FEBS Letters 457 (1999) 144–148 FEBS 22474

# Antagonistic effect of ganglioside GM1 and GM3 on the activity and conformation of sarcoplasmic reticulum $Ca^{2+}$-ATPase

Yuling Wang[a], Zhaochun Tsui[a], Fuyu Yang[b,*]

[a] *Department of Biochemistry, Dalian Medical University, Dalian 116027, China*
[b] *National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, 15 Datun Road, Beijing 100101, China*

Received 12 July 1999

**Abstract It was found that rabbit skeletal muscle sarcoplasmic reticulum (SR) contained two main gangliosides: NeuNAcα 2→3 Galβ 1→4 Glcβ 1→1′ceramide (GM3) and Galβ 1→3 GalNAcβ 1→4(NeuNAcα 2→3) Galβ 1→4 Glcβ 1→1′ceramide (GM1), and that the most abundant ganglioside GM3 could positively modulate the SR $Ca^{2+}$-ATPase activity. In this paper, the effect of GM1 on $Ca^{2+}$-ATPase was further investigated and compared with that of GM3. The study demonstrates that GM1 has an opposite effect with respect to GM3 on the activity of SR $Ca^{2+}$-ATPase. Using assays, including intrinsic and time-resolved fluorescence and fluorescence quenching, the conformational changes of SR $Ca^{2+}$-ATPase induced by GM1 and GM3 were compared. Obtained results indicate that GM1 could make the $Ca^{2+}$-ATPase molecules less compact in the hydrophilic domain but more compact in the hydrophobic domain, while GM3 makes the enzyme more compact in both the hydrophilic and hydrophobic domain. Homogeneous GM1 and GM3 with the same ceramide moiety had similar effects on SR $Ca^{2+}$-ATPase activities compared to their natural counterparts, suggesting that the carbohydrate chain may be the key moiety of the ganglioside molecule to be responsible for the difference of the effect on enzyme activity.**



*Key words:* Sarcoplasmic reticulum $Ca^{2+}$-ATPase; Ganglioside GM3; Ganglioside GM1; Proteoliposomal $Ca^{2+}$-ATPase; Conformation

## 1. Introduction

Gangliosides have been reported to be related to a variety of biological processes such as cellular recognition and adhesion, transmembrane signal transduction, growth regulation, proliferation and differentiation and modulation of enzyme activity [1–4]. Although the detailed mechanism of the above-mentioned ganglioside functions still remains elusive, it is generally accepted that the gangliosides could function at least in some cases through their interactions with their target proteins, changing the conformation of the latter. This postulation is supported by our previous observations that NeuNAcα 2→3 Galβ 1→4 Glcβ 1→1′ceramide (GM3) could alter the conformation of sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase and increase the enzyme's activity [4]. Since Galβ 1→3 GalNAcβ 1→4(NeuNAcα 2→3) Galβ 1→4 Glcβ 1→1′ceramide (GM1) is the second abundant ganglioside in SR, just next to GM3, it is quite logical to ask: does GM1 have any effect on SR $Ca^{2+}$-ATPase? To answer this question, the present study has been carried out. We have found that GM1 had opposite effects with respect to GM3 on the activity and conformation of SR $Ca^{2+}$-ATPase.

## 2. Materials and methods

### 2.1. Materials

GM1 (from bovine brain), NeuNAc, ceramide, neuraminidase and soybean phospholipids were purchased from Sigma. High performance thin layer chromatography (HPTLC) plates were from E. Merck, Darmstadt, Germany. GM3 was prepared from canine erythrocytes by the authors' laboratory [5], 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. GM1 (c18:0-d18:1) was purified from bovine brain with purity over 98% in Dr. Sandro Sonnino's laboratory and was kindly donated to the authors. GM3 (c18:0-d18:1) was purchased from Biorain (Japan). Hypocrellin B (HB) was prepared by Prof. Jiachang Yue of the Institute of Biophysics, Academia Sinica according to the method described in [6]. Other reagents were commercially available in China and were of AR grade.

