Volume 314, number 1, 53–57 FEBS 11828 December 1992

Full activation without calmodulin of calmodulin-dependent cyclic nucleotide phosphodiesterase by acidic glycosphingolipids: GM3, sialosylneolactotetraosylceramide and sulfatide

Hideyoshi Higashi and Tatsuya Yamagata

Laboratory of Glycoconjugate Research, Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194, Japan

Received 20 September 1992; revised version received 15 October 1992

Among calmodulin-non-binding glycosphingolipids, GM3, sialosylneolactotetraosylceramide (LM1), and sulfatide potently activated calmodulin-dependent cyclic nucleotide phosphodiesterase with or without Ca^{2+} showing ED_{50} 1–5 μM. In contrast to calmodulin-binding gangliosides, these glycosphingolipids activated the enzyme up to the maximum level achieved by Ca^{2+}/calmodulin and did not inhibit the activity at higher concentrations. Competition studies with GD1b that bind both to calmodulin and the enzyme suggest that the calmodulin-non-binding glycosphingolipids activate the enzyme through interaction with the same site of the enzyme as GD1b interacts.

GM3; LM1; Sulfatide; Ganglioside; Calmodulin; cAMP-phosphodiesterase

1. INTRODUCTION

Gangliosides with a gangliotetraose core and GM2 were shown in our previous studies to inhibit a CaM-dependent enzyme, cyclic nucleotide phosphodiesterase (PDE), through binding to CaM and the enzyme [3,4]. CaM is expressed in all eukaryotic cells and is essential to calcium signal transduction by modulating a wide variety of enzymes. Gangliosides are present in all vertebrates and higher invertebrates and it is proposed that they modulate several cellular activities [5]. Ganglioside-mediated modulation of cell activities via a CaM-mediated signal transduction pathway has thus been put forward [4].

In non-neural tissues, such a ganglioside-mediated modulation could hardly be expected since CaM-binding gangliosides are usually not abundant in these tissues. However, GM3 and neolacto series gangliosides instead are abundant in these tissues and these gangliosides, when given exogenously to the cells, modulate cellular activities such as differentiation and proliferation in a variety of non-neural and neural cells [6–13]. The gangliosides may regulate these cellular activities by modulating transmembrane signal transductions through binding to certain molecules although until now there has been no evidence of direct interaction of these gangliosides with such molecules. In this study, we have investigated the effects of these and other glycosphingolipids on a typical CaM-dependent enzyme, PDE. Among them thus far tested, GM3, LM1, and sulfatide activated PDE, up to the maximum level achieved by Ca^{2+}/CaM-activation. These effects were compared with those of Ca^{2+}/CaM and CaM-binding gangliosides on the enzyme and it is proposed that these CaM-non-binding glycosphingolipids activate the enzyme through interacting with a site other than CaM-binding site of the enzyme, the same site as CaM-binding gangliosides interact.

Correspondence address: H. Higashi, Laboratory of Glycoconjugate Research, Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194, Japan. Fax: (81) (427) 24-6298.

Abbreviations: CaM, calmodulin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PDE, cyclic nucleotide phosphodiesterase. Abbreviations by Svennerholm [1] for gangliosides and IUPAC-IUB Recommendations for lipids [2] were used: GD1b, II^3(NeuAcα2–8NeuAc)-Gg_4Cer; LM1, sialosylneolactotetraosylceramide, IV^3Sialyl-nLc_4Cer. Structures of the other glycosphingolipids are shown in Table I.

2. MATERIALS AND METHODS

2.1. *Materials*

Cyclic AMP, snake venom from *Crotalus atrox* activator deficient 3′,5′-cyclic nucleotide phosphodiesterase from bovine brain (P-9529), and bovine brain CaM were purchased from Sigma, St. Louis, MO. GM3, GM3(NeuGc), LM1(NeuGc), NeuGc-nLc_6Cer, nLc_4Cer and Gal-nLc_4Cer were prepared as already described [14]. GalCer and sulfatide and Gg_4Cer were prepared from bovine brain as already described [4]. 4-*O*-Ac-GM3(NeuGc) was prepared as already described [15]. LacCer was prepared from equine erythrocytes using Iatrobeads column chromatography by the method described previously [15]. GD1b was purchased from Iatron Lab., Tokyo, Japan.

