Glycoconjugate Journal 17, 173–179, 2000

Glycosphingolipid (GSL) microdomains as attachment platforms for host pathogens and their toxins on intestinal epithelial cells: Activation of signal transduction pathways and perturbations of intestinal absorption and secretion

Jacques Fantini[1]*, Marc Maresca[1], Djilali Hammache[1], Nouara Yahi[2] and Olivier Delézay[3]

[1]*Laboratoire de Biochimie et Biologie de la Nutrition, ESA-CNRS 6033, Faculté des Sciences de St Jérôme, 13397 Marseille Cedex 20, France,* [2]*Laboratoire de Virologie, UF SIDA, Hôpital de la Timone, 13005 Marseille,* [3]*GIMAP, Faculté de Médecine, 42023 St Etienne Cedex 2, France*

Glycosphingolipid (GSL)-enriched microdomains are used as cellular binding sites for various pathogens including viruses and bacteria. These attachment platforms are specifically associated with transducer molecules, so that the binding of host pathogens (or their toxins) to the cell surface may result in the activation of signal transduction pathways. In the intestinal epithelium, such pathogen-induced dysregulations of signal transduction can elicit a severe impairment of enterocytic functions. In this study, we demonstrate that the interaction of a bacterial toxin (cholera toxin) and a viral envelope glycoprotein (HIV-1 gp120) with the apical plasma membrane of intestinal cells is mediated by GSL-enriched microdomains that are associated with G regulatory proteins. These microbial proteins induce a GSL-dependent increase of intestinal fluid secretion by two mechanisms: activation of chloride secretion and inhibition of Na^+-dependent glucose absorption. Taken together, these data support the view that GSL-enriched microdomains in the apical plasma membrane of enterocytes are involved in the regulation of intestinal functions.

Keywords: **glycolipids, HIV-1, cholera toxin, signal transduction, epithelial intestinal cells, air-water interface monolayer**

Introduction

Glycosphingolipids (GSL) are used as cellular binding sites for a wide variety of pathogens, including viruses, bacteria, fungi and parasites [1]. The hydrophilic oligosaccharide residues of GSL protrude into the extracellular space, providing a considerable number of carbohydrate-binding specificities for microbial adhesins. Because of their high melting temperature, GSL are not miscible with glycerophospholipids and are thus clustered to form microdomains in the plasma membrane of mammalian cells [2,3]. These GSL-enriched microdomains (rafts) are not solubilized by Triton X-100 at 4°C and can be purified from the bulk membrane lipids by sucrose-density centrifugation [3]. Pioneer studies from S.-I. Hakomori and coworkers have demonstrated the implication of GSL in signal transduction [4]. The lateral organization of GSL in microdomains of the plasma membrane allows the partitioning of several proteins involved in signal transduction through selective association with these microdomains [5]. Indeed, trimeric G proteins, Ras, and glycosylphosphatidylinositol (GPI)-anchored proteins are co-purified with GSL rafts [3]. Lipids involved in signal transduction (phosphoinositides and sphingomyelin) have also been localized in GSL-enriched microdomains [6]. It has been proposed that the general function of GSL rafts in signal transduction was to concentrate receptors for interaction with ligands and transducers on both sides of the plasma membrane [5]. In epithelial cells, GSL-enriched microdomains are essentially found in the apical plasma membrane [3]. Enteric pathogens and their toxins may therefore attach to GSL

*To whom correspondence should be addressed: Pr. Jacques Fantini, Ph.D., Tel.: + 33 491-288-761; Fax: + 33 491-288-440; E-mail: JACQUES.FANTINI@LBBN.u-3mrs.fr

microdomains on the mucosal surface of enterocytes and activate the signal transduction pathways linked to these microdomains. In this study, we show that HIV-1 surface envelope glycoprotein gp120 and cholera toxin, which bind to the intestinal epithelium through respectively galactosylceramide (GalCer) and ganglioside GM1, induce important perturbations of enterocytic functions by stimulating signal transduction through GSL microdomains.

