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Characteristics and regulation of ganglioside-induced elevation of free cytoplasmic Ca^{2+} in human blood platelets

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We have found that gangliosides G_{D3} and G_{M3} induced rapid, reversible elevation of free cytoplasmic Ca^{2+} in fura-2-loaded human blood platelets. The effect persisted in Ca^{2+}-free medium, indicating that gangliosides stimulated mobilization of intracellular stores. The action of gangliosides was concentration-dependent with EC_{50} of about 1 μM. The Ca^{2+}-mobilizing effects of gangliosides were potentiated by epinephrine and inhibited by substances inducing activation of protein kinase C and cAMP-dependent protein kinases. Acidic phospholipids partially mimicked the Ca^{2+}-mobilizing effects of gangliosides indicating that lipid head charge is essential for this activity. While the elevation of $[Ca^{2+}]_i$ produced by arachidonic acid was almost completely blocked by aspirin pretreatment, the effects of gangliosides were diminished only 2-fold, indicating that gangliosides activate both aspirin-sensitive and aspirin-insensitive mechanisms of $[Ca^{2+}]_i$ elevation.

Introduction

A large body of data indicates that gangliosides, apart from their proposed structural roles in biomembranes, may serve as molecules effecting cell-cell and cell-matrix interactions. It was found that gangliosides took part in numerous cell functions such as differentiation, adhesion [2], modulation of immunological response [3], neuron synaptic transmission [4] and induction of neuritogenesis [5]. The intracellular mechanisms mediating these effects of gangliosides are poorly understood. We have found, previously, that immobilized gangliosides stimulated adherence of human blood platelets [6]. In the present work, we investigated whether this effect was followed by the elevation of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) in platelets. We have found that addition of gangliosides to the suspension of fura-2-loaded platelets produced instant and reversible increase in $[Ca^{2+}]_i$. The characteristics of this Ca^{2+} rise were similar to those produced by platelet agonists, such as thrombin and adenosine diphosphate, and clearly differed from the mode of action of Ca^{2+} ionophores. Therefore, the elevations of $[Ca^{2+}]_i$ produced by gangliosides in platelets may be characterized as 'hormone-like'.

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Abbreviations: The designation of gangliosides follows the IUPAC-IUB recommendations [1]: G_{M3}, II^3-*N*-acetylneuraminosyllactosylceramide; G_{D3}, II^3-di(*N*-acetylneuraminosyl)lactosylceramide.

Materials and Methods

Ganglioside G_{M3} was isolated from human liver [7], ganglioside G_{D3} was isolated from bovine buttermilk [8]. Gangliosides were analysed using HPLC and HPTLC methods [9] and were found to be chromatographically pure. L-α-Phosphatidic acid, dioleoyl and DL-α-phosphatidyl-α-serine, dipalmitoyl were obtained from the Sigma (St. Louis, MO). L-α-Cephaline, dipalmitoyl was obtained from Serva Feinbiochemica (Heidelberg, Germany). Phosphatidylcholine from egg yolk was obtained from Bacterial Preparation Productions (Kharkov, Russia). Fura-2/AM was obtained from Dojin, Japan. All other biochemicals and reagents were obtained from the Sigma (St. Louis, MO).

Preparation of lipid stock solutions

Lipids were added to platelet suspensions from sonicated 100-fold stock solutions in ethanol (phospholipids) or ethanol/water 1 : 1 (gangliosides) in a manner such that final concentrations were 0.5–4 μM. In

some experiments lipids were prepared by sonication of lipid suspensions in water in an immersion ultrasonic bath for 15 min at room temperature.

