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Modification of the adenylate cyclase activity of bovine thyroid plasma membranes by manipulating the ganglioside composition with a nonspecific lipid transfer protein

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Gangliosides (GM_1, GT_{1b}, GD_3) were incorporated in bovine thyroid plasma membranes using the nonspecific lipid transfer protein from beef liver. The transfer of GT_{1b} or GD_3 in the presence of 16 units of transfer protein was twice as high as that of GM_1. However, taking into account the spontaneous exchange ($\approx$ 8% for GT_{1b} or GD_3 and 1% for GM_1) the transfer protein seemed to be more effective for GM_1. Incorporation of these gangliosides in bovine thyroid plasma membranes caused a concentration dependent inhibition of the TSH-stimulated adenylate cyclase activity. The forskolin-stimulated adenylate cyclase activity was not significantly affected by ganglioside modification of the plasma membranes, indicating that the gangliosides do not act at the level of the catalyst of adenylate cyclase. Binding experiments on the other hand revealed that TSH binding to bovine thyroid plasma membranes was inhibited with the same order of efficacy ($GT_{1b} > GD_3 > GM_1$) and to the same extent as their inhibitory effect on TSH stimulation. Therefore, this indicates that the ganglioside induced drop in TSH binding might be an important factor in the decrease in TSH-stimulated adenylate cyclase activity. Incorporation of GT_{1b} or GD_3 ($\approx$ 11 nmol) in bovine thyroid plasma membranes, however, also induced a substantial decrease in cholera toxin-stimulated adenylate cyclase activity ($\approx$ 30%) and to a lesser degree a decrease in NaF-stimulated activity ($\approx$ 17%), whereas GM_1 incorporation did not significantly affect these stimulated activities. These latter inhibitory effects were paralleled by changes in fluorescence steady-state anisotropy: GT_{1b} modification of the plasma membranes provoked a slight increase in TMA-DPH anisotropy, whereas the anisotropy of DPH was substantially enhanced after incorporation of GD_3 or GT_{1b}. These results suggest that gangliosides might also interfere with the coupling between the α-subunit of the stimulatory GTP-binding regulatory protein and the catalyst of the adenylate cyclase system by affecting the membrane fluidity.

Introduction

Several studies have provided evidence indicating that changes in composition as well as in fluidity of the lipid environment of the adenylate cyclase system may modulate the activity of this enzyme [1–6]. Gangliosides, sialic acid containing glycolipids, are important components of plasma membranes. They are incorporated into biological membranes with the lipophylic ceramide portion embedded in the membrane and the hydrophylic carbohydrate portion protruding into the surrounding cytoplasm [7]. This structural feature provides the potential for selective control of membrane characteristics both through fluidity changes and

Abbreviations: G_s, stimulatory GTP binding regulatory protein; TSH, thyroid-stimulating hormone or thyrotropin; GM_1, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; GT_{1b}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylceramide; GD_3, *N*-acetylneuraminyl-*N*-acetylneuraminylgalactosylglucosylceramide; DPPC, dipalmitoylphosphatidylcholine; CH, cholesterol; PL, phospholipid; SUV, sonicated small unilamellar lipid vesicles; buffer A, 0.25 M sucrose in 20 mM Tris-HCl buffer (pH 7.4); DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene; 12-AS, 12-(9-anthroyloxy)stearic acid.

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through specific interaction of the carbohydrate moiety with membrane surface associated components such as hormone receptors. More specifically gangliosides have been shown to affect adenylate cyclase activity [8,9] and may play an important role in the transmission of the TSH signal through the thyroid membrane [10–12]. In a previous paper [4] we have shown that the adenylate cyclase activity of bovine thyroid could be modulated by modification of the membrane phospholipid composition. In the present study the dependence of adenylate cyclase activity on membrane ganglioside composition is investigated. Gangliosides (GM_1, GT_{1b}, GD_3) were incorporated in bovine thyroid plasma membranes using the nonspecific lipid transfer protein (ns-TP) from beef liver and small unilamellar vesicles (SUV). The advantage of this method of incorporation is that the transfer occurs under relatively nonperturbing conditions [1,13,14]. The potential relationship between the effects of ganglioside incorporation on adenylate cyclase activity on one hand and on TSH binding and membrane fluidity on the other hand was examined. Changes in membrane fluidity were monitored using the technique of steady-state fluorescence anisotropy measurements.