Interaction of HIV-1 surface envelope glycoprotein gp120 with microdomains enriched in GalCer and transducer molecules

Folch lower phase glycolipids were extracted from human intestinal HT-29 cells and chromatographed on high performance thin layer chromatography (HPTLC) plates. Orcinol staining of the separated glycolipids showed that these cells expressed a mono, α-hydroxylated form of GalCer (Figure 1A, lane 1). This glycolipid was also found in extracts from normal human ileon and colon epithelia (Figure 1A, lanes 2 and 3). In addition, these normal epithelial cells expressed polyhydroxylated forms of GalCer, as previously reported [7]. Using a HPTLC overlay assay, we found that both mono- and poly-hydroxylated forms of GalCer were recognized by gp120 (Figure 1B), whereas GalCer molecules with a nonhydroxylated fatty acid were not [8]. The binding of gp120 to GalCer was specifically inhibited by an anti-V3 mAb (Figure 1C), in agreement with previous studies involving the V3 domain of gp120 in GalCer recognition [9,10]. These data showed that gp120 interacted specifically to GalCer extracted from the intestinal epithelium. To assess that gp120 binding to intestinal GalCer occured at the level of plasma membrane microdomains, detergent-insoluble membranes (DIMs) were isolated from 5×10^7 intestinal HT-29 cells by centrifugation of Triton X-100 insoluble material on a sucrose density gradient [3]. The microdomains were recovered as molecular complexes from the buoyant fractions (12.8–17.0% sucrose), in agreement with previously characterized DIMs [3]. The glycolipid analysis of DIMs from HT-29 cells revealed that the most abundant GSL was mono-hydroxylated GalCer (Figure 1D). Western blotting analysis of the protein associated with HT-29 microdomains demonstrated the presence of G regulatory proteins in the buoyant fractions enriched in GSL (Figure 1E). This is consistent with previous findings from our laboratory showing that gp120 binding to intestinal GalCer activated a signal transduction pathway resulting in the release of calcium from intracellular stores [11]. Moreover, the interaction of gp120 with GalCer could be measured as an increase of surface pressure when the glycolipid was reconstituted as a monomolecular film in a Langmuir film balance (μTrough SX, Kibron Inc.). The insertion of gp120 within the GalCer monolayer increased with the concentration of gp120 (Figure 2, upper panel), and depended on the initial pressure of the monolayer (Figure 2, inset). The influence of the initial surface pressure on the compressibility of the monolayer demonstrated the specificity of the GalCer/gp120 interaction [12]. In addition, gp120 was able to interact with a monolayer of Folch lower phase glycolipids purified from HT-29 microdomains (Figure 2, lower panel). These data confirmed the presence of biologically active GalCer in the plasma membrane microdomains purified from intestinal HT-29 cells.

