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Gangliosides activate the phosphatase activity of the erythrocyte plasma membrane Ca^{2+}-ATPase

Jie Zhang, Yongfang Zhao, Jianfa Duan, Fuyu Yang, Xujia Zhang *

National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, P.R.China

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Abstract

The previous studies showed that gangliosides modulated the ATPase activity of the PMCA from porcine brain synaptosomes [Yongfang Zhao, Xiaoxuan Fan, Fuyu Yang, Xujia Zhang, Arch. Biochem. Biophys. 427 (2004) 204–212]. The effects of gangliosides on the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP) catalyzed by the erythrocyte plasma membrane Ca^{2+}-ATPase, which was characterized as E_2 conformer of the enzyme, were studied. The results showed that *p*NPPase activity was stimulated up to sevenfold, depending upon the different gangliosides used with GD1b > GM1 > GM2 > GM3 ≈ Asialo-GM1. Under the same conditions, the ATPase activity was also activated, suggesting that gangliosides should modify both E_1 and E_2 conformer of the enzyme. The Ca^{2+}, which drove the enzyme to E_1 conformation, inhibited the *p*NPPase activity, but with the similar half-maximal inhibitory concentrations (IC_{50}) in the presence and the absence of gangliosides. Moreover, the *p*NPPase activity was also inhibited by the raise in ATP concentrations. Gangliosides caused a large increase in V_{max}, but had no effect on the apparent affinity (K_m) of the enzyme for *p*NPP. The kinetic analysis indicated that gangliosides could modulate the erythrocyte PMCA through stabilizing E_2 conformer.

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 the 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. During the catalytic cycle, the PMCA can exist in two different conformations, E_1 and E_2. The E_1 conformer (Scheme 1) has a high affinity for Ca^{2+} and ATP [3], while the E_2 conformer has a low affinity for Ca^{2+} and ATP [4,5]. It has been found that the PMCA is able to hydrolyze *p*-nitrophenyl phosphate (*p*NPP) in the absence of the nucleotide, which is exclusively attributed to the E_2 conformer of the enzyme [6–8] (Scheme 2).

Gangliosides (sialic acid-containing glycosphingolipids) are the ubiquitous vertebrate glycolipids, and are especially abundant in the plasma membrane of neurons [9–11], where they play different roles in controlling cell growth, cell adhesion, and cell–cell interaction [12,13]. Gangliosides are able to regulate Ca^{2+}-homeostasis by interactions with several proteins, such as a nuclear envelope Na^+–Ca^{2+}-exchanger [14], a Ca^{2+} channel in neuroblastoma cells [15], the PMCA [16], and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) [17–19].

We have previously shown that gangliosides modulated the ATPase activity of the PMCA from porcine brain synaptosomes [16]. In the present report, we examined the effect of gangliosides on the *p*NPPase activity of the erythrocyte plasma membrane Ca^{2+}-ATPase. Our results showed that the *p*NPPase activity was greatly enhanced, suggesting that the gangliosides increased the reactivity of the E_2 conformer of the enzyme.

* Corresponding author. Fax: +86 10 6487 2026.
E-mail address: xjzhang@sun5.ibp.ac.cn (X. Zhang).

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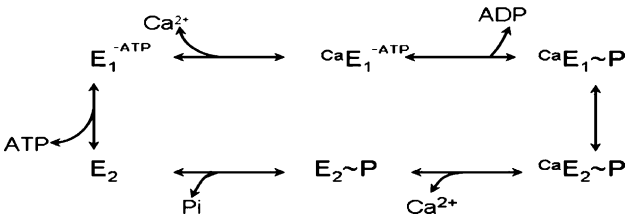
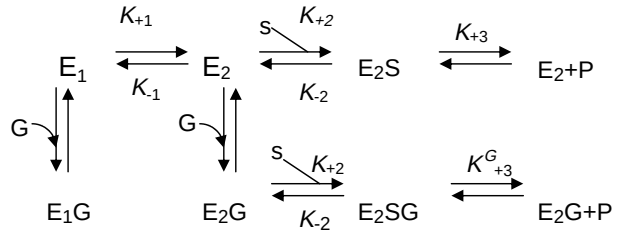


Scheme 1. Simplified catalytic cycle for the plasma membrane Ca^{2+}-ATPase.



