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INHIBITION OF ADENYLATE CYCLASE ACTIVITY OF HUMAN THYROID MEMBRANES BY GANGLIOSIDES

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Gangliosides inhibit basal, thyrotropin-induced and fluoride-induced adenylate cyclase activity of human thyroid membranes in physiological conditions. In contrast neutral glycolipids, phospholipids and neuraminic acid containing oligosaccharides show no effect. The efficacy of inhibition is more dependent upon the position of the sialic acid residues than upon their absolute number. In general gangliosides with disialyl groups are more inhibitory than those with single sialyl moieties. The inhibitory effects of the individual gangliosides on the two modes of stimulation are parallel. This parallelism suggests that the inhibitory effect is located at the postreceptor level and that the gangliosides interact directly with the adenylate cyclase system. A possible role of thyroid membrane gangliosides as suppressive cofactors of adenylate cyclase is discussed in relation to recent findings of stimulating anti-ganglioside antibodies in Graves' disease.

Introduction

Gangliosides, neuraminic acid containing glycosphingolipids, are important components of plasma membranes. The role of G_{M1} as receptor molecule for cholera toxin [1,2] has been established and it has been postulated that gangliosides also function as receptors for tetanus toxin [3], interferon [4] and peptide hormones such as thyrotropin (TSH). It was suggested that gangliosides inhibit TSH binding to thyroid plasma membranes by interacting with the hormone rather than with the membrane [5–7]. However, this interaction is strongly dependent on pH, salt and temperature and is maximal under non-physiological conditions [8–11].

Thyroid membranes contain two distinct classes of binding sites for TSH. First, low-affinity, high-capacity sites present in large numbers are only

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Abbreviations: TSH, thyroid-stimulating hormone or thyrotropin; G_{M3}, *N*-acetylneuraminylgalactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{M1}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; L_{M1}, *N*-acetylneuraminylgalactosyl-*N*-acetylglucosaminylgalactosylglucosylceramide = sialylparagloboside; G_{D3}, *N*-acetylneuraminyl-*N*-acetylneuraminylgalactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; G_{D1b}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylceramide; G_{T1b}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl-*N*-acetylneuraminyl)galactosylglucosylceramide; asialo-G_{M1}, galactosyl-*N*-acetylgalactosaminylgalactosylglucosylceramide; asialo-G_{M2}, *N*-acetylgalactosaminylgalactosylglucosylceramide; G-regulatory protein, guanine nucleotide regulatory protein; NANA, *N*-acetylneuraminic acid.

detectable under non physiological conditions, whereas high-affinity, low-capacity sites can be detected under more physiological conditions [9–13]. Gangliosides do not block TSH binding to the high-affinity binding sites [10,11,13–15]. From these studies it was concluded that gangliosides were not part of the TSH receptor [13].

Apart from binding TSH gangliosides may have a more important role in the transmission of the TSH signal through the membrane [5–7,16], but this hypothesis and in general any role of thyroid gangliosides in activation or regulation of adenylate cyclase has not been examined thoroughly.

In order to get more information and evaluate an eventual role of gangliosides in the regulation of thyroid adenylate cyclase, we tested as a first approach, various gangliosides, and their neutral derivatives, phospholipid and neuraminic acid containing compounds for their ability to affect adenylate cyclase activity of human thyroid membranes under physiological conditions. We investigated their influence on basal, TSH-stimulated activity and also on sodium fluoride-induced activity. This paper reports the specific inhibitory effects of gangliosides on basal and induced adenylate cyclase activity.

Materials and Methods

Membranes. Membranes were prepared from surgically obtained thyroid tissue. All postsurgical manipulations were carried out at 4°C following the method of Orgiazzi et al. [17] with some modifications [18].

Glycolipids. Gangliosides, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} were isolated from normal human brain, G_{M2} was prepared from Tay Sach's brain, G_{M3} from human kidney and G_{D3} from human thyroid tissue.

Gangliosides were extracted from tissues by the method of Tettamanti et al. [19], separated according to the number of sialic acids by chromatography on DEAE-Sephadex or on DEAE-Sepharose [20] and further purified by silica column chromatography and preparative thin-layer chromatography. Ganglioside L_{M1} and the neutral glycolipids lactosylceramide and paragloboside were obtained from human type O erythrocytes. Total glycolipids from erythrocytes were extracted by the procedure of Hakamori and Watanabe [21], gangliosides purified from the 'upper-phase glycolipids' as described above and neutral glycolipids from the 'lower-phase glycolipids' by repeated chromatography on silica columns.

Asialo-G_{M1} and asialo-G_{M2} were obtained by partial hydrolysis of, respectively, G_{M1} and G_{M2} in 1 M formic acid (100°C, 1 h). All gangliosides and neutral glycolipids were identified by gas chromatography and enzymatic analysis. Their purity was assessed by thin-layer chromatography in various solvent systems. All preparations were at least 99% pure and free from phospholipid contaminants.

