Protein Kinase C\(\delta\) Is Activated by Shiga Toxin and Regulates Its Transport\(^*\)

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Protein kinase C (PKC) isozymes regulate different vesicular trafficking steps in the recycling or degradative pathways. However, a possible role of these kinases in the retrograde pathway from endosomes to the Golgi complex has previously not been investigated. We report here the involvement of a specific PKC isozyme, PKC\(\delta\), in the intracellular transport of the glycolipid-binding Shiga toxin (Stx), which utilizes the retrograde pathway to intoxicate cells. Upon binding to cells, Stx was shown to specifically activate PKC\(\delta\) and not PKC\(\alpha\). The involvement of PKC\(\delta\) and PKC\(\alpha\) in the retrograde transport of Stx was then monitored biochemically and by immunofluorescence after inhibition or depletion of the isozymes. PKC\(\delta\), but not PKC\(\alpha\), was shown to selectively regulate the endosome-to-Golgi transport of StxB. Upon inhibition or knockdown of PKC\(\delta\), StxB molecules colocalized less with giantin and more with EEA1, indicating that the molecules were accumulated in endosomes, unable to reach the Golgi complex. The inhibition of Golgi transport of Stx was reflected by a strong reduction in the toxic effect, demonstrating that transport of Stx to the cytosol is dependent on PKC\(\delta\) activity. These results are in agreement with our previous data, which show that Stx is able to stimulate its own transport.

Shiga toxin (Stx)\(^2\) is an AB\(_2\)-toxin consisting of an enzymatically active A-subunit noncovalently linked to a stable pentamer of B-chains (StxB) that binds to the glycosphingolipid GB\(_3\) and mediates transport of the toxin (1). To reach its intracellular target, the toxin has to be endocytosed and transported via early endosomes (EEs) to the Golgi complex. The toxin is then transported further to the endoplasmic reticulum from where the A-subunit is translocated to the cytosol (for reviews see Refs. 1 and 2). In the cytosol the protein synthesis is inhibited by enzymatic modification of the 28 S rRNA. Endocytosis of the toxin is mediated by a clathrin-dependent process in several cell types, although clathrin-independent mechanisms are also partly involved (2). The toxin is transported to the EE, which is considered a sorting station for many endocytosed molecules. Some receptors are recycled to the plasma membrane to be recharged with ligands, like the transferrin receptor, whereas others enter the late endocytic pathway for degradation, like the epidermal growth factor receptor. Stx has been shown to utilize the retrograde pathway, which deviates from the recycling and degradative pathways in the EE and allows the toxin to be directly transported to the trans-Golgi network (TGN) and further to the Golgi and the endoplasmic reticulum (3). This pathway is also used for retrieval of endogenous proteins, like TGN46 and mannose 6-phosphate receptors (reviewed in Ref. 4). Several proteins have been shown to regulate the transport of StxB in this pathway (4). Recently, microtubuli and dynein were also shown to be involved in Stx trafficking, and interestingly, Stx seemed to be able to stimulate microtubule assembly (5). Importantly, the requirements for retrograde transport of various protein toxins such as Stx and ricin differ, suggesting that there is more than one route from EE to the TGN (6, 7).

Stx has been shown to induce signaling cascades leading to apoptosis in several cell types (reviewed in Ref. 8). In some cells the induction of apoptosis is mediated by the ribotoxic stress induced by the A\(_1\)-chain after entry into the cytosol, whereas in other cells both Stx and the Stx B-subunit have been shown to induce apoptosis independent of intracellular transport (8). Several kinases are rapidly activated by Stx, such as the Src kinases Yes (9, 10) and Lyn (11), and the tyrosine kinase Syk (11, 12). In human renal carcinoma-derived ACHN (human, Caucasian, kidney, adenocarcinoma) cells, Stx has been shown to induce phosphorylation of actin-binding proteins such as paxillin and ezrin and also to increase the levels of cortical actin and microtubuli (13). Importantly, we have shown that Stx is able to stimulate its own clathrin-mediated entry in a process dependent on toxin concentration (14, 15) and by induction of signaling cascades that leads to tyrosine phosphorylation of clathrin and activation of Syk (12). Moreover, in the monocytic cell line THP-1, Shiga-like toxin 1 was reported to activate protein kinase C (PKC) (16), and the involvement of PKC in toxin traffic has previously been indicated by the demonstration of...
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increased sensitivity to several toxins after stimulation of cells with 12-O-tetradecanoylphorbol-13-acetate (17), including Stx. In this study we therefore wanted to investigate the role of PKC isozymes in Stx transport and whether Stx is able to activate these kinases to modulate its own transport.

