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DIGLYCERIDE KINASE FROM *ESCHERICHIA COLI*

MODULATION OF ENZYME ACTIVITY BY GLYCOSPHINGOLIPIDS *

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Diglyceride kinase was purified from membranes of *Escherichia coli* K-12 using organic solvents. The enzyme apoprotein depended on lipids, such as cardiolipin (diphosphatidylglycerol), phosphatidylcholine or 1-monooleoylglycerol, for activity with 1,2-dipalmitoylglycerol. Mixed brain cerebrosides and gangliosides as well as defined ganglioside fractions and synthetic lactocerebroside were devoid of lipid cofactor activity. However, all these glycosphingolipids were strong inhibitors of activation by phosphatidylcholine. When cardiolipin was used as lipid activator with the detergent, Triton X-100, as solubilizing agent, the addition of mixed or purified gangliosides first (at about 0.4 mM) resulted in additional activation, but higher ganglioside concentrations were strongly inhibitory. Both effects were absolutely dependent on the presence of lipid-bound sialic acid and were not given by cerebrosides, by free sialic acid or by sialyl-lactose. The stimulating and inhibitory effects of glycosphingolipids could also be demonstrated when 1-monooleoylglycerol was used as substrate, lipid activator and solubilizing agent at the same time. The modulation of kinase activity by glycosphingolipids is discussed at the level of lipid/protein interactions.

Introduction

The glycosphingolipids of mammalian plasma membranes have been recognized as important cellular antigens and differentiation markers, as well as receptor sites for certain toxins, hormones and lectins [1–5]. It is, however, at present unknown how the initial binding events are communicated into the cell so that the often drastic physiological responses are elicited.

Physical studies on glycosphingolipids have revealed a tendency to cluster and self-interact in phospholipid bilayers [6–8]. Interactions with functional membrane proteins may also occur, and the existence of a relatively tight glycosphingolipid 'annulus' around at least certain membrane proteins has been suggested [1]. Glycolipid/protein interactions may therefore be involved in the aforementioned information transfer across the plasma membrane. However, very little experimental data on interactions between glycosphingolipids and functional membrane proteins are available.

Sulfatides are thought to be an essential part of the $(Na^+ + K^+)$-ATPase [9], but evidence to the contrary also exists [10]. A brain microsomal Mg^{2+}-ATPase activity was stimulated by added gangliosides [11] but the $(Na^+ + K^+)$-ATPase of the same membrane was unaffected. Cross-linking experiments have pointed to glycosphingolipid/protein associations in erythrocyte membranes [12]. It has furthermore been suggested that the changed glycosphingolipid patterns of transformed cells may lead to changes in membrane-bound enzyme activities [2]. However, even though

* Dedicated to Professor Dr. H. Holzer on the occasion of his 60th birthday.

extensive studies on the regulation of purified membrane enzymes by phospholipids and detergents have been carried out (for review, see. Ref. 13), no such studies are available for glycosphingolipids.

In the present communication, a highly purified preparation of diglyceride kinase from *Escherichia coli* membranes [14] has been used to search for functional effects of glycosphingolipids. The purified enzyme could be used as a sensitive probe for lipid/protein interactions because its activity was absolutely dependent on lipids [14]. Glycosphingolipids could not activate the enzyme apoprotein by themselves, but they acted as strong modulators of kinase activity restored by other lipids.

Materials and Methods

Materials

Diglyceride kinase (diacylglycerol kinase) was extracted from commercial cells of *E. coli* K-12 (Merck No. 51100) using butanol-1. The enzyme was then purified through step 4 (chromatography on DEAE-cellulose) or, where indicated, step 5 (chromatography on Sephadex LH-60) of the published procedure [14]. Bovine heart cardiolipin, egg phosphatidylcholine and 1-monooleoylglycerol were from Sigma Co., St. Louis, MO, while 1,2-dipalmitoyl-*sn*-glycerol was purchased from Fluka, Neu-Ulm. The mixed brain cerebrosides and gangliosides were obtained from Koch-Light Ltd., Colnbrook. Mean molecular weight values of 728 and 1263 were used for the mixed cerebroside and ganglioside, respectively. Thin-layer chromatography in solvent system A showed only one component (R_f, 0.85) in the case of the cerebrosides, but nine spots of R_f, between 0.09 and 0.85, were present in the gangliosides. A colorimetric assay [15] indicated that the latter preparation contained about 1.2 nmol sialic acid per nmol of ganglioside. The defined mono-, di- and trisialoganglioside fractions, GM_1, GD_1 and GT_1, were purchased from Supelco, Bellefonte, PA. Thin-layer chromatography in solvent system A showed a single spot for GM_1, but GD_1 contained about 10% of GM_1, and GT_1 about 20% of GD_1. Synthetic lactocerebroside was purchased from Miles Co., Frankfurt. All commercial lipids were used without further purification.