Materials and Methods

Materials

Thyrotropin came from Armour, forskolin from Calibiochem and cholera toxin from List Biological Laboratories. Highly purified gangliosides (purity > 99%) were kindly supplied by Dr. G. Dacremont (Ghent State University) and were tritiated in our laboratory by the method of Veh et al. [15]. Gangliosides, GM_1 and GT_{1b} were isolated from normal human brain and GD_3 was prepared from human thyroid tissue. Diphenylhexatriene came from Aldrich and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene was obtained from Molecular Probes Inc. Sigma was the source of 12-(9-anthroyloxy)stearic acid. [α-^{32}P]ATP was provided by NEN Research Products. Highly purified TSH (15–20 IU/mg) came from UCB Bioproducts and was radioiodinated by means of the iodination beads system from NEN, which uses the lactoperoxidase method. Following iodination, ^{125}I-TSH was separated from unreacted iodide by chromatography on Sephadex G-100.

Preparation of plasma membranes

Plasma membranes with a cholesterol to phospholipid molar ratio of 0.40 ± 0.04 were prepared from fresh bovine thyroid glands as described previously [16]. They were stored in liquid N_2 at $-180\,^{\circ}$C immediately after preparation.

Chemical analysis

Protein concentration was estimated by the procedure of Lowry et al. [17] using bovine serum albumin as standard. Phospholipid phosphorus was assayed according to Rouser et al. [18]. Cholesterol was determined by the method of Hanel and Dam [19]. Phospholipid phosphorus and cholesterol were determined on a Bligh and Dyer [20] lipid extract of bovine thyroid plasma membranes. Sphingosine was measured according to the procedure of Kisic and Rapport [21].

Lipid vesicles

Small unilamellar vesicles (SUV) were prepared according to the method of Johnson and Zilversmit [22] except that the lipid suspension was sonicated with a Braun-Sonic 30 homogenizer (Quigley-Rochester, Inc.) using a 4.0 mm diameter titanium probe. SUV were prepared in buffer A (0.25 M sucrose in 20 mM Tris-HCl buffer (pH 7.4)). Before incubation with bovine thyroid plasma membranes and nonspecific lipid transfer protein, SUV were centrifuged at $10\,000 \times g$ for 30 min to remove multilamellar liposomes.

Ganglioside transfer assay

The nonspecific lipid transfer protein from bovine liver was prepared by the method of Crain and Zilversmit [23]. Exchange activity was expressed in units representing the transfer of 1% phosphatidylcholine of total SUV DPPC from [^{14}C]DPPC/cholesterol SUV (0.15 μmol phospholipid, cholesterol to phospholipid molar ratio of 0.4) to bovine thyroid plasma membranes (0.6 μmol phospholipid) in a total volume of 0.5 ml in 1 h at $37\,^{\circ}$C. Ganglioside transfer activity was determined by measuring the transfer of labelled ganglioside from SUV to bovine thyroid plasma membranes in buffer A. In a typical assay bovine thyroid plasma membranes (0.6 μmol phospholipid) were incubated with tritiated ganglioside-containing SUV (0.3 μmol phospholipid, DPPC/ganglioside/cholesterol (1 : 0.1 : 0.4, mol/mol)) and 16 units of transfer protein for 1 h at $37\,^{\circ}$C in a total volume of 0.5 ml. SUV contained also [^{14}C]triolein as a non exchangeable marker. Incubations were always performed in buffer A. Exchange was terminated by centrifugation at $10\,000 \times g$ for 30 min. Bovine thyroid plasma membranes were washed three times with buffer A. The percentage ganglioside transfer was calculated from the radioactivity counted in the pellet (bovine thyroid plasma membranes) as well as in the supernatant. A correction for cosedimentation of vesicles was done by quantitation of the non exchangeable marker [^{14}C]triolein. It was assumed that there was no return of label from the membranes to SUV.