### 2.2. Isolation of SR and purification of $Ca^{2+}$-ATPase

The rabbit SR was prepared according to MacLennan [7] and $Ca^{2+}$-ATPase was purified to homogeneity on SDS-PAGE according to Coll and Murphy [8].

### 2.3. Preparation of proteoliposomes containing SR $Ca^{2+}$-ATPase

Preparation of proteoliposomes was based on the methods described by Gould et al. [9] and Tu and Yang [10]. The lipid/protein ratio was 1000:1 (mol/mol). The incorporation of GM1 or GM3 into SR or proteoliposomes was achieved by adding the desired amounts of gangliosides, followed by incubation at 30°C for 1 h.

### 2.4. Measurement of $Ca^{2+}$-ATPase activities

The ATP hydrolysis activity was monitored at 30°C by using a coupled enzyme assay as described by Froud et al. [11]. $Ca^{2+}$ uptake activity was measured at 30°C by dual wavelength spectrophotometer using arsenazo III as $Ca^{2+}$ indicator as described by Gould et al. [9].

### 2.5. Steady-state intrinsic fluorescence measurement

The intrinsic fluorescence of $Ca^{2+}$-ATPase was measured with a Hitachi F4010 spectrophotometer. The samples were excited at 285 nm and the fluorescence emission spectra were recorded in the 300–400 nm range [12].

### 2.6. Measurement of fluorescence decay lifetime

The intrinsic fluorescence lifetime of $Ca^{2+}$-ATPase was determined from the phase decay at 40 MHz frequency in an Edinburgh 299T nanosecond fluorometer. The samples were excited at 285 nm and the emissions were monitored at 335 nm. The protein concentration was 0.3 mg/ml. Time-resolved data were analyzed as described by Vos et al. [13], the lifetimes yielded were referred to as $\tau_1$, $\tau_2$ and $\tau_3$ with their proportions $f_1$, $f_2$ and $f_3$, respectively.

*Corresponding author. Fax: (86) (10) 64872026.
E-mail: yangfy@sun5.ibp.ac.cn

*Abbreviations:* Gal, galactose; NeuNAc, *N*-acetylneuraminic acid; Glc, glucose; GM1, Galβ 1→3 GalNAcβ 1→4(NeuNAcα 2→3) Galβ 1→4 Glcβ 1→1′ceramide; GM3, NeuNAcα 2→3 Galβ 1→4 Glcβ 1→1′ceramide; HB, hypocrellin B; HPTLC, high performance thin layer chromatography; SR, sarcoplasmic reticulum


PII: S0014-5793(99)01024-8

Table 1
Effect of GM1 on the activities of SR $Ca^{2+}$-ATPase in SR membrane

| Concentration of GM1 (nmol/mg protein) | ATP hydrolysis (μmol/min·mg) | $Ca^{2+}$ uptake[a] (nmol/min·mg) |
|---|---|---|
| 0 | 1.31 ± 0.03 | 92 ± 5 |
| 2 | 1.04 ± 0.01 | 85 ± 4 |
| 4 | 0.81 ± 0.03 | 62 ± 1 |
| 8 | 0.64 ± 0.02 | 47 ± 2 |
| 12 | 0.60 ± 0.02 | 45 ± 1 |

Values are means ± S.D. of four experiments.
[a]Initial rate.

*2.7. Determination of intrinsic fluorescence quenching*

Quenching experiments were carried out by using KI and HB. Stock solutions of 5 M KI (containing a trace amount of thiosulfate to retard $I_3^-$ formation) and 2 mM HB (in dimethylformamide) were prepared. The fluorescence intensity was measured as a function of quencher concentration at 335 nm emission wavelength.