General methods

Determinations of protein, lipid phosphorus and radioactivity were carried out as previously described [14]. Thin-layer chromatography was performed on precoated Silica gel G plates (Merck No. 5553) using solvent system A, chloroform/-methanol/0.2% (w/v) $CaCl_2$ in water (60:40:9, v/v). Ascending paper chromatography on Whatman 3MM paper was performed in solvent system B, diisobutyl ketone/acetic acid/water (20:15:2, v/v).

Standard kinase assay

Unless noted otherwise, the following assay procedure was employed. Aliquots of chloroform/methanol or hexane solutions of 1,2-dipalmitoylglycerol, lipid activator and/or glycosphingolipid were pipetted into a test-tube and solvents were removed in vacuo. Enzyme (1–10 μg protein) was added in butanol-1 solution [14], followed again by solvent removal in vacuo. The dry enzyme/lipid film was shaken with about 50 μl water and the sample was again dried in vacuo (about 30 min, 20°C, vacuum pump). The purpose of the addition and subsequent removal of water was to eliminate trace of butanol-1. Buffer (95 μl; 50 mM Tris-phosphate, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, pH 6.6, containing 0.70% (w/v) Triton X-100) was added, and the enzyme/lipid film was dispersed by mild sonication (Bandelin sonic bath RK 255, 30 s, 25°C). The reaction was started by addition of 5 μl 80 mM [γ-^{32}P]ATP (0.05–0.1 μCi). After 30 min at 40°C in a shaking water-bath, the incubation was terminated by addition of 50 μl chloroform/-methanol (1:1, v/v). The entire reaction mixture was fractionated in solvent system B, and the amount of [^{32}P]phosphatidic acid was determined by liquid scintillation counting [14].

Prolonged storage of the enzyme at −70°C and thawing-freezing of the enzyme solution led to losses in kinase activity so that the specific kinase activities in different experiments showed some variation.

Results

Lipid activation

The purified kinase apoprotein was inactive

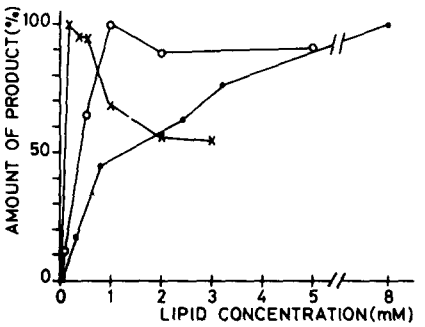

Fig. 1. Activation of diacylglycerol kinase apoprotein by bovine heart cardiolipin (×———×), egg phosphatidylcholine (○———○), and 1-monooleoylglycerol (●———●). Cardiolipin and phosphatidylcholine were tested in the standard assay procedure in the presence of 0.1 mM 1,2-dipalmitoylglycerol and 0.66% (w/v) Triton X-100. These components were omitted in assays involving 1-monooleoylglycerol. Cardiolipin and phosphatidylcholine were tested with 12 μg step 4 enzyme [14], whereas 1-monooleoylglycerol was tested with 0.8 μg step 5 enzyme [14]. The maximal amounts of product obtained in each case were set 100%. These values were 33 nmol/min per mg protein for cardiolipin, 16 nmol/min per mg protein for phosphatidylcholine and 1040 nmol/min per mg protein for 1-monooleoylglycerol.

with 1,2-diacylglycerols unless recombined with lipids such as cardiolipin, phosphatidylcholine or 1-monooleoylglycerol (Fig. 1). These lipid activators had in principle at least two functions, viz. (i) solubilization of the water-insoluble substrate and apoprotein, and (ii) specific interaction, with the apoprotein. Since only the latter was to be studied, the detergent, Triton X-100, was routinely included in the incubation mixture at 0.66% (w/v) (about 11 mM). Triton X-100 did not activate the apoprotein by itself, although it enhanced the activity restored by cardiolipin, phosphatidylcholine and other lipid activators (not shown).