PKC isozymes are serine-threonine kinases that are implicated in diverse cellular functions, such as growth, differentiation, apoptosis, motility, ruffling, and vesicular trafficking (for reviews see Refs. 18 and 19). The isoforms can be divided into subgroups based on structure and cofactor requirements. The classic PKC (cPKC) isozymes (α, β₁, β₂, γ, and δ) are activated by Ca²⁺ and 1,2-diacylglycerol, whereas the novel PKC (nPKC) isozymes (ε, η, θ) lack the Ca²⁺-binding domain, but are activated by 1,2-diacylglycerol, whereas the atypical PKC (aPKC) isozymes (ζ and λ/ι) are both Ca²⁺- and 1,2-diacylglycerol-independent but might be activated by inositol 1,4,5-trisphosphate (18, 19).

Different PKC isozymes have been shown associated with several intracellular structures involved in vesicular trafficking such as caveolae, multiple endocytic compartments, the Golgi complex, and lysosomes. PKC activity has been shown to regulate endocytosis of an increasing number of ligands and to regulate different vesicular trafficking steps in the recycling or degradative pathways (reviewed in Ref. 20). However, a role of PKC isozymes in the retrograde pathway has previously not been reported. Recently, both PKCα and PKCβ were shown to mediate correct trafficking of FcαR vesicles (21), and we therefore wanted to investigate whether PKCα or δ activity were implicated in targeting Stx into the retrograde pathway. First, the ability of StxB to rapidly activate these isoforms was determined, and we found that PKCβ was specifically activated by StxB binding but not PKCα. Then, PKCα and δ were inhibited or depleted by the use of specific drugs or siRNA oligonucleotides, and the main Stx transport steps were monitored; the endocytosis, the endosome-to-Golgi transport, and the toxicity exerted in the cytosol. PKCβ was found to regulate the endosome-to-Golgi transport of Stx, whereas PKCα activity did not seem to be involved in any of the intracellular transport steps. Upon PKCβ inhibition or depletion the StxB reen route to the Golgi accumulated in endocytic structures early in the transport pathway. The inhibition of endosome-to-Golgi transport of Stx was reflected by reduced toxicity of Stx. These results show that PKCβ is an important regulator of Stx trafficking and are in agreement with the idea that Stx is able to stimulate its own intracellular transport.

EXPERIMENTAL PROCEDURES

Materials—Hepes, bovine serum albumin, MESNa (mercaptoethanesulfonic acid), n-octylglycyopyranoside, and tetracycline were purchased from Sigma. Na₂SO₄ and [³H]leucine were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Mouse anti-Stx antibodies (13C4 and 3C10) were from Toxin Technology (Sarasota, FL), rabbit anti-PKCα antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and mouse anti-α-tubulin antibody and mouse anti-ricin anti-

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solved in 0.1 M KOH. The incorporation of radioactivity was finally quantified.

Endocytosis Experiments—Measurements of internalization of $^{125}$I-labeled ricin and transferrin were performed essentially as described previously (24). The endocytosis of Stx was quantified using a modified version of the procedure described previously (12, 15), which takes advantage of a special ruthenium label quantitated in a highly specialized electro-chemiluminescence detection instrument provided by BioVeris Corp. (Gaithersburg, MD). Briefly, Stx was biotinylated with the reducible EZ-Link Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Rockford, IL), and a monoclonal antibody against Stx (3C10, Toxin Technology, Sarasota, FL) was labeled with the special BV-TAG® label containing a Tris(bipyridine)-chelated ruthenium(II) atom (BioVeris Corp.). To measure the uptake of biotinylated Stx in cells treated with different PKC inhibitors, the cells were washed once in Hepes-buffered minimal essential medium and then preincubated with the inhibitors before biotin-labeled Stx (0.33 nM) was added. The incubation was continued at 37 °C for the indicated time periods, still in the presence of the inhibitors. Control cells were treated with Me2SO alone. To distinguish internalized toxin from total cell-associated toxin (bound plus internalized), the cells were washed (0.14 M NaCl, 2 mM CaCl$_2$, 20 mM Hepes, pH 8.6), and one half of the plate was treated with 0.1 nM MESNa in the same buffer for 30 min on ice to reduce the SS-linked biotin in cell surface-bound toxin. The other half was mock treated. The cells were washed in cold buffer (0.14 M NaCl, 2 mM CaCl$_2$, 20 mM Hepes, pH 7.0) and lysed (1% Triton, 60 mM n-octylglucopyranoside, 100 mM NaCl, 5 mM MgCl$_2$, 50 mM Hepes). The cell lysate was incubated with the BV-TAG®-labeled anti-Stx antibody (0.5 µg/ml), and simultaneously, to selectively quantify biotinylated Stx, the biotin-labeled Stx was captured by streptavidin-coated magnetic beads (0.1 mg/ml, Invitrogen) by gentle shaking for 1.5 h in assay diluent (0.2% bovine serum albumin, 0.5% Tween 20 in phosphate-buffered saline). The amount of streptavidin-captured Stx complexed to the BV-TAG®-labeled antibody was quantified by an M1R Analyzer (BioVeris Corp.). Counts from cells treated with MESNa represent the amount of internalized toxin, whereas counts from untreated cells represent the total amount of toxin associated with the cells (bound plus internalized). Endocytosis of Stx was reported as internalized toxin in percentage of total cell-associated toxin.

