the plane of excitation. The polarization (P) was calculated by the equation: $P = (I_{0,0} - GI_{0,90})/(I_{0,0} + GI_{0,90})$, where $G = (I_{90,0}/I_{90,90})$, and I is the intensity.

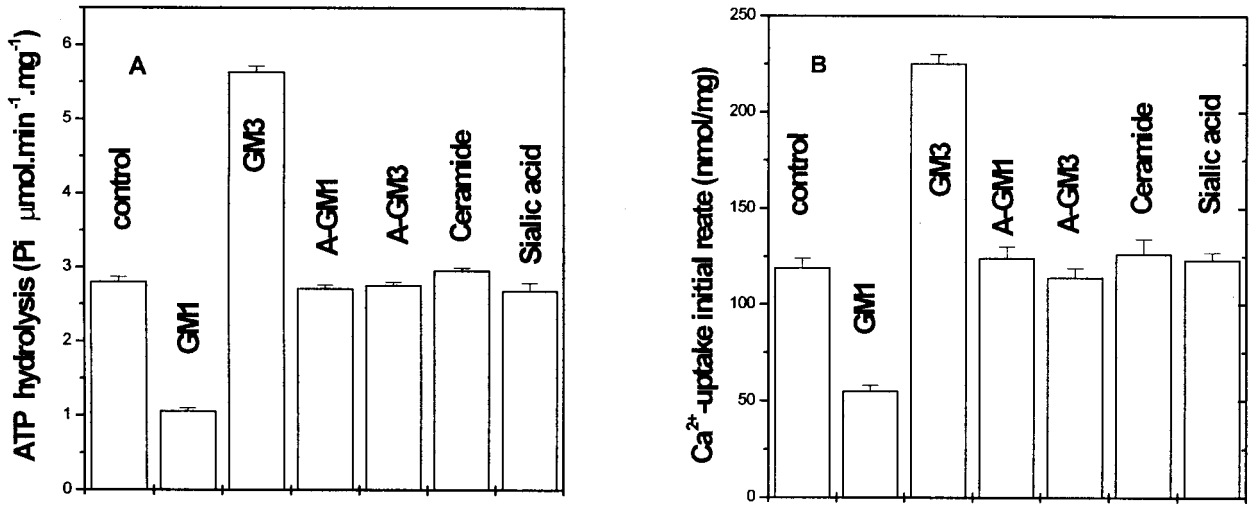

Figure 1. Effect of intact GM1 and GM3 and some components on the activity of reconstituted SR Ca^{2+}-ATPase. In the assay of hydrolysis activity, the reaction mixture containing PEP, NADH, PK, LDH, buffer system and the sample was incubated at 30°C for 5 min, Ca^{2+} was added to trigger the reaction, and the absorbance of 340 nm was measured by a Hitachi UVPC 2010 spectrophotometer. In the assay of Ca^{2+}-uptake activity, the reaction mixture containing enzyme, arsenazo III as indicator, and Ca^{2+} was incubated at 30°C for 5 min, ATP was added to trigger the reaction, the dual wave-length absorbance of 675–685 nm was measured by a Shimadzu UV-3000 spectrophotometer. The ganglioside added was 10 nmole GM1/mg protein or 15 nmole GM3/mg protein. Panel A: ATP hydrolysis, Panel B: Ca^{2+}-uptake. Each datum represents the mean ± SD for 3 experiments.

Results

Effects of intact molecules of GM1 and GM3 and their components on proteoliposomal Ca^{2+}-ATPase activity

GM1 inhibited both ATP hydrolysis and calcium uptake activities (Figure 1) in a concentration dependent manner. Maximum inhibition (ca. 60%) was reached at concentration above 10 nmol GM1/mg protein. However, GM3 stimulated the enzyme also in a concentration dependent manner. When the GM3 concentration was above 15 nmol GM3/mg protein, the enzyme activity doubled. The components of GM1 and GM3 used in this experiment include asialo-GM1, asialo-GM3, sialic acid (NeuNAc) or ceramide failed to show any significant stimulation or inhibition. These results strongly suggest that intact molecules of GM1 and GM3 with their specific conformations are needed for them to function.