Modification of bovine thyroid plasma membranes by ganglioside incorporation

Gangliosides were incorporated in bovine thyroid plasma membranes (0.6 μmol phospholipid) using the same conditions as described for the ganglioside transfer assay. Several experiments required the incorpora-

tion of an equal amount of each of the gangliosides in bovine thyroid plasma membranes. To this end, the number of units of transfer protein and the SUV concentration in the incubation mixture were adapted according to the transfer activity measured for each of the gangliosides. Control incubations contained bovine thyroid plasma membranes with or without transfer protein only. After incubation the mixture was centrifuged at $10\,000 \times g$ for 30 min. Bovine thyroid plasma membranes were washed three times with the appropriate volume of buffer and aliquots were taken for adenylate cyclase assay, binding assay, anisotropy measurements and Blight and Dyer lipid extraction.

Adenylate cyclase assay

Adenylate cyclase activity was measured by adapting the methodology of Salomon [24]. Incubations were performed for 30 min at 30°C in a final volume of 60 μl containing approx. 100 μg of thyroid plasma membrane protein, approx. $3 \cdot 10^6$ cpm of [α-^{32}P]ATP (30–35 Ci/mmol), 5 mM creatine phosphate, 50 IU/ml creatine phosphokinase, 25 mM Tris-acetate buffer (pH 7.4), 5 mM magnesium acetate, 0.5 mM ATP, 0.05 mM cAMP, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.01 mM GTP, 10 mM theophyline. Reactions were terminated by the addition of 110 μl stopping solution (45 mM ATP and 1.3 mM cAMP in 2% (w/v) sodium laurylsulfate (pH 7.5)), 50 μl [8-^{3}H]3′,5′-cAMP ($2 \cdot 10^5$ cpm/ml) and heating for 3 min in a boiling water bath.

TSH binding assay

TSH binding was assayed in 40 mM Tris-acetate buffer (pH 6.0); 0.25% bovine serum albumin, approx. 100 000 cpm ^{125}I-TSH (about 1 ng of protein) and 20 μg of membrane protein in a final volume of 100 μl. Specific binding of 20 μg of membranes gave a value that is on the linear part of the binding curve when cpm bound is plotted against amount of membrane protein. Incubations were performed for 1 h at 4°C. The incubation was terminated by addition of 1 ml 40 mM Tris-acetate (pH 6.0) containing 2.5% bovine serum albumin. Bound radioligand was separated from free ^{125}I-TSH by filtration through 0.45 μm cellulose acetate filters placed in a 12-place vacuum filtration manifold VFM3 (Amicon). The filters were presoaked in 2.5% bovine serum albumin and the wash buffer was 40 mM Tris-acetate (pH 6.0) containing 2.5% bovine serum albumin. Nonspecific binding as measured by the addition of a 10 000-fold excess of unlabelled TSH ($4 \cdot 10^{-6}$ M) amounted to 7% of the specific binding.

Fluorescence measurements

Diphenylhexatriene was made up in tetrahydrofuran at a concentration of $4 \cdot 10^{-4}$ M, whereas TMA-DPH was made up in chloroform at the same concentration. For labelling, these solutions were diluted 1000-fold by adding to vigourously stirred buffer A. A stock solution of $4 \cdot 10^{-7}$ M 12-AS was prepared by diluting 1000-fold a $4 \cdot 10^{-4}$ M 12-AS solution in ethanol with buffer A. The fluorescent probes were added to plasma membranes at a ratio of one molecule fluorophore for every 500 phospholipid molecules. The final lipid phosphorus concentration was always adjusted to 50 μM. This was important in securing constant 'dilute' anisotropy values. Anisotropy measurements were performed at 37°C on a SLM 4800 spectrofluorometer. Temperature was controlled by Lauda thermostated water-bath and measured inside the cuvette with an AD590 probe (Analog Devices). The fluorophores DPH, TMA-DPH and 12-AS were excited at 360 nm and Schott KV399 filters were used in both emission beams.

