Filipin-dependent Inhibition of Cholera Toxin: Evidence for Toxin Internalization and Activation through Caveolae-like Domains

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Abstract. The mechanism by which cholera toxin (CT) is internalized from the plasma membrane before its intracellular reduction and subsequent activation of adenylyl cyclase is not well understood. Ganglioside G_{M1}, the receptor for CT, is predominantly clustered in detergent-insoluble glycolipid rafts and in caveolae, noncoated, cholesterol-rich invaginations on the plasma membrane. In this study, we used filipin, a sterol-binding agent that disrupts caveolae and caveolae-like structures, to explore their role in the internalization and activation of CT in CaCo-2 human intestinal epithelial cells. When toxin internalization was quantified, only 33% of surface-bound toxin was internalized by filipin-treated cells within 1 h compared with 79% in untreated cells. However, CT activation as determined by its reduction to form the A$_1$ peptide and CT activity as measured by cyclic AMP accumulation were inhibited in filipin-treated cells. Another sterol-binding agent, 2-hydroxy-β-cyclodextrin, gave comparable results. The cationic amphiphilic drug chlorpromazine, an inhibitor of clathrin-dependent, receptor-mediated endocytosis, however, affected neither CT internalization, activation, nor activity in contrast to its inhibitory effects on diphtheria toxin cytotoxicity. As filipin did not inhibit the latter, the two drugs appeared to distinguish between caveolae- and coated pit–mediated processes. In addition to its effects in CaCo-2 cells that express low levels of caveolin, filipin also inhibited CT activity in human epidermoid carcinoma A431 and Jurkat T lymphoma cells that are, respectively, rich in or lack caveolin. Thus, filipin inhibition correlated more closely with alterations in the biochemical characteristics of CT-bound membranes due to the interactions of filipin with cholesterol rather than with the expressed levels of caveolin and caveolar structure. Our results indicated that the internalization and activation of CT was dependent on and mediated through cholesterol- and glycolipid-rich microdomains at the plasma membrane rather than through a specific morphological structure and that these glycolipid microdomains have the necessary components required to mediate endocytosis.

The mechanism of action of cholera toxin (CT)$^1$ in target cells involves a distinct lag period between toxin binding and adenyl cyclase activation (reviewed in Fishman et al., 1993). During this time, the toxin must gain entry into the cell and undergo processing to generate small amounts of the A$_1$ peptide (CT-A$_1$), an ADP-ribosyltransferase that catalyzes the transfer of ADP-ribose from NAD$^+$ to the stimulatory G protein, Gs. However, the molecular mechanism of intracellular toxin transport during these intervening sequences is not well understood.

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1. Abbreviations used in this paper: BFA, brefeldin A; CAD, cationic amphiphilic drug; CT, cholera toxin; CT-A$_1$ and -B, A subunit, A$_1$ peptide, and B subunit of CT, respectively; DIG, detergent-insoluble glycolipid-enriched complexes; DT, diphtheria toxin; IBMX, 3-isobutyl-1-methylxanthine; Rh-CTB, rhodamine-conjugated CT-B.

Toxin internalization from the plasma membrane has been the focus of many studies. We have previously shown that upon binding through its B subunit (CT-B) to cell surface ganglioside G_{M1}, the intact holotoxin is internalized from the plasma membrane (Orlandi and Fishman, 1993). In the human intestinal epithelial cell line, CaCo-2, the disappearance of both A and B subunits from the cell surface precedes CT-A$_1$ formation and the activation of adenyl cyclase (Orlandi and Fishman, 1993; Orlandi, 1997).

Using cultured liver cells labeled with CT absorbed to colloidal gold (gold-CT), Montesano et al. (1982) initially identified the plasma membrane structures involved in the binding and subsequent internalization of CT as small, noncoated microinvaginations. Tran et al. (1987) obtained similar results with murine 3T3-L1 fibroblasts. They found that CT internalized through these noncoated regions subsequently enters a tubulovesicular compartment and finally multivesicular bodies. In addition, they observed that in these latter structures, CT colocalizes with ligands known...
to enter the cell through coated pits. Their results suggested a common intracellular pathway for ligands that enter a cell either through coated or noncoated invaginations on the plasma membrane. The involvement of these smooth membrane invaginations known as caveolae in the internalization of CT was further supported in a study of the ultrastructural distribution of G\textsubscript{M1} in human A431 cells (Parton, 1994). G\textsubscript{M1} is enriched fourfold in caveolae as identified by the colocalization of gold-CT-B with VIP-21/caveolin, an integral membrane protein frequently associated with caveolar structure (Dupree et al., 1993). Consequently, G\textsubscript{M1} and caveolin have become common markers for the identification and purification of caveolae.