## 3. Results

*3.1. Antagonistic effect of GM1 and GM3 on the activity of SR $Ca^{2+}$-ATPase*

Firstly, the effect of GM1 on the activity of $Ca^{2+}$-ATPase in natural SR was examined. Table 1 shows that GM1 markedly decreased both the APT hydrolysis activity, and $Ca^{2+}$ uptake. Both the inhibitions were concentration-dependent within the range of 0–8 nmol GM1/mg protein, and the inhibition approached a maximum value at about 8 nmol GM1/mg protein, with an inhibition of 51% for ATP hydrolysis and 49% for $Ca^{2+}$ uptake. No significant further decrease was seen at concentrations above 8 nmol GM1/mg protein.

Secondly, the $Ca^{2+}$-ATPase was purified to homogeneity from rabbit SR and reconstituted into soybean phospholipid liposomes. Then the changes of ATP hydrolysis and $Ca^{2+}$ uptake of proteoliposomal $Ca^{2+}$-ATPase were monitored as a function of ganglioside concentration. As shown in Fig. 1A, GM1 markedly inhibited the ATP hydrolysis by reconstituted $Ca^{2+}$-ATPase within the range of 1–10 nmol GM1/mg protein, while the effect of GM3 on ATP hydrolysis was opposite within the range of 1–15 nmol/mg protein. Both were concentration-dependent. The inhibition approached a maximum of 60% at 10 nmol GM1/mg protein, and the activation approached a maximum of 101% at 15 nmol GM3/mg protein. Fig. 1B shows that $Ca^{2+}$ uptake of reconstituted SR $Ca^{2+}$-ATPase was also inhibited by GM1, but activated by GM3.

*3.2. Conformational changes of reconstituted SR $Ca^{2+}$-ATPase induced by GM1 or GM3*

*3.2.1. Intrinsic fluorescence measurement.* Fig. 2 shows the intrinsic fluorescence emission spectra of reconstituted SR $Ca^{2+}$-ATPase in the presence of GM1 or GM3. It can be seen that GM1 caused decrease of the intrinsic fluorescence intensity $Ca^{2+}$-ATPase, while GM3 caused increase. These results suggest that the microenvironments of Trps in reconstituted SR $Ca^{2+}$-ATPase were changed after the incorporation of GM1 and GM3, and the changes were opposite to each other.

*3.2.2. Time-resolved fluorescence studies.* The different Trps have different lifetimes according to their microenvironments, as reflected by time-resolved fluorescence measurement. So, time-resolved emissions were measured in order to explore the different behavior of Trps in reconstituted SR $Ca^{2+}$-ATPase incorporated with GM1 or GM3. A three-exponential fit was required to describe the intrinsic fluorescence intensity decays of reconstituted SR $Ca^{2+}$-ATPase. The lifetimes shown in Table 2 demonstrate that SR $Ca^{2+}$-ATPase contains two main populations of Trps, which are characterized by different fluorescence lifetimes, i.e. a longer fluorescence lifetime $\tau_1$ (3.91 ns) and a shorter fluorescence lifetime $\tau_2$ (1.04 ns). A third group of Trps with a longest fluorescence lifetime $\tau_3$ (8.83 ns) was also observed, but only as a small fraction of the fluorescence (9%). According to the report of MacLennan et al., 10 out of 13 tryptophan residues are located in the intramembranous hydrophobic portion of the protein [14,15]. Table 2 shows that GM1 shortened the fluorescence lifetimes (particularly in the case of $\tau_1$ and $\tau_2$), while GM3 prolonged these parameters. So, from the time-resolved fluorescence results it may be deduced that in consequence of the interaction of the hydrophilic oligosaccharide chain and the hydrophobic ceramide moiety of GM1 or GM3 with their