2.2. *Phosphodiesterase assay*

The Cheung method [16] was used with a slight modification as described previously [3]. For an assessment of the effect of the enzyme reaction by glycosphingolipids, the glycosphingolipids were added at an appropriate concentration to the incubation mixture consisting of cAMP and PDE. Little, if any CaM-independent phosphodiesterase activity could be detected in the enzyme preparation.

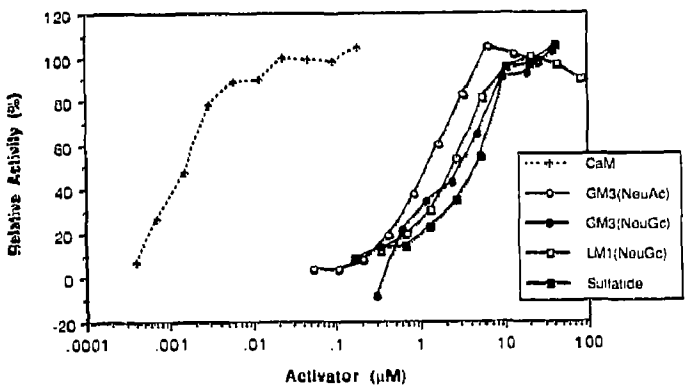

Fig. 1. Effects of glycosphingolipids on CaM-dependent phosphodiesterase. Different glycosphingolipids at various concentrations were added to reaction mixtures of phosphodiesterase without CaM. Activity is expressed as activity relative to maximum activity stimulated by Ca^{2+}/CaM.

Trypsin-treatment of phosphodiesterase was performed by the method of Davis and Daly [17] with a modification as already described [4].

2.3. *Other methods*

Lipid-bound sialic acids were measured by the method of Jourdian et al. [18].

3. RESULTS

3.1. *Effects of various glycosphingolipids on PDE*

GM3, LM1, and sulfatide activated the enzyme in the absence of CaM (Fig. 1). The activation was Ca^{2+} independent (data not shown). Besides GM3 and sulfatide [3,4], LM1 showed no effect on Ca^{2+}/CaM-activated PDE or fluorescence spectrum of dansyl-CaM (data not shown), indicating that it also did not bind to CaM. As shown in Table I, ED_{50} of these glycosphingolipids were 1–5 μM. Higher concentrations were required for activation compared to gangliosides that interact with both CaM and PDE; GD1b, GD1a, and GT1b (Group 1) [4]. However, in contrast to Group 1 gangliosides, it should be emphasized that they showed no inhibition at all even at higher concentrations. They were thus shown to act as CaM agonists to PDE though higher concentrations by three orders of magnitude are required compared to CaM. Now, we would like to classify these glycosphingolipids to Group 3 glycosphingolipids.

GM3 derivatives were examined with special reference to sialyl residues. NeuAc was found to be slightly more efficient than NeuGc which was followed by *O*-acetylation at 4 position of sialyl residue (Fig. 1 and Table I).

The efficiency of NeuGc-nLc$_6$Cer which has a longer sugar chain than LM1(NeuGc) was remarkably diminished, showing nine times as much as ED_{50} of LM1 while LM1(NeuGc) was slightly more effective than GM3(NeuGc) (Table I). Neutral glycosphingolipids, nLc$_4$Cer and Gg$_4$Cer showed activation to some extent even though their efficiency was less than 1/10 that of their sialyl derivatives. Gal-nLc$_4$Cer, Gb$_4$Cer, LacCer, and GalCer showed little, if any, effect.

3.2. *Effects of Group 1 gangliosides on PDE activity stimulated by Group 3 glycosphingolipids*

Ca^{2+}/CaM-stimulated PDE activity was shown in the previous study to be inhibited by Group 1 gangliosides such as GD1b [4]. GD1b showed inhibition even at the concentrations where it showed activation of the enzyme in the absence of CaM. Excess CaM overcame inhibition. To compare the nature of PDE stimulation by Group 3 glycosphingolipids with that by Ca^{2+}/CaM, the effects of GD1b on the glycosphingolipid-stimulated enzyme activity were examined. Fig. 2 shows the effects of GD1b on LM1(NeuGc)-stimulated PDE. The activity stimulated by LM1(NeuGc) at the concentrations above 10 μM was inhibited by GD1b at all the concentrations used. Inhibition was manifest even at the concentrations where GD1b showed activation of the enzyme in the absence of other stimulators, while the activity stimulated by LM1(NeuGc) at concentrations below 10 μM was additively activated by the stimulative