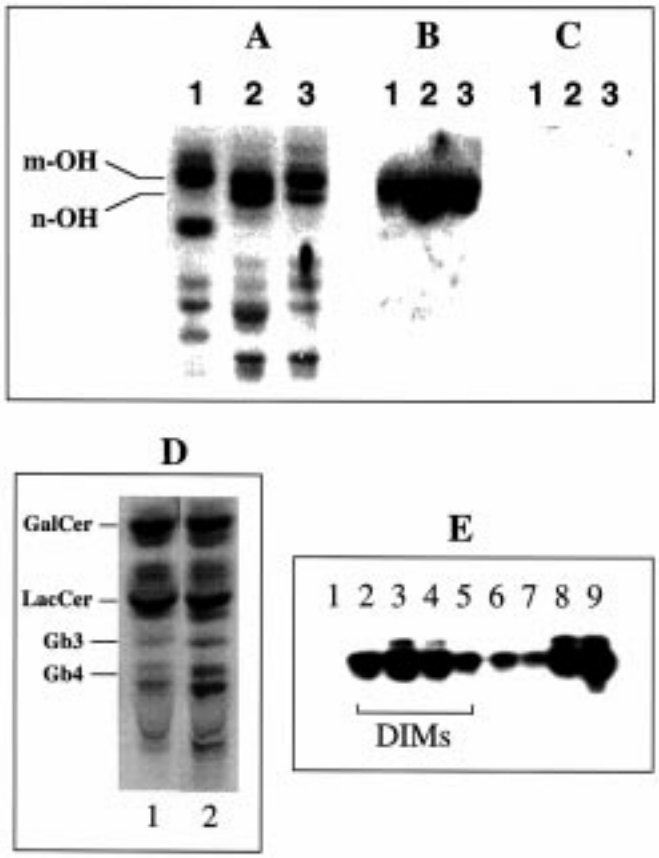

Figure 1. Characterization of GalCer-enriched microdomains purified from human intestinal epithelial cells. Upper panels: Folch lower phase lipids were extracted from HT-29 cells (lane 1), human ileal epithelium (lane 2) or human colonic epithelium (lane 3). After chromatographic separation on HPTLC plates, the glycolipids were either orcinol-stained (A) or probed with recombinant gp120 alone (B) or gp120 pre-incubated with 0.5β, an anti-V3 mAb (C) using the HPTLC overlay assay. Standard glycolipids were run in parallel on the same plate for glycolipid identification. Two forms of GalCer were characterized: mono-hydroxylated GalCer (mOH) and poly-hydroxylated GalCer (nOH), which were both recognized by recombinant gp120. The binding of gp120 to GalCer was abrogated by the 0.5 β mAb. Lower panels: (D) Folch lower phase glycolipids extracted from HT-29 cells (lane 1) or from detergent-insoluble membranes (DIMs) purified from HT-29 cells by sucrose density gradient (lane 2). (E) Western blot detection of transducer molecules (α subunit of Gs) in various fractions separated on sucrose density gradient centrifugation. The buoyant fractions (2–5) correspond to the GSL-containing DIMs analyzed in panel D, lane 2. The primary antibody was a rabbit polyclonal antibody against the α subunit of Gs. The labeling was revealed by radioiodinated goat anti-rabbit antibodies followed by autoradiography.

Binding of HIV-1 gp120 to GalCer-enriched microdomains induces intracellular calcium release and microtubule depolymerization

As shown in Figure 3, the addition of recombinant gp120 to isolated HT-29-D4 cells (a clonal cell line derived from HT-29) induced a dose-dependent increase of intracellular calcium concentration ([Ca^{2+}]i). The calcium response resulted from release of calcium from caffeine-sensitive intracellular stores that are also involved in the cellular response to neurotensin [11]. Following gp120 stimulation, the cells were no longer sensitive to neurotensin, suggesting that the viral glycoprotein and neurotensin activated a common pathway of calcium mobilization. Therefore, it is likely that HIV-1 gp120 stimulated inositol triphosphate-mediated calcium mobilization, as previously demonstrated for neurotensin in HT-29 cells [13]. In addition, the calcium response induced by gp120 was blocked by anti-V3 antibodies, and was mimicked by anti-GalCer antibodies [11], which strongly suggests that it was triggered by the binding of gp120 to GalCer-enriched microdomains. The presence of regulatory G proteins in the microdomains purified from intestinal cells (Figure 1E) is consistent with this hypothesis. In addition, one should consider the possibility that HIV-1 coreceptors CCR5 and CXCR4, which are expressed by both normal and transformed intestinal epithelial cells [14,15], may also be involved in gp120-induced signal transduction. In this respect, it would be interesting to study the potential association of these G protein-coupled seven transmembrane domains receptors with GSL-enriched microdomains of intestinal cells. Whatever the molecular interactions between HIV-1 gp120 and these microdomains, we observed that the gp120-induced rise in calcium concentration affected the integrity of the microtubular network of HT-29 cells [16]. These data shed some light on the cellular mechanisms by which HIV-1 impaired the differentiation of intestinal epithelial cells [17,18]. Interestingly, these mechanisms do not seem to require the infection of enterocytes by HIV-1, which remains controversial *in vivo* [19]. Instead, we suggest that HIV-1 attachment to plasma membrane microdomains is sufficient to alter the morphological and functional maturation of intestinal epithelial cells. This concept of a viral toxin-like effect exerted during virus-cell contact is supported by recent data showing that rotavirus diarrhea is probably caused by nonreplicating viral particles [20]. Therefore, the following sequence of events can be proposed to explain how HIV-1 could directly interfere with the maturation process of enterocytes: i) binding of gp120 to the plasma membrane of enterocytes through GalCer-enriched microdomains; ii) activation of a G protein-linked signal transduction pathway leading to the release of calcium from intracellular stores, iii) depolymerization of microtubules. Since microtubules are necessary for the correct targeting of apical proteins in epithelial cells [21], these data suggest that

