Effect of gangliosides on membrane permeability studied by enzymic and fluorescence-spectroscopy techniques

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The effect of gangliosides on membrane permeability was investigated by studying the kinetic properties of cytochrome *c* oxidase, the activity of which, when the enzyme is reconstituted in phospholipid vesicles, is dependent on membrane permeability to H^+ and K^+ . The experiments indicate that three different gangliosides (G_{M1} , D_{D1a} , G_{T1b}) incorporated into cytochrome *c* oxidase-containing phospholipid vesicles stimulate enzymic activity, in the absence of ionophores, most probably by disorganizing the bilayer lipid assembly and increasing its permeability to ions. This interpretation was confirmed by fluorescence-spectroscopy experiments in which the rate of passive leakage of carboxyfluorescein entrapped in the vesicles was measured. Cholera toxin, or its isolated B-subunit, added to G_{M1} -containing proteoliposomes inhibited cytochrome *c* oxidase activity, indicating the lack of formation, under these experimental conditions, of channels freely permeable to H^+ or K^+ .

INTRODUCTION

Gangliosides are normal components of the plasma membrane of vertebrate cells [1,2]. They are known to modify the physico-chemical properties of the membranes where they are inserted, such as fluidity [3,4] or thermotropic properties [5] and affect the activity of a number of membrane-bound enzymes [6]. A valid model to inspect in detail the ganglioside influence on membrane properties is represented by artificial membranes (phospholipid vesicles) [7,8] or reconstituted systems where a protein, inserted in phospholipid vesicles, can be functionally studied with reference to the lipid environment [9]. Aiming to provide more information about the influence of gangliosides on membrane permeability, we tested the model of cytochrome *c* oxidase reconstituted into phospholipid (asolectin) vesicles. In fact the enzyme redox and H^+ -pumping activities are very sensitive to the structure and assembly of the proteo-membrane unit and, in particular, to permeability to H^+ and K^+ ions [10–12]. The comparison between cytochrome *c* oxidase activity in the presence and in the absence of gangliosides in the phospholipidic bilayer may represent a valid indication of their influence on membrane properties. Moreover, since cholera toxin (CT) is known to bind specifically to G_{M1} ganglioside, causing remarkable modifications of the G_{M1} distribution in the membrane and the possible formation of channels [13], the effect of the toxin on cytochrome oxidase reconstituted into phospholipid vesicles containing G_{M1} was also investigated.

MATERIALS AND METHODS

Chemicals

Triton X-100 was from Merck (Darmstadt, Germany) and Sephadex G-50 was from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Asolectin [*L*- α -phosphatidylcholine type IIS, from soybean (*Glycine max*)], valinomycin, nigericin, carbonyl

cyanide *m*-chlorophenylhydrazine (CCCP), carboxyfluorescein (CF) and CT were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Stock solutions of the ionophores were made in 95% (v/v) ethanol. Asolectins were purified by means of acetone/diethyl ether fractionation cycles. To remove azide, used as a preservative of CT, samples were extensively dialysed against 0.1 M-potassium Hepes, pH 7.4, before use. The B-subunit of CT was kindly given by Dr. Maurizio Tomasi (Istituto Superiore di Sanita', Rome, Italy). When used, CT was added in a stoichiometric (1:5) molar ratio to G_{M1} ganglioside [14], assuming that 50% of the total cytochrome-oxidase-containing vesicle glycolipid content in the vesicles is accessible to the toxin. Gangliosides G_{M1} , G_{D1a} and G_{T1b} were extracted and purified from ox brain according to the procedure described by Tettamanti *et al.* [15]. The identification, structural analysis and purity of the samples were assessed as described by Sonnino *et al.* [16]. Gangliosides were 3H -labelled at C-3 of the long-chain-base moiety and prepared as the *erythro* form as described by Gazzotti *et al.* [17].