Our knowledge of the characteristics and cellular function of caveolae has increased considerably in recent years (reviewed in Parton, 1996; Kurzchalia and Parton, 1996). Originally caveolae were thought to function only in receptor-mediated potocytosis (Anderson et al., 1992); however, speculation of their biological role has since expanded to include such diverse functions as endocytosis independent of the coated pit pathway, sorting and internalization of GPI-anchored proteins, transcytosis, calcium signaling, and signal transduction. Purified endothelial caveolae possess elements essential for intracellular vesicular transport and signal transduction including heterotrimeric G proteins, SNAP, NSF, and GTPases and Src-family kinases (Sargiacomo et al., 1993; Schnitzer et al., 1995a). Of particular interest is their distinct lipid composition which is enriched in cholesterol, sphingomyelin, and glycosphingolipids but devoid of phospholipids (Brown and Rose, 1992; Fiedler et al., 1993). As such, a fundamental property of these structures is their isolation in low density, detergent-insoluble complexes. The use of sterol binding agents such as filipin, nystatin, and digitonin as well as inhibitors of cholesterol metabolism, has shown that in addition to VIP-21/caveolin, cholesterol is essential for maintaining caveolar shape and their ability to pinch off to form intracellular vesicles (Rothberg et al., 1992; Smart et al., 1994). Depletion, redistribution, or removal of plasma membrane cholesterol results in the flattening and disassembly of these invaginations, unclustering of receptors, and loss of caveolae-mediated endocytosis (Chang et al., 1992; Schnitzer et al., 1994).

Although it has been inferred from the electron microscopic studies using CT-B to localize G\textsubscript{M1} to caveolae that the latter may be the major vehicle for toxin internalization, no evidence has been provided as yet to either directly link caveolae to toxin activation or to rule out the involvement of other subpopulations of G\textsubscript{M1} at the cell surface. The latter are randomly distributed in minute amounts in coated pits or in glycolipid microdomains (see Parton, 1994). An earlier study by Sofer and Futerman (1995) suggested that CT is not excluded from clathrin-coated pits and may in fact represent a means by which toxin gains access to the endocytic pathway before its intracellular activation. They reported that inhibitors of the clathrin-dependent pathway, such as cationic amphiphilic drugs (CADs), also acted as partial inhibitors of CT activity. Although only a small percentage of surface-bound CT has been identified in coated pits, only minute quantities of the active CT-A\textsubscript{1} have to be generated from the bound CT in order to elicit its cytotoxic effects (Kassis et al., 1982; Orlandi et al., 1993; Orlandi, 1997).

Glycolipid microdomains, though lacking caveolin, bear a striking similarity in composition to caveolae and are similarly enriched in detergent-insoluble extracts (Schnitzer et al., 1995b). The significance of these domains is best illustrated in cells such as lymphocytes and neuroblastoma cells. Such cells do not express morphologically distinct caveolae and lack any detectable levels of caveolin (Fra et al., 1994; Gorodinsky and Harris, 1995; Parton and Simons, 1995). However, even in the absence of defined caveolae these cells display endocytotic and signal transduction events quite similar to caveolae-mediated functions in nonlymphoid cells (Deckert et al., 1996). As such cells bind and respond to CT (Fishman and Atikkan, 1980; Kassiss et al., 1982), the mechanism by which these cells internalize and activate the toxin remains unclear. That these cells contain detergent-insoluble domains rich in cholesterol and glycosphingolipids (Parton and Simons, 1995; Parton, 1996; Simons and Ikonen, 1997), raises the possibility that such caveolae-like glycolipid domains may play a role in toxin action.

In this study, we explored the relationship between CT internalization and intracellular activation in human intestinal CaCo-2 cells as well as human Jurkat T lymphoma cells that lack caveolin and well-defined caveolae (Fra et al., 1994) and human A431 epidermoid carcinoma cells that are rich in both caveolin and caveolae (Parton, 1994). Through the use of several drugs that target their effects on the function and structural characteristics of caveolae, caveolae-like glycolipid rafts, and clathrin-coated pits, we demonstrated that the mechanism of internalization that leads to toxin activation was highly dependent on the clustering of cholesterol within glycolipid domains on the plasma membrane. Our results suggested that both caveolae and caveolae-like glycolipid rafts devoid of caveolar shape or caveolin are indistinguishable with regard to their ability to act as a vehicle for CT entry and activation.