Table I

Stimulative effects of glycosphingolipids on phosphodiesterase

Glycosphingolipid	Structure	ED_{50} (μM)
GM3(NeuAc)	NeuAcα2 → 3Galβ1 → 4Glc-Cer	1.2
GM3(NeuGc)	NeuGcα2 → 3Galβ1 → 4Glc-Cer	2.9
4-*O*-Ac-GM3(NeuGc)	4-*O*-Acetyl-NeuGcα2 → 3Galβ1 → 4Glc-Cer	4.1
LM1(NeuGc)	NeuGcα2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-Cer	2.4
Sulfatide	H_2SO_3 → 3Gal-Cer	4.5
NeuGc-nLc$_6$Cer	Siaα2 → 3(Galβ1 → 4GlcNAcβ1 → 3)$_2$Galβ1 → 4Glc-Cer	21
nLc$_4$Cer	Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-Cer	30
Gal-nLc$_4$Cer	Galα1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc-Cer	> 100
Gg$_4$Cer	Galβ1 → 3GalNAcβ1 → 4Galβ1 → 4Glc-Cer	24
Gb$_4$Cer	GalNAcβ1 → 3Galα1 → 4Galβ1 → Glc-Cer	> 100
LacCer	Galβ1 → 4Glc-Cer	> 100
GalCer	Gal-Cer	> 100

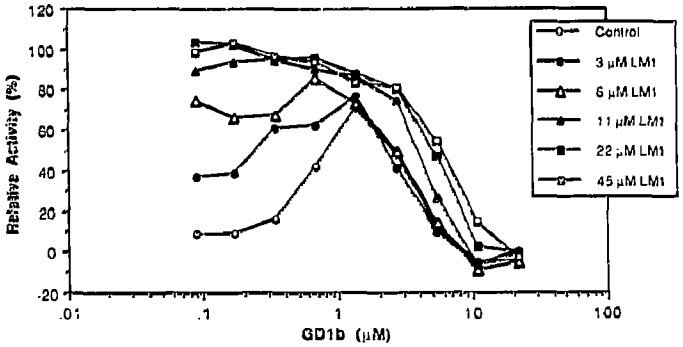

Fig. 2. Effects of GD1b on LM1(NeuGc)-stimulated phosphodiesterase activity. Various concentrations of GD1b were added to reaction mixtures of phosphodiesterase stimulated by the indicated concentrations of LM1(NeuGc). Activity is expressed as activity relative to that stimulated by 45 μM LM1(NeuGc) without GD1b.

concentration (below 1 μM) of GD1b. Direct inhibition of the enzyme activity by GD1b shown at the concentrations above 1 μM was eliminated by excess LM1(NeuGc). Similar results were obtained using GM3(NeuGc) or sulfatide instead of LM1(NeuGc) (data not shown). Their activation properties were thus shown to be very similar to Ca^{2+}/CaM (refer to Fig. 1C in [4]).

To investigate further a relationship between activation by LM1 and inhibition by GD1b, activation of PDE by LM1(NeuGc) in the presence of GD1b at inhibiting concentrations was examined. The Lineweaver-Burk plots indicate it to be a competitive inhibition with a constant maximum activity and increasing ED_{50} corresponding to GD1b concentration (Fig. 3). Thus GD1b was shown to inhibit the enzyme by competing with LM1(NeuGc). K_i of GD1b was calculated as 1.6–2.8 μM. The above results suggest that an LM1-interacting site of the enzyme is either the GD1b-interacting site or CaM-binding site.