Results

Ganglioside incorporation in thyroid plasma membranes

As shown in Table I, the nonspecific lipid transfer protein from bovine liver was able to promote the exchange of gangliosides (GM_1, GT_{1b}, GD_3) between ganglioside-containing SUV and bovine thyroid plasma membranes. Gangliosides GT_{1b} and GD_3 exhibited nearly the same transfer activity which was twice as high as that of GM_1. The spontaneous, nonprotein mediated transfer of GD_3 of GT_{1b} was approximately 8-times higher than that of GM_1. Transfer of a particular ganglioside in the absence of transfer protein was always less than 25% of the total transfer observed in the presence of 16 units of transfer protein. Taking into account the spontaneous exchange ($\approx 8\%$ for GT_{1b} or GD_3 and 1% for GM_1) the transfer protein seemed to be more effective for GM_1 (exchange activity was enhanced 18-times versus 4.5-times). The donor vesicles in

TABLE I

Ganglioside transfer from SUV to bovine thyroid plasma membranes catalyzed by transfer protein

For incubation conditions see Materials and Methods. Ganglioside transfer is expressed as percent of the indicated radioactive labelled ganglioside. SUV compositions are expressed as molar ratios. Cholesterol/phospholipid molar ratios were determined on a Bligh and Dyer lipid extract of bovine thyroid plasma membranes. Results are averages ±S.E. of duplicate determinations of three separate experiments. CH, cholesterol; PL, phospholipid; ns-TP, nonspecific lipid transfer protein.

SUV	ns-TP	% ganglioside transfer	CH/PL
DPPC/CH/[^{3}H]GM_1	–	1.0±0.2	0.40±0.04
(1:0.4:0.1)	+	18.5±1.5	0.38±0.03
DPPC/CH[^{3}H]GT_{1b}	–	8.5±0.8	0.39±0.05
(1:0.4:0.1)	+	37.3±2.3	0.37±0.03
DPPC/CH/[^{3}H]GD_3	–	8.0±0.7	0.38±0.04
(1:0.4:0.1)	+	36.3±2.1	0.37±0.03

all experiments had a cholesterol to phospholipid molar ratio of 0.4, a ratio identical to that of bovine thyroid plasma membranes [16]. Incubation of bovine thyroid plasma membranes with SUV of this composition, in the absence or presence of transfer protein, did not affect the molar ratio of cholesterol to phospholipids in the plasma membranes (Table I). This was important since changes in the cholesterol content of membranes have been shown to affect adenylate cyclase activity [25]. The plasma membrane phospholipid content was hardly affected after incubation with SUV and transfer protein indicating that ganglioside incorporation must be ascribed to ganglioside exchange rather than to absorption of liposomes to the plasma membranes. Moreover, we always corrected for cosedimentation of SUV with thyroid plasma membranes by quantitation of non exchangeable marker [^{14}C]triolein.

Effect of modification of membrane ganglioside composition on the TSH-stimulated adenylate cyclase activity

Incubations of bovine thyroid plasma membranes with lipid-exchange protein only did neither significantly affect basal nor TSH-stimulated activity. On the other hand incorporation of approx. 11 nmol GT_{1b} in thyroid plasma membranes resulted in an inhibition of 47% of the TSH-stimulated adenylate cyclase activity (Table II). Since gangliosides comprise up to approx. 1% of the total lipid content of thyroid plasma membranes [26] and incorporation of 11 nmol gangliosides resulted in an additional 2%, the ganglioside content of the plasma membranes was tripled after ganglioside-incorporation. Incorporation of an equal amount of GD_3 or GM_1 ($\approx$ 11 nmol) resulted in an inhibition of respectively, 37% and 23% of the TSH-stimulated adenylate cyclase activity (Table II). Therefore, the efficacy of inhibition of TSH stimulation had the following order: $GT_{1b} > GD_3 > GM_1$. The percentage inhibition of TSH-stimulated adenylate cyclase activity varied linearly with the amount of each ganglioside incorporated (Fig. 1). The data were fit to straight lines by the method of linear regression. The correlation coefficient was always around 0.8 and the correlation was significant at a $P < 0.005$ level (Student's t-test). Calculation of the slopes of the regression lines revealed that GT_{1b} was 2-tmes more effective as inhibitor of TSH stimulation than GM_1 and 1.3-times more effective than GD_3.

