Gangliosides activate the phosphatase activity of the erythrocyte plasma membrane Ca\textsuperscript{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

Received 20 June 2005, and in revised form 12 July 2005
Available online 8 August 2005

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 (pNPP) catalyzed by the erythrocyte plasma membrane Ca\textsuperscript{2+}-ATPase, which was characterized as E\textsubscript{2} conformer of the enzyme, were studied. The results showed that pNPPase 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\textsubscript{1} and E\textsubscript{2} conformer of the enzyme. The Ca\textsuperscript{2+}, which drove the enzyme to E\textsubscript{1} conformation, inhibited the pNPPase activity, but with the similar half-maximal inhibitory concentrations (IC\textsubscript{50}) in the presence and the absence of gangliosides. Moreover, the pNPPase activity was also inhibited by the raise in ATP concentrations. Gangliosides caused a large increase in V\textsubscript{max}, but had no effect on the apparent affinity (K\textsubscript{m}) of the enzyme for pNPP. The kinetic analysis indicated that gangliosides could modulate the erythrocyte PMCA through stabilizing E\textsubscript{2} conformer.

Keywords: Plasma membrane Ca\textsuperscript{2+}-ATPase; Gangliosides; Monosialoganglioside-G\textsubscript{M1}; Monosialogangliosides-G\textsubscript{M2}; Monosialogangliosides-G\textsubscript{M3}; Disialogangliosides-GD1b

The plasma membrane Ca\textsuperscript{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\textsuperscript{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\textsubscript{1} and E\textsubscript{2}. The E\textsubscript{1} conformer (Scheme 1) has a high affinity for Ca\textsuperscript{2+} and ATP [3], while the E\textsubscript{2} conformer has a low affinity for Ca\textsuperscript{2+} and ATP [4,5]. It has been found that the PMCA is able to hydrolyze p-nitrophenyl phosphate (pNPP) in the absence of the nucleotide, which is exclusively attributed to the E\textsubscript{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\textsuperscript{2+}-homeostasis by interactions with several proteins, such as a nuclear envelope Na\textsuperscript{+}–Ca\textsuperscript{2+}-exchanger [14], a Ca\textsuperscript{2+} channel in neuroblastoma cells [15], the PMCA [16], and the sarcoplasmic reticulum Ca\textsuperscript{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 pNPPase activity of the erythrocyte plasma membrane Ca\textsuperscript{2+}-ATPase. Our results showed that the pNPPase activity was greatly enhanced, suggesting that the gangliosides increased the reactivity of the E\textsubscript{2} conformer of the enzyme.
Materials and methods

Materials

\( pNPP \) (disodium, hexahydrate) was purchased from Amresco. 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 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 times in 130 mM KCl, 10 mM Tris–HCl (pH 7.4) (buffer I). Red cells were then lysed in 10 \( v/v \) 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 mM 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 \( \mu \)M 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 pNPP activity, release of \( p \)-nitrophenol (\( p \)-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 \( p \)-nitrophenol 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 pNPPase activity was measured in buffer V with different amounts of CaCl\(_2\).
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.stanford.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 pNPPase 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 pNPP, 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 pNPPase activity (Fig. 1B), although to various extents, depending upon gangliosides (Fig. 1). Among GD1b (two sialic acid residues), GM1 (one sialic acid residue), and Asialo-GM1 (no sialic acid residue), the pNPPase activity could be stimulated over 7 folds from 0.027 μmol pNPP/mg min to 0.18 μmol pNPP/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 pNPPase 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 pNPPase activity of the PMCA to various extents, suggesting that sialic acid residue(s) and oligosaccharide of gangliosides be important in the modulation of E2 conformer of the PMCA by gangliosides. So far, no experimental data about the relative concentrations of E1 and E2 in the resting ATPase are yet available. Nevertheless, the results shown in Fig. 1 suggest that gangliosides should bind to both E1 and E2, based on the observation that gangliosides stimulated both ATPase and pNPPase activity. The equilibrium between E1 and E2 was not largely altered in the presence of gangliosides.