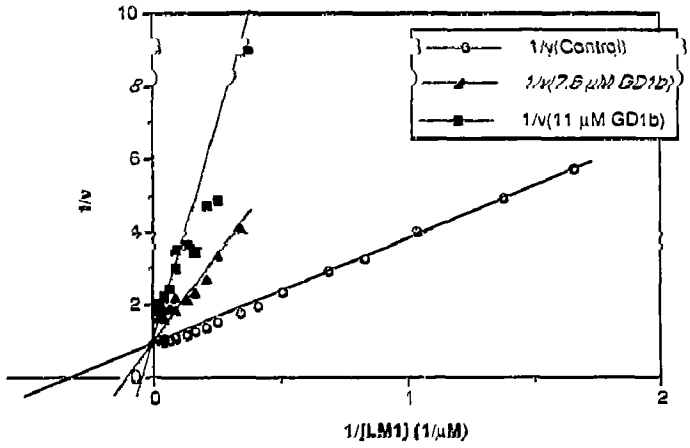

Fig. 3. Inhibition by GD1b on LM1(NeuGc)-stimulation of phosphodiesterase activity. Activation by LM1(NeuGc) of phosphodiesterase was assessed in the presence of indicated concentrations of GD1b. Activation by LM1(NeuGc) alone was measured for the control. Activity (v) is expressed as relative activity to maximum activity stimulated by LM1(NeuGc) alone. Lineweaver-Burk plots of the data are shown.

3.3. *Effects of Group 3 glycosphingolipids on trypsin-activated PDE*

To determine whether LM1, GM3, and sulfatide interact with the CaM-binding site or the same site as GD1b does, the effects of these glycosphingolipids on trypsin-treated PDE, which has lost CaM-binding domain and expressed full activity without CaM, were examined. The three glycosphingolipids showed no effect on the trypsin-activated enzyme (data not shown). However, they reversed inhibition by GD1b of the trypsin-activated enzyme activity (Fig. 4). These results indicate that they interact either with GD1b or the same site of the enzyme as GD1b does, which is presumably different from the CaM-binding site, to recover the activity. Since they failed to increase the fluorescence intensity of dansyl-CaM by GD1b (data not shown), it is likely they did not interact directly with GD1b. It is thus suggested that these glycosphingolipids interact with the same site as GD1b does.

4. DISCUSSION

CaM-binding gangliosides are classified into two groups according to their effects on PDE [4]: Group 1 gangliosides such as GD1b, GT1b, and GD1a show strong direct activation and inhibition of the enzyme; Group 2 gangliosides such as GM1, GM2, and GQ1b show little direct inhibition of the enzyme. As shown in the present study, GM3, LM1, and sulfatide did not bind to CaM showing only a positive effect on the enzyme and should thus be classified into another group, Group 3. They activated PDE to the maximum level achieved by Ca^{2+}/CaM (Fig. 1) and showed no effect on Ca^{2+}/CaM-stimulated PDE. PDE activity stimulated by Group 3 glycosphingolipids was inhibited by a Group 1 ganglioside GD1b (Fig. 2). The GD1b inhibition was competitive with Group 3 glycosphingolipids (Fig. 3) as in the case of inhibition of Ca^{2+}/CaM-stimulated enzyme by GD1b where GD1b inhibits the activity competitively with CaM [4]. Furthermore, as in the case of inhibition of Ca^{2+}/CaM-stimulated PDE [4], GD1b, even at activating concentrations, inhibited activity stimulated by Group 3 glycosphingolipids (Fig. 2). While Group 3 glycosphingolipids showed no effect on the activity of trypsin-stimulated PDE, they eliminated inhibition by GD1b of trypsin-stimulated enzyme (Fig. 4). Thus the effects of Group 3 glycosphingolipids on PDE were quite similar to those of Ca^{2+}/CaM. However, in contrast to CaM, Group 3 glycosphingolipids provided no evidence of binding to GD1b as described above. Thus, it is proposed that the glycosphingolipids bind to the same site as GD1b binds, CaM-like binding site [19] of the enzyme for activation. They may release a suppressive domain, CaM-binding site from CaM-like