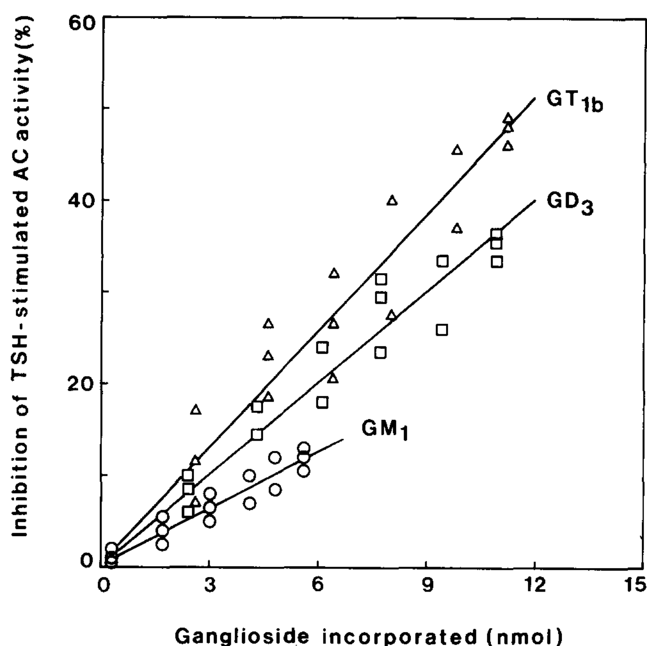

Fig. 1. Inhibition of TSH-stimulated adenylate cyclase activity as a function of the amount of each ganglioside incorporated: GT_{1b} (△), GD_3 (□) and GM_1 (○). Bovine thyroid plasma membranes (0.6 μmol phospholipid) were incubated with SUV (0.3 μmol phospholipid/ganglioside/cholesterol (1 : 0.1 : 0.4, mol/mol)) and 1.1; 4.6; 7.7; 11; 13.5 and 16 units of transfer activity. Controls contained bovine thyroid plasma membranes with the same units of exchange activity.

Effect of ganglioside incorporation on TSH binding

Inhibition of TSH stimulation could be due to gangliosides affecting TSH receptor binding. Therefore, the gangliosides were tested for their ability to affect the binding of ^{125}I-TSH to bovine thyroid plasma membranes. An equal amount ($\approx$ 11 nmol) of each of the gangliosides was incorporated in the plasma membranes. From these experiments it appeared that TSH binding to bovine thyroid plasma membranes was in-

TABLE II

Adenylate cyclase activity in unmodified and ganglioside-modified bovine thyroid plasma membranes

For incubation conditions see Materials and Methods. Percent inhibition was determined relative to control incubations which contained bovine thyroid plasma membranes with transfer protein alone. An equal amount of each of the gangliosides (approx. 11 nmol) was incorporated in the plasma membranes. Agonists were added to the incubation mixture to give the indicated final concentrations. Cholera toxin was preactivated by treatment with 20 mM dithiothreitol for 1 h at 30 °C. Results are averages ±S.E. of duplicate determinations of three different experiments.

Agonist	cAMP (pmol/mg protein per 30 min)				% inhibition		
	control	GM_1	GD_3	GT_{1b}	GM_1	GD_3	GT_{1b}
–	0.30±0.03	0.30±0.03	0.29±0.02	0.28±0.02	<1	3	7
TSH (50 mU/ml)	0.60±0.04	0.47±0.05	0.38±0.03	0.32±0.03	23	37	47
Cholera toxin (5 μM)	1.80±0.16	1.62±0.15	1.28±0.13	1.22±0.12	10	29	32
Forskolin (10 μM)	1.20±0.13	1.16±0.12	1.15±0.12	1.14±0.12	3	4	5
NaF (10 mM)	3.00±0.31	2.79±0.28	2.52±0.24	2.46±0.23	7	16	18

TABLE III

Influence of ganglioside-incorporation in bovine thyroid plasma membranes by transfer protein on TSH binding

For incubation conditions see Materials and Methods. Percent inhibition was determined relative to control incubations which contained bovine thyroid plasma membranes with transfer protein alone. An equal amount of each of the gangliosides (approx. 11 nmol) was incorporated in the plasma membranes. SUV compositions are expressed as molar ratios. Results are means ± S.D. of three separate experiments. CH, cholesterol.