Scheme 2. A kinetic scheme for the effects of gangliosides on the PMCA; G is gangliosides.

Materials and methods

Materials

*p*NPP (disodium, hexahydrate) was purchased from Amresco. Phosphatidylcholine and phosphatidylethanolamine were from Sigma. Calmodulin–Sepharose CL-4B came from Pharmacia. GD1b, GM1[1], 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 pig erythrocyte ghosts

Pig erythrocyte ghosts were prepared from fresh pig blood according to the procedure described by Haaker and Racker [20] with some modifications. Fresh pig blood (1 liter, containing heparin sodium of 0.1–0.2 mg/ml blood) was centrifuged at 5800*g* for 10 min and washed 4× 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 in every step, and the milky pellet (ghosts) was kept on the top of the red part. Finally, the ghosts was 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 used.

Ghosts (6 mg/ml) in 10 mM Hepes–KOH (pH 7.4), 300 mM KCl, 1 mM $MgCl_2$, 100 M $CaCl_2$, 0.1 mM PMSF, 10 mM 2-mercaptoethanol, and 15% (w/v) glycerol was solubilized by the addition of Triton X-100 to a final concentration of 0.6% (w/v) and agitated slowly on ice for 15 min. After centrifugation at 1,25,000*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+} and 0.06% (w/v) of Triton X-100. After the chromatography column, fractions containing maximum protein concentration and ATPase activity were collected. $MgCl_2$ and $CaCl_2$ were added to the collections to a concentration of 1 and 0.1 mM, respectively. Aliquots of the purified PMCA were quickly frozen in liquid N_2, and stored at −80 °C. The protein concentration was determined 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. [21], 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 pNPPase activity

For measurements of *p*NPP activity, release of *p*-nitrophenyl (*p*NP) from *p*-nitrophenylphosphate was detected spectrophotometrically at 425 nm [6]. The reaction mixture contained 40 mM Hepes (pH 7.4), 120 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM *p*NPP (buffer V), and incubated for 60 min at 37 °C,unless otherwise indicated. The reaction was stopped by the addition of two volumes of 1 M NaOH. The samples were then centrifuged at 2460*g* for 15 min to remove the membrane debris, and the *p*NP in the supernatant was estimated spectrophotometrically at 425 nm. A standard curve of *p*-nitrophenyl was prepared to convert optical density into micromoles of substrate split per minute per milligram of the enzyme.

Ca^{2+} dependence of the pNPPase activity in the presence of gangliosides

The Ca^{2+} dependence of the *p*NPPase activity was measured in buffer V with different amounts of $CaCl_2$

[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; *p*NPP, *p*-nitrophenyl phosphate.

to result in various free Ca^{2+} concentrations in the presence or absence of 10 μM gangliosides. Defined concentrations of free Ca^{2+} were established with the aid of $CaCl_2$ and EGTA solutions (determined using an algorithm [22] and software available at http://www.stadford.edu/~cpatton/maxc.html). The reaction was started by the addition of the proteoliposomes at 37 °C. The data were fitted to Bolzman equation, and the half-maximal inhibitory concentrations (IC_{50}) for Ca^{2+} were derived. The values shown in the figure are means ± SD for 3 different experiments, using different enzyme preparations.

ATP dependence of the pNPPase activity in the presence of gangliosides

The ATP dependence of the *p*NPPase activity was measured in buffer V with the addition of different concentrations of ATP. The following procedure was similar to the above mentioned.

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 $MgCl_2$, 2 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 0.5%. The phosphate produced by ATP hydrolysis was determined according to the method of Hergenrother and Martin [23]. Appropriate blanks were included to correct any interference with the colorimetric method.

Results and discussion

The impetus for the current study was our earlier observation that gangliosides modulated the ATPase activity of the PMCA from porcine brain synaptosomes [16]. To characterize the catalytic cycle of the PMCA regulated by gangliosides, the hydrolysis of *p*NPP, which has been exclusively attributed to the E_2 conformer of the enzyme was performed in the presence of gangliosides.