Sulfation of StxB-sulf$_2$ or Ricin-sulf$_2$—Ricin-sulf$_2$, a modified ricin A-chain containing a tyrosine sulfation site reconstituted with ricin B-chain, was produced in Escherichia coli BL21(DE3) cells as previously described (25). The StxB containing a tandem of sulfation sites in the C terminus (StxB-sulf$_2$) was produced in Escherichia coli BL21(DE3) cells as previously described (26). For sulfation experiments, HeLa, HEP-2, or Vero cells (2 × 10$^5$/well in 6-well plate) were washed twice in sulfate-free Dulbecco’s modified Eagles’ medium supplemented with 2 mM l-glutamine, before they were preincubated with 0.2 mM Na$_2$S$^{35}$SO$_4$ for 3 h in sulfate-free Dulbecco’s modified Eagles’ medium. For the inhibitor studies, the drugs were added for the last 30 min of this preincubation. Then StxB-sulf$_2$, or ricin-sulf$_2$, was added (2 µg/ml), and the incubation continued for 45 min (StxB) or 2 h (ricin), in the presence or absence of the inhibitors.

To accumulate StxB in endosomes, the cells were starved in sulfate-free medium for 2 h, and then StxB-sulf$_2$ was internalized at 19.5 °C for 1 h, before the cells were transferred to 37 °C and the incubation continued for 45 min. The cells were washed in cold phosphate-buffered saline, lysed (0.1 M NaCl, 10 mM Na$_2$HPO$_4$, 1 mM EDTA, 1% Triton X-100, and 60 mM n-octylglucopyranoside, supplemented with a mixture of protease inhibitors (Roche Applied Science), pH 7.4), and scraped. The lysate was cleared by centrifugation (10,000 × g, 10 min), and StxB or ricin was immunoprecipitated from the supernatant with monoclonal antibodies prebound to protein-A/ Sepharose beads (Amersham Biosciences) overnight at 4 °C. The beads were washed twice in phosphate-buffered saline/0.35% Triton X-100, before the adsorbed material was analyzed by 4–20% SDS-PAGE under reducing conditions and transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA). The bands were detected by exposing the membrane to a PhosphorImager screen, and the signal intensities were quantified by using ImageQuaNT 5.0 software (Amersham Biosciences). To determine the level of protein sulfation in general in cells treated with the different inhibitors or transfected with siRNA, the amount of $^{35}$S-labeled proteins in the lysate was measured by trichloroacetic acid precipitation. Only minor changes in the sulfation of proteins in general were observed under the different conditions. In addition, the amount of protein in each well of transfected cells was determined by the BCA protein assay (Pierce) with the use of bovine serum albumin as the standard. Only minor changes in the level of proteins were observed.

Immunoprecipitation and Immunoblotting—PKCδ detection was performed as follows. HeLa cells were starved for 4 h and treated with StxB (250 ng/ml) for the indicated time, before lysates were prepared (1% Triton X-100, 0.5 M Tris, 20 mM EDTA, 10 mM NaF, and 30 mM sodium pyrophosphate decahydrate, supplemented with a mixture of protease inhibitors (Roche Applied Science) and a mixture of phosphatase inhibitors (Inhibitor Mixture 1, Sigma), pH 7.4). The lysates were sonicated for 10 s, and then the cleared supernatants were used for immunoprecipitation with mouse anti-PKCδ (BD Biosciences) precoated on protein-G beads overnight at 4 °C. The immunoprecipitation was then washed twice in the lysis buffer, and the adsorbed material was analyzed by 4–20% SDS-PAGE under reducing conditions and transferred onto a polyvinylidene difluoride membrane. The phosphorylation state of PKCδ was determined by rabbit anti-phospho-PKCδ (Thr$^{505}$, Cell Signaling Technology) and the enhanced signal chemiluminescence reagent (Pierce Biotechnology). The total level of PKCδ was monitored by rabbit anti-PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA). The phosphorylation state of PKCa was analyzed in parallel. A fraction of the whole cell lysate from the PKCδ experiment was analyzed by Western blot using anti-phospho-PKCa (Ser-657, Millipore, Billerica, MA). Total PKCa was determined by mouse anti-PKCa (BD Biosciences). To determine the level of PKCa or PKCa upon siRNA treatment, lysates were prepared and PKC content was analyzed by Western blotting. The level of PKCδ was detected by rabbit anti-PKCδ (Santa Cruz Biotechnology). PKCa was detected by mouse anti-PKCa (BD Biosciences). The level of α-tubulin was
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detected by mouse anti-α-tubulin (Sigma) and was used as a loading control. Signal intensities of the bands were quantified using ImageQuaNT 5.0.