The conformational changes of Ca^{2+}-ATPase induced by GM1 and GM3

1. Intensity of Fluorescence emitted from Ca^{2+}-ATPase labeled IAEDANS

IAEDANS is known to covalently modify the sulfhydryl groups of Cys 670/674 located in the hydrophilic region of Ca^{2+}-ATPase [16], and is also known as an extrinsic fluorophore. Figure 2 shows the intensity of fluorescence (Ex = 340 nm, Em = 460 nm) emitted from Ca^{2+}-ATPase labeled with IAEDANS. The enzyme treated by GM1 showed a higher intensity Ca^{2+}-ATPase labeled with IAEDANS. The enzyme treated by GM1 showed a higher intensity than that of control, while that of GM3 was lower. The results indicate that the accessibility of IAEDANS to the sulfhydryl groups in hydrophilic domain of Ca^{2+}-ATPase was increased by GM1, but was decreased by GM3. These changes could be explained

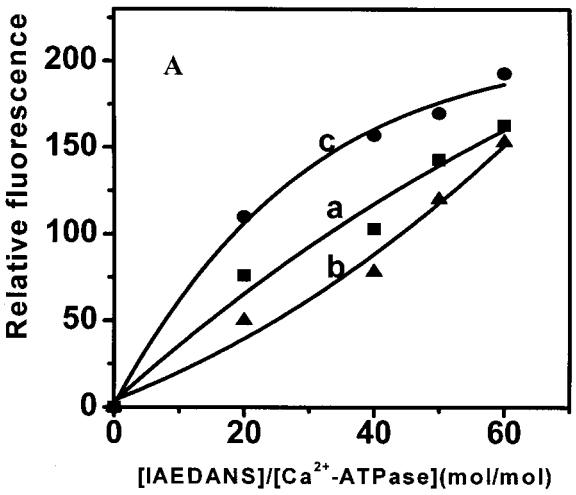

Figure 2. Relative fluorescence of reconstituted SR Ca^{2+}-ATPase labeled with IAEDANS Proteoliposomal Ca^{2+}-ATPase was incubated with 10 nmole GM1/mg protein or 15 nmole GM3/mg protein for 1 hr at 30°C, then followed by labeling with IAEDANS in the medium (pH 7.4) containing IAEDANS for 30 min at 25°C. Unreacted label molecules were removed by dialyzing the mixture against the 10 mM Na_2HPO_4 buffer (containing 0.85% NaCl, pH 7.4) for 24 hr at 4°C. Assay medium contained 40 mM Hepes (pH 7.2), 5 mM $MgCl_2$, 1.1 mM EGTA, 100 μg of Ca^{2+}-ATPase/ml. Ex = 340 nm., Em = 465 nm. The data are average of 5 experiments.

by that GM1 tended to make the microenvironment of Cys 670/674 more accessible to IAEDANS in consequence of conformation change, while GM3 had the opposite effect.

2. The FRET measurement

The emission spectrum of Ca^{2+}-ATPase labeled with IAEDANS at Cys 670/674 can well overlap with the absorption spectrum of FITC labeled at Lys 515, and their distance is less than 100 Å, so energy transfer would happen between IAEDANS and FITC [13,16]. In the Ca^{2+}-ATPase molecule, the distance between Cys 670 and Cys 674 is so small that when they are labeled with IAEDANS, the two donor fluorophores can be considered as one point source [16]. Figure 3 shows the energy transfer efficiency from IAEDANS to FITC. The results indicate that GM1 decreased the energy transfer, while GM3 increased it, suggesting that GM1 lengthened the distance between Cys 670/674 and the ATP binding site Lys 515 (from 56 to 60 Å), while GM3 shortened it (from 56 to 54 Å). Again this could be explained that GM1 made the conformation of the hydrophilic region less compact, and GM3 made it more compact with respect to the control. This is consistent with the results shown in IAEDANS labeling (Figure 2).