Gangliosides stimulate both the ATPase and pNPPase activity of the erythrocyte PMCA

Incubation of the highly purified erythrocyte PMCA reconstituted into liposomes containing phosphatidylcholine with increasing concentrations of gangliosides activated both the Ca^{2+}-dependent ATPase (Fig. 1A) and the *p*NPPase activity (Fig. 1B), although to various extents, depending upon gangliosides (Fig. 1). Among

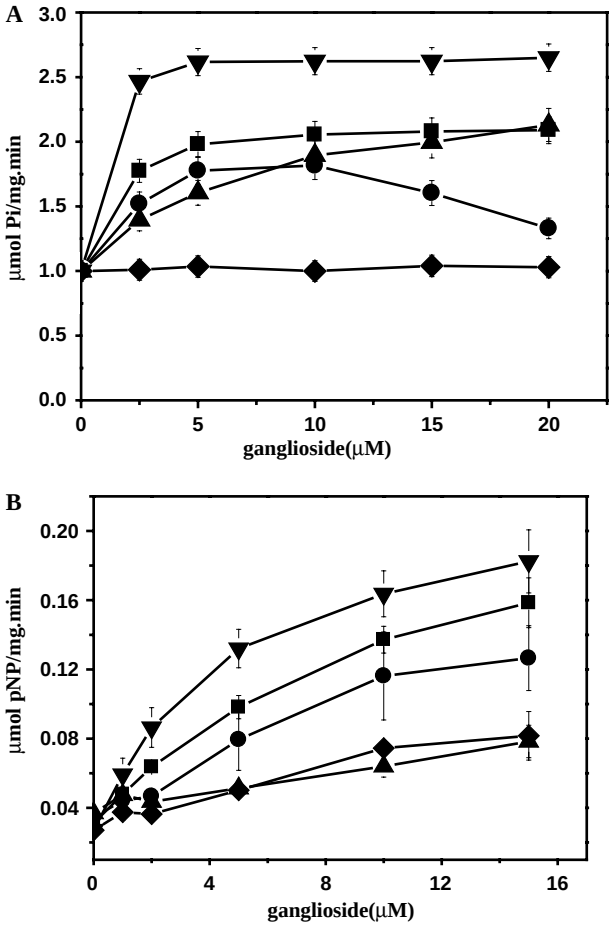


Fig. 1. Comparison of the effects of gangliosides on the ATPase (A) and *p*NPPase (B) activities of the erythrocyte Ca^{2+}-ATPase. (A) ATPase activity. The reaction medium contained 130 mM KCl, 20 mM Hepes–KOH, (pH 7.4), 2 mM ATP, 1 mM $MgCl_2$, 1 mM EGTA, and the amount of $CaCl_2$ to give a final Ca^{2+} concentration of 10 μM and the indicated concentrations of gangliosides. The reaction was started by the addition of 25 μg/ml proteoliposomes in a final volume of 0.1 ml. After 30 min incubation, the reaction was stopped by the addition of 0.5% SDS. (■) GM1, (●) GM2, (▲) GM3, (▼) GD1b, and (◆) Asialo-GM1.(B) *p*NPP activity. The reaction medium contained 40 mM Hepes–KOH, pH 7.4, 130 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM *p*NPP, and the indicated concentrations of gangliosides. Five microliters of proteoliposomes was added to the mixture in a final volume of 200μl. Then the reaction was started by transferring the tube to be incubated at 37 °C for 60 min. Two volumes of NaOH were added into the medium to quench the reaction. After 15 min of centrifugation, the supernatant was spectrophotometrically detected at OD_{425}, (■) GM1, (●) GM2, (▲) GM3, (▼) GD1b, and (◆) Asialo-GM1.