**Materials and Methods**

**Materials**

CT, rhodamine-conjugated CT-B (Rh-CTB), diphtheria toxin (DT) and *Pseudomonas* exotoxin A were obtained from List Biological Laboratories (Campbell, CA). Other reagents were obtained as follows: filipin complex, chlorpromazine, and 3-isobutyl-1-methylxanthine (IBMX) from Sigma; brefeldin A (BFA) from Epicentre Technologies (Madison, WI); Na\textsuperscript{125}I (carrier-free) and Na\textsuperscript{125}I-protein A (9.5 μCi/mg) from Dupont-New England Nuclear (Boston, MA). 2-hydroxypropyl-β-cyclodextrin was graciously provided by Dr. Peter Pentchev (National Institutes of Health, Bethesda, MD) and additionally purchased from Research Plus Inc. (Bayonne, NJ).

**Cells and Cell Culture**

Cells were obtained from the American Type Culture Collection (Rockville, MD). CaCo-2 cells were grown in MEM supplemented with nonessential amino acids, sodium pyruvate, 2 mM glutamine, and 20% NuSerum IV (Collaborative Biomedical, Bedford, MA; Orlandi and Fishman, 1993). A431 and Jurkat cells were grown in DME and RPMI-1640 media supplemented with 10% FBS, respectively. For assaying cAMP accumulation, CaCo-2 cells were grown in 24 × 16-mm clusters; for assaying the formation of CT-A\textsubscript{1}, in 6 × 35-mm clusters; for CT internalization and degradation experiments in 12 × 22-mm clusters; for detergent extraction, in 75 cm\textsuperscript{2} flasks; and for fluorescence microscopy, in 8-well chamber slides (Lab-Tek from Nune, Naperville, IL).
Filipin for 1 h, and then incubated with 2-hydroxypropyl-
\text{-cyclo-dextrin, cells were entered through a filipin-sensitive mechanism,}
\text{and analyzed for cAMP by radioimmune assay (Zaremba and Fishman,}
\text{unrelated otherwise indicated. The cells then were extracted with 0.1 M HCl, and the extracts assayed for cAMP by radioimmunone assay (Zaremba and Fishman,}
\text{8C, and washed three times in PBS. The slides were then mounted with a coverslip and the cells observed by fluorescence mi-
\text{croscope using a Zeiss Axioshot microscope equipped with a Plan-APO-
\text{PH-2D, serum-free medium buffered with 25 mM Hepes containing 0.01% for the
\text{first time-cultured for 48 h in serum-free MEM containing 0.1% fatty acid-free
\text{without 1 g/ml filipin and a mixture of protease inhibitors (5 g/ml each of leupeptin, soybean trypsin inhibitor, and benzamidine; and 1 mM
\text{phenylmethylsulfonylfluoride). The extracts were adjusted to 40% su-
\text{crose, 2 mM phenylmethylsulfonylfluoride, and 1% Triton X-100 with or
\text{without 1 g/ml filipin for 30 min at 4°C. The samples were then centrif-
\text{uged at 10,000 g for 10 min, and the supernatants, designated as the soluble
\text{fractions, were counted for 125I-CT. Analysis of detergent extracts by
\text{floatation on continuous sucrose gradients was adapted from previously
\text{described procedures (Smart et al., 1994; Fra et al., 1994). CaCo-2 and
\text{Jurkat cell pellets (~1 × 10^7 cells) were prepared as described above, and ex-
\text{tracted for 30 min at 4°C in 1 ml of 50 mM Tris, pH 7.4, 150 mM NaCl, 1%}
\text{Triton X-100 ± 1 g/ml filipin and a mixture of protease inhibitors (5 g/ml each of leupeptin, soybean trypsin inhibitor, and benzamidine; and 1 mM
\text{phenylmethylsulfonylfluoride). The extracts were adjusted to 40% su-
\text{crose and 2-ml portions were layered under a 10-ml 10–30% linear sucrose
\text{gradient. Samples were centrifuged for 16 h at 38,000 rpm at 4°C using a
\text{SW40 rotor. Fractions (~0.5ml) were collected, counted for 125I-CT, and
\text{analyzed for caveolin by immunoblotting using a dot-blot apparatus
\text{(Schleicher & Schuell, Inc., Keene, NH), anti-caveolin and anti-rabbit-HRP
\text{as described below.}
\text{Other Methods}