1-Monooleoylglycerol was unique in serving as substrate, lipid cofactor and solubilizing agent at the same time (Fig. 1), although relatively high concentrations were required for full activation.

The following glycosphingolipids were tested at up to 4 mM in the presence of Triton X-100, mixed brain cerebrosides and gangliosides, synthetic lactocerebroside and the defined ganglioside fractions, GM_1, GD_1 and GT_1. No activation of the apoprotein could be obtained.

Effects of glycosphingolipids

Initial experiments were performed with egg phosphatidylcholine as lipid activator and Triton X-100 as solubilizing agent. Addition of glycosphingolipids led to strong inhibition, as shown in Fig. 2 for mixed brain gangliosides and for synthetic lactocerebroside. Similar inhibition curves were obtained with the defined ganglioside fractions, GM_1, GD_1 and GT_1 and with mixed brain cerebrosides. The mixed brain gangliosides and synthetic lactocerebroside were also studied in the absence of Triton X-100, and at least at protein amounts of >2 μg strong inhibition was again observed for both glycolipids (1 mM egg phosphatidylcholine used as activator).

When cardiolipin was used as lipid activator with Triton X-100 as solubilizing agent, only glycosphingolipids containing sialic acid were inhibitory. This became already apparent in a comparison of mixed brain cerebrosides and gangliosides (Fig. 3). To further study the effect of sialic acid, the mixed brain gangliosides were subjected to mild acid hydrolysis (50 mM HCl, 60 min, 80°C). The resulting glycolipid fraction contained only about 0.1 nmol sialic acid/nmol lipid (colorimetric assay [15]). The hydrolysed glycolipid was without effect when tested at up to 1 mM under the conditions of Fig. 3. When the inhibition by increasing amounts of mixed brain gangliosides was studied at several fixed concentrations of 1,2-dipalmitoylglycerol, no change in the apparent K_m value of 1,2-dipalmitoylglycerol (25 μM) was ob-

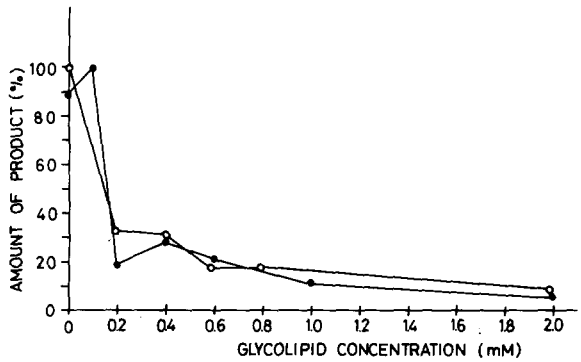

Fig. 2. Effect of glycosphingolipids on phosphatidylcholine-activated diacylglycerol kinase. The standard incubation mixture contained 1 mM egg phosphatidylcholine, 0.1 mM 1,2-dipalmitoylglycerol, 0.66% (w/v) Triton X-100, 3 μg step 4 enzyme [14] and, in addition, the indicated concentrations of lactocerebroside (●———●) or mixed brain gangliosides (○———○). Two independent experiments are shown and the maximum amounts of product obtained are set 100%.

