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Ganglioside GM2 modulates the erythrocyte Ca^{2+}-ATPase through its binding to the calmodulin-binding domain and its 'receptor'

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Abstract

We have previously demonstrated that gangliosides were able to modulate the plasma membrane Ca^{2+}-ATPase (PMCA) from porcine brain synaptosomes and porcine erythrocytes [Y. Zhao, X. Fan, F. Yang, X. Zhang, Arch. Biochem. Biophys. 427 (2004) 204–212 and J. Zhang, Y. Zhao, J. Duan, F. Yang, X. Zhang, Arch. Biochem. Biophys. 444 (2005) 1–6]. The results indicated that the PMCA from porcine erythrocytes responded to gangliosides was different from that from synaptosomes, suggesting that the effects of gangliosides on the PMCA are isoform specific. Most interestingly, GM2 activated the PMCA from porcine erythrocytes at lower concentrations, but inhibited it at higher concentrations. In the present study, we found that GD1b, GM1 and GM3 did not affect the calpain digested PMCA from porcine erythrocytes or the intact enzyme in the presence of calmodulin, while GM2 inhibited it. Moreover, a synthetic peptide of 17 amino acid residues corresponding to the 'receptor' of the calmodulin-binding domain of the enzyme interfered with the inhibition of the enzyme by GM2 in competition assays. Taken together, our results suggested that gangliosides GD1b, GM1, GM2 (lower concentrations) and GM3 stimulated the PMCA by the interaction with calmodulin-binding domain, while the interaction of GM2 with the 'receptor' of the calmodulin-binding domain of the enzyme led to the inhibition of the enzyme.

Keywords: Plasma membrane Ca^{2+}-ATPase; Gangliosides; Monosialoganglioside-G_{M1}; Monosialogangliosides-G_{M2}; Monosialogangliosides-G_{M3}; Disialogangliosides-G_{D1b}

Plasma membrane calcium ATPases (PMCAs)[1] belong to the family of P-type ATPases, and are responsible for the expulsion of the excess Ca^{2+} from the cytosol of all eukaryotic cells during intracellular Ca^{2+} signaling [1,2]. A particularly distinctive feature of the PMCAs is the existence of the calmodulin-binding domain (CBD) located in the C-terminal region [3–5]. The calmodulin-binding domain of the PMCAs acts as an auto-inhibitory domain, binding to 'receptor' sites, i.e. CaM-like sites (CLS) in the second and third cytoplasmic units of the ATPase, in the absence of calmodulin. The interaction of calmodulin would then remove the binding site from its 'receptor', permitting full expression of the ATPase activity.

Gangliosides (sialic acid-containing glycosphingolipids) act as structural components of cell membranes, involving in numerous events, such as cell–cell interaction, adhesion, differentiation, and growth regulation [6,7]. Recent studies have demonstrated that gangliosides have been implicated in regulating Ca^{2+}-homeostasis [8–12]. Of the gangliosides, GM2 is a minor ganglioside in most normal tissues, but is highly expressed in pathological conditions. The massive accumulation of GM2 in the brain causes both Tay–Sachs and Sandhoff disease [13]. Impairments of endoplasmic reticulum Ca^{2+}-ATPase (SERCA) upon GM2 accumulation, subsequent affecting Ca^{2+}-homeostasis were suggested to be of significance for the diseases [14].

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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; CBD, calmodulin-binding domain; CLS, CaM-like sites.

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Since both the SERCA and PMCA are two major regulators of Ca^{2+} clearance mechanisms in cells, it is thus interesting to study the effect of gangliosides, especially GM2 on PMCAs. We have previously shown that gangliosides modulated the ATPase activity of the PMCAs from porcine brain synaptosomes [15] and porcine erythrocytes [16], the molecular mechanisms, however, remained to be elucidated. In the current study we examine the effects of GM2 and other gangliosides on the erythrocyte plasma membrane Ca^{2+}-ATPases, which mainly express the PMCA4 isoform [17,18]. GD1b, GM1 and GM3 stimulated the enzyme, while GM2's effects were biphasic: PMCA was activated at lower concentrations of GM2 and then reversed when GM2 was added at higher concentrations. We also demonstrated that only GM2 was able to bind to the CLS of the PMCA, indicating that the GM2 and other gangliosides modulated the PMCA via different mechanisms.