Isolation of platelets and measurements of $[Ca^{2+}]_i$

Human blood was taken by venipuncture into 1/6 volume of acid citrate–dextrose anticoagulant (85 mM trisodium citrate, 71 mM citric acid, 9 mM D-glucose). Platelet-rich plasma (PRP) was obtained by centrifugation (200 × *g*, 15 min). PRP was centrifuged (1200 × *g*, 10 min) and pelleted platelets resuspended in an equal volume of modified Tyrode's solution, containing 150 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $CaCl_2$, 5 mM D-glucose, 10 mM Hepes-NaOH (pH 6.55), 0.1 mg/ml apyrase, 0.35% human serum albumin fraction V (HSA) and 2.5 μM fura-2/AM. After incubation for 1 h at 30°C the cells were pelleted (1200 × *g*, 10 min) and resuspended in an equal volume of modified Tyrode's solution (pH 7.4), containing HSA and apyrase and without fura-2/AM. The measurements of fura-2 fluorescence were performed on a Hitachi 3000 spectrofluorimeter at 340 and 500 nm. The temperature was maintained at 30°C. Immediately before measurements an aliquot of cell suspension was diluted 1:10 with the same buffer, without HSA and apyrase. Calibration of fluorescent signal and calculations of $[Ca^{2+}]_i$ were performed, as described in Refs. 10 and 11.

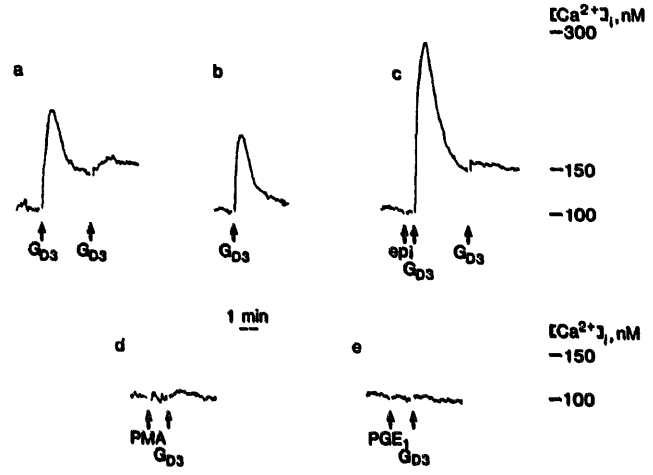

Fig. 1. Characteristics and regulation of ganglioside-induced $[Ca^{2+}]_i$ elevation in fura-2-loaded platelets. Platelets ($5 \cdot 10^7$ cells/ml) were suspended in Ca^{2+}-free modified Tyrode's solution. 1 min before the experiment 1 mM $CaCl_2$ (a, c, d, e) or 1 mM EGTA (b) was added. The platelets were exposed to ganglioside G_{D3} ($2 \cdot 10^{-6}$ M) in the absence (a, b) and after preincubation for 30 s at 30°C with either 10^{-6} M epinephrine (c), 10^{-8} M phorbol myristate acetate (d) or for 60 s with 10^{-7} M prostaglandin E_1 (e). G_{D3} was added from stock solution in ethanol/water 1:1, giving a final ethanol concentration of 0.5%. Ethanol alone at such a concentration was without effect. The tracings depicted utilized platelets from a single subject and were typical of identical experiments carried out in platelets from two other subjects. Very similar results were obtained using sonicated water dispersions of ganglioside.

Results

We have found that addition of G_{D3} ganglioside to the suspension of fura-2-loaded platelets instantly produced a reversible increase in $[Ca^{2+}]_i$ (Fig. 1a). Repeated addition of ganglioside did not result in additional change in $[Ca^{2+}]_i$ (Fig. 1a) indicating that the effects of gangliosides did not result from interference of these lipids with the method of $[Ca^{2+}]_i$ monitoring used in our experiments. Very similar changes were observed after treatment of platelets with G_{M3} (results not shown). In most cases the lipids were added from ethanolic stock solutions giving a final alcohol concentration of 1%. To avoid possible influence of ethanol, sonified water suspensions of gangliosides were used in a number of experiments. These experiments gave essentially similar results and, therefore, are not described separately.

The effect of gangliosides in Ca^{2+}-free medium has been studied to show the mechanism of free cytoplasmic Ca^{2+} elevation. The effect of gangliosides remained in Ca^{2+}-free medium indicating that at least a part of observed $[Ca^{2+}]_i$ elevation resulted from mobilization of intracellular stores (Fig. 1b).