Preparation of cytochrome *c* oxidase-containing vesicles

Cytochrome oxidase was prepared as described by Yonetani [18] with minor modifications. The final suspending buffer contained either 0.2% sodium cholate or 0.2% Tween 80. Cytochrome oxidase-containing vesicles (COV) were prepared by the cholate-dialysis method [19]. The buffer internal to the vesicles was 0.1 M-potassium Hepes, pH 7.4. Increasing molar contents of ganglioside were mixed with the cholate/phospholipid suspension before sonication. Care was taken in maintaining the COV interior iso-osmotic with the external medium by adding suitable amounts of KCl and sucrose. The incorporation of gangliosides into the vesicles after dialysis was checked as described in [20], and the amount of ganglioside incorporated was established by addition of a small amount of a

Abbreviations used: COV, cytochrome oxidase vesicles; RCR, respiratory-control ratio; CF, carboxyfluorescein; CT, cholera toxin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

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[³H]ganglioside during the preparation of the samples and radioactivity counting.

The respiratory-control ratio (RCR) of different COV preparations, determined by the spectroscopic or polarographic methods [21], ranged between 5 and 10. The fraction of COV exposing cytochrome *c* to the external medium (sidedness) was 85 (± 5) % [22].

In some experiments detergent-solubilized cytochrome oxidase was directly exposed to gangliosides by using the same protein/ganglioside ratio as in the vesicles.

Determination of cytochrome *c* oxidase activity

The activity of cytochrome *c* oxidase was assayed by measuring the rate of ferrocytochrome *c* oxidation or O₂ consumption.

The oxidation of ferrocytochrome *c* was monitored at 550 or 563 nm in a thermostatically controlled Durrum-Gibson stopped-flow apparatus equipped with a 2 cm-light-path observation cell. O₂ consumption was assayed polarographically with a Clark-type oxygen electrode (Ysi 5300; Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.).

Fluorescence measurements: release of CF from liposomes

For fluorescence experiments, liposomes made of purified asolectin and containing increasing proportions of G_{M1} ganglioside (up to 10 % mol/mol) were prepared as follows. Phospholipid/ganglioside mixtures (3 μmol total), dissolved in chloroform/methanol (2:1, v/v), were dried under a gentle stream of N₂ and then in vacuo. The dried lipids were suspended and vortexed in 0.1 ml of aq. 0.2 M-CF pH 7.4. The lipid suspension was sonicated in a bath sonicator (model G112 SPIG; Laboratory Supplies, Hicksville, NY, U.S.A.) for 30 min. Untrapped CF was removed by passage of the vesicle dispersion through a column (1 cm × 40 cm) of Sephadex G-50 (Pharmacia, Uppsala, Sweden) equilibrated with a buffer containing 0.015 M-NaCl/0.01 M-Hepes, pH 7.4, at 4 °C.

The fluorescence of CF was measured with an FP 770 spectrofluorimeter (Jasco, Tokyo, Japan), the method described by Guo *et al.* [23], with minor modifications, being used. Self-quenching is present at CF concentrations above 0.01 M, whereas in dilute solutions fluorescence intensity is proportional to concentration [24,25]. An aliquot (300 μl) of CF-containing liposomes was rapidly mixed at zero time with 700 μl of buffer at 25 °C and the fluorescence intensity was recorded continuously for about 20 min. At the end of the experiment, 100 μl of 20 % Triton X-100 was added to the mixture in order to release CF still trapped in the liposome population.

The rate of leakage was determined from analysis of the semilogarithmic time course of the CF release. Corrections were made for Triton X-100 quenching of the CF fluorescence value.

RESULTS

The effect of G_{M1} ganglioside on passive permeability of the vesicles was assessed by monitoring the time course of leakage of the bulk of CF trapped in the vesicles. Table 1 reports the first-order rate constants for CF release measured for every batch of vesicles at any given G_{M1} concentration; in the same Table the rate constants of cytochrome *c*²⁺ oxidation catalysed by COV containing the same amount of G_{M1} are reported for comparison. A 40 % increase in the CF release rate is observed at the highest G_{M1} concentration (10 %); concomitantly the rate of cytochrome *c*²⁺ oxidation increases by a factor of 2 (see below).

The influence of gangliosides on vesicle-bound cytochrome oxidase activity was probed by monitoring either the oxidation of cytochrome *c* in the stopped-flow, for two to five turnovers, or the O₂ consumption by polarography, for > 100 turnovers (see

Table 1. Influence of ganglioside-to-phospholipid ratio on CF permeability, and on the rate constant for cytochrome *c* oxidation, in COVs

G _{M1} (%)	<i>k'</i> _{obs.} (min ⁻¹)*	<i>k'</i> _{obs.} (s ⁻¹)†
0	0.070	0.55
2	0.080	0.74
7.5	0.084	1.1
10	0.100	1.2

* First-order rate constant for release of CF in the bulk phase.