**Immunofluorescence**—HeLa cells grown on coverslips were washed in Hepes medium and preincubated with or without rottlerin (2.5 μM) for 30 min, then StxB-sulf2 (2 μg/ml) was added and the incubation continued for 10 min. The cells were washed three times in warm Hepes medium, before incubation for a further 20 min at 37 °C in warm Hepes medium with or without rottlerin. The cells were fixed in 3% paraformaldehyde in phosphate-buffered saline, permeabilized in 0.1% Triton X-100, and blocked in 5% fetal calf serum. Stx was stained by mouse anti-Stx antibodies (13C4, Toxin Technology, Sarasota, FL) followed by Cy2-labeled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and the cells were labeled with either rabbit anti-giantin antibodies (BabCO, Berkeley, CA) and Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories), or human anti-EEA1 serum (a gift from Dr. Harald Stenmark, The Norwegian Radium Hospital, Norway) and rhodamine-labeled donkey anti-human IgG (Jackson ImmunoResearch Laboratories). The cells were mounted in Mowiol and analyzed in a Zeiss LSM 510 Meta confocal microscope (Zeiss, Jena, Germany) equipped with a Neo-Fluar 100 ×/1.45 oil immersion objective. Images were taken of thin single plane sections, and the signal intensities were quantified by using the histogram-function in the Zeiss LSM Image Browser software (Version 3).

**RESULTS**

**PKCδ Is Specifically Activated by StxB**—We have recently shown that the tyrosine kinase Syk is activated by Stx and regulates toxin uptake (12). We therefore wanted to investigate whether other key regulators of intracellular transport are activated by Stx and have a role in toxin transport. PKC isozymes are involved in sorting and intracellular transport of several receptors, and recently both PKCα and δ activity were implicated in key transport steps. First, we wanted to study whether Stx binding would activate these specific PKC isozymes. Several PKC isozymes have been detected in HeLa cells, including α, βII, δ, ε, η, and ζ (27, 28). Phosphorylation of Thr657 located at the activation loop of PKCδ is known to increase the activity of this kinase (29). As shown in Fig. 1, upon addition of StxB, PKCδ was rapidly phosphorylated at Thr657. Quantification of the amount of phosphorylated PKCδ from three independent experiments revealed that there was an increase already after 10 min, and that the peak was observed at 20 min with an increase to ~300% of unstimulated control cells. The phosphorylation was then reduced, but did not return to control levels after 60 min.3 Notably, there was a basal PKCδ phosphorylation even in unstimulated control cells (Fig. 1). The phosphorylation status of PKCα was determined in parallel. Autophosphorylation of Ser657 is a hallmark of PKCα activation (29). However, no increase in PKCα phosphorylation was detected in the lysate (Fig. 1). From these data we conclude that PKCδ is specifically activated by StxB.

**HeLa Cells Are Protected against Stx Toxicity by the PKCδ Inhibitor Rottlerin**—Knowing that Stx was able to activate PKCδ, we wanted to study whether this kinase had a role in intracellular transport of the toxin. First, the general toxicity of Stx, determined as inhibition of cellular protein synthesis, was measured in HeLa cells treated with different PKC inhibitors. Fig. 2A shows a typical toxicity experiment, and Fig. 2B illustrates the -fold protection against Stx obtained by pretreatment with the different inhibitors. Treatment with the general PKC inhibitor BLM, which inhibits both classic and novel PKC isozymes, gave a 2-fold protection against Stx, whereas treatment with rottlerin, which is specific for PKCδ, induced a 5-fold protection. In contrast, treatment with G6976, which selec-
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Stx in HeLa cells, whereas PKCα activity did not seem to be involved in this process.

The Endocytic Uptake of Stx Is Slightly Reduced by Rottlerin—To intoxicate cells, Stx has to be transported all the way from the plasma membrane, via EE to the Golgi, and further to the endoplasmic reticulum before translocation to the cytosol. To investigate which of the one or more steps in this intracellular transport might be regulated by PKCδ activity and lead to the protection observed in Fig. 2. First, we measured the very first step in the process, the endocytic uptake of Stx. HeLa cells were pretreated with the different PKC inhibitors used above, and the internalization of labeled Stx was measured after 15 min of incubation. As shown in Fig. 3A, although G66976 did not affect Stx endocytosis, both BIM and rottlerin gave a small, but significant reduction in toxin uptake after 15 min, indicating that PKCδ activity is partially involved in the initial uptake of Stx. The endocytic rates of Stx in control and rottlerin-treated cells are compared in Fig. 3B. Rottlerin did not seem to increase the recycling of Stx, because an increased recycling would lead to a reduction in the amount of internalized Stx over time. In contrast, the reduction in endocytosis upon PKCδ inhibition was observed already from early time points, and it was constant over time (Fig. 3B). However, it is unlikely that the small reduction in Stx uptake would lead to the 5-fold protection against Stx observed in rottlerin-treated cells, and we therefore went on to investigate whether the next step in the internalization process of Stx, the endosome-to-Golgi transport, would also be reduced by rottlerin.