We have found that the ganglioside-induced elevations of $[Ca^{2+}]_i$ were regulated in a specific way. Our data indicate that the effect of G_{D3} was significantly increased in the presence of epinephrine, while this catecholamine alone was without effect (Fig. 1c). As shown in Fig. 1a, the second addition of G_{D3} to epinephrine-treated platelets did not significantly elevate $[Ca^{2+}]_i$, indicating the development of desensitisation (Fig. 1c). In contrast to the action of epinephrine, pretreatment of platelets with protein kinase C activator, phorbol myristate acetate, or with platelet adenylate cyclase activator, prostaglandin E_1, completely blocked the effects of gangliosides (Fig. 1d and e). The sensitivity of platelets to G_{M3} changed in a similar way (results not shown).

The effects of gangliosides, both in the presence or absence of epinephrine, were concentration-dependent, reaching saturation at concentrations above 2 μM (Fig. 2). Half-maximal effective concentrations and maximum $[Ca^{2+}]_i$ levels are presented in Table I.

In order to clarify whether the observed effects were specific to sialic acid-containing lipids we tested the action of some other polar lipids. The data of Table II indicate that the effects of gangliosides were partially mimicked by the acidic phospholipids, while the effects of the neutral ones were less pronounced.

We assessed the possible involvement of arachidonic acid metabolites in the Ca^{2+}-mobilizing action of gangliosides. The platelet cyclooxygenase was blocked by pretreatment with aspirin. Such treatment did not change loading of platelets with fura-2 or basal $[Ca^{2+}]_i$

TABLE I

Characteristics of ganglioside-induced $[Ca^{2+}]_i$ elevations in human platelets

Fura-2-loaded platelets ($5 \cdot 10^7$ cells/ml) were treated with varying concentrations of gangliosides in the absence or presence of epinephrine (10^{-6} M), as described in the legend to Fig. 2 and half-maximal effective concentrations of gangliosides (EC_{50}) and maximal $[Ca^{2+}]_i$ increases (E_{max}) were determined. The data are expressed as mean ± S.D. Numbers in parentheses indicate the number of independent experiments utilising platelets of different donors.

Addition	EC_{50} (μM)		Maximum $[Ca^{2+}]_i$ level (nM)	
	ganglioside alone	epinephrine + ganglioside	ganglioside alone	epinephrine + ganglioside
G_{M3}	1.3 ± 0.69 ($n = 3$)	0.9 ± 0.43 ($n = 4$)	61 ± 29.3 ($n = 3$)	219 ± 32 ($n = 4$)
G_{D3}	0.9 ± 0.57 ($n = 3$)	0.7 ± 0.47 ($n = 4$)	65 ± 46.9 ($n = 3$)	123 ± 78.7 ($n = 4$)

TABLE II

Comparison of increase in Ca^{2+}-mobilizing activity by G_{D3} and phospholipids

The lipids were added to a suspension of fura-2-loaded platelets from 100-fold stock solution in ethanol (phospholipids) or ethanol/water 1:1 (G_{D3}) to a final concentration of $2 \cdot 10^{-6}$ M in the absence or presence of epinephrine (10^{-6} M). The effects of lipids are normalized to those of the G_{D3} effects which are 84 ± 26 nM and 245 ± 90 nM in the absence or presence of epinephrine, respectively. Epinephrine alone did not change the $[Ca^{2+}]_i$ level. The data are presented as mean ± S.D. of three independent experiments, performed on platelets of different donors. Very similar results were obtained using sonicated water dispersions of lipids.

Addition	Increase in $[Ca^{2+}]_i$ (% of G_{D3})	
	lipid only	epinephrine + lipid
G_{D3}	100	100
Phosphatidic acid	58 ± 6	61 ± 20
Phosphatidylserine	22 ± 18	38 ± 12
Phosphatidylcholine	10 ± 1	14 ± 8
Phosphatidylethanolamine	18 ± 4	12 ± 7

TABLE III

The influence of aspirin pretreatment on ganglioside- and agonist-induced elevations of $[Ca^{2+}]_i$ in fura-2-loaded human blood platelets

Human platelets were incubated in modified Tyrode's solution (pH 6.55) with or without aspirin (10^{-3} M) for 30 min at 37°C. After fura-2 loading the $[Ca^{2+}]_i$ increases induced by G_{D3} ($2 \cdot 10^{-6}$ M) in the absence or presence of epinephrine (10^{-6} M), by adenosine diphosphate (10^{-6} M), platelet activating factor ($2 \cdot 10^{-9}$ M) or arachidonic acid (10^{-6} M) were determined. The data are expressed as mean ± S.D. of two independent experiments performed using platelets of different donors.