Materials and methods

Materials

Phosphatidylcholine and phosphatidylethanolamine were from Sigma. Calmodulin-Sepharose CL-4B came from Pharmacia. GD1b, GM1, GM2, GM3, asialo-GM1, ATP and heparin sodium were from Sigma. Bio-Beads were from Bio-Rad. All other reagents used were of analytical grade.

Purification of plasma membrane (Ca^{2+}–Mg^{2+})-ATPase from porcine erythrocyte ghosts

Porcine erythrocyte ghosts were prepared from fresh blood according to the procedure described by Huub Haaker et al. [19] with some modifications. Fresh pig blood (1 L, containing heparin sodium of 0.1–0.2 mg/ml blood) was centrifuged at 5800*g* for 10 min and washed 4 times in 130 mM KCl, 10 mM Tris/HCl (pH 7.4) (Buffer I). Red cells were then lysed in 10× volumes of 1 mM EDTA, 10 mM Tris/HCl (pH 7.4) (Buffer II). The hemolysate was then centrifuged at 20,000*g* for 35 min. The pellet was thoroughly washed several times with Buffer II and 10 mM Hepes/KOH (pH 7.4) (Buffer III). The very firm red pellet (blood blot) was discarded during every step, and the milky pellet (ghosts) on the top of the red part was kept. Finally, the ghosts were resuspended in 10 mM Hepes/KOH (pH 7.4), 130 mM KCl, 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$ (Buffer IV), and stored at −80 °C until use.

Ghosts (6 mg/ml) in 10 mM Hepes/KOH (pH 7.4), 300 mM KCl, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 0.1 mM PMSF, 10 mM of 2-mercaptoethanol and 15% (W/V) glycerol were solubilized by the addition of 0.6% (W/V) Triton X-100 and agitated slowly on ice for 15 min. After centrifugation at 125,000*g* for 30 min, the supernatant was applied onto a CaM-affinity column and washed roughly with a buffer containing 100 μM Ca^{2+}. PMCA was eluted from the column with a buffer containing 2 mM EDTA instead of any Ca^{2+}. After the chromatography column, fractions containing maximum protein concentration and ATPase activity were collected. $MgCl_2$ and $CaCl_2$ were added to the collection to a concentration of 2 mM and 0.05 mM, respectively. Aliquots of the purified PMCA were quickly frozen in liquid N_2, and stored at −80 °C. The protein concentration was determined by using the slight modification of the Lowry et al. procedure to avoid any 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. [20], except that 80 mg/ml Bio-Beads were added to the mixture of phospholipids solution and ATPase every hour for three times and the mixture was agitated slowly at room temperature.

Determination of Ca^{2+}-ATPase activity

Aliquots of reconstituted PMCA (about 10 μg of protein/ml) were incubated in a medium containing 130 mM KCl, 20 mM Hepes/KOH, pH 7.4, 1 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and the appropriate quantity of $CaCl_2$, to obtain the desired free calcium concentrations. The reaction was carried out for 30 min at 37 °C and was stopped by addition of SDS at 1%. The phosphate produced by ATP hydrolysis was determined according to the method of Hergenrother et al. [21]. Appropriate blanks were included to correct any interference with the colorimetric method.

Results

Regulation of the reconstituted PMCA activity by gangliosides

Previous studies have shown that gangliosides modulated the ATPase activity of the PMCA from porcine brain synaptosomes, which contains the mixture of PMCA1–PMCA4 isoforms [15]. To see if the regulation of the PMCA by gangliosides was isoform specific, we have therefore examined the effects of gangliosides on the PMCA from porcine erythrocytes [16], which presumably mainly express the PMCA4 because the isoform composition of PMCA in porcine erythrocytes is not known, but erythrocytes from human contain PMCA1 and PMCA4 in a ratio of about 1:4 [22].

Comparing the regulations of the PMCA from porcine brain synaptosomes with porcine erythrocytes by gangliosides, we found that GD1b activated the PMCA from both synaptosomal plasma membranes and porcine erythrocytes. Unexpectedly; however, after activated at lower concentrations of GM2, the increased activity of enzyme from porcine erythrocytes was then fallen down when GM2 was added at higher concentrations. Moreover, GM1 and GM3 stimulated the enzyme from the erythrocyte, whereas inhibited the PMCA from synaptosomes. The effect of asialo-GM1 on the PMCA from porcine erythrocytes and synaptosomes also behaved differently, i.e. no apparent effect on the erythrocyte PMCA was observed, while asialo-GM1 significantly reduced the PMCA activity from synaptosomes.