GD1b (two sialic acid residues), GM1 (one sialic acid residue), and Asialo-GM1 (no sialic acid residue), the *p*NPP activity was stimulated in the order of GD1b > GM1 > Asialo-GM1, *p*NPPase activity could be stimulated over 7 folds from 0.027 μmol *p*NPP/mg min to 0.18 μmol *p*NPP/mg min in the presence of 15 μM GD1b. Meanwhile, the oligosaccharide effects by using GM1, GM2, and GM3 whose only difference was in the length of their oligosaccharide chain were

also assessed. The results showed that the GM1, GM2, and GM3 activated the *p*NPPase activity, whereas GM1 was the most potent activator. Taken together, gangliosides, i.e., GD1b, GM1, GM2, GM3, and Asialo-GM1 are able to stimulate the *p*NPPase activity of the PMCA to various extents, suggesting that sialic acid residue(s) and oligosaccharide of gangliosides be important in the modulation of E_2 conformer of the PMCA by gangliosides. So far, no experimental data about the relative concentrations of E_1 and E_2 in the resting ATPase are yet available. Nevertheless, the results shown in Fig. 1 suggest that gangliosides should bind to both E_1 and E_2, based on the observation that gangliosides stimulated both ATPase and *p*NPPase activity. The equilibrium between E_1 and E_2 was not largely altered in the presence of gangliosides.

The effects of gangliosides on the hydrolysis of *p*NPP in the presence of calmodulin were also performed (Fig. 2). It shows that gangliosides inhibit the *p*NPPase activity, which is activated in the absence of calmodulin (Fig. 1B). After modification of E_1 and E_2 conformer, gangliosides affect the E_1 and E_2 conformer differently, and the equilibrium between E_1 and E_2 would be favor to the E_1 in the presence of gangliosides.

It should also 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, but PMCA4 represents at least 80% of the total erythrocyte pump; while PMCA2 and PMCA3 are essentially restricted to the nervous cells with high concentrations of PMCA2 in the cerebellum [24]. The previous study demonstrated that GM1, GM2, and GM3 inhibited the ATPase activity of the PMCA from porcine brain synaptosomes [16]. On the contrary, the current experiments showed that GM1, GM2, and GM3 activated the PMCA. Apparently, our preliminary results indicated that the effects of gangliosides were related to the PMCA isoforms, if presumably, similar isoforms present in the pig erythrocyte to that in the human erythrocyte. Similar observations have been reported that the activation of the PMCA by ethanol was isoform-specific [25].

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

Ca^{2+} is able to drive the PMCA to the E_1 conformation which has the high affinity sites for Ca^{2+} [3,6,8]. Accordingly, the *p*NPPase activity is inhibited by the raise in free Ca^{2+} concentration due to the binding of Ca^{2+} directly to the enzyme, leading to a Ca^{2+} bound form unable to hydrolyze *p*NPP (Fig. 3). The half-maximal inhibitory concentrations (IC_{50}) are $0.233 \pm 0.015\ \mu M$ for GD1b, $0.254 \pm 0.027\ \mu M$ for GM1, and $0.225 \pm 0.028\ \mu M$ for GM3 with respect to the control ($0.278 \pm 0.054\ \mu M$ in the absence of gangliosides). At Ca^{2+} concentrations higher than 1 μM, the *p*NPPase activity was almost completely inhibited. This result indicated that the affinity of E_1 conformer for Ca^{2+} remained unchanged in the presence of gangliosides, suggesting that Ca^{2+} and gangliosides bind different sites of the enzyme.

It is known that *p*NPP and ATP compete for the low affinity site of the PMCA [6,26]. In the presence of gangliosides, the *p*NPPase activity was still inhibited by ATP at a concentration similar to that observed in the absence of gangliosides (Fig. 4). This result shows that

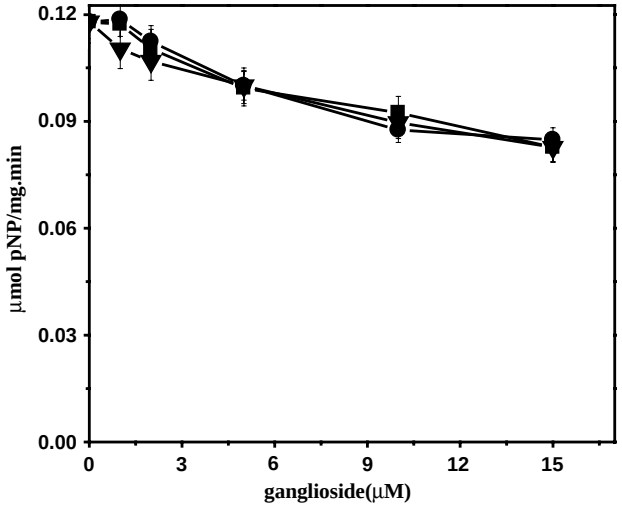


Fig. 2. Inhibition of the hydrolysis of *p*NPP by gangliosides in the presence of calmodulin. Experimental conditions were as in Fig. 1, except that 60 nM calmodulin and appropriate calcium were added into the medium to maximally stimulate the *p*NPPase activity of the plasma membrane Ca^{2+}-ATPase. (■) GM1, (●) GM2, (▼) GD1b.