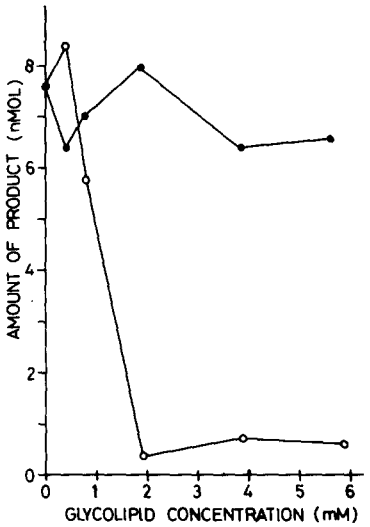

Fig. 3. Effect of glycosphingolipids on cardiolipin-activated diacylglycerol kinase. The standard incubation mixture contained 0.41 mM cardiolipin, 0.1 mM 1,2-dipalmitoylglycerol, 0.66% (w/v) Triton X-100, 6 μg step 4 enzyme [14] and, in addition, the indicated concentrations of mixed brain cerebrosides (●———●) or gangliosides (○———○). The amounts of product are plotted against glycolipid concentration.

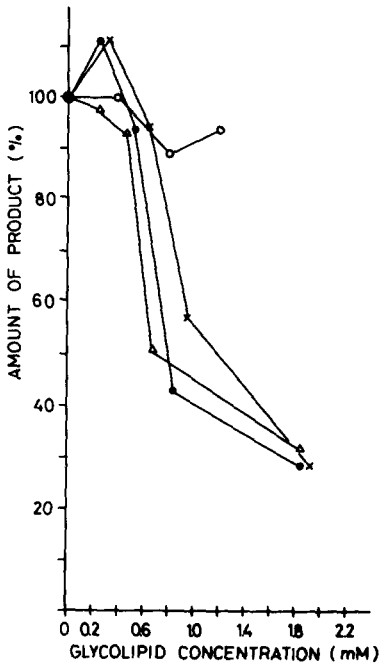

Fig. 4. Effect of defined glycosphingolipids on cardiolipin-activated diacylglycerol kinase. The standard incubation mixture contained 0.41 mM cardiolipin, 0.1 mM 1,2-dipalmitoylglycerol, 0.66% (w/v) Triton X-100, and the indicated concentrations of lactocerebroside (○———○), or ganglioside fractions GM_1 (×———×), GD_1 (●———●) or GT_1 (△———△). The amounts of step 4 enzyme [14] were 3 μg protein in the case of lactocerebroside and 11 μg protein in the case of the gangliosides. The amounts of product obtained in the absence of glycosphingolipid (= 100%) were 66 nmol/min per mg protein in the lactocerebroside experiments and 20 nmol/min per mg protein in the ganglioside experiments.

served (tested with 0.41 mM cardiolipin, 0.66% (w/v) Triton X-100; not shown).

At low ganglioside concentrations some stimulation of kinase activity appeared to occur (Fig. 3). More extensive experiments with mixed gangliosides and with the defined GM_1 and GD_1 ganglioside fractions showed that the 10–20% stimulation at 0.4 mM ganglioside was real. A representative experiment where the three ganglioside curves are directly comparable is shown in Fig. 4. At concentrations > 0.6 mM all three ganglioside fractions were strongly inhibitory, but synthetic lactocerebroside was without significant effect. The latter was also found for free sialic acid (up to 1 mM) or sialyl lactose (up to 4 mM) or NaCl (tested up to 500 mM) under the conditions of Fig. 4.

In further experiments (Fig. 5) it was examined whether an increase in the negatively charged activator lipid, cardiolipin, would abolish the effects of the negatively charged gangliosides. This was not the case, since both the stimulatory and the inhibitory effects of gangliosides occurred even when the concentration of cardiolipin was increased 5-fold, to 2.2 mM.

It was next examined, whether the effects of gangliosides were in some way linked to properties of the water-insoluble substrate, 1,2-dipalmitoylglycerol. The alternative substrate, 1-monooleoylglycerol, had drastically different physical properties and could also serve as lipid activator and solubilizing agent. Addition of lactocerebroside to 1-monooleoylglycerol led to a pronounced stimulation of the reaction (Fig. 6A).