GM2 inhibited the reconstituted PMCA activity in the presence of calmodulin

To understand the mechanism by which gangliosides modulated the PMCA activity, the effects of gangliosides on the porcine erythrocyte PMCA in the presence of CaM were studied (Fig. 1). Surprisingly, gangliosides i.e. GD1b, GM1, GM3 and asialo-GM1 had no significant effects on the enzyme, while GM2 reduced the PMCA activity. The result suggested that the stimulation of the PMCA found in our previous study [16] could be similar to that of CaM, i.e. gangliosides interacted with the C-terminal region in the

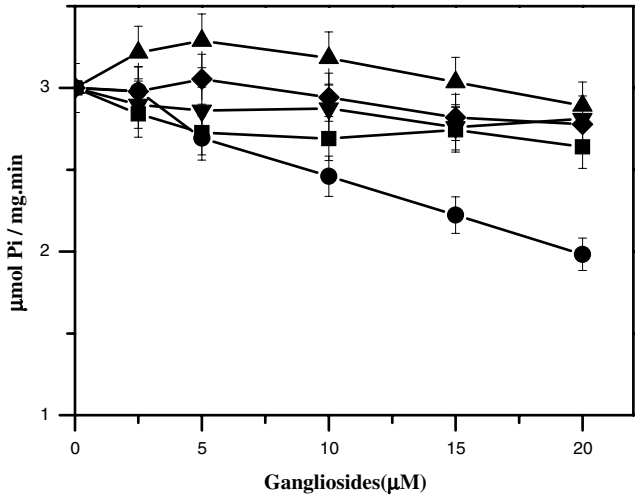

Fig. 1. Effect of gangliosides on the ATPase activity of the reconstituted PMCA from porcine erythrocyte in the presence of calmodulin (2 µg/ml). The reaction medium contained 130 mM KCl, 20 mM Hepes/KOH, (pH 7.4), 1 mM ATP, 1 mM $MgCl_2$, 1 mM EGTA, the amount of $CaCl_2$ to give a final Ca^{2+} concentration of 10 µM with the aid of $CaCl_2$ and EGTA solutions (determined using software available at http://www.stanford.edu/~cpatton/maxc.html) and the indicated concentrations of gangliosides. The reaction was started by the addition of 2.5 µg/ml PMCA-containing proteoliposomes in a final volume of 0.1 ml. After 30 min incubation at 37 °C, the reaction was stopped by the addition of 1.0% SDS. (■), GM1; (●), GM2; (▲), GM3; (▼), GD1b; (◆), asialo-GM1.

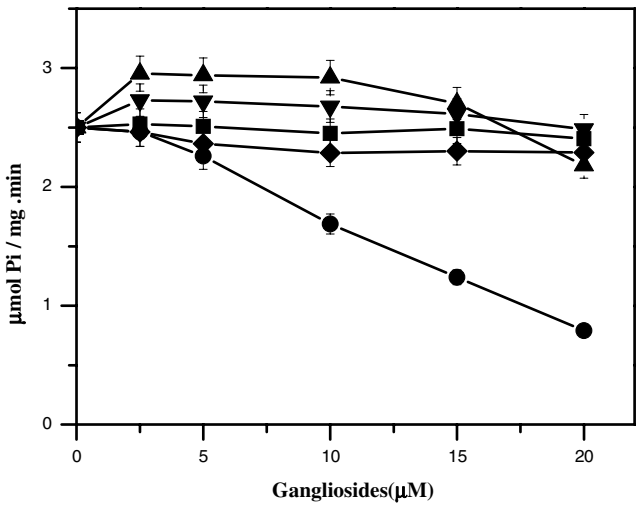

Fig. 2. Effects of gangliosides on the calpain digested PMCA. Different gangliosides at various concentrations were added to the reaction mixture of PMCA stimulated by calpain digestion, the proteolysis reaction was arrested by adding 10-fold (w/w) excess of leupeptin. ATP hydrolysis was measured as in Fig. 1. (■), GM1; (●), GM2; (▲), GM3; (▼), GD1b; (◆), asialo-GM1.

absence of CaM. The inhibition of PMCA activity by GM2 could be caused by its direct binding to CaM or PMCA.