Established methods were used to determine the generation of A peptide by intact cells (Kassiss et al., 1982) and the degradation of bound 125I-CT (Fishman, 1982). The immunological detection of cell surface CT using anti-CT-A antibodies (Orlandi and Fishman, 1993) was performed as previously described (Fishman, 1982). DT cytotoxicity was assayed as before (Orlandi, 1997).

**Results**

**Effects of Filipin and Chlorpromazine on the Internalization of CT**

To investigate the pathway(s) of CT internalization and activation in human intestinal epithelial CaCo-2 cells, we first examined the effects of two classes of drugs on the up-

**Indirect Immunofluorescence Microscopy**

CaCo-2 cells were labeled with Rh-CTB using a modification of the procedure of Sofer and Futerman (1995). In brief, cells were washed to remove any serum, incubated for 1 h at 37°C in MEM buffered with 25 mM Hepes plus 0.01% BSA with no addition; 1 μg/ml filipin; 10 μg/ml chlorprom-

**Accumulation of cAMP**

Cells (CaCo-2 and A431 in monolayer; Jurkat in suspension) were incubated at 37°C in serum-free medium buffered with 25 mM Hepes and containing 1 mM IBMX and 0.01% BSA with 30 pM CT for 2 h unless other-

**Triton X-100 Solubility and Analysis of Detergent-insoluble Extracts**

CaCo-2 or Jurkat cells (~1 × 10^7) were treated with and without 1 μg/ml filipin for 1 h, and then incubated with 125I-CT at 4°C for 1 h. After wash-

**Differential Effects of Filipin and Chlorpromazine on CT Activation in CaCo-2 Cells**

We next examined the effects of filipin on toxin activation and activity. Although the majority of surface-bound toxin entered the cell through a filipin-sensitive mechanism, repeated experiments suggested that even in the presence of filipin, ~30–40% of the toxin appeared to be internal-

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Caveolae, and Cholesterol in CT Activation

Examination of the Relationship Between Caveolin, Caveolae, and Cholesterol in CT Activation

Thus far, the results with filipin indicated that CT internalization and activation occurred through cholesterol-rich glycolipid microdomains to include the possible involvement of caveolae. To examine this relationship further, we next compared the effects of filipin on CT activation in CaCo-2, A431, and Jurkat T-lymphoma cells. While it is well established that A431 cells contain caveolin and caveolae, Jurkat cells express neither. Studies with CaCo-2 cells, however, have produced conflicting reports on the presence of caveolin and caveolae (Mayor et al., 1994; Mirre et al., 1996). In agreement with Mayor et al. (1994), we confirmed the presence of low levels of caveolin in these cells by RT-PCR, indirect immunofluorescence, and immunoblotting albeit at a considerably lower level than that expressed by A431 cells and in contrast to its known absence in Jurkat cells (data not shown). For detection of caveolin by RT-PCR, synthetic primers were designed from the 5′ and 3′ sequences of human caveolin mRNA that encompassed a portion of the 5′-flanking region and the first 97 amino acids of the protein (Glenney, 1992). The 5′-nucleotide sequence was (sense strand from 5′ to 3′) TTCATCCAGCCACGGGCCAGCATGTCTGGG. The 3′-nucleotide sequence was (antisense strand from 5′ to 3′) CTTCGAAGCGACGGGAGCACTCATGTCTGGG. Indirect immunofluorescence and immunoblotting assays were performed using affinity-purified polyclonal rabbit antibodies against the first 97 amino acids of human caveolin obtained from Transduction Laboratories (Lexington, KY).