† Pseudo-first-order rate constant for cytochrome *c* oxidation in the absence of ionophores.

Fig. 1. Time course of the oxidation of ferrocytochrome *c* in air by COV in the absence (●) or presence (○) of ionophores

Reactant concentrations after mixing were as follows: cytochrome *c* oxidase, 1.25 μM (haem a); ferrocytochrome *c*, 10 μM; buffer, 10 mM-potassium Hepes, pH 7.3, containing 39.6 mM-KCl and 40.6 mM-sucrose. Ionophores were added to the COV-containing syringe at the following concentrations: valinomycin, 10 μM; CCCP, 5 μM. The temperature was 20 °C.

[21]). As shown in Fig. 1, the time course of cytochrome *c* oxidation by COV monitored by stopped flow at high oxidase concentration is biphasic in the absence of ionophores; in the presence of valinomycin and CCCP (or nigericin) the reaction is monoexponential and approx. 5 times faster (RCR = 5). The significance of the fast phase in the absence of ionophores is discussed elsewhere [26] and will not be considered any further. When similar experiments are carried out with G_{M1}-containing COV, clear effects are observed both in the absence [*k'*(-)] and in the presence [*k'*(+)] (where *k'* is the pseudo-first-order rate constant for cytochrome *c* oxidation) of ionophores (see Fig. 2a). The value of *k'*(-) increases with the G_{M1} content from 0.5 up to 1.3 s; data points smoothly approach a maximum value when the content of G_{M1} is approx. 5–10 % of the total lipid content. In the presence of ionophores the value of *k'*(+) increases very slightly up to 2 % G_{M1}, and thereafter decreases monotonically, approaching the value achieved by *k'*(-). The same trend is observed polarographically, as shown in Fig. 2b. As a result, the value of the RCR decreases dramatically as a function of G_{M1} concentration (see the inset to Fig. 2a).

The same type of effect was observed with G_{D1a} and G_{T1b} gangliosides. The results are shown in Figs. 3(a) and 3(b) in terms of fractional change of the same kinetic parameters with respect to the control. As observed for G_{M1} (reported again for comparison), the value of *k'*(-) and *VO*₂(-) (*VO*₂ is the rate of O₂ consumption) doubles, at least, and approaches a maximum

Fig. 2. Variation of cytochrome oxidase activity with the concentration of the G_{M1} ganglioside

The pseudo-first-order rate constant values for the oxidation of cytochrome *c* in air is shown in (a); the rate of O_2 consumption (arbitrary units, a.u.) in (b). Open (○) and closed (●) symbols refer to data collected in the presence or in the absence of ionophores respectively. Experimental conditions were as for Fig. 1. The inset shows the decrease of RCR (from 5 to 1) as a function of G_{M1} concentration.

Fig. 3. Variation of cytochrome oxidase activity with the concentration of the G_{D1a} and G_{T1b} gangliosides

The ordinate in (a) is the fractional value of the pseudo-first-order rate constant for cytochrome *c* oxidation and in (b) the rate of oxygen consumption, with respect to control (CTR) COV, at any given G_{D1a} (△, ▲) and G_{T1b} (□, ■) concentration. The broken lines and relative data points (○, ●) refer to G_{M1} (reported for comparison). Open and closed symbols refer to data collected in the presence or absence of ionophores respectively. All experimental conditions were as for Fig. 1.

value when the concentration of G_{D1a} or G_{T1b} in the vesicle is about 5% of total lipids; on the other hand the value of k' (+) and VO_2 (+) (after a slight increase observed at the lowest concentration) decreases as a function of the gangliosides content.

In an attempt to obtain more information on the influence of gangliosides on membrane organization we have carried out polarographic experiments to probe the effect of CT. For these experiments both the native toxin and its isolated B-subunit have been used in the presence or absence of G_{M1} ganglioside. Tests were carried out with cytochrome *c* oxidase solubilized in detergent as well as with the enzyme reconstituted into vesicles. Table 2 summarizes the results obtained.