SUV	% inhibition of TSH binding
DPPC/CH/GT_{1b} (1:0.4:0.1)	40 ± 4
DPPC/CH/GD_3 (1:0.4:0.1)	34 ± 3
DPPC/CH/GM_1 (1:0.4:0.1)	21 ± 4

hibited by ganglioside incorporation with the same order of efficacy ($GT_{1b} > GD_3 > GM_1$) and to the same extent as their inhibitory effect on TSH stimulation (Table III).

Influence of ganglioside incorporation on other stimulated states of adenylate cyclase

Although it was shown that incorporation of gangliosides into bovine thyroid plasma membranes inhibited TSH binding, it is possible that they could also exert an effect at a point beyond hormone binding. Therefore, their effects on NaF-, cholera toxin- and forskolin-stimulated adenylate cyclase activities were investigated. As shown in Table II, basal as well as forskolin-stimulated adenylate cyclase activities were not significantly affected by ganglioside modification of the plasma membranes, indicating that the gangliosides do not act at the level of the catalyst of adenylate cyclase. Incorporation of GT_{1b} or GD_3 in thyroid plasma membranes gave rise to a substantial decrease in cholera toxin-stimulated adenylate cyclase activity (approx. 30%0 and to a lesser degree to a decrease in NaF-stimulated activity (approx. 17%), whereas GM_1 incorporation did not significantly affect these stimulated activities (Table II).

TABLE IV

Effect of ganglioside-incorporation in bovine thyroid plasma membranes by transfer protein on membrane fluidity

For incubation conditions see Materials and Methods. Control incubations contained bovine thyroid plasma membranes with transfer protein alone. An equal amount of each of the gangliosides (approx. 11 nmol) was incorporated in the plasma membranes. SUV compositions are expressed as molar ratios. All anisotropy measurements were performed at 37 °C. Results are means ± S.E. of duplicate determinations of three separate experiments. CH, cholesterol.

SUV	r_s		
	DPH	12-AS	TMA-DPH
–	0.172 ± 0.005	0.132 ± 0.003	0.225 ± 0.004
DPPC/CH/GM_1 (1:0.4:0.1)	0.173 ± 0.005	0.133 ± 0.003	0.228 ± 0.004
DPPC/CH/GT_{1b} (1:0.4:0.1)	0.195 ± 0.006	0.136 ± 0.004	0.239 ± 0.005
DPPC/CH/GD_3 (1:0.4:0.1)	0.205 ± 0.006	0.138 ± 0.005	0.232 ± 0.003

Effect of ganglioside incorporation on membrane fluidity

Gangliosides might interfere with the coupling between $G_{s\alpha}$ and the catalyst of the adenylate cyclase system by affecting the membrane fluidity or by a direct interaction with the G_s-protein. In order to study the influence of ganglioside incorporation on the fluidity of bovine thyroid plasma membranes we measured the steady-state fluorescence anisotropy (r_s) of DPH, known to probe the hydrophobic interior of the membrane [27]; TMA-DPH, located at the hydrophobic/hydrophylic interface of the membrane [28] and 12-AS, a probe of which the fluorescent reporter group is located near the centre of the bilayer [29]. Simultaneous evaluation of the steady-state anisotropies of these fluorescent probes allowed the estimation of changes in membrane fluidity in different regions of the bilayer.

TABLE V

Effect of cooling on bovine thyroid adenylate cyclase activation

For incubation conditions see Materials and Methods. The incubation time was 30 min at the indicated temperature. Agonists were added to the incubation mixture to give the indicated final concentrations. Results are expressed in pmol cAMP/mg protein per 30 min and are averages ± S.D. of three separate experiments. *S*, stimulation factor.