Addition of mixed gangliosides first (at approx. 0.5 mM) led to about 50% stimulation of kinase activity, but higher ganglioside concentrations were strongly inhibitory. These experiments were repeated in the additional presence of cardiolipin in order to examine whether the observed effects were sensitive to charge (Fig. 6B). Both glycolipids

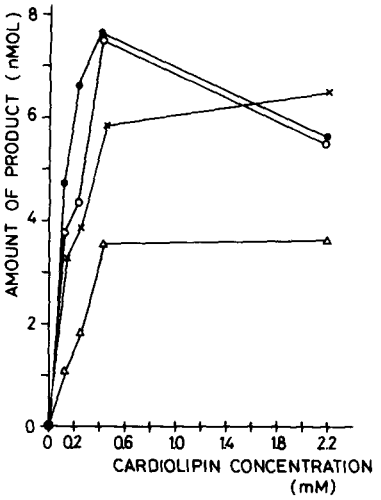

Fig. 5. Effect of ganglioside on cardiolipin-activated diacylglycerol kinase. The standard incubation mixture contained the indicated concentration of cardiolipin as well as 0.1 mM 1,2-dipalmitoylglycerol, 0.66% (w/v) Triton X-100, 7 μg step 4 enzyme [14] and, in addition, 0.39 (●——●), 0.79 (×——×), 2.4 mM (△——△) or no (○——○) mixed brain ganglioside. The amount of product (nmol) is plotted against cardiolipin concentration (mM).

retained their effects in the presence of cardiolipin, the ganglioside inhibiting at even lower concentration.

Discussion

A possible mechanism for kinase modulation by glycolipids

The main purpose of the present study was to search for functional effects of glycosphingolipids, using the lipid-dependent bacterial diacylglycerol kinase apoprotein as a probe. The purified apoprotein could be activated by a variety of phospholipids, fatty acids and certain detergents (unpublished data). In contrast, the glycosphingolipids tested were devoid of cofactor activity. Their main effect was to inhibit the activation given by other lipids. Under certain conditions, glycosphingolipids could also enhance the activation by other lipids.