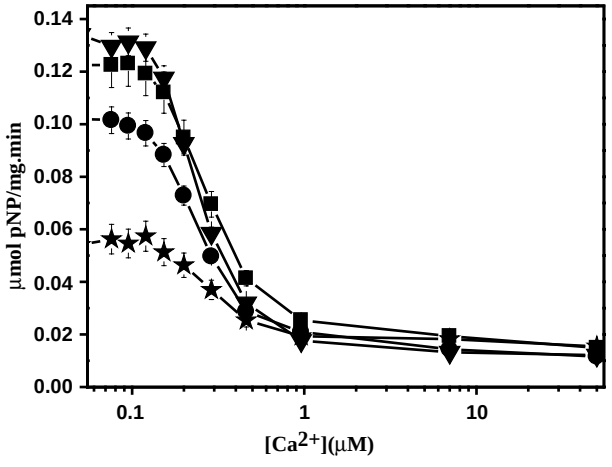


Fig. 3. Ca^{2+} inhibition of the *p*NPPase activity of the erythrocyte Ca^{2+}-ATPase in the presence of gangliosides. Experimental conditions were as in Fig. 1, except that 10 μM gangliosides was added into the medium at indicated free Ca^{2+}concentrations. The final free Ca^{2+} concentration was obtained upon the addition of 1mM EGTA and the calculated quantity of $CaCl_2$. (★) Without gangliosides, (■) GM1, (●) GM2, and (▼) GD1b.

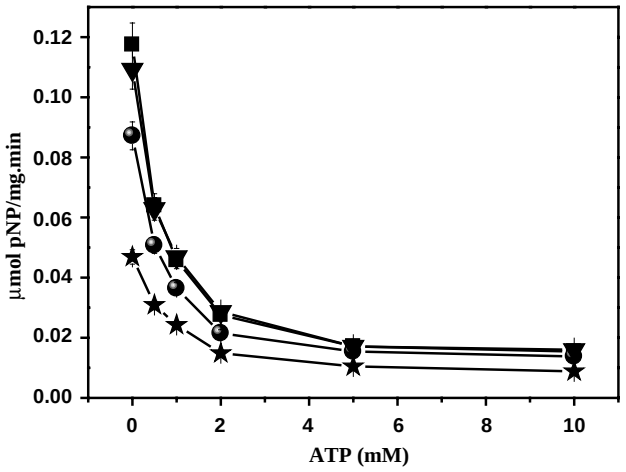


Fig. 4. Inhibition of the hydrolysis of *p*NPP by ATP in the presence of gangliosides. Experimental conditions were as in Fig. 1, except that 10µM gangliosides was added into the medium with indicated ATP concentration in the medium. (★) Without gangliosides, (■) GM1, (●) GM2, (▼) GD1b.

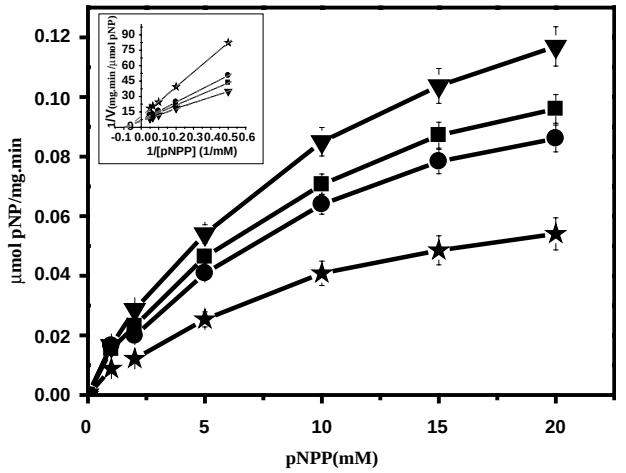


Fig. 5. *p*NPP concentration dependence for hydrolysis of the erythrocyte Ca^{2+}-ATPase in the presence of gangliosides. Experimental conditions were as in Fig. 1, except that 5 µM gangliosides was added into the medium at indicated *p*NPP concentrations. Inset: The double-reciprocal plot of $1/V$ vs $1/[p\text{NPP}]$. (★) Without gangliosides, (■) GM1, (●) GM2, and (▼) GD1b.