To confirm that gangliosides could interact with C-terminal region of the PMCA to stimulate the enzyme, the effects of gangliosides on the enzyme activated by calpain treatment were examined, with the understanding that selective removal of the CaM-binding domain by calpain causes its activation independently on Ca^{2+}/CaM [23,24]. As shown in the Fig. 2, the calpain-treated enzyme was inhibited by GM2, whereas no apparent effects were observed in the presence of GD1b, GM1 and GM3. This result supported that the binding region of GD1b, GM1 and GM3 may locate at C-terminal region which was removed by calpain. Moreover, Fig. 2 also rules out a possible interaction of GM2 with CaM leading to the inhibitory effects shown in Fig. 1.

Interaction of GM2 with the 'receptor' of the CaM-binding domain

Because it was reported that gangliosides could interact with CaM-like sites (CLS) [25], we hypothesize that the inhibitory effects of GM2 could be due to the interaction of GM2 with the 'receptor' of the CaM-binding domain containing the CLS. Therefore, a peptide (CLS17) consisting of 17 amino

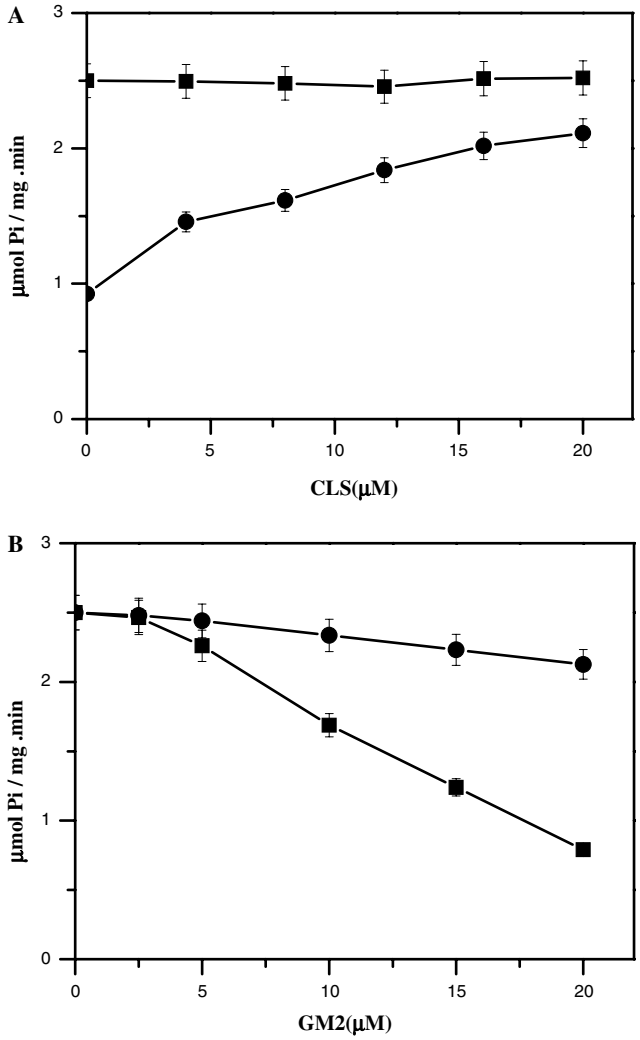

Fig. 3. Interaction of CLS17 with GM2. (A) ATP hydrolysis was measured in the absence (■) and presence of 20 µM GM2 (●). (B) ATP hydrolysis was measured in the absence (■) and presence of 20 µM CLS17 (●). The experimental conditions were as Fig. 1, except calpain digested PMCA was used.