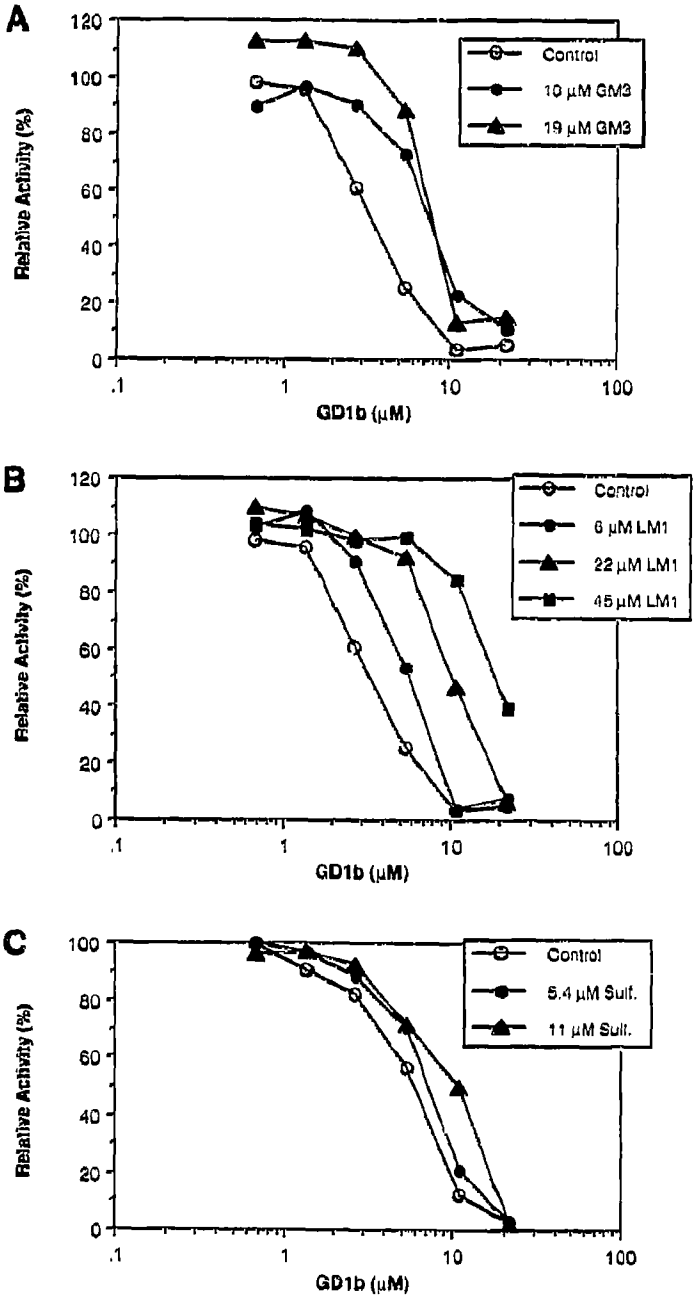

Fig. 4. Effects on glycosphingolipids on GD1b inhibition of phosphodiesterase activated by trypsin-treatment. Various concentrations of GD1b were added to reaction mixtures of the trypsin-stimulated phosphodiesterase in the presence of indicated concentrations of (A) GM3(NeuGc), (B) LM1(NeuGc) and (C) sulfatide. Activity is expressed as activity relative to that without gangliosides. The trypsinized enzyme underwent no change in activity when glycosphingolipids were added alone.

binding site to activate PDE. They may also release GD1b from CaM-like binding site by binding to this site competitively with GD1b to eliminate enzyme inhibition caused by GD1b.

The present results suggest that sialyl residue of the Group 3 glycosphingolipids is not essential but acidic group and a certain length of saccharide chain which keeps the distance between acidic group and hydrophobic ceramide portion are necessary for the activation, since sulfatide was potent activator besides GM3 and LM1, and sialyl nLc_6Cer was much less effective than LM1 (Table I). In addition, difference in activation potential of neutral glycosphingolipids (Table I) indicates conformation of saccharide chain may also affect activation potency. It is noteworthy that Group 3 glycosphingolipids are much more efficient than PDE activating lipids so far known, such as fatty acids and lysophospholipids (ED_{50}=20 μM or more) [20,21].

Since in vitro interactions of GM3, LM1, and sulfatide with PDE have been shown in the present study, in vivo interactions of these CaM-non-binding glycosphingolipids and PDE may occur, as has been postulated in the case of CaM-binding gangliosides and CaM. Some glycosphingolipids are reported to be present in cytosol [22–27] and oriented to the cytosolic side in a membrane [28–31], such glycosphingolipids, therefore, possibly interact with CaM-dependent enzymes such as PDE to regulate them at low Ca^{2+} concentrations or give some bias toward these activities to modulate cell activities.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (No. 02259103) from the Ministry of Education, Science and Culture of Japan. We express our appreciation to S. Yoshida for her competent technical assistance.