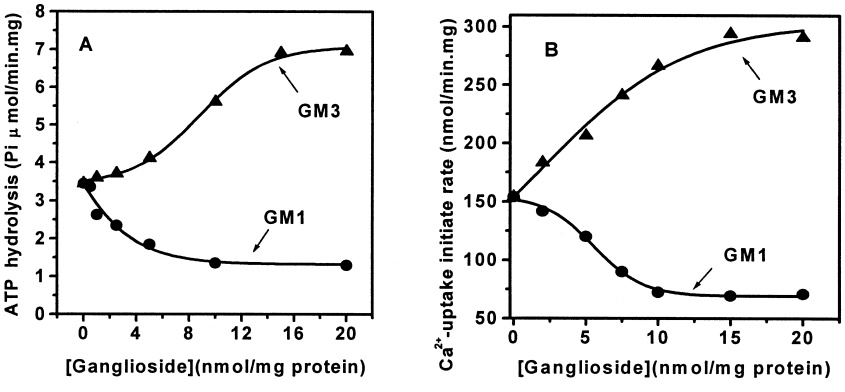


Fig. 1. Effect of GM1 or GM3 on the activity of reconstituted SR $Ca^{2+}$-ATPase. A: ATP hydrolysis. B: $Ca^{2+}$ uptake.

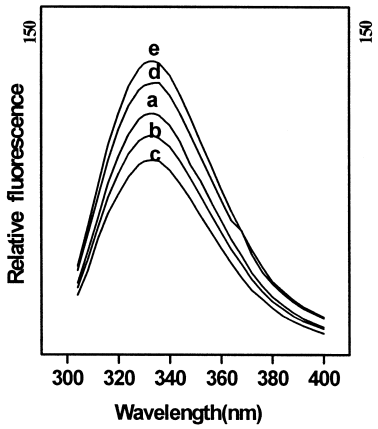


Fig. 2. Intrinsic fluorescence spectra of reconstituted SR $Ca^{2+}$-ATPase. a: Without GM1 or GM3; b: 5 nmol/mg GM1; c: 10 nmol/mg GM1; d: 5 nmol/mg GM3; e: 10 nmol/mg GM3. Assay medium consisted of 40 mmol/l HEPES (pH 7.2), 5 mmol/l $MgCl_2$ 1.01 mmol/l EGTA, 100 μg of $Ca^{2+}$-ATPase/ml. The excitation and emission wavelengths were 285 and 335 nm.

counterparts in the SR $Ca^{2+}$-ATPase, the conformation of the enzyme molecule had been changed, and the changes were of opposite direction. In the following experiments, quenchers which selectively quench Trps belonging to different domains were further used to compare the conformational changes of SR $Ca^{2+}$-ATPase induced by GM1 or GM3.

*3.2.3. Fluorescence quenching studies.* The water-soluble iodide is known to quench fluorescence emitted from Trps located in the hydrophilic domain of the protein molecule. It can be seen from Fig. 3A that the quenching degree of KI to fluorescence of Trps in reconstituted $Ca^{2+}$-ATPase increased with GM1, but decreased with GM3, suggesting that the accessibility of KI was increased with the addition of GM1, while decreased with GM3.

HB is lipid-soluble and is often used to quench fluorescence emitted from Trps located in the hydrophobic domain of the protein. Fig. 3B shows that the HB quenchings of reconstituted SR $Ca^{2+}$-ATPase with GM1 or GM3 were all decreased, but that with GM3 was more decreased than that with GM1. The results suggest that the conformational changes of the hydrophobic domain of reconstituted SR $Ca^{2+}$-ATPase induced by GM1 and GM3 were not of the same extent.

Table 2
Effect of GM1 or GM3 on time-resolved fluorescence lifetimes of tryptophan residues in reconstituted SR $Ca^{2+}$-ATPase

| | Intrinsic fluorescence lifetime (ns) | | |
|---|---|---|---|
| | $\tau_1$ ($f_1$) | $\tau_2$ ($f_2$) | $\tau_3$ ($f_3$) |
| Control | 8.83 (9.06) | 3.91 (62.31) | 1.04 (28.63) |
| +GM1 | 7.21 (14.39) | 3.60 (60.05) | 0.91 (25.57) |
| +GM3 | 10.7 (5.02) | 4.07 (65.87) | 1.05 (29.2) |

$f$: percentage of different fluorescence lifetime components.
The data are average of four experiments, S.E.M. $< 0.1$ ns.
The proteoliposomes were incubated with 10 nmol GM1/mg protein or 15 nmol GM3/mg protein at 30°C for 1 h. Assay medium consisted of 40 mmol/l HEPES (pH 7.2), 5 mmol/l $MgCl_2$, 1.01 mmol/l EGTA, 300 μg of $Ca^{2+}$-ATPase/ml. The excitation and emission wavelengths were 295 nm and 335 nm.