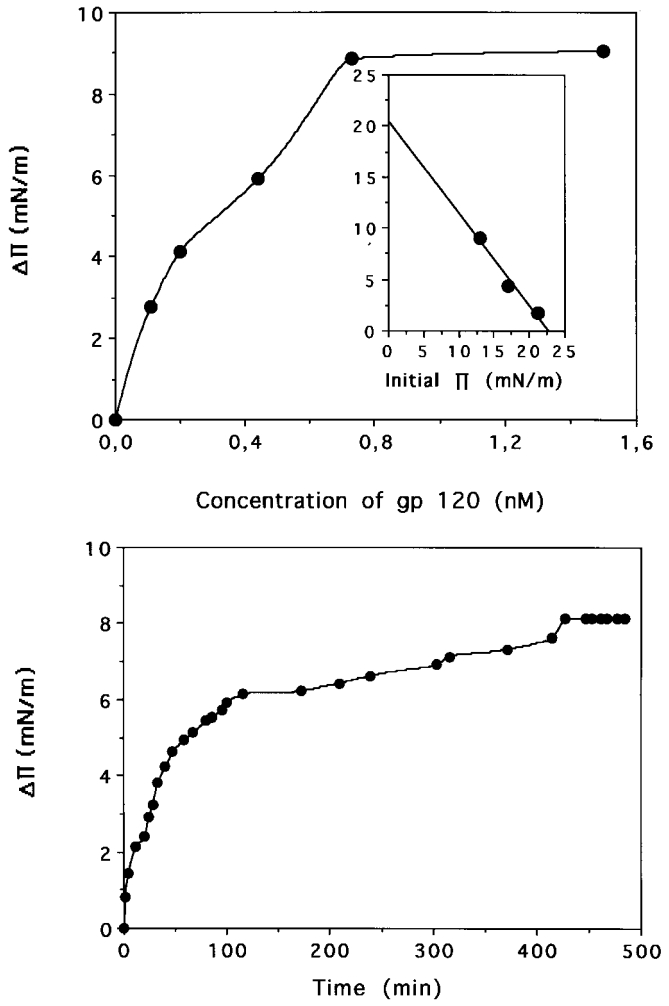

Figure 2. Interaction of HIV-1 gp120 with GalCer monolayers. Upper panel: Variations in surface pressure of a monolayer of monohydroxylated GalCer after injection of the recombinant gp120. Inset: Maximal surface pressure increase reached after injection of gp120 (1.5 nM) under a GalCer film at various initial surface pressures. Lower panel: Folch lower phase glycolipids extracted from HT-29 microdomains (DIMs) were spread at the air-water interface and recombinant gp120 was then added in the aqueous phase at a concentration of 2 nM. The kinetics of the surface pressure increase induced by the viral glycoprotein are shown.

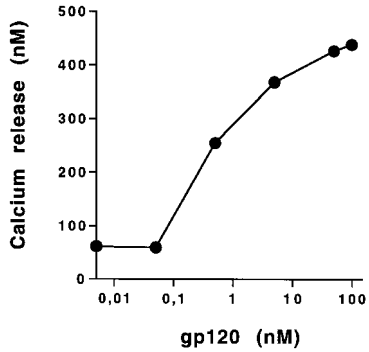

Figure 3. Effect of various concentrations of gp120 on [Ca^{2+}]i rise in isolated HT-29-D4 cells.

HIV-1 gp120 may directly impair the protein composition of the apical plasma membrane of enterocytes.

HIV-1-induced inhibition of glucose absorption in human intestinal epithelial cells

Sugar malabsorption is frequently reported in HIV-1-infected individuals [17]. In mature enterocytes, the apical sodium/glucose cotransporter (SGLT1) is the main sugar transport system [22]. Thus, we studied whether HIV-1 could modulate this active glucose transport function, which gradually appears during the course of enterocytic differentiation along the crypt-villus axis of the intestinal epithelium [23,24]. We found that the activity of SGLT1 was 51% ($\pm$ 7%, n = 4) lower in HT-29-D4 cells cultured in the presence of HIV-1 than in control cells [16]. It should be underscored that the decreased activity of the sodium/glucose cotransporter may not only result in sugar malabsorption, but also in diarrhea, since SGLT1 has the properties of a water channel [22]. In this respect, it has been estimated that, in the human small intestine, secondary active transport of water through the sodium/glucose cotransporter could account for as much as 5 liters of water absorption per day [22]. A diminished transepithelial water absorption due to a defect of SGLT1 activity may therefore potentiate a diarrhea syndrom. Taken together, these data support the concept of an etiologic role for HIV-1 in the malabsorption and diarrhea syndroms associated with AIDS [11,16] and strongly suggest the involvement of GalCer-enriched microdomains in these dysregulations.