3. Effects of GM1 and GM3 on the spectra of CD of Ca^{2+}-ATPase

Figure 4 shows the CD spectra of reconstituted Ca^{2+}-ATPase, the two negative peaks at 210 and 223 nm shows the characteristic spectrum of α-helixes. The figure shows that by GM1 and GM3 treatment of the proteoliposomes, the degrees of ellipse were lowered, i.e., the contents of α-helical structures were decreased in both GM1 and GM3, but GM1 caused a stronger decrease than that of GM3. It may further indicate that the conformations of proteoliposomal Ca^{2+}-ATPase were different in the presence of GM1 or GM3.

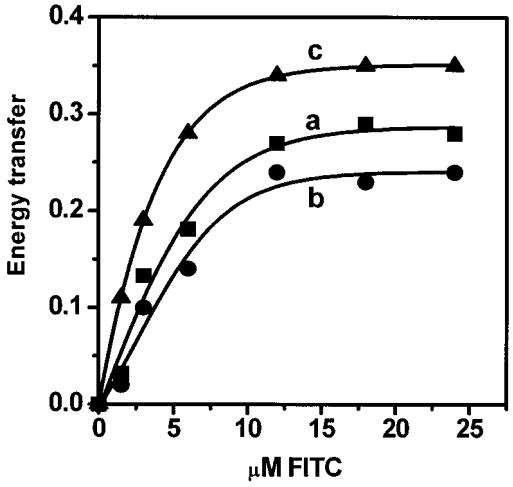

Figure 3. Energy transfer from IAEDANS to FITC in reconstituted SR Ca^2-ATPase Proteoliposomal Ca^2-ATPase was incubated with 10 nmole GM1/mg protein or 15 nmole GM3/mg protein for 1 hr at 30°C. Then the mixture was incubated with both IAEDANS and FITC for 30 min at 25°C (pH 7.4). Un-reacted label molecules were removed by dialyzing the mixture against the 10 mM Na_2HPO_4 buffer (containing 0.85% NaCl, pH 7.4), for 24 hr at 4°C. Assay medium contained 40 mM Hepes (pH 7.2), 5 mM $MgCl_2$, 1.01 mM EGTA, 100 μg of Ca^{2+}-ATPase/ml. Ex = 340 nm., Em = 465 nm. Each datum is the average of 4 experiments.

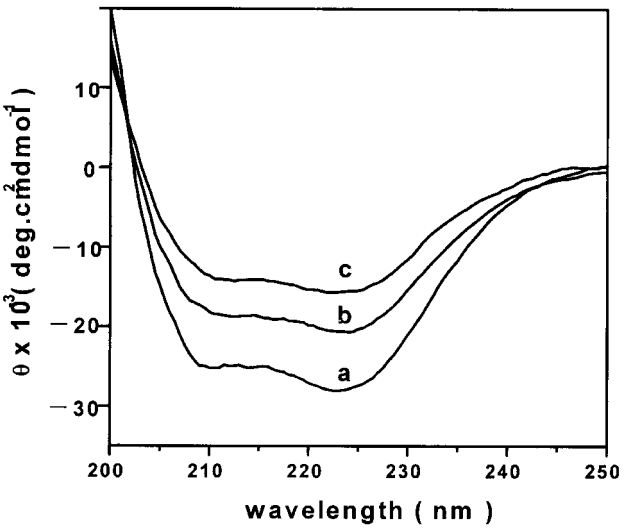

Figure 4. CD spectra of reconstituted Ca^{2+}-ATPase Proteoliposomal Ca^{2+}-ATPase was incubated with 10 nmole GM1/mg protein or 15 nmole GM3/mg protein. The assay medium contained 20 mM Tris, 5 mM $MgCl_2$, pH 7.2, 0.5 mg protein/ml. Hitachi JASCO-J-720 spectrometer was used to monitor the CD spectra. Each spectrum was the average of 6 scans recorded between 190 and 250 nm in a quartz cell 0.1 mm in width.

Effects of GM1 and GM3 on the membrane lipid fluidity on proteoliposomes

The results shown in Table 1 indicate that GM1 increased the fluorescence polarization values of proteoliposomes, while GM3 did not induce significant decrease when compared with the control, but it induced a significant decrease when compared with the GM1 groups. Thus, GM1 decreased the membrane lipid fluidity and GM3 tends to increase it.