### *3.3. Antagonistic effect of GM1 and GM3 with the same ceramide moiety on the activities of reconstituted SR $Ca^{2+}$-ATPase*

The GM1 and GM3 used in the above experiments were all from natural sources and they were heterogeneous in ceramide moiety. It is necessary to clarify which part of the GM1 and GM3 molecule was responsible for the different effect on $Ca^{2+}$-ATPase. So, the homogeneous GM1 (c18:0-d18:1) and GM3 (c18:0-d18:1) were used instead of natural ones. The results obtained show that GM1 (c18:0-d18:1) and GM3 (c18:0-d18:1), which are different only by two sugar residues, exert antagonistic effect on the ATP hydrolysis and calcium uptaking of reconstituted SR $Ca^{2+}$-ATPase (Table 3).

## 4. Discussion

The present study shows that GM1 inhibited the activities of $Ca^{2+}$-ATPase both in SR vesicles and in reconstituted proteoliposomes, while GM3 had an opposite effect to GM1 under the same experimental conditions (Table 1, Fig. 1). Comparing the known structures of natural GM1 and GM3, it can be seen that GM1 is different from GM3 in carbohydrate chain by having two more hexose residues, and the heterogeneous ceramide moiety might have a different fatty acid and sphingosine composition. It is logical to ask which part of the ganglioside structure is mainly responsible for the difference in modulating the enzyme activity. To answer this question, homogeneous GM1 and GM3 with the

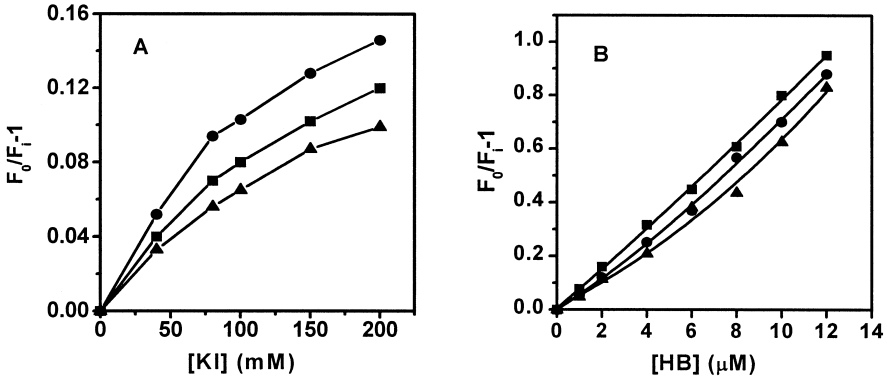


Fig. 3. Fluorescence quenching of reconstituted SR $Ca^{2+}$-ATPase with GM1 or GM3. A: Quenching by KI. B: Quenching by HB. (■) Without GM1 or GM3; (●) GM1; (▲) GM3. $F$ and $F_0$ are the intrinsic fluorescence values with and without quenching agent, respectively. Assay medium consisted of 40 mmol/l HEPES (pH 7.2), 5 mmol/l $MgCl_2$, 1.01 mmol/l EGTA, 100 μg of $Ca^{2+}$-ATPase/ml. The excitation and emission wavelengths were 285 and 335 nm.