Interaction of cholera toxin with GSL-enriched microdomains is mediated by ganglioside GM1

The enterotoxin of Vibrio cholerae, cholera toxin, is constituted of two types of subunits, namely CT-A (28 kDa) and CT-B (11.5 kDa) [25]. In the active form of the toxin, five CT-B molecules are associated in a pentameric ring responsible for the interaction of the toxin with its cellular receptor, the monosialoganglioside GM1 [26]. CT-A is constituted of two peptidic chains (A1 and A2) linked by a disulfide bridge.

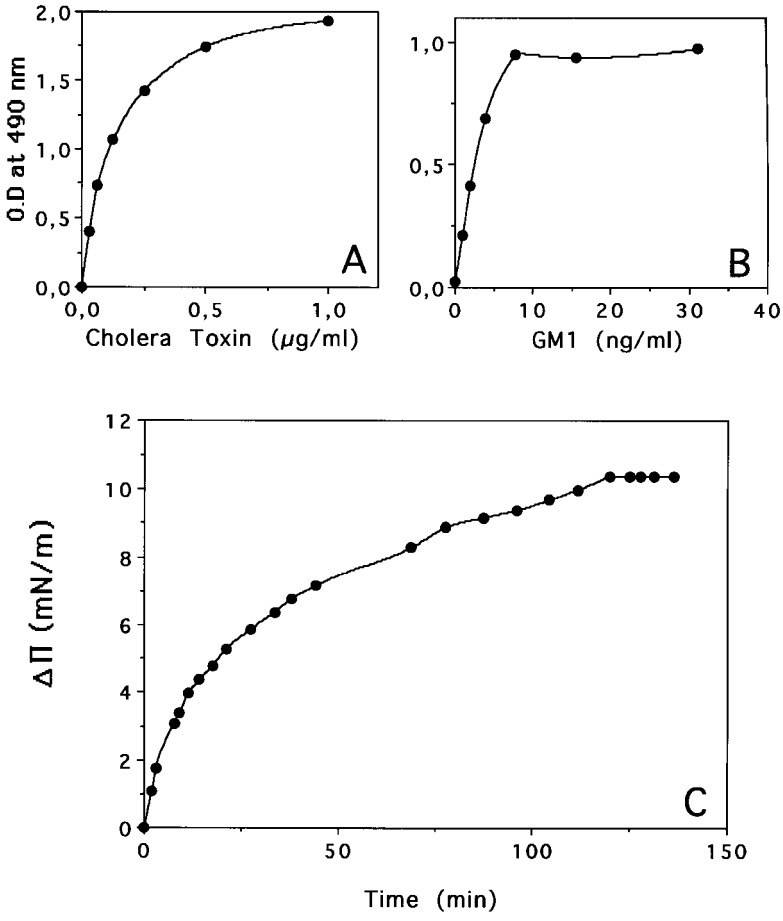

Figure 4. Interaction of cholera toxin (B-subunit) with ganglioside GM1. A- Binding of peroxidase-conjugated CT-B to GM1 (100 ng/ml) bound on ELISA multiwell plates. B- Binding of peroxidase-conjugated CT-B (0.125 μg/ml) to various amounts of GM1 adsorbed on ELISA multiwell plates. C- Surface pressure increase induced by cholera toxin (1 μg/ml) added under a monolayer of Folch upper phase glycolipids extracted from HT-29 microdomains (DIMs). The kinetics of the surface pressure increase induced by the bacterial toxin are shown.

The A2 peptide (5.5 kDa) binds to CT-B through noncovalent interactions allowing the association of the A1 peptide to the CT-B pentameric ring. The A1 peptide (23 kDa) corresponds to the enzymatic subunit of cholera toxin, i.e. ADP-ribosyltransferase and NAD-glycohydrolase activities [27]. The interaction of CT-B with GM1 can be visualized by ELISA by using peroxidase-conjugated CT-B as a probe. Under these conditions, the binding of the toxin to GM1 adsorbed on ELISA plates increased with the toxin concentration (Figure 4A) and with the amount of GM1 (Figure 4B). To ensure that GM1 was recovered with the microdomain fraction purified from HT-29 cells, the glycolipids from HT-29 DIMs (Figure 1) were partitioned according to the Folch procedure, and the aqueous upper phase was reconstituted as a monomolecular film in a Langmuir film balance. The interaction of cholera toxin with these glycolipids was then measured as a toxin-induced increase in the surface pressure (Figure 4C). Overall, these data strongly suggested that the intestinal receptor for cholera toxin is indeed GM1, and that this ganglioside is localized in plasma membrane microdomains of intestinal HT-29 cells.

