Protein kinase C bound to the Golgi apparatus supports the formation of constitutive transport vesicles

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Constitutive secretion of heparan sulphate proteoglycans (HSPGs) was stimulated in human hepatoma HepG2 cells by phorbol 12-myristate 13-acetate (PMA) and inhibited by calphostin C, a specific inhibitor of protein kinase C (PKC). To delineate more closely the site of PKC action, the packaging *in vitro* of $^{35}SO_4$-labelled HSPGs into transport vesicles was investigated. Formation of transport vesicles at the trans-Golgi network was stimulated by PMA and inhibited by calphostin C or Ro 31-8220 by using a post-nuclear supernatant. Treatment of either isolated Golgi-enriched membranes or cytosolic proteins with calphostin C provided evidence that membrane-bound PKC forms strongly supported vesicle formation, whereas cytosolic PKC forms showed a marginal effect. The PKC isoforms PKC-α and PKC-ζ were attached to highly purified Golgi membranes, as shown by Western blotting. Both isoforms were localized by confocal immunofluorescence microscopy in the Golgi area of HepG2 cells. Immunoelectron microscopy of ultrathin cryosections of HepG2 cells showed that PKC-ζ predominantly attaches to the trans-Golgi region, whereas PKC-α binds to the cis- and trans-Golgi area.

INTRODUCTION

Protein kinase C (PKC) isoforms are a class of ubiquitous, phospholipid-dependent, serine/threonine kinases that play a pivotal role in cellular signal transduction. Besides an involvement in mitotic signalling and numerous other cellular functions, activation of PKC results in the secretion of hormones and neurotransmitters by the regulated secretory pathway. Several lines of evidence support this conclusion. First, receptor-mediated activation of phospholipase C, which activates PKC, stimulates secretion in COS-7 and GH3 cells [1,2]. Secondly, direct stimulation of PKC by phorbol 12-myristate 13-acetate (PMA) induces exocytosis in rat basophilic cells [3]. Thirdly, secretion of permeabilized RBL cells [4] or PC12 cells [5] is restored by the addition of purified PKC-β or PKC-δ. Fourthly, overexpression of PKC-ϵ stimulates prolactin secretion by rat pituitary GH4C1 cells [6]. Lastly, down-regulation of PKC by prolonged treatment with PMA decreases secretion by RBL-2H3 cells [3,4]. Indirect evidence supports the notion that PKC might also modulate constitutive secretion. ADP-ribosylation factor, a small GTP-binding protein essential for the formation of both constitutive and regulated transport vesicles [7,8], becomes bound to Golgi membranes on stimulation by PMA in RBL cells [9]. Secretion of glycosaminoglycans by RBL and MDCK cells is stimulated by phorbol ester [9,10]. Moreover, transport of vesicular stomatitis virus (VSV) G protein from the trans-Golgi network (TGN) to the cell membrane is stimulated by PMA [10] and inhibited by calphostin C [11] or Ro 31-8220 [10].

To study the role of PKC in constitutive secretion, human hepatoma HepG2 cells were chosen that predominantly transport proteins via the constitutive secretory pathway. Extracts from HepG2 cells are able to package sulphate-labelled heparan sulphate proteoglycan (HSPG) efficiently into constitutive post-Golgi vesicles in a cell-free system adapted from [12]. Here we show that PKC activity is essential for the formation of constitutive transport vesicle at the TGN. PKC-α and PKC-ζ are both bound to the TGN and might contribute to the formation of constitutive transport vesicles.