Table 3
Effect of GM1 and GM3 with the same ceramide moiety on the activities of reconstituted SR $Ca^{2+}$-ATPase

| | ATP hydrolysis (Pi μmol/min·mg) | $Ca^{2+}$ uptake[a] (nmol/min·mg) |
|---|---|---|
| Control | 2.80 ± 0.07 | 119 ± 5 |
| GM1 | 1.06 ± 0.04 | 55 ± 3 |
| c18:0-d18:1-GM1 | 1.20 ± 0.05 | 57 ± 2 |
| GM3 | 5.63 ± 0.08 | 225 ± 5 |
| c18:0-d18:1-GM3 | 5.46 ± 0.11 | 203 ± 4 |

Values reported here refer to the activity of the maximum inhibition/activation and all data are the means ± S.D. for three experiments.
[a]Initial rate.

same ceramide structure were used in place of natural gangliosides. Each homogeneous ganglioside showed a similar effect as its natural counterpart, i.e. c18:0-d18:1-GM1 decreased the activity of reconstituted SR $Ca^{2+}$-ATPase, while c18:0-d18:0-GM3 increased it (Table 3). These results suggest that the difference observed in the modulation of activity of SR $Ca^{2+}$-ATPase by GM1 or GM3 mainly came from the difference of the carbohydrate chain. In addition, the effect of some ganglioside components including asialo-GM1, asialo-GM3, ceramide or *N*-acetylneuraminic acid on SR $Ca^{2+}$-ATPase were also studied, none of these components had a significant effect on the enzyme activities within comparable concentration ranges (data not shown). Most likely, an intact molecule of the ganglioside with definite conformation was needed in performing its function to modulate SR $Ca^{2+}$-ATPase.

In the steady-state and time-resolved intrinsic fluorescence studies, the results (Fig. 2, Table 2) mutually support each other. The message drawn from these results is that GM1 and GM3 induced $Ca^{2+}$-ATPase to have conformational changes of the opposite direction. Quenching studies could give more information on the conformational changes occurred in both hydrophilic and hydrophobic domains. The quenching experiments demonstrate that GM1 caused the conformation of the hydrophilic domain to be less compact, and the hydrophobic domain became more compact, while GM3 caused both domains to be more compact.

Based on the 8 Å structure of SR $Ca^{2+}$-ATPase, obtained by electron microscopy of decavanadate tubes, the enzyme molecule consists of a large cytoplasmic domain, connected by a narrow stalk to the transmembrane helices [16]. The cytoplasmic domain, in which ATP hydrolysis takes place, stretches 65 Å above the plane of the membrane [17] and the calcium-binding site is expected to be located in the middle of the membrane (~12 Å from the cytoplasmic/lipid bilayer junction [16]). As amphipathic molecules, both GM1 and GM3 are incorporated into biological membranes with the lipophilic ceramide moiety embedded in the outer leaflet of the lipid bilayer and the hydrophilic oligosaccharide portion protruded toward the surrounding environment [18]. It is reasonable to assume that the effects of GM1 and GM3 on SR $Ca^{2+}$-ATPase may result from their direct interactions and the hydrophilic oligosaccharide chain and the hydrophobic ceramide moiety of ganglioside with corresponding domains of the enzyme molecule. GM1 could make the $Ca^{2+}$-ATPase molecule less compact in the hydrophilic domain, leading to lower enzyme activity, while GM3 made the whole enzyme more compact with higher activity. Here, the steric hindrance of GM1 due to its longer sugar chain might also affect the accessibility of ATP to its binding site and thus results in the lower activity SR $Ca^{2+}$-ATPase. The conformational changes in the hydrophilic region of SR $Ca^{2+}$-ATPase induced by GM1 or GM3 could be transmitted to the hydrophobic region by domain-domain communication.

To sum up, the present paper clearly shows an antagonistic effect of GM1 and GM3 on the activity and conformation of SR $Ca^{2+}$-ATPase. However, the detailed molecular mechanism of how GM1 induced a different conformational change of SR $Ca^{2+}$-ATPase from that of GM3 is still an open question and deserves further investigation.