Oligosaccharides. Sialic acid containing oligosaccharides were isolated and purified from human urine by combined anion and cation exchange chromatography as described by Strecker et al. [22].

Egg yolk phosphatidylcholine was obtained from Sigma Chemical Co. St. Louis, MO, U.S.A., and sphingomyeline from Koch and Light Laboratories Ltd. Colnbrook, U.K. Dicetylphosphate was purchased from K and K Laboratories Inc., Planview, NY, U.S.A.

Adenylate cyclase assay. The effect of the various glycolipids and oligosaccharides on stimulated adenylate cyclase activity of thyroid membranes was evaluated by the method of Orgiazzi et al. [17] with some modifications described below. In standard experiments NaF (10 μM) or TSH (24.6 U/l, UCB Belgium) and increasing amounts of gangliosides were preincubated for 30 min at 34°C in a total volume of 100 μl ATP buffer (containing 20 mM Tris-HCl buffer (pH 7.4), 6 mM $MgCl_2$, 3 mM ATP, 10 mM theophylline, 10 mM creatinine phosphate and 43 μg of rabbit muscle creatine kinase). When the temperature of the reaction reached 34°C 50 μl of thyroid membrane suspension (100 μg of protein) in the same buffer was added and incubation continued for 15 min with TSH or NaF. The reaction was stopped by adding 200 μl of boiling 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA. The mixture was then centrifuged and cAMP determined on an aliquot of the supernatant by a competitive protein binding assay (Amersham International).

The influence on basal adenylate activity was measured under the same conditions in the ab-

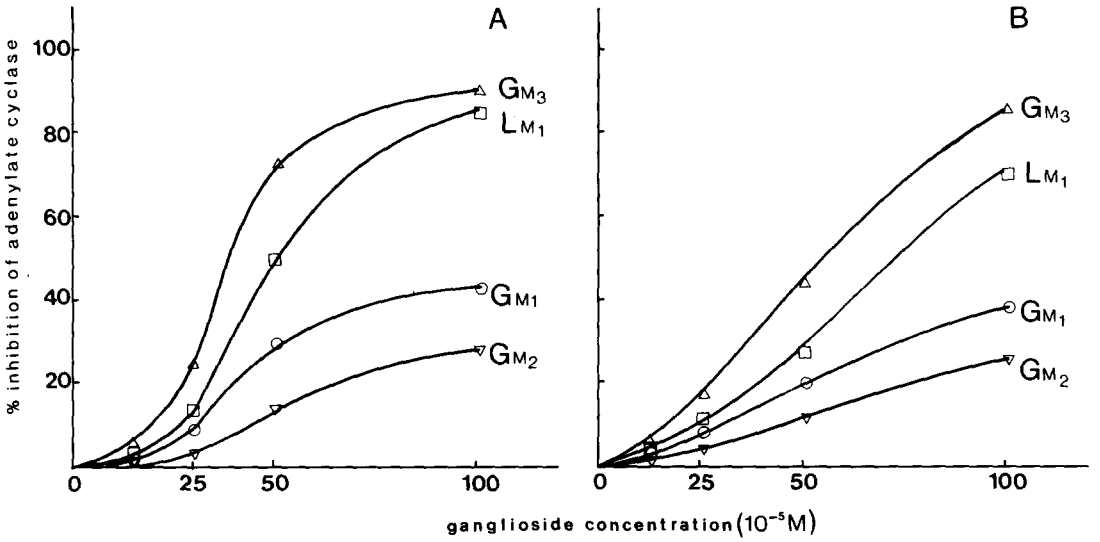

Fig. 1. Inhibitory effect of monosialogangliosides on (A) TSH-induced, (B) NaF-induced adenylate cyclase activity.

sence of stimulators. Gangliosides, sulphatides, neutral glycolipids and phospholipids were tested in the concentration range of 0 to 1 mM and neuraminic acid containing oligosaccharides in the range of 0 to 2 mM neuraminic acid. Results are expressed as % of inhibition of adenylate cyclase activity (in the figures) or as absolute amount of cAMP produced per 15 min per mg protein (in Table I).

Results

All monosialogangliosides tested inhibit basal, TSH- and NaF-stimulated adenylate cyclase activity of human thyroid membranes. The efficacy of inhibition of monosialogangliosides had the following order: $G_{M3} > L_{M1} > G_{M1} > G_{M2}$ (Fig. 1, Table I). Monosialogangliosides with neuraminic acid attached to the terminal galactose were in general more inhibitory than those with neuraminic acid on the internal galactose.