EXPERIMENTAL

Materials

A monoclonal antibody against PKC-ζ, as well as antibodies against rabbit IgG labelled with fluorescein isothiocyanate (FITC) or against mouse IgG labelled with Cyanine 3 (Cy3), were obtained from Dianova (Hamburg, Germany). A monoclonal antibody specific for PKC-α was obtained from UBI (Lake Placid, NY, U.S.A.), and the antibody recognizing cytochrome *c* reductase was from Amersham Buchler. Antibodies specific for Rab5 and TGN-38 were gifts from Dr. Marino Zerial (EMBL, Heidelberg, Germany) and Dr. G. Banting (University of Bristol, Bristol, U.K.), respectively. An antibody recognizing the C-terminal peptide of TGN38 (FALEGKRSKVTRRPK-ASDYQRLNLKL) was raised in rabbits by immunization with the peptide conjugated with keyhole limpet hemocyanin and purified as described [13]. Anti-mouse IgG-specific antibodies coupled to 12 nm gold particles were from Dianova (Hamburg, Germany), and Protein A bound to 5 nm gold particles was from Utrecht University (School of Medicine, Department of Cell Biology, Utrecht, The Netherlands). PMA, Ro 31-8220 and calphostin C were from Calbiochem (Neu Isenburg, Germany). Pefabloc in combination with a set of protease inhibitors was used as recommended by the supplier (Boehringer Mannheim, Mannheim, Germany).

Cell culture and metabolic labelling

HepG2 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) [14] with glutamine, 10% (v/v) fetal calf serum and 10% (v/v) horse serum in cell culture dishes coated with collagen S (Boehringer Mannheim). For immuno-

Abbreviations used: Cy3, Cyanine 3; DMEM, Dulbecco's modified Eagle's medium; DMF, dimethylformamide; FITC, fluorescein isothiocyanate; HSPG, heparan sulphate proteoglycan; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PNS, post-nuclear supernatant; TGN, trans-Golgi network; VSV, vesicular stomatitis virus.

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fluorescence studies, cells were grown in chamber slides (Nunc, Naperville, IL, U.S.A.). HSPGs were labelled by incubating nearly confluent HepG2 cells in sulphate-free minimal essential medium with 10 % (v/v) fetal calf serum for 20 min at 37 °C, followed by a 5 min incubation in 4 ml of fresh sulphate-free minimal essential medium containing 1 mCi of carrier-free $^{35}SO_4$ (Du Pont de Nemours, Bad Homburg, Germany). Thereafter, cells were processed as described below.

Secretion of HSPGs by HepG2 cells

HepG2 cells were labelled as above with 0.1 mCi of carrier-free $^{35}SO_4$ and rinsed once with DMEM containing 4 mM sodium sulphate. Secretion of labelled sulphated HSPGs was measured during three consecutive incubations, each for 15 min in 3 ml of fresh DMEM at 37 °C. Cellular PKC was stimulated by adding 100 nM PMA to the medium. To inhibit PKC, cells were treated after labelling with 4 μM calphostin C and irradiated with light (300–400 nm) for 5 min at 4 °C. Controls were treated with the corresponding concentration of DMSO. Medium withdrawn after secretion intervals of 15 min was centrifuged for 10 min at 1000 ***g*** to remove cells. This and all subsequent centrifugation procedures were carried out at 4 °C. Secreted HSPGs were then precipitated with cetylpyridinium chloride as described [15] and collected on nitrocellulose (Hybond, Amersham). Radioactivity was counted in a toluene-based scintillator.

Isolation of Golgi fractions

Preparation of post-nuclear supernatant (PNS) was adapted as described elsewhere [12]. Briefly, cells from two 150 mm dishes were rinsed with ice-cold buffer A [10 mM Hepes/KOH (pH 7.4)/1 mM $MgCl_2$/1 mM EDTA] containing 0.25 M sucrose and scraped with a rubber 'policeman' into 0.5 ml of buffer A. After the addition of protease inhibitors, cell suspensions were passed six times through a cell cracker (EMBL Workshop, Heidelberg, Germany) with a clearance of 18 μm. Cell debris and nuclei were pelleted at 1000 ***g*** for 10 min and the PNS was saved. To isolate Golgi-enriched membranes, PNS was underlaid with 0.8 and 1.1 M sucrose in buffer A. Step gradients were spun for 30 min at 30000 rev./min (100000 ***g***; SW60 rotor, Beckman Instruments) at 4 °C. Golgi membranes were collected from the 0.8/1.1 M sucrose interface, diluted with buffer A containing 0.25 M sucrose and pelleted for 15 min at 15000 ***g***. Highly purified Golgi membranes were obtained by flotation of Golgi-enriched membranes through 1.18 M sucrose as described [16]. Galactosyltransferase [17] and sialyltransferase [18] were assayed as described and protein concentrations were determined as described in [19]. Specific galactosyltransferase activity was increased 20–25-fold after gradient centrifugation of the PNS, and 80–100-fold after flotation of Golgi-enriched membranes in comparison with the PNS. The absence of contaminating endoplasmic reticulum, endosomes or plasma membrane was proved by Western blotting with antibodies against cytochrome *c* reductase, Rab5 or transferrin receptor respectively (results not shown).