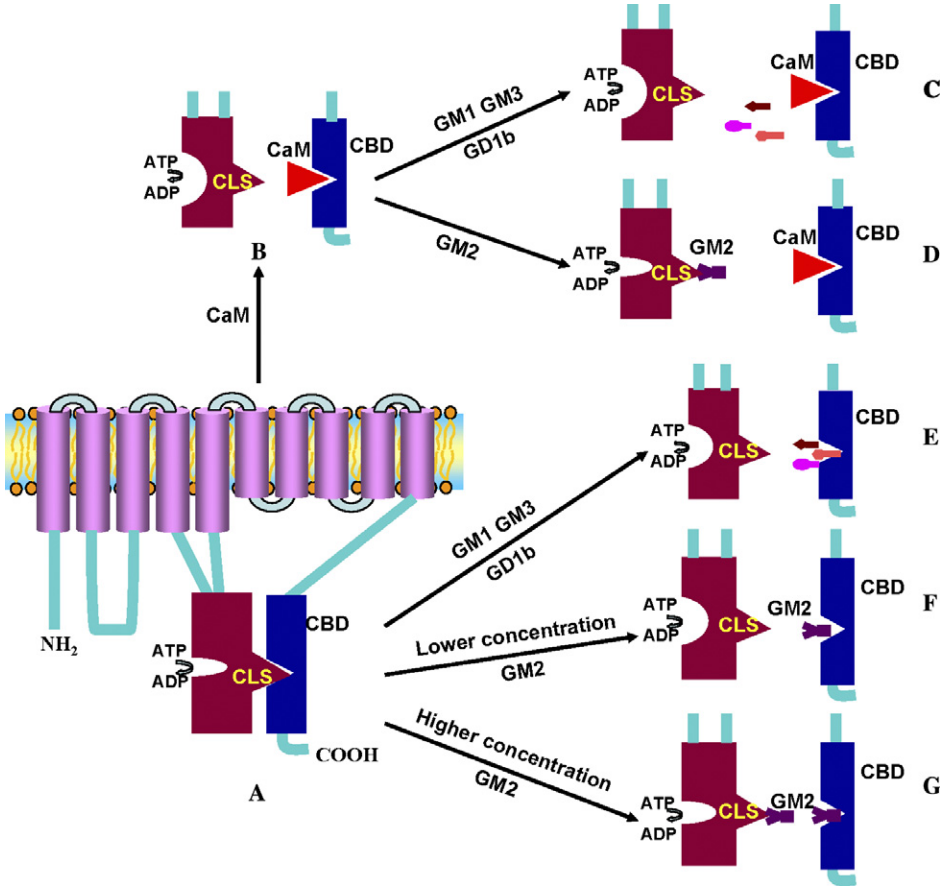

Fig. 4. Proposed mechanism for the modulation of the PMCA by gangliosides. (A) and (B) represent the basal state and CaM stimulated PMCA, respectively. In the presence of CaM, GD1b, GM1 and GM3 no longer interact with CBD (C); GM2 interact with CLS, and result in the inhibition of the PMCA (D). In the absence of CaM, gangliosides stimulated the PMCA by mimicking CaM and interacting with CBD (E); GM2 at lower concentrations mainly interacted with CBD to activate the PMCA (F); GM2 at higher concentrations interacted with both CBD and CLS (G).

acid residues (VGNKTECALLGFVTDLK) corresponding to the 531–547 residues of the PMCA 4b was synthesized [26]. This peptide has been identified to be part of the 'receptor'. Then, competitive experiments were performed to examine whether or not the synthetic peptide could cancel the inhibition of the calpain digested PMCA by GM2. As expected, the enzyme activity was recovered with increasing the peptide concentrations in the presence of GM2 (Fig. 3A), suggesting the interaction of CLS17 with GM2. Moreover, GM2 has no apparent effect on PMCA in the presence of the peptide (Fig. 3B), giving further support that GM2 competes the same region of the PMCA, i.e. the CLS with the peptide.

Discussion

We recently found that gangliosides differently modulated the PMCAs from porcine brain synaptosomes [15] and porcine erythrocytes [16]. Because the same experimental conditions were used in the tow sets of experiments, and the purified PMCAs were reconstituted into the same liposomes, it was unlikely that gangliosides differently interfered with the lipid bilayer surrounding the enzyme in the membrane. Actually, we found that gangliosides had no effects on calpain digested PMCAs (Fig. 2), suggesting that gangliosides mainly affect the cytosolic portions of the enzyme. The observed differences, therefore, could be caused by different isoform compositions in these two tissues. In other words, PMCA isoforms were differently affected by gangliosides. It was reported that the brain contains the most complicated PMCAs ranging from PMCA1–PMCA4, while the PMCA in erythrocytes is relatively simple [17]. Although the isoform composition from porcine erythrocytes is not known yet, it has been determined that the human erythrocytes contain PMCA1 and PMCA4 in a ratio of 1:4. It should be noted that the effect of ethanol on the PMCA was also isoform specific with the greatest effect observed on PMCA2 [27]. We made a comparison of sequence analogy of the PMCA1, PMCA2 and PMCA4. It was found that the C-terminal region of PMCA1 and PMCA4 is very similar, but that of PMCA2 is 36 amino acids less than PMCA4, which might be responsible for the interactions with calmodulin. This analysis suggests that the shorter C-terminal region of PMCA2 would lead to the different response of the PMCA from synaptosomes and erythrocytes to gangliosides.