The effects of gangliosides on the hydrolysis of pNPP in the presence of calmodulin were also performed (Fig. 2). It shows that gangliosides inhibit the pNPPase activity, which is activated in the absence of calmodulin (Fig. 1B). After modification of E1 and E2 conformer, gangliosides affect the E1 and E2 conformer differently, and the equilibrium between E1 and E2 would be favor to the E1 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 Ca2+ and ATP dependencies of pNPPase activity

Ca2+ is able to drive the PMCA to the E1 conformation which has the high affinity sites for Ca2+ [3,6,8]. Accordingly, the pNPPase activity is inhibited by the raise in free Ca2+ concentration due to the binding of Ca2+ directly to the enzyme, leading to a Ca2+ bound form unable to hydrolyze pNPP (Fig. 3). The half-maximal inhibitory concentrations (IC50) are 0.233 ± 0.015 μM for GD1b, 0.254 ± 0.027 μM for GM1, and 0.225 ± 0.028 μM for GM3 with respect to the control (0.278 ± 0.054 μM in the absence of gangliosides). At Ca2+ concentrations higher than 1 μM, the pNPPase activity was almost completely inhibited. This result indicated that the affinity of E1 conformer for Ca2+ remained unchanged in the presence of gangliosides, suggesting that Ca2+ and gangliosides bind different sites of the enzyme.

It is known that pNPP and ATP compete for the low affinity site of the PMCA [6,26]. In the presence of gangliosides, the pNPPase activity was still inhibited by ATP at a concentration similar to that observed in the absence of gangliosides (Fig. 4). This result shows that
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 pNPP was examined (Fig. 5). By fitting the Michaelis–Menten equation (the inset of Fig. 5), $V_{\text{max}}$ and $K_m$ are summarized in Table 1. Fig. 5 shows that gangliosides cause a large increase in the $V_{\text{max}}$, but have no effects on the apparent affinity ($K_m$) of the PMCA for pNPP. 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 pNPP hydrolysis, then

\[
\begin{align*}
E_1 & \xrightleftharpoons[k_1]{K_1} E_2 \\
& \quad \overset{s}{\longrightarrow} K_2 E_2S \\
& \quad \overset{K_3}{\longrightarrow} E_2+P
\end{align*}
\]

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

\[
K_m = \frac{[(k_{-2} + k_3)/k_2](1 + k_{-1}/k_1)}{K_1 K_2 K_3}.
\]  

On the basis of the observation that gangliosides stimulate both the ATPase and the pNPPase 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 $K_m$ should keep unchanged in the course of gangliosides. Considering normally $k_3 \ll k_{-2}$, the independence of the $K_m$ for pNPP upon gangliosides suggests that $E_2$–E$_2$S are not affected, i.e., $k_{-2}/k_2$ is not changed. Therefore, the increase in the $V_{\text{max}}$ indicated that the modified $E_2$ conformer by gangliosides hydrolyzed pNPP 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$_2$G) has the same affinity for the substrate (pNPP), therefore, the $K_m$ is not affected. The larger $K_m$ leads to the increase in the $V_{\text{max}}$.

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GM1</th>
<th>GM2</th>
<th>GD1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (μM)</td>
<td>13.11 ± 0.498</td>
<td>10.71 ± 0.244</td>
<td>12.15 ± 0.234</td>
<td>9.77 ± 0.825</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>0.092 ± 0.003</td>
<td>0.147 ± 0.002</td>
<td>0.141 ± 0.002</td>
<td>0.168 ± 0.011</td>
</tr>
</tbody>
</table>

The values for $K_m$ and $V_{\text{max}}$ 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. $V_{\text{max}}$ is expressed in μmol pNPP/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 pNPPase activity [6,27–29]. Following the same line of calmodulin vs the pNPPase 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

This work was supported by Chinese National Science Foundation (30230120) and National Basic Research Program of China (2004CB720000).

References