Agonist	Temperature of the adenylate cyclase incubation (°C)					
	16		23		30	
	activity	*S*	activity	*S*	activity	*S*
None	0.030 ± 0.006		0.18 ± 0.04		0.30 ± 0.05	
Cholera toxin (5 μM)	0.080 ± 0.017	2.8	0.76 ± 0.12	4.2	1.86 ± 0.26	6.2
TSH (50 mU/ml)	0.069 ± 0.015	2.3	0.40 ± 0.06	2.2	0.59 ± 0.11	2.0
NaF (10 mM)	0.24 ± 0.04	8.0	1.64 ± 0.22	9.1	3.06 ± 0.32	10.2
Forskolin (10 μM)	0.13 ± 0.02	4.4	0.76 ± 0.14	4.2	1.22 ± 0.18	4.1

Incorporation of the various gangliosides in bovine thyroid plasma membranes by transfer protein did not significantly affect the fluorescence anisotropy of 12-AS (Table IV). However, GT_{1b}-modification of the plasma membranes provoked an increase in TMA-DPH anisotropy, whereas the anisotropy of DPH was substantially enhanced after incorporation of GD_3 or GT_{1b} (Table IV). GM_1-modification of the plasma membranes did not affect the fluorescence anisotropy of any of the probes tested. These results demonstrate that the inhibitory effects of the gangliosides on cholera toxin- and NaF-stimulated were paralleled by changes in DPH anisotropy.

The higher sensitivity of the cholera toxin activated adenylate cyclase towards changes in membrane fluidity is also suggested by the effect of temperature on the stimulating effect of this toxin. When the temperature of the adenylate cyclase incubation was lowered from 30°C to 16°C, basal as well as stimulated adenylate cyclase activities were decreased (Table V). The stimulation by forskolin and that induced by TSH (S-factors) were barely modified or slightly increased. The fluoride activation however was diminished upon cooling, whereas the activation by cholera toxin was substantially decreased (Table V).

Discussion

Spontaneous incorporation of gangliosides in membranes is a well established phenomenon [30–32]. However, less information excists on the protein-mediated transfer of gangliosides between membranes. Bloj and Zilversmit [33] reported that GM_1 exchange between membranes is catalyzed by the nonspecific lipid transfer protein from beef liver. The present study demonstrates that transfer of other gangliosides such as GD_3 and GT_{1b} is also catalyzed by this transfer protein. The protein-mediated exchange of GT_{1b} or GD_3 was twice as high as that of GM_1. The spontaneous, nonproteinmediated transfer of GT_{1b} or GD_3 was approx. 8-times higher than that of GM_1. The latter results are in agreement with those of Felgner et al. [32], who observed that the rate of spontaneous transfer for GT_{1b} is substantially larger than that for GM_1.

According to Felgner et al. [32] the spontaneous lipid transfer occurs through the aqueous phase and not upon collision of lipid-containing structures. Therefore, the higher transfer activity of negatively charged lipids might be due to their higher degree of hydrophylicity. Our results, together with those of Felgner et al. [32], suggest that ganglioside transfer activity is dependent upon the structure of the ganglioside molecules.

Incorporation of gangliosides in bovine thyroid plasma membranes was found to have a concentration-dependent inhibitory effect on TSH-stimulated adenylate cyclase activity with the following order of efficacy: $GT_{1b} > GD_3 > GM_1$. These data support the view that certain gangliosides might play a functional role in TSH receptor expression [11,12,34]. Our results are in agreement with those of Dacremont et al. [8] except that in human thyroid membranes GD_3 was more inhibitory than GT_{1b}. However, comparison with their results is difficult as they incubated gangliosides with the plasma membranes whereas in out study, gangliosides were incorporated into the plasma membranes using transfer protein. Since gangliosides form micelles even at low concentrations in aqueous media [35–37], thyrotropin might be partially captured by the micelles formed by the gangliosides which were not incorporated into the plasma membranes.

Binding experiments demonstrated that TSH binding to bovine thyroid plasma membranes was inhibited by ganglioside incorporation with the same order of efficacy ($GT_{1b} > GD_3 > GM_1$) and to the same extent as their inhibitory effect on TSH stimulation. Therefore, it is suggested that the decrease in TSH-stimulated adenylate cyclase activity upon ganglioside incorporation is mainly the result of a drop in TSH binding.