The Sulfation of StxB Is Strongly Inhibited by Rottlerin—To quantitate the transport of toxin molecules to the TGN biochemically, we employed StxB molecules with tandem sulfation sites recombinantly added to the C terminus. These sites are sulfated by a sulfotransferase upon arrival to the TGN (31, 32).
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FIGURE 4. Sulfation of StxB in cells treated with different PKC inhibitors. After 2.5 h of starvation in sulfate-free medium containing 0.2 mcCi/ml of Na$_2$SO$_4$, HeLa cells were preincubated with rottlerin (1–2.5 μM), BIM (10 μM), or Go6976 (50 nM or 1 μM) in sulfate-free medium for 30 min, then StxB-sulf$_2$ (5 μg/ml) was added, and the incubation continued for 45 min, still in the presence of the inhibitors. The cells were lysed, StxB was immunoprecipitated from the lysate, and the sulfated StxB was visualized by SDS-PAGE and autoradiography. In the upper panel, a typical autoradiogram is shown for cells treated with rottlerin (2.5 μM), BIM (10 μM), and Go6976 (50 nM). In the lower panel data from three to seven independent experiments were pooled. The autoradiograms were quantified by densitometry, and the band intensity of sulfated StxB in untreated control cells was normalized to 100%, and the amount of sulfated StxB was calculated as percentage of control for each condition. The data shown are mean values ± S.D. Statistical significance of difference between control and rottlerin (2.5 μM) treated with rottlerin resulted in a marked, concentration-dependent inhibition of StxB sulfation, whereas treatment with Go6976 (50 nM) or BIM (10 μM) did not show any significant effect (Fig. 4).

Sulfation of StxB Is Inhibited by siRNA-induced Knockdown of PKCα—To verify that PKCα is involved in endosome-to-Golgi transport of StxB, the inhibition of this step is large enough to account for the protection against Stx in rottlerin-treated cells (Fig. 2). Again, PKCα activity did not seem to be involved. The possibility existed that pretreatment with the PKC inhibitors would reduce the general level of protein sulfation in the cells, and as described under “Experimental Procedures,” we routinely measured the total amount of sulfated proteins and found it unchanged under the different conditions presented herein.

To investigate whether the StxB transport was sensitive to rottlerin also in other cell types, StxB sulfation was measured both in Vero and HEp-2 cells. In the two cell types the StxB sulfation was reduced to 30.0 ± 4.4% and 16.0 ± 7.9% of untreated control, respectively (mean ± S.D., n = 3 independent experiments) upon rottlerin treatment (5 μM) without affecting the total sulfation of proteins. Clearly, the involvement of PKCα in StxB sulfation is not restricted to HeLa cells.

Sulfation of StxB Is Inhibited by siRNA-induced Knockdown of PKCα—To verify that PKCα is involved in endosome-to-Golgi transport of Stx, HeLa cells were depleted for PKCα by transfection with three different siRNA oligonucleotides complementary to non-overlapping regions of PKCα mRNA. The effect of the siRNAs was tested by immunoblotting, and as shown in Fig. 5A, 48 h after transfection of HeLa cells with siRNA 1, the PKCα protein level was strongly reduced (down to 37% of control-transfected cells) already at 25 nM siRNA oligonucleotide. The protein level was further reduced in a concentration-dependent manner until it was virtually knocked out at 100 nM siRNA 1 (down to 16% of control-transfected cells). The blot was stripped and reprobed with anti-α-tubulin to show equal loading. Then StxB sulfation was determined in the siRNA-transfected cells. As shown in Fig. 5B for one typical experiment and in Fig. 5C from several pooled experiments, upon transfection with siRNA 1, the sulfation of StxB was strongly reduced already at 25 nM and was further reduced in a concentration-dependent manner at 50 and 100 nM. Thus, there seemed to be a strong correlation between the PKCα protein level and the extent of StxB sulfation. Transfection with two other siRNAs against PKCα, siRNA 2 and 3, reduced the protein level of PKCα to ~45% of that in control-transfected cells (Fig. 5D), and in these cells the StxB sulfation was reduced to ~65% of the control (Fig. 5E).

To investigate whether PKCα activity is a general regulator of retrograde transport routes or whether such activity might be specific for the pathway used by StxB, we compared the endosome-to-Golgi transport of StxB with that of ricin in cells treated with rottlerin or PKCα siRNA. The sulfation of the two toxins was measured in parallel. As shown in supplemental Fig. S1A, although the StxB sulfation was inhibited in cells treated with rottlerin, the ricin sulfation was not reduced. In most experiments the ricin sulfation was actually slightly increased. The same result was obtained when the ricin sulfation was determined after 45 min of incubation, although the band intensity was lower. Moreover, as shown in supplemental Fig. S1B, although transfection with PKCα siRNA 1 strongly reduced the StxB sulfation, again the ricin sulfation was slightly increased. In addition, the small inhibitory effect that rottlerin seemed to exert on the initial uptake of Stx seems to be specific to this toxin. Rottlerin treatment reduced neither the general clathrin-mediated endocytosis, as measured by uptake of $^{125}$I-labeled transferrin (113 ± 13% of control, n = 4 independent experiments) nor the uptake of $^{125}$I-ricin (101 ± 10% of control, n = 5 independent experiments). Taken together, the retrograde transport of Stx and ricin seem to be differentially regulated with respect to PKCα.