In order to assess the role of the negatively charged sialic acid residue we also tested the asialo derivatives of G_{M1}, G_{M2}, G_{M3} and L_{M1}. None of these neutral glycosphingolipids had any effect on basal nor stimulated adenylate cyclase activity nor had sulphatides. These data suggest the relative importance of the neuraminic acid residue. Neuraminic acid containing oligosaccharides, however, had no inhibitory effect indicating also the role of the lipid moieties.

Since human thyroid membranes (Ref. 23 and our own observations) contain more complex gangliosides we also tested the effect of disialo- and trisialogangliosides in our system. As seen in Fig. 2 and Table I both TSH- and NaF-stimulated as well as the basal adenylate cyclase activity of human thyroid membranes were inhibited. The efficacy of inhibition had the following order: $G_{D3} > G_{D1b} = G_{T1b} > G_{D1a}$. Gangliosides with a disialosyl group (G_{D3}, G_{D1b}, and G_{T1b}) are the

TABLE I

EFFECT OF VARIOUS GANGLIOSIDES ON BASAL ADENYLATE CYCLASE ACTIVITY

Increasing amounts of gangliosides were suspended in ATP buffer (see Methods) and incubated for 15 min at 34°C with human thyroid membranes in the absence of stimulator. The amount of cAMP produced as measured by competitive protein binding assay is expressed in pmol per 15 min per mg membrane.

	Activity		
Concn. (M)($\times 10^5$):	0	50	100
G_{M3}	99	42	9
G_{M2}	103	96	90
G_{M1}	101	88	66
L_{M1}	112	58	22
G_{D3}	87	3	-
G_{D1a}	93	85	59
G_{D1b}	103	12	4
G_{T1b}	95	44	11

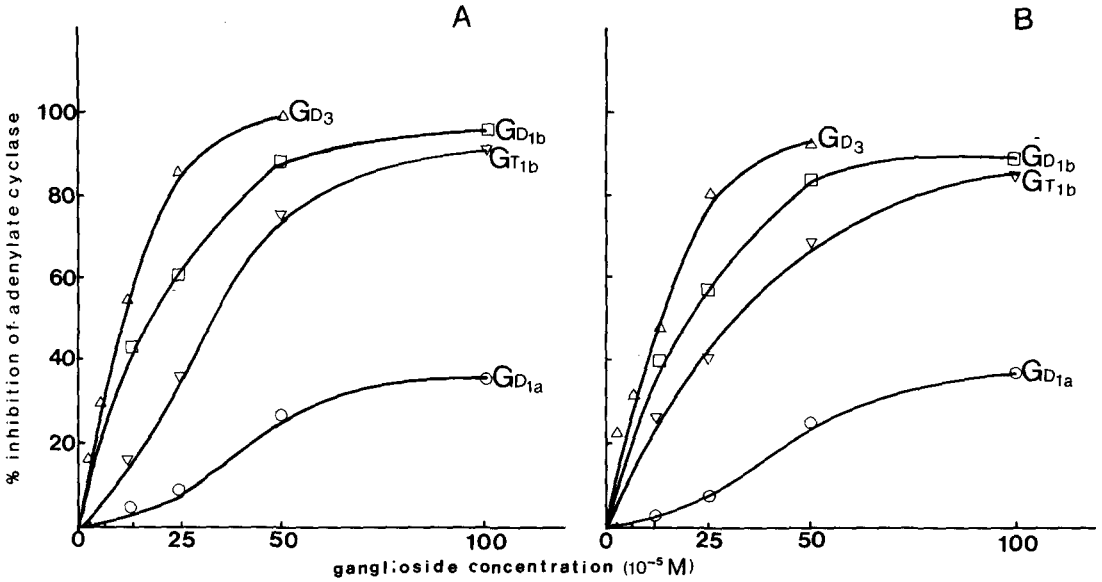

Fig. 2. Inhibitory effect of di- and trisialogangliosides on (A) TSH-stimulated, (B) NaF-stimulated adenylate cyclase activity.

strongest inhibitors. The inhibitory effects of mono, di- and trisialogangliosides were parallel in both fluoride- and TSH-stimulated adenylate cyclase activities. These results suggest that the inhibitory effects by gangliosides were located at the postreceptor level, since fluoride is believed to act via the G-regulatory protein.

The phospholipid composition affects the hormonal response in many tissues including thyroid cells [24]. However, in our system phospholipids such as sphingomyelin or phosphatidylcholine and dicetylphosphate had no inhibitory effect on basal nor TSH- and fluoride-stimulated adenylate cyclase activity.

Discussion

The present report demonstrates that purified ganglioside preparations inhibit basal, TSH- and NaF-stimulated adenylate cyclase activity of human thyroid membranes. In contrast asialyl derivatives of these gangliosides, neuraminic acid-containing oligosaccharides and phospholipids had no inhibitory effect, indicating that the inhibition is dependent on both neuraminic acid and lipid residues of the gangliosides.