Cholera toxin inhibits glucose absorption by human intestinal epithelial cells

As shown in Figure 5, cholera toxin was able to inhibit the activity of the sodium/glucose cotransporter, and this inhibition increased with the time of incubation of the cells with the toxin. The maximal effect was reached for a concentration of 120 nM of CT after 24 hours of incubation, with an inhibition of SGLT1 activity of 55 ± 9 % ($n = 5$). Previous studies from our laboratory have demonstrated that the activity of SGLT1 is electrogenic for HT-29-D4 cells [28]. Accordingly, apical addition of increasing concentrations of either D-glucose or its nonmetabolizable analog α-methyl-D-glucose (AMG) on HT-29-D4 cells caused a stepwise variation in transepithelial potential difference (TPD) (Figure 6). This increase of TPD was reversed by apical addition of phlorizin, a selective inhibitor of SGLT1. Thus, we investigated whether the inhibition of SGLT1 activity induced by cholera toxin could be visualized by means of electrophysiological approaches. In this experiment, a saturating concentration of AMG (10 mM) was used to study the electrogenic effect associated with the activity of SGLT1. We found that the apical incubation of the cells with cholera toxin (120 nM) for 20 hours was associated with a decrease of short-circuit current (Isc) variation induced by AMG ($34.2 \pm 10.5\%$ ($n = 4$), with a 100% value of $0.76 \pm 0.02\ \mu A.cm^{-2}$ ($n = 4$)). Most importantly, the transepithelial resistance (Rt) of treated cells was similar to the one of control cells, i.e 980.0 ± 96.8 and $1014.0 \pm 64.6\ \Omega.cm^2$ ($n = 6$), respectively. Since the level of Rt is representative for the integrity of tight junctions overall the epithelial monolayer [16], these data demonstrated that cholera toxin did not affect cellular viability. Taken together, these data showed that

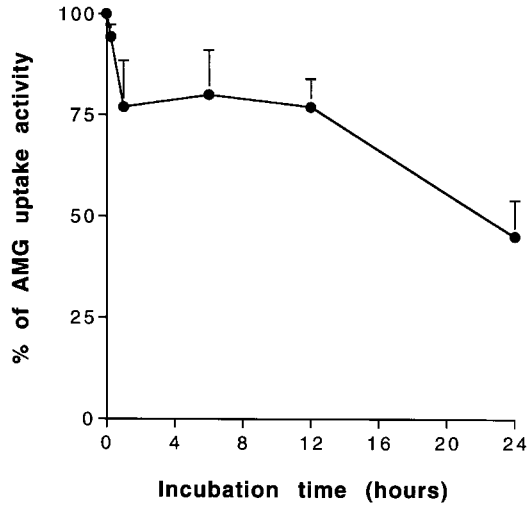

Figure 5. Cholera toxin-induced inhibition of sodium/glucose cotransport in intestinal cells. Differentiated HT-29-D4 cells were grown at confluency on polyester filters (Costar Transwell chambers) and treated apically by 120 nM cholera toxin for the indicated time. To measure the Na^+-dependent sugar uptake, the cells were incubated 20 min at 37°C with [^{14}C]-AMG ($0.15\ \mu Ci.ml^{-1}$) at the concentration of 0.1 mM in the apical compartment. At the end of the incubation, the apical and basolateral media were removed and the cells were washed three times. The cells were then disrupted with 0.1 N NaOH and SDS 0.1%. The radioactivity was measured in a Beckman beta counter by liquid scintillation (Ultimagold, Packard). The results are expressed as the relative percentage of control uptake measured with nontreated cells ($\pm$ SD, $n = 5$).