Determination of PKC activity

Aliquots containing 5 μg of membrane protein were assayed for H1 histone kinase activity in a 100 μl assay containing 40 μg of H1 histone (Sigma, Deidenhofen, Germany) or 4 μg of PKC-α-pseudosubstrate-derived peptide (RFARLGSLRQKNV; Peninsula), 5 μg of creatine phosphokinase and 1.0 μCi of [γ-^{32}P]ATP (NEN) in buffer C [20 mM Tris/HCl (pH 7.5)/10 mM magnesium acetate/1 mM $CaCl_2$/10 μM ATP/10 mM creatine phosphate/10 mM KF/100 nM ammonium molybdate/100 nM nitrophenyl phosphate/250 mM sucrose]. PMA (150 nM), EGTA (1 mM) or calphostin C (1 μM) was added as indicated. Assays containing calphostin C were exposed to light (300–400 nm) while being kept on ice for 5 min before incubation [20]. Assays were incubated at 37 °C for 10 min and stopped by the addition of 0.5 ml of ice-cold 10 % (w/v) trichloroacetic acid. Precipitated proteins were collected on glass-fibre filters type GFA (Macherey and Nagel) and dried; the radioactivity was counted in a toluene-based scintillation mixture.

Cell-free formation of constitutive secretory vesicles

Method A

Post-Golgi vesicle formation was assayed in a PNS obtained from $^{35}SO_4$-labelled HepG2 cells, essentially as described in [12]. After incubation of PNS, containing 10^5 d.p.m. of carbohydrate-bound sulphate, with an ATP-regenerating system and GTP for 30 min at 37 °C, Golgi membranes were pelleted for 15 min at 14000 ***g*** and vesicles were sedimented for 20 min at 100000 ***g***. Pellets were solubilized in buffer A containing 1 % Triton X-100, and HSPGs were precipitated by the addition of cetylpyridinium chloride and chondroitin sulphate as carrier, as described in [15].

Method B

Vesicle formation *in vitro* was performed as decribed in [21]. Briefly, Golgi membranes (0.1 mg of protein) containing $^{35}SO_4$-labelled HSPGs (10^5 d.p.m.) and 1.0 mg of unlabelled cytosolic proteins, prepared by centrifugation of PNS for 30 min at 100000 ***g***, were assayed in 250 μl of buffer A containing 0.25 M sucrose supplemented with 0.2 mM $MgCl_2$, 1 mM ATP, 0.1 mM GTP, 1 mM creatine phosphate and 1.25 μg of creatine phosphokinase. After incubation for 30 min at 37 °C, Golgi membranes and vesicles were isolated and the labelling of HSPGs was determined as above. Alternatively, proteins of sedimented Golgi or vesicle fractions were separated by SDS gel electrophoresis and labelled HSPGs were determined by autoradiography and scanning with a Phosphorimager (Raytest, Sprockhavel, Germany).