REFERENCES

[1] Svennerholm, L. (1964) J. Lipid Res. 5, 145–155.
[2] IUPAC-IUB Commission on Biochemical Nomenclature (1977) Eur. J. Biochem. 79, 11–21.
[3] Higashi, H., Omori, A. and Yamagata, T. (1992) J. Biol. Chem. 267, 9831–9838.
[4] Higashi, H. and Yamagata, T. (1992) J. Biol. Chem. 267, 9839–9843.
[5] Hakomori, S. (1990) J. Biol. Chem. 265, 18713–18716.
[6] Bremer, E.G. and Hakomori, S. (1982) Biochem. Biophys. Res. Commun. 106, 711–718.
[7] Bremer, E.G., Hakomori, S., Bowen-Pope, D.F., Raines, E. and Ross, R. (1984) J. Biol. Chem. 259, 6818–6825.
[8] Bremer, E.G., Schlessinger, J. and Hakomori, S. (1986) J. Biol. Chem. 261, 2434–2440.
[9] Nojiri, H., Takaku, F., Terui, Y., Miura, Y. and Saito, M. (1986) Proc. Natl. Acad. Sci. USA 83, 782–786.
[10] Rodrig, N., Osanai, T., Iwamori, M. and Nagai, Y. (1987) FEBS Lett. 221, 315–319.
[11] Byrne, M.C., Ledeen, R.W., Roisen, F.J., Yorke, F., and Sclafani, J.R. (1983) J. Neurochem. 41, 1214–1222.
[12] Nojiri, H., Kitagawa, S., Nakamura, M., Kirito, K., Enomoto, Y. and Saito, M. (1988) J. Biol. Chem. 263, 7443–7446.
[13] Nojiri, H., Stroud, M. and Hakomori, S. (1991) J. Biol. Chem. 266, 4531–4537.
[14] Higashi, H., Sugii, T. and Kato, S. (1988) Biochim. Biophys. Acta 963, 333–339.
[15] Miyoshi, I., Higashi, H., Hirabayashi, Y., Kato, S. and Naiki, M. (1986) Mol. Immunol. 23, 631–638.
[16] Cheung, W.Y. (1971) J. Biol. Chem. 246, 2859–2869.
[17] Davis, C.W. and Daly, J.W. (1980) Mol. Pharmacol. 17, 206–211.
[18] Jourdian, G.W., Dean, L. and Roseman, S. (1971) J. Biol. Chem. 246, 430–435.
[19] Jarrett, H.W. and Madhavan, R. (1991) J. Biol. Chem. 266, 362–371.

[20] Pichard, A.-L. and Cheung, W.Y. (1977) J. Biol. Chem. 252, 4872–4875.
[21] Tai, H.-H. and Tai, C.L. (1982) Arch. Biochem. Biophys. 214, 622–627.
[22] Ledeen, R.W., Skrivanek, J.A., Tirri, L.J., Margolis, R.K. and Margolis, R.U. (1976) Adv. Exp. Med. Biol. 71, 83–103.
[23] Sonnino, S., Ghidoni, R., Marchesini, S. and Tettamanti, G. (1979) J. Neurochem. 33, 117–121.
[24] Miller-Podraza, H. and Fishman, P.H. (1983) J. Neurochem. 41, 860–867.
[25] Chigorno, V., Valsecchi, M., Acquotti, D., Sonnino, S., and Tettamanti, G. (1990) FEBS Lett. 263, 329–331.
[26] Chigorno, V., Valsecchi, M., Sonnino, S. and Tettamanti, G. (1990) FEBS Lett. 277, 164–166.
[27] Sakakibara, K., Momoi, T., Uchida, T. and Nagai, Y. (1981) Nature 293, 76–79.
[28] Leskawa, K.C. and Rosenberg, A. (1980) Adv. Exp. Med. Biol. 125, 125–135.
[29] Trinchera, M., Fabbri, M. and Ghidoni, R. (1991) J. Biol. Chem. 266, 20907–20912.
[30] Futerman, A.H. and Pagano, R.E. (1991) Biochem. J. 280, 295–302.
[31] Jeckel, D., Karrenbauer, A., Burger, K.N.J., van Meer, G. and Wieland, F. (1992) J. Cell Biol. 117, 259–267.