The efficacy of inhibition of the adenylcyclase activity by gangliosides was more dependent upon the position of the sialic acid residues than on their absolute number (Fig. 3). In general gangliosides with a disialosyl group (G_{D3}, G_{D1b}, G_{T1b}) were more inhibitory than those with a monosialosyl residue (G_{M1}, G_{M2}, L_{M1}, G_{M3}, G_{D1a}). A

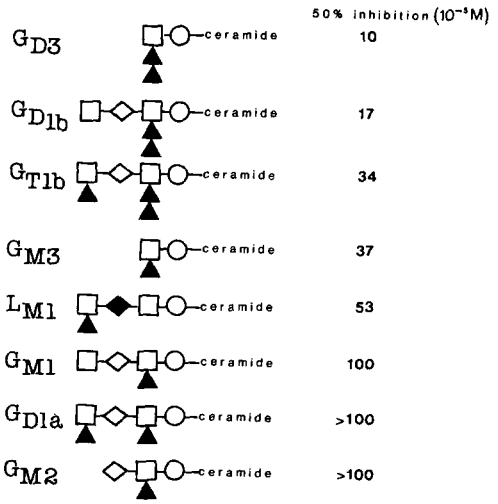

Fig. 3. Schematic representation of the structure of the different gangliosides used in relation to their inhibitory capacities on adenylate cyclase activity. In the right column the amount of gangliosides (nmol) necessary to inhibit the TSH- or NaF-stimulated adenylate cyclase by 50% is indicated. Symbols: □, galactose; ◇, *N*-acetylgalactosamine; ○, glucose; ◆, *N*-acetylglucosamine; ▲, *N*-acetylneuraminic acid.

shorter carbohydrate core clearly enhances the inhibitory effect of both mono- or disialogangliosides.

The concentration of gangliosides required to inhibit the adenylate cyclase activity in our system were above physiological concentration, except for G_{D3} which showed already an inhibitory effect at $3 \cdot 10^{-5}$ M. It is not excluded that in human thyroid membranes more potent ganglioside inhibitors, possibly as minor components, are present. The ganglioside composition of human thyroid is until now not analysed systematically. The presence of different types of gangliosides has been described but identification was only performed by TLC and was based on the relative migration rates with respect to ganglioside standards from brain [23]. In regard to molecular species of gangliosides, human thyroid appears to be more complex than bovine thyroid. In the latter five molecular species were identified: G_{M3}, G_{M1}, fucosyl-G_{M1}, G_{D3} and G_{D1a} [25,26].

In regard to the mechanism by which gangliosides inhibit thyrotropin action and subsequent activation of the adenylate cyclase system, our data are suggestive that the inhibitory effect is the result of a direct interaction of the gangliosides with the adenylate cyclase system rather than with the hormone, since both basal and stimulated adenylate cyclase activities are inhibited by gangliosides. The parallelism in the dose inhibition curves for both TSH- and fluoride-stimulation of the individual gangliosides points to a similar mechanism. Fluoride acts directly on the G-regulatory protein-adenylate cyclase complex [27] and there is no interaction between fluoride and gangliosides. Consequently, the inhibitory effect of gangliosides can only be explained by a direct interaction with the adenylate cyclase system, bypassing the glycoprotein component of TSH receptor. Therefore endogenous more internally located gangliosides in human thyroid cells may be associated to the adenylate cyclase system and be involved as constant suppressors or in general as regulating 'cofactors' of the adenylate cyclase activity. A possible role of gangliosides as cofactors of membrane adenylate cyclase has already been suggested by Partington and Daly [28].

Further evaluation of the role of gangliosides on thyrotropin receptor functions will be possible with the use of antibodies to these sphingoglycolipids. It has been reported that immunization of rabbits with gangliosides results in thyroid hypofunction of the immunized animals suggesting that antibodies against thyroid gangliosides produce impairment of thyroid function [29]. In contrast the effect of thyroid stimulating immunoglobulins present in patients with active Graves' disease can be blocked by G_{M1} [18]. These patients have circulating antibodies against asialo G_{M1} (Ref. 30, and unpublished observations), suggesting that a population of antibodies can remove the inhibiting effects of gangliosides in adenylate cyclase activity. The specific effector role of these anti-ganglioside antibodies has been clarified by monoclonal antibody technology. Some human monoclonal antibodies derived from hybridization of peripheral blood lymphocytes of patients with Graves' disease exerting stimulatory effects on thyroid cells, interact with human thyroid gangliosides, but do not inhibit TSH binding to the glycoprotein component of TSH receptors. A second set of monoclonal antibodies with no stimulatory action block the binding of TSH to the glycoprotein part of its receptor [31].

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