As one of the markers predominantly used to define the presence of caveolar structure, caveolin expression in CaCo-2 cells suggested the presence of caveolae and hence their possible role in facilitating toxin entry. When CaCo-2, A431 and Jurkat cells were treated with various concentrations of filipin before their exposure to CT, the concentration-dependent effects of filipin on the activity of CT were found to differ only slightly (Fig. 4). Thus, the interaction of filipin with plasma membrane cholesterol and its inhibitory effects on CT internalization and activation were found to occur in cells that do not express caveolin and caveolae as well as those that express high levels of caveolin (and well-defined caveolae). Furthermore, we found that in filipin-treated Jurkat cells, CT-A1 formation was totally blocked (data not shown), indicating that the same mechanism of inhibition was occurring in both caveolin-positive and -negative cells.
Further Differentiation of CT Internalization Through Coated and Noncoated Pathways

We next used filipin and chlorpromazine to further differentiate the distinct pathways of internalization for CT and DT, another ADP-ribosylating toxin. DT enters the endocytic pathway of target cells through clathrin-coated pits (Moya et al., 1985; Beaumelle et al., 1992). Whereas CT activity was inhibited >95% in filipin-treated CaCo-2 cells, DT activity was slightly enhanced (~110%; Fig. 5). However, the converse was observed when cells were exposed to 25 μg/ml (70 μM) chlorpromazine. DT cytotoxicity was inhibited by ~88% whereas CT activity exhibited only a 20% decrease. A similar pattern of results was obtained when A431 cells were treated with either filipin or chlorpromazine and then DT (Fig. 5). Comparable results also were obtained with a third ADP-ribosylating toxin, *Pseudomonas* exotoxin A, that enters cells through clathrin-coated pits but then follows the same retrograde pathway as CT to the ER, both toxins being blocked by BFA in contrast to DT which is not (see Orlandi et al., 1993). Exotoxin A blocked protein synthesis in control and filipin-treated A431 cells with EC50 values of 90 and 71 ng/ml whereas in chlorpromazine-treated cells, the EC50 was shifted to 402 ng/ml.

The Effects of a Cyclodextrin on CT Internalization and Activation

To further explore the role of plasma membrane cholesterol, particularly that associated with detergent-insoluble glycolipid microdomains and caveolae, in mediating CT internalization and activation, we performed similar experiments using another sterol binding agent, 2-hydroxypropyl-β-cyclodextrin. Cyclodextrin has been shown to specifically remove cholesterol from the plasma membrane (Neufeld et al., 1996). Whereas treatment of CaCo-2 cells with cyclodextrin at 100 μg/ml had no significant effect on toxin binding, the internalization of CT was inhibited (Fig. 6A). As a consequence of this effect, CaCo-2 cells exposed to cyclodextrin no longer responded to CT as measured by cAMP accumulation (Fig. 6B). As was observed with filipin treatment, the inhibitory effects of cyclodextrin were also selective as the treated CaCo-2 cells retained their sensitivity to DT (Fig. 6B).

Filipin-induced Inhibition of CT Activity Was Time-dependent and Reversible

Filipin was an effective inhibitor when added to cells ei-
Filipin prevented toxin degradation

While Filipin also compared the rates of CT degradation in untreated and Filipin-treated cells. Although only a small percentage of bound CT is required to exert its cytotoxic effects, the majority of internalized toxin is degraded (Fishman, 1982; Orlandi et al., 1993). To measure the rates of CT degradation, cells were treated with or without Filipin for 60 min at 37°C, incubated with 125I-CT at 4°C, washed, and warmed to 37°C for the indicated times in fresh medium with or without Filipin. The medium was then analyzed for TCA-soluble radioactivity (Fishman, 1982). Consistent with the reduced ability of CT to become internalized from the cell surface in the presence of Filipin, toxin degradation was likewise inhibited. Whereas 21% of CT was degraded in untreated cells over a 6 h period at 37°C, no more than 1% was degraded over the same time period in cells continually exposed to Filipin (Fig. 8).

The effects of Filipin on the Detergent Extraction of CT Bound to Cells and on CT in Detergent-resistant Complexes

The interactions between CT and its receptor GM1 at the cell surface were further assessed by examining the effects of Filipin on the Triton X-100 solubility of CT bound to cells at 4°C. Hagmann and Fishman (1982) had shown that the majority of CT-ganglioside complexes formed in membranes and intact cells are resistant to extraction with Triton X-100. Likewise, caveolae and glycolipid microdomains, enriched in both ganglioside GM1 and cholesterol have been characterized in terms of their detergent insolu-
bility in the presence of Triton X-100 (Brown and Rose, 1992). Similar to the results of Hagmann and Fishman (1982), when $^{125}$I-CT was bound to CaCo-2 cells at 4°C and extracted with 1% Triton X-100 for 30 min at 4°C, 33.7 ± 3.7% of the toxin was found in the soluble fraction. In contrast, 46.6 ± 3.5% of the bound toxin was extracted into the soluble phase in cells treated with 1 μg/ml filipin. The effects of filipin on membrane-bound toxin solubility was even more pronounced in Jurkat cells. In the presence of filipin, >97% of the membrane-bound toxin was found in the soluble fraction compared with 47% in untreated cells.