gangliosides do not impair the binding of ATP to the low affinity site of the PMCA. The stimulation of the ATPase activity observed in the presence of gangliosides (Fig. 1B) could be due to the increase in the enzyme catalytic efficiency of the enzyme to hydrolyze ATP.

Effect of gangliosides on the affinity of the PMCA for pNPP

The effect of gangliosides on the affinity of the PMCA for *p*NPP was examined (Fig. 5). By fitting the Michaelis–Menten equation (the inset of Fig. 5), V_{max} and K_m are summarized in Table 1. Fig. 5 shows that gangliosides cause a large increase in the V_{max}, but have no effects on the apparent affinity (K_m) of the PMCA for *p*NPP. If we consider that two enzymatic forms, i.e., E_1 and E_2 are in equilibrium, and only the E_2 conformer is active in the *p*NPP hydrolysis, then

$$E_1 \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} E_2 \underset{K_{-2}}{\overset{S\ K_{+2}}{\rightleftharpoons}} E_2S \overset{K_{+3}}{\rightleftharpoons} E_2{+}P$$

On solving the rate equations for the steady state, the K_m can be described by the equation

$$K_m = [(k_{-2} + k_3)/k_2](1 + k_{-1}/k_1). \quad (1)$$

On the basis of the observation that gangliosides stimulate both the ATPase and the *p*NPPase activity (Fig. 1), and the affinity for the Ca^{2+} is independent upon the gangliosides, i.e., IC_{50} is not changed (Fig. 3), the k_{-1}/k_1 should keep unchanged in the course of gangliosides. Considering normally $k_3 \ll k_{-2}$, the independence of the K_m for *p*NPP upon gangliosides suggests that E_2–E_2S are not affected, i.e., k_{-2}/k_2 is not changed. Therefore, the increase in the V_{max} indicated that the modified E_2 conformer by gangliosides hydrolyzed *p*NPP with a higher rate than the unmodified E_2, suggesting the increase in the catalytic constant (k_3 in Eq. (1)).

Conclusion

Taken together, we propose a kinetic scheme for the effects of gangliosides on the PMCA.

The kinetic analysis indicates that gangliosides could bind to both E_1 and E_2 conformer, but the modified E_2 (E_2G) has the same affinity for the substrate (*p*NPP), therefore, the K_m is not affected. The larger K^G_{+3} leads to the increase in the V_{max}.

In summary, gangliosides could modulate the erythrocyte PMCA through modifying E_2 conformer. The previous studies provided evidence that the effect of gangliosides could be due to the direct interaction with

Table 1
Kinetic parameters for *p*NPP hydrolysis by the erythrocyte Ca^{2+}-ATPase in the presence of gangliosides

	Control	GM1	GM2	GD1b
K_m (µM)	13.11 ± 0.498	10.71 ± 0.244	12.15 ± 0.234	9.77 ± 0.825
V_{max}	0.092 ± 0.003	0.147 ± 0.002	0.141 ± 0.002	0.168 ± 0.011

The values for K_m and V_{max} were obtained from fitting Michaelis–Menten equation by a double-reciprocal plot (inset of Fig. 4). The data are presented as means± SD of experiments with three different preparations. K_m is expressed in µM. V_{max} is expressed in µmol *p*NPP/mg min.

Calmodulin-binding domain in a similar manner with calmodulin to stimulate the PMCA [16]. It has been reported that calmodulin could stabilize E_2 conformer, and activated the *p*NPPase activity [6,27–29]. Following the same line of calmodulin vs the *p*NPPase activity of the PMCA, it is reasonable to propose that the stabilization of the E_2 conformer with gangliosides would lead to the activation of the erythrocyte PMCA.

Acknowledgments

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