Table 1. Effects of GM1 and GM3 on the fluorescence polarization of DPH in proteoliposomes

	P value
Control	0.151 ± 0.006
+ GM1	0.165 ± 0.005*
+ GM3	0.144 ± 0.007**

Each datum is the mean ± SD of five experiments.
* $p < 0.05$ as compared with control. ** $p < 0.05$ as compared with GM1.

Discussion

It is known that 70% of the mass of the Ca^{2+}-ATPase molecule is located in the hydrophilic region, 25% in the transmembrane region [17]. This amphipathic property provides the topological basis for the interaction with another amphipathic molecule, e.g., ganglioside. From Figure 1, we could see that although asialo-GM1 and asialo-GM3 satisfied the amphipathic requirement, they were no longer effective in modulating the activity of Ca^{2+}-ATPase. This points to the importance of sialic acid residues of the intact molecules. Because sialic acid is negatively charged under the experimental conditions (pH 7.4), we propose that the electrostatic forces between the ganglioside and a positive charge in Ca^{2+}-ATPase molecule might play a major role in modulating the activity of the enzyme. However, the detailed molecular mechanism of how GM1 induced a different conformational change of Ca^{2+}-ATPase still remains an open question and deserves further investigation.

The IAEDANS labeling experiment (Figure 2) indicates that GM1 increased and GM3 decreased the accessibility of the extrinsic fluorophore IAEDANS to Cys 670/674 and other sulfhydryl groups. Again when we calculate the stoichiometry of IAEDANS labeled per molecule of Ca^{2+}-ATPase, we got 1.9 (Approx. 2) for the control group, and 3.9 (approx. 4) for the GM1 group, and 1.8 for the GM3 group. This is compatible to the results shown Figure 2 which have been reinforced by the FRET measurements (Figure 3). From Figure 3, GM1 reduced the FRET between Cys 670/674 and Lys 515, and GM3 increased it. These results support the observations shown previously [7]. It reminds us that the changes of enzyme conformations in the opposite directions could match the antagonistic effects of GM3 and GM1 on the activity of Ca^{2+}-ATPase.

The action of gangliosides on Ca^{2+}-ATPase might also be mediated through influencing the membrane matrix where the enzyme is situated. So the physical state of the matrix might play a role in changing the conformation of the membrane enzyme, thus we measured the membrane fluidity of the proteoliposomes. The results indicate that GM1 reduced the membrane fluidity of proteoliposomes, and GM3 had a tendency to increase the membrane fluidity (Table 1) albeit not significant. Since it is the ceramide moiety that is inserted into the membrane, and the degree of unsaturation in ceramide would influence the membrane fluidity. GM1 and GM3 had different effects on this physical parameter. So a question is raised here: is the ceramide moiety of GM1 and GM3 playing the major role in modulating the enzyme activity? From our previous observation [7] that when we used homogeneous GM1 and GM3, which had exactly the same ceramide moiety (fatty acid, 18 : 0; sphingosine, 18 : 1), we got the same antagonistic effects on Ca^{2+}-ATPase activity as the natural heterogeneous ones did. So we can postulate that the ceramide moiety of the ganglioside is not playing the major role. The difference of GM1 and GM3 on the membrane lipid fluidity might come from their difference of the carbohydrate moieties, and this difference can exert its effect through intramolecular cross-talk between the hydrophilic and the hydrophobic moieties. Thus our results support the postulation that the influence of membrane fluidity on the conformation (also the activity) of the Ca^{2+}-ATPase is less important than that of the direct ganglioside-Ca^{2+}-ATPase interactions, and this postulation deserves future investigation.

Here we conclude that: 1) Intact molecules of GM1 made the conformation of the hydrophilic region of the Ca^{2+}-ATPase less compact, and this conformation is not favored by the enzyme for its activity. 2) Intact molecules of GM3 made the Ca^{2+}-ATPase more compact, and this conformation is conducive to the enzyme activity.

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