Although it has been reported previously that changes in membrane fluidity are able to modulate the affinity of TSH receptors [38], as well as the number of receptors in other systems [39], it is highly unlikely that the ganglioside induced changes in membrane fluidity are responsible for the decrease in TSH binding. This follows mainly from the observation that whereas incorporation of GM_1 causes a significant reduction in TSH binding the membrane fluidity is not significantly affected. Therefore, we believe that incorporated gangliosides directly interact with receptors decreasing their ability to bind TSH.

Incorporation of GT_{1b} or GD_3 in bovine thyroid plasma membranes gave however also rise to a substantial decrease in cholera toxin-stimulated adenylate cyclase activity and to a lesser degree in a decrease in NaF-stimulated activity, whereas GM_1 incorporation did not significantly affect these stimulated activities. Forskolin stimulation of adenylate cyclase was not significantly affected by ganglioside-modification of the plasma membranes. These results indicate that gangliosides might also interfere with the efficiency of the activating coupling between $G_{s\alpha}$ and the catalyst of the adenylate cyclase system. This might be due to changes in membrane fluidity or to a direct interaction of the gangliosides with the G_s-protein.

GT_{1b} modification of bovine thyroid plasma membranes provoked an increase in TMA-DPH anisotropy, whereas the anisotropy of DPH was substantially enhanced after incorporation of GD_3 or GT_{1b}. Thus, incorporation of GD_3 or GT_{1b} in thyroid plasma membranes resulted in a substantial decrease in fluidity in the hydrophobic core of the membrane. These results are in agreement with other physicochemical studies in

which it was shown that gangliosides might have a significant influence on molecular motions in phosphatidylcholine bilayers: their presence generally resulting in a rigidification of the bilayer and an increase in lipid order [40–43]. In a previous paper [26] we have also shown that incorporation of bovine thyroid membrane gangliosides in reconstituted vesicles provoked an increase in the lipid order parameter of DPH. The observed rigidification effect might be the consequence of interactions (formation of extra hydrogen bounds) among gangliosides and/or interactions between gangliosides and other membrane constituents [40,43]. These changes in membrane fluidity after ganglioside-modification of the plasma membrane might be responsible for the decrease in cholera toxin- and NaF-stimulation suggesting that gangliosides might interfere with the efficiency of the activating coupling between $G_{s\alpha}$ and the catalyst of the adenylate cyclase system by affecting the membrane fluidity. However, the possibility of a direct interaction of the gangliosides with the G_s-protein cannot be excluded.

Several other studies have also supported the view that the adenylate cyclase activity is sensitivity to alterations in membrane fluidity. More specifically it has been demonstrated that increase in bilayer fluidity achieved by either neutral [44] or charged [45] anaesthetics lead to an activation of adenylate cyclase. On the other hand, decreass in bilayer fluidity achieved by manipulating the cholesterol content of isolated rat liver plasma membranes [46,47] or treatment with dimethylnitrosamine [48], quinidine and mellitin [49] lead to a substantial inhibition of the adenylate cyclase. A potential role of membrane fluidity has also been suggested for the adenylate cyclase of euthyroid multinodular goiter plasma membranes [50].

The more pronounced inhibition of the cholera toxin-stimulated enzyme activity might be due to an additional effect of gangliosides on the catalytic activity (NAD^+-dependent ADP-ribosylation of $G_{s\alpha}$) of the active part of cholera toxin [51].

A role of membrane fluidity in the activating coupling between $G_{s\alpha}$ and the catalyst of the adenylate cyclase system is also suggested by the effect of lowering the temperature on the activation of adenneylate cyclase by cholera toxin and NaF. Whereas stimulation of the adenylate cyclase by TSH was not affected a substantial decrease in NaF- and cholera toxin-stimulated activity was observed.

The more pronounced effect of temperature on the cholera toxin-stimulated activity might be due to an additional effect on the enzymatic activity of the toxin.

Insensitivity to low temperatures on TSH-stimulated adenylate cyclase activity has also been previously reported for dog thyroid slices and their homogenates [52]; therefore one must assume that the interaction between the occupied TSH receptor and the G_s protein is not fluidity dependent and that this interaction is probably the rate-limiting step in the overall process.

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