Addition	Increase in $[Ca^{2+}]_i$ (nM)	
	control cells	aspirin-treated cells
G_{D3}	88 ± 15	36 ± 6
Epinephrine + G_{D3}	250 ± 39	120 ± 19
ADP	284 ± 44	131 ± 20
PAF	401 ± 20	261 ± 13
Arachidonic acid	217 ± 9	9 ± 0.4

levels (data not shown), but significantly decreased the sensitivity of platelets to the arachidonic acid action (Table III), possibly due to inhibition of thromboxane

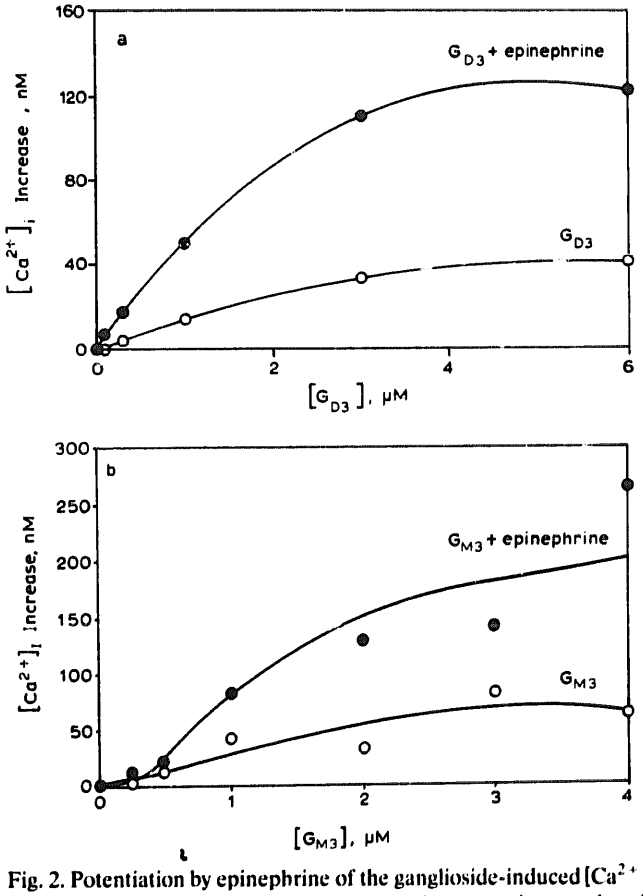

Fig. 2. Potentiation by epinephrine of the ganglioside-induced $[Ca^{2+}]_i$ elevation in fura-2-loaded platelets. Peak increases in cytoplasmic Ca^{2+} concentrations (maximum $[Ca^{2+}]_i$ levels minus basal levels) due to various amounts of G_{D3} (a) or G_{M3} (b) were determined in platelets suspended in modified Tyrode's solution in the absence and presence of epinephrine (10^{-6} M). Epinephrine was added 30 s prior to the ganglioside. The catecholamine alone was without effect. Gangliosides were added from 100-fold stock solutions in ethanol/water, 1:1. Very similar results were obtained using sonicated water dispersions of gangliosides. The results presented are typical of four(G_{D3}) and five(G_{M3}) independent experiments performed using platelets of different donors.

A_2 formation [12]. The effects of adenosine diphosphate and platelet activating factor were also partially inhibited (Table III) indicating that prostaglandin endoperoxide/thromboxane A_2 generation enhanced platelet activation produced by these agonists. This proposal is in agreement with previously published data [12].

Discussion

We have found previously that immobilized gangliosides stimulated adhesion of human blood platelets [6]. We proposed that this effect might be mediated by the elevation of free cytoplasmic calcium, known to be the main intracellular regulator of platelet functions [13]. Evidence supporting this hypothesis is presented in Fig. 1, demonstrating that the addition of ganglioside to fura-2-loaded platelets instantly produced reversible elevation of $[Ca^{2+}]_i$ levels.