To demonstrate that rottlerin is specific for PKCα in our system, we performed sulfation experiments with the combined treatments of rottlerin and PKCα siRNA. We reasoned that, if the inhibition of StxB sulfation was not increased in the presence of both treatments compared with the inhibition observed with one treatment alone, rottlerin acts only on PKCα. In other words, the removal of the drug’s target should abolish the effect of the drug. As shown in Fig. 5 (F and G), no significant additive effect was observed in cells treated with both rottlerin and PKCα siRNA compared with both treatments alone. This confirms that rottlerin is specific for PKCα in our assay.

The possibility existed that the strong reduction in StxB sulfation we observed upon transfection with the PKCα-specific siRNAs could be due to reduced binding and/or uptake of the toxin. To exclude this, PKCα siRNA 1–3 were tested in endocytosis-experiments. The transfection process itself did not
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The amount of sulfated StxB in control-transfected cells was normalized to 100%, and the amount of sulfated StxB in cells transfected with nonspecific siRNA was normalized to 100%, and the amount of sulfated StxB after knockdown of PKCδ-specific siRNA 1 (100 nM, 3 days) was calculated as percentage of control-transfected cells for each of the three concentrations used. The data shown are mean ± S.D. of three independent experiments.

Knockdown of PKCδ depletion, 3 indicating that the reduction in sulfation was caused by reduced endocytic uptake of the toxin but rather a specific inhibition of the endosome-to-Golgi transport step. The amount of cell-associated StxB in PKCδ siRNA-transfected cells was further investigated by measuring the cell-associated 125I-StxB after 45 min at 37 °C. The amount of 125I-StxB was only slightly reduced upon PKCδ depletion, 3 indicating that the reduction in sulfation was not associated with an increased degradation of StxB.

StxB Sulfation Is Unchanged by Knockdown of PKCα by siRNA Oligonucleotides—Data from StxB sulfation experiments upon treatment with Go6976 (Fig. 4) suggested that PKCα was not involved in endosome-to-Golgi transport of StxB. We wanted to confirm this by depletion of PKCα by two different
siRNA oligonucleotides complementary to non-overlapping regions of PKCδ mRNA. As shown by immunoblotting in Fig. 6A, the two siRNAs virtually knocked out the PKCα protein at 50 nM 48 h after transfection. However, as shown for one typical experiment in Fig. 6B and from several pooled experiments in Fig. 6C, the StxB sulfation proceeded as normal despite the knockdown of the PKCα protein, which is in accordance with the inhibitor data.

StxB Accumulates in Endosomes and Does Not Colocalize with Giantin upon Treatment with Rottlerin or PKCδ-specific siRNA—From the biochemical data, PKCδ, and not PKCα, seemed to be involved in the endosome-to-Golgi transport of StxB, and we wanted to confirm these data by another approach. To this end HeLa cells were either pre-treated with rottlerin to inactivate PKCδ, or the cells were depleted of PKCδ by siRNA, and the transport of StxB was monitored by confocal microscopy. As shown in Fig. 7, both rottlerin treatment (B) and transfection with the PKCδ-specific siRNA 1 (D) resulted in an almost complete loss of colocalization between StxB and the Golgi-marker giantin, compared with the extensive colocalization of these markers in both untreated control cells (A) and cells transfected with control siRNA (C). In the rottlerin-treated or siRNA 1-transfected cells StxB showed a punctate cytosolic staining, which was presumably labeling of endosomal structures. In the PKCδ-depleted cells the StxB labeling was weaker, and many of the StxB-positive structures were actually smaller than in the rottlerin-treated cells. The colocalization between StxB and giantin was quantified in each of the four conditions, and calculated as a percentage of total giantin staining. Giantin was chosen as the reference, because the staining pattern was equal under all conditions. As shown in Fig. 7 (right panels) both rottlerin and siRNA1 transfection reduced the colocalization with giantin strongly, indicating that StxB was unable to reach the Golgi under these conditions. Similar phenotypes were obtained by the PKCδ siRNA 2 and 3 (Fig. 7, F and G), although under these conditions the phenotype was less obvious, in accordance with the partial knockdown of the PKCδ protein using these siRNAs (see Fig. 5D). Inhibition or depletion of PKCδ did not seem to increase the recycling of StxB, as there was no detectable increase in StxB staining on the cell surface of treated cells compared with controls (Fig. 7, B and D).