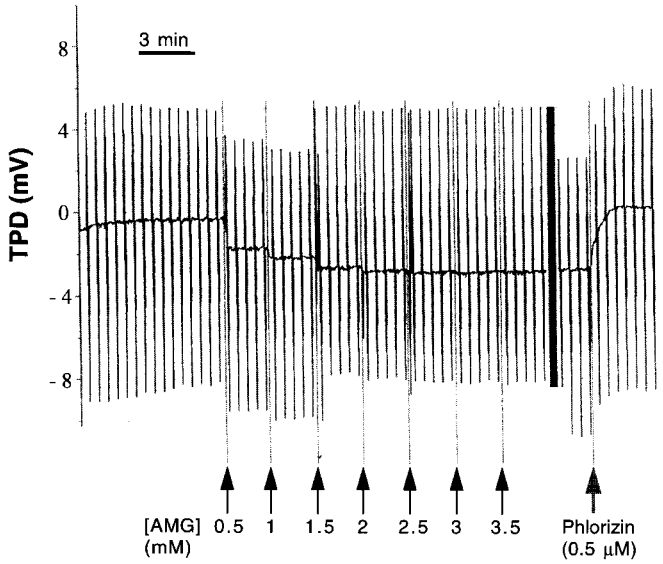

Figure 6. Demonstration of the electrogenic effect of AMG. The apical additions of 0.5 mM of AMG in the apical compartment of differentiated HT-29-D4 cells induced a stepwise increase of the transepithelial potential difference. Phlorizin was then used at a final concentration of 0.5 μM in the apical compartment of Ussing chamber.

cholera toxin induced a specific inhibition of apical glucose absorption by affecting the activity of SGLT1.

Impairment of intestinal absorption and secretion by activation of signal transduction through GSL-enriched microdomains

The prominent effect of cholera toxin on the intestinal epithelium is an activation of chloride secretion. This secretion is a complex physiological phenomenon that requires the concerted activity of four membrane proteins: i) a regulated apical chloride conductance (namely the CFTR channel), ii) a basolateral $Na^+/K^+/2Cl^-$ cotransporter, iii) a basolateral K^+ conductance, and iv) the Na^+K^+ ATPase [29]. Conceptually, cholera toxin can activate chloride secretion by two distinct mechanisms. The most dramatic effect is the well-established stimulation of the cAMP pathway by CT-A, which results in the protein kinase A-dependent activation of CFTR. The second mechanism may involve a Ca^{2+} response induced by interaction of GM1 microdomains with CT-B [30]. This potential increase of $[Ca^{2+}]i$ could activate Ca^{2+}-regulated K^+ channels (Figure 7, left panel), which are known to potentiate chloride secretion [29]. Regulatory G proteins associated with plasma membrane microdomains enriched in GM1 could mediate these effects. Similarly, it can be postulated that HIV-1 gp120, which activates the Ca^{2+} pathway in intestinal epithelial cells, may also induce a slight activation of chloride secretion. Preliminary electrophysiological data from our laboratory are consistent with this hypothesis (data not shown).

Moreover, we demonstrated that HIV-1 gp120 and cholera toxin could also inhibit the intestinal Na^+-dependent absorption of glucose. In both cases, the effect was mediated by the binding of the toxin (either gp120 or cholera toxin) to a GSL-enriched microdomain (Figure 7, right panel). Activation of the Ca^{2+} signal transduction pathway through microdomain-associated G proteins would then induce i) microtubule depolymerization, and ii) an inhibition of AMG uptake, as demonstrated for HIV-1 gp120 [16]. Since treatment of the cells with nocodazole, a microtubule-disrupting agent, also resulted in an inhibition of AMG uptake (Table 1), these data suggested that gp120 and cholera toxin may not directly inhibit the activity of SGLT1, but would rather act by decreasing the amount of functional SGLT1 proteins in the apical plasma membrane. Also, our data do not formally exclude the possibility of a cAMP-mediated inhibition of SGLT1 activity by the A subunit of cholera toxin [31]. Finally, we observed that three distinct inhibitors of sphingolipid biosynthesis (fumonisine B1, PDMP, and L-cyclo-serine) decreased the activity of SGLT1 in intestinal epithelial cells