Triton X-100 detergent extracts of CaCo-2 and Jurkat cells labeled with $^{125}$I-CT at 4°C were also analyzed by floatation on continuous sucrose gradients (Fig. 9). Under these conditions, CT-GM$_1$ complexes formed at the cell surface were identified in a peak of radioactivity in the upper regions of the gradient representing detergent-insoluble glycolipid domains (Fig. 9). However, when both cell lines were treated with filipin this detergent-insoluble region displayed a noticeable shift to heavier buoyant densities. The shift in density of the CT-bound peaks indicated that the filipin-sterol complexes formed within cell surface caveolae and glycolipid microdomains may have altered the compositional or biophysical characteristics of these domains. The formation of large peristomal rings of sterols in the presence of filipin as described by Simionescu et al. (1983) may have contributed to this shift in buoyant density. We also analyzed the gradient fractions derived from CaCo-2 cells for caveolin by immunoblotting and found that caveolin colocalized with $^{125}$I-CT in extracts from both control and filipin-treated cells (data not shown).

Discussion

Based on recent studies of CT activation and intracellular transport (Orlandi et al., 1993, 1997; Lencer et al., 1995; Majoul et al., 1996; Sandvig et al., 1996), a clearer understanding is slowly emerging of the molecular events that occur between toxin binding to ganglioside GM$_1$ on the cell surface and its ultimate activation of adenylate cyclase. Of particular interest is the mechanism by which bound CT is internalized and ultimately targeted to those intracellular sites necessary for its activation. Although most of the bound CT is rapidly internalized from the cell surface, only a small percentage of the internalized toxin is actually responsible for the activation of adenyl cyclase, the majority of internalized toxin being destined for degradation (Fishman, 1982; Fishman et al., 1993; Orlandi et al., 1993). In this regard, the elegant electron micrographic studies on the internalization and localization of CT and the cell surface distribution of GM$_1$ (Montesano et al., 1982; Tran et al., 1987; Parton, 1994) do not resolve whether more than one pathway exists for toxin entry and subsequent activation.

In this study, we used the polyene antibiotic filipin to show not only that the internalization of CT was mediated by caveolae or caveolae-like domains on the cell surface but that the activation of CT was dependent on its entry through these structures. Filipin interacts with 3β-hydroxysterols such as cholesterol in the plasma membrane to form planar sterol–filipin complexes and peristomal rings of sterols. Cholesterol is a major component of cell-surface microdomains frequently described in the literature as detergent-insoluble glycolipid-enriched complexes, DIGs, and caveolae (Simons and Ikonen, 1997). The endocytic functions of these entities are influenced by the presence and state of cholesterol (Rothberg et al., 1990; Schnitzer et al., 1994).

We observed that in the presence of filipin, the internalization of surface bound CT in CaCo-2 cells was inhibited using two different assays, a quantitative one using anti-CT-A$_1$ antibodies and $^{125}$I-protein A, and direct fluorescence with Rh-CT-B. This inhibition in turn resulted in the blocking of subsequent steps in the intracellular processing of the toxin. These included the inability of the toxin to be reduced to the active A$_1$ peptide, and thereby to activate adenyl cyclase. In addition, the degradation of CT was blocked in filipin-treated CaCo-2 cells. These effects of filipin are consistent with its primary action being the disruption of the cholesterol-rich microdomains localized to caveolae and caveolae-like structures on the cell surface. In this regard, another sterol binding agent, cyclodextrin, had similar effects on CT internalization and activity.