Addition of CT to the enzyme in solution or reconstituted, with or without 5–10 min preincubation, does not affect the rate of O_2 consumption in the absence of G_{M1} . Addition of G_{M1} to the

soluble enzyme is likewise ineffective. Most interestingly, if soluble cytochrome *c* oxidase is preincubated for several minutes (typically 5–10) with G_{M1} and then CT is added to the mixture before assay, a clear inhibition of the O_2 consumption rate is recorded. Parallel experiments carried out with COV also showed that the addition of CT to G_{M1} containing COV (10%) causes a dramatic increase of both $VO_2(-)$ and $VO_2(+)$. As compared with the controls, the residual enzyme activity after addition of CT is 22% in the absence of ionophores and 37% in their presence. When the isolated cholera toxin B-subunit was used, virtually identical results were obtained.

DISCUSSION

Insertion of gangliosides (namely G_{M1} , G_{D1a} and G_{T1b}) into small unilamellar vesicles changes the permeability of the mem-

Table 2. Effect of G_{M1} ganglioside and CT on the rate of O_2 consumption by soluble and reconstituted cytochrome c oxidase (COV)

The rates (in arbitrary units) were measured polarographically in the absence [$VO_2(-)$] and in the presence [$VO_2(+)$] of ionophores, as described in the Materials and methods section and in the legend to Fig. 3.

Cytochrome c oxidase	G_{M1} (%)	CT (μM)	$VO_2(-)$	$VO_2(+)$
COV	0	0	24	—
	0	3	23	—
	10	0	23	—
	10	3	13	—
Soluble	0	0	5	30
	0	3	6	30
	10	0	17	20
	10	3	4	8

brane. Compared with controls, the passive membrane permeability of G_{M1} -containing vesicles is increased, as demonstrated by a 40% increase of the CF release rate and suggested by the increased rate of cytochrome c oxidation observed in the absence of ionophores. The increase in membrane permeability could arise at the interface between bulk lipid and 'interfacial lipid' associated with the incorporated oxidase. Moreover, gangliosides are known to increase the rigidity of the bilayer [3] and cause lateral separation phenomena [5]. The decrease of membrane order at the interface between bulk lipid and laterally phase-separated regions could be another source of increased permeability (although these are not the only possible interpretations of the phenomenon). The changes in cytochrome c oxidase activity observed in the absence and in the presence of ionophores accounts for the large decrease of the RCR on increasing the ganglioside content in the vesicles and is related to an inhibition of cytochrome c oxidase activity exerted by ganglioside; this is better seen in the presence of ionophores, i.e. when any membrane potential-linked effect is virtually absent. This effect is best interpreted by assuming that gangliosides reside in the structural domain(s) of the enzyme. The sialic acid moiety (negatively charged at pH 7.4) may contribute to a 'localized' electrostatic effect (perturbing cytochrome c to cytochrome oxidase e^- transfer), comparable with that observed with phosphate and other anions [27]. This view is also consistent with the fact that G_{D1a} and G_{T1b} , which carry higher numbers of charges, seem to exert the same inhibition at lower concentrations as compared with G_{M1} .

The lack of effect of CT on the activity of soluble and reconstituted cytochrome oxidase in the absence of G_{M1} demonstrates that the toxin, by itself, does not interfere with catalysis. However, when G_{M1} is preincubated with the solubilized enzyme or is already present in COV, addition of CT leads to a very large (3–4-fold) decrease of the catalytic activity. The experiments carried out with the detergent-solubilized enzyme indicate that a 5–10 min preincubation is necessary for the effect to manifest itself, presumably enabling exchange of ganglioside molecules. The rate of O_2 consumption in the absence of ionophores, $VO_2(-)$, measured after addition of CT to G_{M1} -containing COV, is smaller by a factor of 2 than the maximal rate achieved upon addition of ionophores, $VO_2(+)$ (see Table 2). This unequivocal finding excludes the possibility that the toxin is acting as, or producing, an ionic 'channel' freely permeable to H^+ or K^+ , as suggested on the basis of the proposed capability of CT to induce

the formation of ganglioside patches [14,28] under conditions similar to, but not identical with, ours.

In conclusion, the data reported above show that gangliosides induce functional modifications of the membrane as shown by fluorescence data and, indirectly, by changes of cytochrome oxidase activity. It should be pointed out that the present investigation uses cytochrome oxidase activity as an indicator and that the results are relevant to this defined model system and not to the mitochondrial inner membrane.

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