The mechanism by which gangliosides modulate the PMCA activity from erythrocytes has also been investigated. It has been reported that gangliosides modulated

CaM-dependent enzymes, i.e. cyclic nucleotide phosphordiesterase via the direct interaction with the CaM-binding site [25,28]. Therefore, it was expected that the direct interaction between gangliosides and the PMCA would be occurred in view of the C-terminal region of the PMCA containing the CBD. This conclusion was supported by the experiments on the truncated pump, which strongly suggests that the effect of gangliosides was mediated by the C-terminal region encompassing the CBD (Fig. 2). The CBD of the PMCA acts as an auto-inhibitory domain, binding to the CLS in the second and third cytoplasmic units of the pump, in the absence of CaM. The binding of calmodulin is assumed to displace the calmodulin-binding domain from the receptor site, freeing the pump from the auto-inhibition [29]. In our case, the GD1b, GM1 and GM3 could directly interact with the CBD in a similar manner with CaM to stimulate the PMCA.

Surprisingly, GM2 behaved differently with GD1b, GM1 and GM3 on the PMCA, i.e. it stimulated the enzyme at lower concentrations and the increase was reversed when GM2 was added at higher concentrations. Moreover, GM2 reduced activity of the PMCA in the presence of CaM or the truncated pump. Different experiments have indicated that the CBD interacts with two sites on the resting molecule, locates in the first and second large cytosolic loops. GM2 could influence the association of the CBD with the CLS by directly interacting with the CLS. This conclusion could be supported by the competitive experiment (Fig. 3). The synthetic peptide, corresponding to the 531–547 residues of the PMCA 4b competitively interacts with GM2, leads to the recovery of the PMCA activity. The interaction of GM2 with the CLS would also explain the reduced activity of the PMCA in the presence of CaM or the truncated pump, because GM2 could behave similarly as the CBD binding to the CLS.

Why only GM2 interacted with the CLS is so far not clear. Our previous results [15,16] indicated that the regulation of the PMCA by gangliosides depended upon the defined structural features of the gangliosides. A ganglioside consists of sialic acid residue(s), oligosaccharide and ceramide. Comparing the effects of GD1b, GM1 and asialo-GM1, whose difference is the sialic acid residues on the PMCA, GD1b containing two sialic acid residues showed the greatest stimulation, whereas asialo-GM1 containing no sialic acid had no effect. This result indicated the importance of sialic acid in the regulation of the PMCA. Moreover, GM1, GM2 (lower concentrations) and GM3 increased the PMCA activity with the order of GM1 > GM2 > GM3. Since these monosialogangliosides differ only in the length of their oligosaccharide chain, this suggested the importance of the saccharide structure.

In summary, we proposed a mechanism by which gangliosides modulate the PMCA (Fig. 4). In the absence of CaM, gangliosides, i.e. GD1b, GM1 and GM3 stimulated the PMCA via the interaction with the CBD at the C-terminal tail (Fig. 4E). GM2 behaved similarly at lower concentrations (Fig. 4F), but it could bind to the inhibitory sites-'receptor' of CBD located at CLS at higher concentrations, and reverse the increased enzymatic activity (Fig. 4G). In the presence of CaM, because of the interaction of CaM with CBD, the gangliosides, i.e. GD1b, GM1 and GM3 no longer interact with CBD (Fig. 4C). However, GM2 was able to bind to the 'receptor' of CBD at the CLS, result the inhibition of the PMCA activity (Fig. 4D). The different mechanism by which GM2 and other gangliosides, i.e. GD1b, GM1 and GM3 modulate erythrocyte PMCA remains elusive.

Acknowledgments

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