Further supporting such a mode of action are our observations that the effects of filipin were both rapid and reversible. We found that even when the cells were exposed to filipin and CT at the same time, their response to the toxin was effectively inhibited. When filipin was added after CT, the cells became resistant to its inhibitory effects. Thus, once the toxin has become internalized, it is no longer sensitive to the effects of filipin. The cells also recovered rapidly from the effects of filipin; when they were exposed to CT immediately after being removed from filipin-containing medium, they accumulated almost as much cAMP as control cells. In this regard, CT bound to the surface of filipin-treated cells did not become inactive as once the medium was replaced with filipin-free medium, the cells were able to respond to the toxin by accumulating cAMP. Taken together with the surface labeling experiments, it is clear that filipin treatment has little effect on the ability of CT to bind to its receptor GM$_1$.
the toxin–ganglioside complexes from entering the internalization pathway.

In contrast to the importance of caveolae and caveolae-like microdomains, clathrin-coated pits did not play a significant role in the activation of CT. While the majority of bound CT remained at the cell surface in the presence of filipin, a small percentage still appeared to be internalized (as judged by the loss of cell surface immunoreactivity with CT-A1 antisera). These results may reflect the inability of filipin-treated cells to form fully competent endocytic vesicles capable of transporting toxin from the cell surface. Consequently, a portion of the bound toxin may have become trapped in incompletely-formed vesicles at or near the plasma membrane and were inaccessible to antibody. Another possible explanation may be that a portion of the membrane-bound toxin in these cells was internalized through the coated-pit dependent pathway. We addressed this possibility using CADs that have been shown to reduce the number of cell-surface coated pits and inhibit receptor-mediated endocytosis. When CaCo-2 cells were exposed to CADs such as chlorpromazine, slightly depressed levels of toxin binding and uptake were observed while the combination of chlorpromazine and filipin resulted in the nearly complete inhibition of toxin uptake from the cell surface. These findings suggested some CT was taken up through coated pits, although its entry via this mechanism was not a functional pathway for toxin activation. This was consistent with an early study demonstrating that CT entering cells via transferrin receptors is unable to activate adenylyl cyclase (Pacuszka and Fishman, 1992). Additionally, the contrasting effects of chlorpromazine and filipin (as well as cyclodextrin) on the activity of both CT and DT emphasized the distinct differences between the internalization of DT through clathrin-coated pits and the apparent dependence of CT on caveolae or caveolae-like domains.

Caveolin is both a marker for caveolae and appears to be essential for caveolae formation (Parton, 1996). Not only does caveolin have a high affinity for cholesterol, but

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**Table I. Effects of Filipin on the Activity of Surface-Bound CT in CaCo-2 Cells**

| Treatments | CT stimulation of cAMP (pmol/well) |
|------------|-----------------------------------|
| CT         | 2 h                               | 3 h                            |
| −          | 9 ± 0.4                           | 12 ± 2                         |
| +          | 261 ± 7.3 (100)                   | 485 ± 15 (100)                 |
| +          | 40 ± 6 (12)                       | 96 ± 10 (18)                   |
| ±          | 42 ± 2 (13)                       | 197 ± 23 (39)                  |

CaCo-2 cells were chilled to 4°C, incubated without (−) and with (+) 0.03 mM CT for 1 h at 4°C, washed and incubated at 37°C for the times indicated in the presence (+) or absence (−) of 1 μg/ml filipin. After 1 h at 37°C, one set of filipin-treated cells (±) were washed and further incubated in filipin-free medium at 37°C for 1 or 2 h. All cells were then assayed for cAMP accumulation as described in Materials and Methods. Values in parentheses denote the net accumulation of cAMP in response to CT as a percent of untreated cells.
appears to interact with G\textsubscript{M1} in caveolae (Fra et al., 1995). In this study, we used three different cell lines (CaCo-2, A431, and Jurkat) that express varying levels of caveolin and caveolae to examine the relationship between CT internalization and caveolae and caveolae-like function. While it had previously been reported that CaCo-2 cells do not express caveolin, we have shown here that low levels of the protein are in fact present in this cell line. In all likelihood, the discrepancy between our results and those reported by Mirre et al. (1996) may be attributed to the specificity of the reagents and sensitivity of the assays used. Although CaCo-2 cells express low levels of caveolin, our results indicated that it was not required for the activation of CT. We also found that CT was reduced to its A\textsubscript{1} peptide and stimulated cAMP accumulation in human Jurkat T lymphoma cells that lack caveolin and caveolae (Fra et al., 1994). The ability of filipin to block the activation and action of CT in Jurkat cells indicates that the complex lipid microdomains of cholesterol, sphingomyelin and glycolipids are the essential plasma membrane entities for a cellular response to the toxin. In this regard, the detergent-resistant properties of cell surface-bound \textsuperscript{125}I-CT were dramatically altered in both Jurkat and CaCo-2 cells treated with filipin.