Immunofluorescence microscopy

HepG2 cells were rinsed with PBS [100 mM sodium chloride/20 mM sodium phosphate (pH 7.2)] and then fixed with 3 % (w/v) paraformaldehyde in 0.1 M sodium phosphate (pH 7.0) for 30 min. Specimens were quenched with 0.1 M Tris/glycine buffer, pH 7.4, and blocked with 1 mg/ml BSA in PBS. Slides were incubated with a monoclonal antibody specific for PKC-α (1:100) or PKC-ζ (1:100), followed by three 5 min washes with PBS and incubation with Cy3-conjugated anti-(mouse IgG) antibody (1:100). After being washed with PBS, the samples were incubated with the peptide-specific TGN38 antibody (1:200) and stained with anti-(rabbit IgG) labelled with FITC (1:800). Fluorescence was detected with either a conventional fluorescence microscope (Axioplan, Carl Zeiss, Oberkochen, Germany) or a confocal laser scan microscope (Nikon Diaphot, Bio-Rad MRC600) operating with an argon laser. Omission of antibodies specific for PKC isoforms or the use of preimmune sera in the case of TGN38 completely abolished fluorescent staining (results not shown).

Table 1 Secretion of HSPG by HepG2 cells

Cells labelled with $^{35}SO_4$ were incubated in DMEM. The medium was replaced after 15, 30 or 45 min and secreted HSPGs were precipitated with cetylpyridinium chloride [15]. Results are shown as means ± S.E.M. for five independent experiments.

	10^{-3} × Secretion of HSPG (d.p.m.)		
Time after labelling of cells ...	0–15 min	15–30 min	30–45 min
Control HepG2 cells	81 ± 11	219 ± 18	98 ± 16
HepG2 cells treated with 100 nM PMA during incubation	103 ± 14	274 ± 21	151 ± 14
HepG2 cells treated with calphostin before incubation	32 ± 6	75 ± 13	39 ± 9

Table 2 PKC activity of Golgi-enriched fraction

Golgi membranes purified as described were assayed for kinase activity by incubation with [γ-^{32}P]ATP and H1 histone. Experimental details are given in the Experimental section. To compare individual experiments, the incorporation of $^{32}PO_4$ under control conditions was normalized to 100. Results are shown as means ± S.E.M. for five independent experiments.

	Kinase assay additions			
Golgi prepared from	None	100 nM PMA	1mM EGTA	1 μM calphostin C
Control HepG2 cells	100 ± 12	113 ± 15	48.6 ± 7.5	13.0 ± 4.5
HepG2 cells treated for 10 min with PMA	129 ± 15	148 ± 22	71 ± 9	20.0 ± 5.2
HepG2 cells treated for 16 h with PMA	161 ± 13.5	254 ± 21	157 ± 16.5	23.1 ± 2.2

Immunoelectron microscopy

Cryosections of HepG2 cells were prepared as described [22], with some modifications. Cells were resuspended and fixed for 2 h on ice in 4% (w/v) formaldehyde/0.25% (w/v) glutaraldehyde/0.18 M sucrose/100 mM sodium phosphate (pH 7.4), washed four times with sucrose–phosphate buffer, pelleted and equilibrated for 6 h with 1.8 M sucrose in 20% (w/v) polyvinylpyrrolidone [23]. Cells that had been frozen in liquid nitrogen were sectioned with a Reichert FC4E cryo-ultramicrotome at −110 °C. Ultrathin sections were collected on carbonized Formvar-coated copper/palladium grids, quenched in 1% (w/v) BSA/0.12% glycine in PBS. Sections were incubated in the following order: first, anti-TGN38 antibodies; secondly, 5 nm Protein A–gold particles; thirdly, PKC-ζ specific monoclonal antibody; fourthly, anti-(mouse IgG) coupled to 12 nm gold particles. Incubations were done in PBS containing 1% (w/v) BSA, followed by five wash steps on every occasion. Finally, after being rinsed with water four times, sections were stained with 0.3% tungstosilicic acid in 2.8% (w/v) polyvinyl alcohol for 10 min [24] and examined using a Zeiss 900 microscope. Background labelling in the absence of the monoclonal anti-PKC antibodies or TGN38 antibodies was extremely low and was therefore not taken into consideration.