The main object of this study was the investigation of mechanisms of ganglioside-induced elevation of $[Ca^{2+}]_i$. We examined two main possibilities. First, the effect could be linked with the amphiphilic nature of ganglioside molecules and their ability to bind Ca^{2+} ions [14]. These properties indicate that the Ca^{2+}-elevating action of gangliosides could result either from nonspecific disturbance of plasma membrane or from the generation of membrane-soluble complexes with Ca^{2+}, in a way similar to Ca^{2+}-ionophores, such as ionomycin and A23187 [15]. The other hypothesis suggested that gangliosides might activate complex pathways of intracellular Ca^{2+}-signalling, in a way similar to action of platelet agonists, such as thrombin, platelet activating factor, adenosine diphosphate, etc. The action of these substances on platelets is mediated by plasma membrane receptors and is usually referred to as 'hormone-like' [13]. To examine the two hypotheses we investigated the sources and regulation of ganglioside-induced elevation of $[Ca^{2+}]_i$.

Our data indicate that, in a number of characteristics, the action of gangliosides parallelled that of platelet agonists. The similar features were as follows: (1) the action of gangliosides was reversible (Fig. 1a, b and c), resembling, in this respect, the action of platelet agonists [13]. The repeated addition of ganglioside did not produce additional elevation of $[Ca^{2+}]_i$ (Fig. 1a and c), suggesting the development of desensitisation, known to be the characteristic feature of hormone action [13]; (2) the effect of gangliosides remained in Ca^{2+}-free medium, suggesting that intracellular Ca^{2+} stores were mobilized (Fig. 1b). It is well known that the action of platelet agonists is also accompanied by the efflux of Ca^{2+} ions from reticulum into the cytoplasm [13,16,17]; (3) the action of gangliosides depended on concentration and reached saturation at concentrations above 2 μM (Fig. 2, Table I). The maximum $[Ca^{2+}]_i$ elevations observed after addition of saturating concentrations of gangliosides were far below the level of Ca^{2+} in the incubation medium (Fig. 2), indicating that gangliosides did not produce permanent permeabilization of the plasma membrane; (4) the strongest evidence of the specificity of ganglioside action came from the experiments studying the pharmacological regulation of the Ca^{2+}-mobilizing effects. We have found that the effects of gangliosides were potentiated by epinephrine (Fig. 1a and c, Fig. 2) and blocked by substances inducing activation of protein kinase C and cAMP-dependent protein kinases (Fig. 1a, d and e). It is well known that similar regulation is a characteristic of hormone action [18–21].

At present we do not know what particular physicochemical properties of gangliosides underlie their ability to elevate $[Ca^{2+}]_i$. The data of Table II suggest that negative charges in polar lipid heads may be essential for this activity. The effects of gangliosides were observed in the concentration range (Fig. 2) where these lipids exist in micellar form [22]. Therefore, the ability of gangliosides to elevate $[Ca^{2+}]_i$ may result from interaction of micelles with the platelet surface. It was shown previously that adhesion of platelets to certain substrates could stimulate intracellular processes of activation [6]. An alternative explanation may be that of the ability of gangliosides to influence activity of enzymes involved in regulation of cellular metabolism. The premise for such a hypothesis is the evidence indicating the regulation by gangliosides and other acidic lipids of activity of phospholipase A_2 [23], protein kinase C [24] and other protein kinases [25,26]. We have found that pretreatment of platelets with aspirin, known to inhibit generation of active metabolites of free arachidonic acid [27], partially blocked the Ca^{2+}-mobilizing activity of the ganglioside. The percentage inhibition was equal to that of PAF and ADP and was less marked than inhibition of arachidonic acid action (Table III). These data suggest that the elevation of $[Ca^{2+}]_i$ in platelets by gangliosides may be a complex process, including both arachidonic acid-dependent and -independent stages, in a way similar to well-known platelet agonists, such as PAF and ADP [12,13].

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