The mechanism of CT internalization may be related to the cross-linking of ganglioside G\textsubscript{M1} by the pentavalent binding of CT-B. The clustering of G\textsubscript{M1}–CT complexes in turn may facilitate and enhance G\textsubscript{M1}–cholesterol interactions and subsequently lead to sequestration within caveolae or caveolae-like domains. In his study on the distribution of G\textsubscript{M1} in A431 cells, Parton (1994) suggested that the increased colocalization of G\textsubscript{M1} and caveolin within caveolae structures may be related to toxin binding. Thus, 44% of the plasma membrane gold-CT-B is found associated with caveolae in cells labeled at 8°C and then fixed and embedded compared with 22% by post-embedding labeling techniques. This observation is wholly consistent with studies on the distribution of GPI-anchored proteins on the plasma membrane (Mayor et al., 1994; Schnitzer et al., 1995\textsuperscript{b}). Whereas GPI-anchored proteins were found to reside in microdomains distinct from caveolae, enrichment or partitioning into or near these structures has been observed only upon cross-linking. The increased localization of surface-bound CT into these microdomains initiated by cross-linking of its ganglioside G\textsubscript{M1} receptor may trigger toxin internalization. In the presence of filipin, G\textsubscript{M1} as well as CT–G\textsubscript{M1} complexes may be less able to interact with cholesterol in these microdomains. The increase in detergent extraction of \textsuperscript{125}I-CT bound to filipin-treated cells and the change in its buoyant density are consistent with such an effect.

Although additional studies are necessary to fully understand the molecular mechanisms that drive the formation and internalization of caveolae and caveolae-like domains, it is quite apparent that they play a significant role in clathrin-independent receptor-mediated endocytosis and signal transduction. The inherent biochemical characteristics of glycolipids and their interactions with other membrane components have long been suspected of aiding in directed intracellular membrane trafficking, particularly in

![Figure 8](image-url)  
*Figure 8.* Filipin inhibits degradation of CT by CaCo-2 cells. Untreated cells (●) and cells treated with 1 µg/ml filipin (○) were incubated with \textsuperscript{125}I-CT at 4°C, washed to remove any unbound toxin, and incubated with fresh medium with or without filipin at 37°C for the indicated times. Toxin degradation was determined by the levels of TCA-soluble radioactivity that accumulated in the culture medium with time.

![Figure 9](image-url)  
*Figure 9.* Analysis of Triton X-100 extracts from CaCo-2 and Jurkat cells on sucrose gradients. Untreated (●) and 1 µg/ml filipin-treated (○) CaCo-2 and Jurkat cells were incubated with \textsuperscript{125}I-CT for 1 h at 4°C, washed in PBS, scraped, and pelleted. After the cells were extracted for 30 min at 4°C in 1% Triton X-100 solution ± 1 µg/ml filipin, the extracts were centrifuged on a 10–30% linear sucrose gradient as described in Materials and Methods. Fractions (~0.5 ml) were collected from the top of the gradient and counted for \textsuperscript{125}I.
epithelial cells in light of their polar distribution among apical and baso-lateral membrane domains (Simons and van Meer, 1988). It is not surprising then that the glycosphingolipid and cholesterol components of caveolae and caveolae-like domains play a similar role in mediating the endocytic function of these entities. This is evident from the results presented here as well as within the literature that alterations in plasma membrane cholesterol affect clathrin-independent receptor-mediated endocytosis. Likewise, as a core component of caveolae and caveolae-like domains, ganglioside GM1 possesses a similar influence. Although GM1-oligosaccharides provide the recognition site for CT binding, Pacuszka et al. (1991) demonstrated that the nature of the lipid moiety plays an equally essential role in directing CT internalization and activation. Thus, a cholera derivative of GM1 is a more effective receptor than native GM1 whereas phospholipid derivatives of GM1 are less effective receptors. The latter observation may be particularly relevant in glycolipid-rich domains are depleted of phospholipids (Fiedler et al., 1993).

Whereas this event probably occurs through specific morphological structures such as caveolae in certain cell lines, DIGs also appear to contain all the necessary components to mediate toxin endocytosis. The mechanism by which toxin internalized through these structures is subsequently targeted to sites necessary for activation awaits further study.

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