The glycosphingolipids could conceivably act

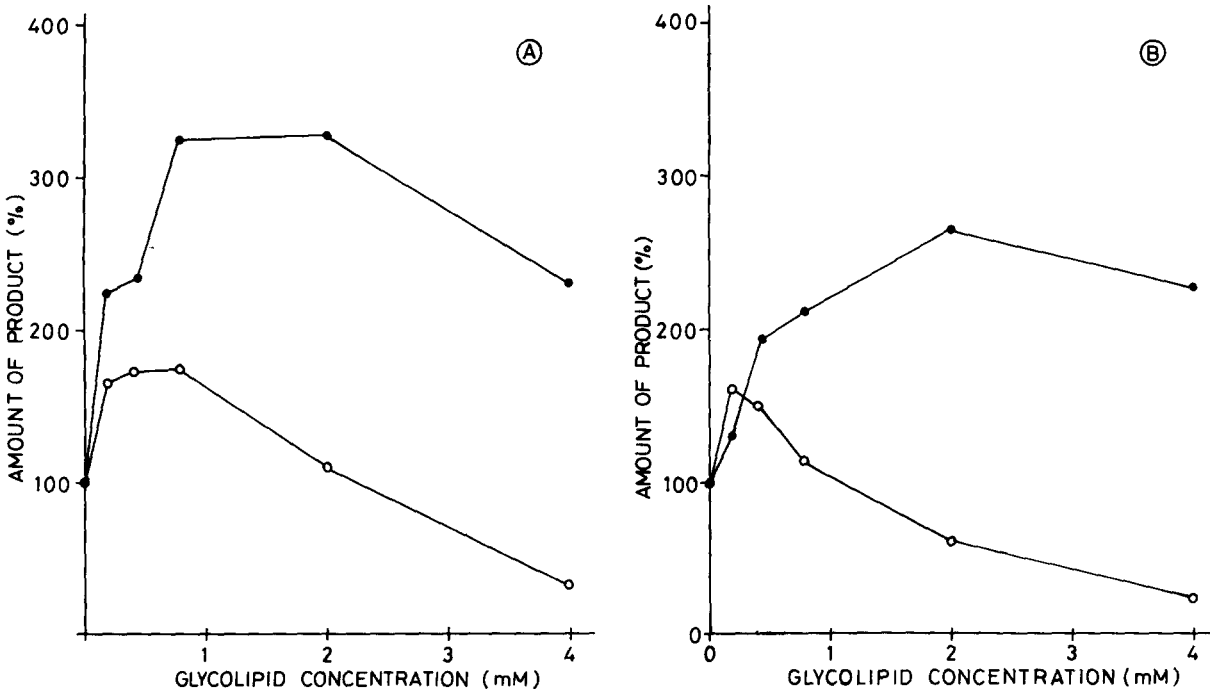

Fig. 6. Effect of glycosphingolipids on 1-monooleoylglycerol-activated diacylglycerol kinase (A) in the absence, and (B) in the presence of cardiolipin. The standard incubation mixture contained 8 mM 1-monooleoylglycerol with or without 0.41 mM cardiolipin, 1 μg step 5 enzyme [14] and the indicated concentration of lactocerebroside (●——●) or mixed brain ganglioside (○——○). The amount of product (%) is plotted against glycosphingolipid concentration. The amounts of product formed in the absence of glycosphingolipid (=100%) were 9.5 nmol in the absence and 16.5 nmol in the presence of cardiolipin.

by two possible mechanisms. They could change lipid/protein interactions, or they could influence the simple aqueous solubilization of the water-insoluble enzyme apoprotein and the substrate, 1,2-dipalmitoylglycerol. In order to reduce mere solubilization problems, an excess (about 11 mM) of the solubilizing agent, Triton X-100, was included in the standard incubation mixture. The presence of excess detergent micelles probably also reduced differences in physical properties, such as fluidity, amongst the different lipids tested. The best evidence that simple solubilization did not become rate-limiting could be obtained by using 1-monooleoylglycerol as substrate, lipid activator and solubilizing agent at the same time. In spite of the high 1-monooleoylglycerol concentration required, the stimulatory and inhibitory effects of glycosphingolipids were still present (Fig. 6). The modulation of kinase activity by glycolipids will therefore be discussed at the level of lipid/protein interactions.

The positive or negative modulation of enzyme activity did not depend on the exact chemical structure of the glycosphingolipids, because the effects obtained with the defined ganglioside fractions GM_1, GD_1 and GT_1, or with synthetic lactocerebroside, were also obtained at the same concentrations with the mixed brain gangliosides and cerebrosides, respectively. The glycolipid concentrations for either stimulation or inhibition were in the same range of 0.1 to 4 mM that was also typical for activation or inhibition by phospholipids, fatty acids or certain detergents (unpublished results). No evidence for high-affinity binding such as envisaged in the glycosphingolipid 'annulus' model [1] could be obtained for the bacterial diacylglycerol kinase. The modulating effects of glycosphingolipids therefore appeared to be related to the general problem of inserting an amphiphilic protein into a bilayer or micelle structure. This type of specific solubilization has previously been discussed in detail [13]. Because of the extended and highly irregular protein surface structure, multiple lipid binding sites are expected so that a mixed lipid phase should give a better fit around the protein than a chemically uniform lipid phase. This effect may explain the stimulation exerted by glycosphingolipids under certain defined conditions. The inhibitory effect of glycosphingolipids could be due to non-productive binding or to a displacement of the activating lipid from the protein. The latter process may be facilitated by the relatively long and rigid hydrocarbon chains and the high hydrogen bonding capacity of glycosphingolipids [1], and by the negatively charged sialic acid residues in the case of gangliosides.

More complete explanations should also consider lateral lipid exchange, protein/protein interactions and, most important, conformational changes of the apoprotein which are probably responsible for its lipid activation. The considerable qualitative and quantitative differences in the effects given by the various lipids and glycolipids studied may be related to their different binding constants for association with the protein relative to lipid/lipid association. Such binding data are, however, at present not available.

Interfacial regulation

In the case of kinase apoprotein activated by cardiolipin both the stimulatory and the inhibitory effects of glycosphingolipids were absolutely dependent on the presence of lipid-bound sialic acid but not on the exact chemical structure of the ganglioside. Both effects persisted when the concentration of cardiolipin was increased (Fig. 5), and free sialic acid or sialyl-lactose were without effect. Gangliosides therefore appeared to act by a specific amphiphilic binding process rather than simple ion exchange, so that the present polar group effect constitutes another example of 'interfacial regulation' [13].

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