To investigate whether the Golgi transport of StxB and the Shiga holotoxin was affected in the same way by depletion of PKCδ, HeLa cells transfected with PKCδ siRNA 1 or 2 were treated with Stx and processed for immunofluorescence as for StxB. In control-transfected cells Stx showed a dense, perinuclear staining identical to that observed for StxB (Fig. 7, compare C and H), and in the PKCδ siRNA-transfected cells the staining pattern of Stx was equal to that of StxB, a punctate cytosolic distribution (Fig. 7, compare D–F with I–J).

To verify the lack of effect on Golgi transport by PKCα depletion (the sulfation data), cells transfected with PKCα siRNA was also monitored by immunofluorescence. As shown in Fig. 8 (A and B), transfection with PKCα siRNA 1 did not lead to an altered localization of StxB compared with control-transfected cells. Similar results were obtained with the other PKCα siRNA. This is in accordance with the sulfation data and supports the conclusion that PKCα is not involved in the endosome-to-Golgi transport of StxB.

The structure of the Golgi itself, as judged by the giantin staining, did not seem to be affected by rottlerin or the lack of PKCδ. However, the TGN46 staining was slightly redistributed under these conditions. A partial redistribution of TGN38/46 to endosomes has previously been shown in cells where endosome-to-Golgi transport is strongly inhibited, a situation that would also inhibit the retrieval of Golgi proteins (33, 34). Note that transfection with PKCα siRNA, which had no effect on StxB sulfation (Fig. 6), did not induce a redistribution of TGN46 (Fig. 8B).

In an attempt to identify the cytosolic structures in which the StxB accumulated upon PKCδ inhibition, control- and rottlerin-treated cells were labeled for the EE-marker EEA1 after internalization of StxB. As shown in Fig. 8C, the control cells revealed as expected, a dense, perinuclear staining of StxB and hardly any colocalization with the punctate EEA1 staining. Upon rottlerin treatment the StxB staining was punctate and no longer perinuclear, and in addition, there was a higher degree of colocalization between StxB and EEA1 (Fig. 8D). The colocalization of StxB and EEA1 was quantified and presented as the percentage of total EEA1 staining (Fig. 8, right panel). The
FIGURE 7. Localization of StxB or Stx in cells treated with rottlerin or transfected with PKCδ siRNA. A and B, after 30 min of preincubation with (B) or without (A) rottlerin (2.5 μM), HeLa cells were incubated with StxB (2 μg/ml) for 10 min at 37 °C. Then the cells were washed and chased for 20 min at 37 °C in fresh medium with or without rottlerin. The cells were fixed and labeled with antibodies against StxB (green) and giantin (red) as described under “Experimental Procedures.” The right panel shows quantification of the extent of colocalization between StxB and giantin (as the percentage of total giantin staining) in cells incubated with or without rottlerin. Data were pooled from three independent experiments and show mean values ± S.D. (control, n = 67; rottlerin, n = 57). C and D, HeLa cells were transfected with nonspecific siRNA (C) or PKCδ siRNA 1 (D) (50 nM, 48 h) and then incubated with StxB (2 μg/ml) for 10 min, washed, and chased for 20 min. The cells were fixed and processed for immunofluorescence as described above. The right panel shows quantification of the extent of colocalization between StxB and giantin (as the percentage of total giantin staining) in control and PKCδ siRNA 1-transfected cells. Data were pooled from three independent experiments and show mean values ± S.D. (nonspecific siRNA, n = 23; PKCδ siRNA 1, n = 17). E–G, HeLa cells were transfected with nonspecific siRNA (E), PKCδ siRNA 2 (F), or PKCδ siRNA 3 (G) (100 nM, 72 h) and then incubated with StxB exactly as described for C and D. The cells were fixed and labeled with antibodies against StxB (green) and giantin (red) as described under “Experimental Procedures.” The images are representative of data from three independent experiments. Bar, 20 μm.
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 FIGURE 8. Localization of StxB in cells treated with rottlerin or transfected with PKCδ siRNA. A and B, HeLa cells were transfected with nonspecific siRNA (A) or PKCα siRNA 1 (B) (50 nM, 48 h) and then incubated with StxB as described in Fig. 7C. The cells were fixed and labeled with antibodies against StxB (green) and TGN46 (red) as described under “Experimental Procedures.” C and D, HeLa cells were preincubated with rottlerin (2.5 μM) and incubated with StxB as described in Fig. 7A. The cells were fixed and labeled with antibodies against StxB (green) and EEA1 (red) as described under “Experimental Procedures.” The right panel shows quantification of the extent of colocalization between StxB and EEA1 (as the percentage of total EEA1 staining) in cells incubated with or without rottlerin. Data were pooled from three independent experiments and show mean values ± S.D. (control, n = 16; rottlerin, n = 23). Bar, 20 μm.