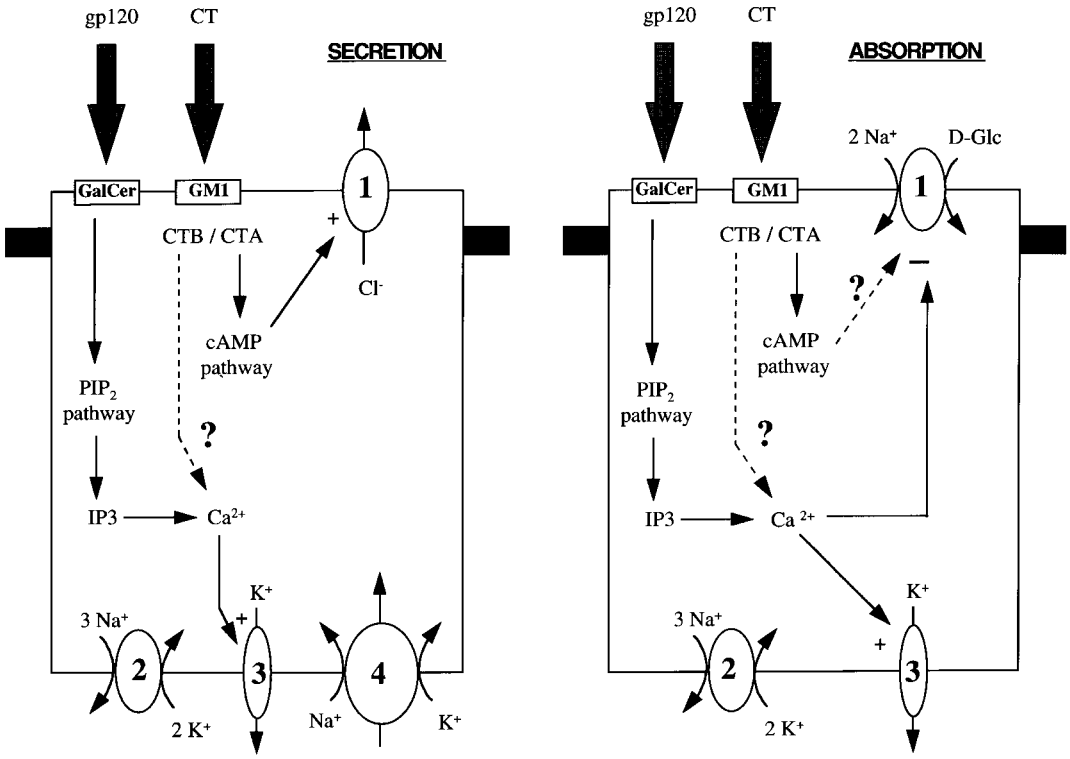

Figure 7. GSL-dependent perturbations of intestinal secretion and absorption by cholera toxin and HIV-1 gp120. Left panel: Perturbation of intestinal chloride secretion induced by the binding of cholera toxin (CT) and HIV-1 gp120 to plasma membrane microdomains enriched in GM1 and GalCer, respectively. (1) apical chloride conductance, i.e. CFTR chloride channel, (2) Na^+/K^+ ATPase, (3) basolateral K^+ conductance, (4) basolateral $Na^+/K^+/2Cl^-$ cotransporter. Right panel: GSL- and Ca^{2+}-mediated perturbation of Na^+/glucose absorption induced by cholera toxin and HIV-1 gp120. (1) SGLT1, (2) Na^+/K^+ ATPase, (3) basolateral K^+ conductance.

Table 1. Inhibition of Na^+-dependent AMG uptake by inhibitors of GSL biosynthesis and nocodazole

Treatment	*Na^+-dependent AMG uptake (% of inhibition)*
Fumonisine B1 (50 μg/ml)	33 ± 1
PDMP (40 μg/ml)	48 ± 2
L-cyclo-serine (100 μg/ml)	53 ± 9
Nocodazole (100 μM)	52 ± 3

The activity of the Na^+/glucose cotransporter in differentiated HT-29-D4 cells was evaluated by AMG uptake measurements over an incubation of 10 min with radioactive AMG. The cells were treated for 48 hr with the indicated concentration of inhibitor before the assay.

(Table 1). These data, which support the view that GSL-enriched microdomains are involved in the regulation of intestinal functions, warrant further investigation.

Conclusion

In this study, we demonstrated that the intestinal hydric balance may be perturbated by two pathogens (HIV-1 and Vibrio cholerae) that interact with GSL-enriched microdomains. In both cases, the impairment of enterocytic functions is initiated by the binding of a pathogenic protein (i.e. HIV-1 gp120 or cholera toxin) to a specific glycolipid clustered in a plasma membrane microdomain. This interaction triggers the activation of signal transduction pathways by regulatory G proteins that are selectively associated with the microdomains. The toxins increase intestinal fluid secretion by two mechanisms: activation of chloride secretion and inhibition of Na^+-dependent glucose absorption, leading to acute diarrhea.

Acknowledgement

We thank C. Langlet and H.-T. He for helpful discussions and stimulating advice. This work was supported by a grant (to J.F.) and a post-doctoral fellowship (to D.H.) from the Fondation pour la Recherche Médicale (SIDACTION).

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