The results presented here may further suggest that GM1 or GM3, present in SR membrane, is not just acting as a 'passer-by' in its transport within the cell, but is actively involved in the modulation of SR $Ca^{2+}$-ATPase activity [19]. Although the physiological ganglioside concentrations in natural SR are much lower than we used in the present study, we postulate that the gangliosides may cluster before functioning, and therefore higher concentrations of GM1 and GM3 might be attained locally. This postulation has been reinforced by the separation of a GM3 rich-domain from plasma membrane of lymphocytes by Sorice et al. recently [20]. The two gangliosides might, in vivo, cooperatively modulate the activity of SR $Ca^{2+}$-ATPase, and contribute to maintain the calcium homeostasis of muscle cells.

*Acknowledgements:* This work was financially supported as external project (3973010) by the National Laboratory of Biomacromolecules, Academia Sinica and partly financed by the National Natural Science Foundation of China. We sincerely thank Dr. Sandro Sonnino (Department of Medical Chemistry and Biochemistry, University of Milan, Italy) for his kind donation of GM1 homogeneous in ceramide moiety. We also express our thanks to Dr. Lihua Wang (Postdoctorate of NIH) and Dr. Xiaoyi Yang (Postdoctorate of NIH) for their helpful suggestions for the project.

## References

[1] Hakomori, S. (1993) Biochem. Soc. Trans. 21, 583–595.
[2] Hakomori, S. (1990) J. Biol. Chem. 265, 18713–18716.
[3] Yates, A.J., Agudelo, J.D. and Sung, C.C. (1992) Lipids 27, 308–310.
[4] Wang, L.H., Yang, X.Y., Tu, Y.P., Tsui, Z.C. and Yang, F.Y. (1996) FEBS Lett. 388, 128–130.
[5] Tsui, Z.C., Hou, W.H. and Zhu, Z.M. (1990) Prog. Biochem. Biophys. (Beijing) 17, 206–209.
[6] Yue, J.C. and Pang, S.Z. (1997) Sci. China (C) 140, 194–201.
[7] MacLennan, D.H. (1970) J. Biol. Chem. 245, 4508–4518.
[8] Coll, R.H. and Murphy, A.J. (1984) J. Biol. Chem. 259, 14249–14254.
[9] Gould, G.W., Mewhirter, J.M., East, J.M. and Lee, A.G. (1987) Biochim. Biophys. Acta 904, 36–44.
[10] Tu, Y.P. and Yang, F.Y. (1993) Biochem. Biophys. Res. Commun. 196, 561–568.
[11] Froud, R.J., Earl, C.R.A., East, J.M. and Lee, A.G. (1986) Biochim. Biophys. Acta 860, 354–360.
[12] Kitchell, B.B. and Henkens, R.W. (1978) Biochim. Biophys. Acta 534, 89–98.

[13] Vos, K., Van Hoek, A.A. and Visser, A.J.W.G. (1987) Eur. J. Biochem. 165, 55–63.
[14] Clarke, D.M., Loo, T.W., Inesi, G. and MacLennan, D.H. (1989) Nature 339, 476–478.
[15] MacLennan, D.H., Brandi, C.J., Korezak, B. and Green, N.M. (1985) Nature 316, 696–700.
[16] Zhang, P., Toyoshima, C., Yonekura, K., Green, N.M. and Stokes, D.L. (1998) Nature 392, 835–839.
[17] Stokes, D.L. and Green, N.M. (1990) Biophys. J. 57, 1–14.
[18] Irwin, L.N. (1974) Rev. Neurosci. 1, 137–180.
[19] Yang, F.Y., Wang, L.H., Yang, X.Y., Tsui, Z.C. and Tu, Y.P. (1997) Biophys. Chem. 68, 137–146.
[20] Sorice, M., Parolini, I., Sansolini, T., Garofalo, T., Dolo, V., Sargiacomo, M., Tai, T., Peschle, C., Torrisi, M.R. and Pavan, A. (1997) J. Lipid Res. 38, 969–980.