It was demonstrated that the activity of the Golgi-resident enzyme sulfotransferase was not regulated by PKCδ. Furthermore, inhibition or depletion of PKCδ did not seem to affect degradation of Stx or recycling to the plasma membrane. Under control conditions both the degradation and the recycling of Stx have been shown to be marginal in HeLa cells (39), and neither of the processes seemed to be changed by the inhibition or depletion of PKCδ.3

Stx has been shown to activate several kinases leading to apoptosis in different cell types (reviewed in Ref. 8). We have recently demonstrated that Stx-induced signaling also stimulates its entry (12). Here we report that Stx specifically activated PKCδ, and the same kinase was found to regulate the endosome-to-Golgi transport of the toxin. In contrast, PKCα was neither activated nor involved in toxin to the plasma membrane. This indicates that Stx is able to increase its own retrograde transport by specific activation of PKCδ. However, it cannot be excluded that the basal activity of PKCδ in HeLa cells (see Fig. 1) is sufficient to mediate the retrograde transport of Stx. It should also be noted that a potential role of the atypical PKC isoforms in Stx transport has not been determined by the treatments employed in this study.

PKCδ has many potential targets at different cellular locations. The kinase might act directly on a key regulator of Stx transport, or alternatively, components important for endosome-to-Golgi transport of Stx might be relocated due to PKCδ inhibition. PKC isoforms are known to translocate to the plasma membrane upon activation (18). In this study, although only a fraction of the initial Stx uptake seemed to be dependent on PKCδ, active translocated PKCδ might be important for recruitment of specific proteins or lipids to the area of Stx internalization. This microenvironment could determine the destination of Stx-containing vesicles. Correct retrograde targeting of both Stx and cholera toxin (CT) have been shown to require localization of these toxins to lipid rafts (39–41), and the fraction of Gb3 that is raft-associated was recently shown to increase upon Stx binding (40). Interestingly, activated PKCα and -δ have been shown to translocate to plasma membrane rafts upon initiation of an immunoreceptor signaling cascade activated by cross-linking of FcαR (21). The signaling was shown to be required for correct trafficking of FcαR vesicles. Moreover, retrograde transport of CT has been shown to require an association between CT-GM1 and actin mediated via lipid rafts (42). The link between CT in the outer leaflet of the plasma membrane with actin...
StxB, despite the fact that depolymerization of actin by cytochalasins was reported to have little effect on this step (51). Calmodulin does not seem to be involved in the endosome-to-Golgi transport of StxB, however, any potential cross-talk between PKC and MARCKS, actin, or calmodulin at the plasma membrane that could regulate the subsequent sorting of StxB in endosomes cannot be excluded.

Stx stimulation has been shown to induce microtubuli assembly both in ACHN cells (13) and in Vero cells (5). In Vero cells, Stx was suggested to stimulate its own trafficking, based on the observed Stx-induced assembly of microtubules and the requirements of both microtubules and dynemin in the Golgi transport of Stx (5). Notably, it was shown that the Stx-induced signaling activating microtubuli assembly was not mediated via Syk, suggesting that multiple signaling pathways are induced by Stx. Whether PKCζ activity is involved in the increased assembly of microtubuli is unknown.

PKCζ might regulate the exit of Stx from EEs into the retrograde pathway by affecting target proteins on the EE. The retromer complex has been detected on EE and is implicated in exit of mannose 6-phosphate receptors from EEs into the retrograde pathway (52). The yeast homolog of the retromer complex sorting nexin 1 and 2, Vps5p, is a phosphoprotein (53). Therefore it might be speculated that sorting nexin 1 and/or 2 are potential candidates for PKCζ-mediated regulation of Stx transport. Moreover, a proposed complex of the sorting nexins 4, 41, and 42 is shown to mediate retrieval of the v-SNARE Snc1p from endosomes to the Golgi complex in yeast (54). A role for this complex in Stx transport awaits determination.

PKCζ may also affect Stx transport at the Golgi level. The SNARE proteins are regulators of vesicle biology and known PKC substrates (55, 56). The t-SNAREs involved in StxB transport are mostly localized to the TGN or Golgi, and PKCζ has been shown to translocate to the Golgi under certain conditions (57, 58). The endosome-to-Golgi transport of StxB and TGN46 has been shown to implicate Syntaxin 6 and Syntaxin 16 (59). Syntaxin 16 is a phosphoprotein, thus, PKCζ could regulate the endosome-to-Golgi transport of StxB by acting at the SNARE complex. Sed5, the yeast homolog of Syntaxin 5, is also shown to be a phosphoprotein. However, only a conserved PKA phosphorylation site was described (60). Interestingly, Syntaxin 5 was reported to regulate the retrograde transport of StxB in a pathway parallel to the Syntaxin 16-regulated route (61).

In conclusion, PKCζ is specifically activated by Stx and regulates the endosome-to-Golgi transport of the toxin. Clearly, future studies are required to fully understand the mechanisms of the different intracellular transport routes followed by Stx and to further elucidate the toxin’s own role in cellular entry and transport.

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