Protein electrophoresis and Western blotting

Pelleted Golgi fractions (30 μg of protein in each) were dissolved in SDS sample buffer for 15 min at 40 °C and proteins were separated on a 10% (w/v) polyacrylamide gel [25]. Proteins were blotted on Hybond nitrocellulose membranes (Amersham Buchler) at 10 V for 90 min with a semi-dry blotter (Phase, Braunschweig, Germany). Blots were blocked with 5% (w/v) skimmed milk overnight and probed with antibodies for 2 h. Antibodies recognizing PKC-α, PKC-ζ, Rab5 or transferrin receptor were incubated with anti-(mouse IgG)–peroxidase conjugate and blots were stained with Fast stain (Sigma). Antibodies specific for cytochrome *c* reductase were probed with anti-(rabbit IgG)–peroxidase conjugate (Boehringer Mannheim).

RESULTS

Constitutive secretion of [^{35}S]sulphate-labelled proteins depends on PKC

HSPGs in the TGN were labelled by a brief incubation of cells in the presence of $^{35}SO_4$ [26]. Cells were then chased in the presence of unlabelled sulphate after the addition of 100 nM PMA or DMSO as a control. Labelled proteins transported to the cell surface and secreted during chasing were analysed either by gel electrophoresis (results not shown) or by precipitation of labelled HSPGs with cetylpyridinium chloride (Table 1). Both assays gave consistent results. The addition of PMA stimulated the secretion of sulphated proteins, most clearly between 15 and 45 min after labelling. Secretion during the first time interval was not stimulated significantly, indicating a time lag between the addition of PMA and the onset of increased secretion. Inhibition of PKC by treating cells with calphostin C strongly decreased secretion. To delineate the PKC-dependent transport step(s) more clearly, cell extracts were prepared and vesicle formation at the TGN was assayed *in vitro* as described below.

Isolation of Golgi-enriched membrane fractions from HepG2 cells

Centrifugation of a PNS obtained from HepG2 cells through a step gradient of 0.8 and 1.1 M sucrose in buffer A resulted in a Golgi-enriched membrane fraction. Activities of galactosyltransferase and sialyltransferase, which were assayed as Golgi markers, increased by 20–25-fold and 10–12-fold respectively in comparison with the activities in PNS. Additional flotation of Golgi-enriched membranes through 1.18 M sucrose [16] resulted in a highly enriched Golgi fraction showing galactosyltransferase

Table 3 Effects of PMA or PKC inhibitors on packaging of ^{35}S-labelled HSPG into constitutive transport vesicles *in vitro*

Budding assays were performed *in vitro* with PNS of HepG2 cells as described in the Experimental section. Budding efficiencies shown in rows 2–5 and 9–12 are represented as ratios between labelling of vesicles formed *in vitro* with PNS and additions as indicated, and labelling obtained with a control assay (row 1). Budding efficiencies in rows 6–8 are calculated as ratios between vesicle labelling in the presence and in the absence of Ro 311-8220, both at the indicated ATP concentrations. Experiments with different PNS preparations varied in budding efficiencies; between 15 and 20% of total labelled HSPG was packaged into vesicles under standard conditions. To compare different experiments, ratios of budding efficiencies, obtained with and without additions, were calculated for each experiment; ratios are shown as means ± S.E.M for four independent experiments.

PNS isolated from	ATP concentration (mM)	Additions	Budding efficiency
Control HepG2 cells	1.0	–	1.00
	1.0	100 nM PMA	1.24 ± 0.12
	1.0	100 nM PMA + 1 μM calphostin C	0.60 ± 0.072
	1.0	1 μM calphostin C	0.51 ± 0.044
	1.0	10 μM Ro 31-8220	0.90 ± 0.058
	0.1	10 μM Ro 31-8220	0.86 ± 0.062
	0.05	10 μM Ro 31-8220	0.70 ± 0.15
	0.02	10 μM Ro 31-8220	0.68 ± 0.13
HepG2 cells treated with 100 nM PMA for 10 min	1.0	–	1.38 ± 0.16
	1.0	1 μM calphostin C	0.44 ± 0.062
HepG2 cells treated with 100 nM PMA for 16 h	1.0	–	0.61 ± 0.051
	1.0	1 μM calphostin C	0.37 ± 0.048

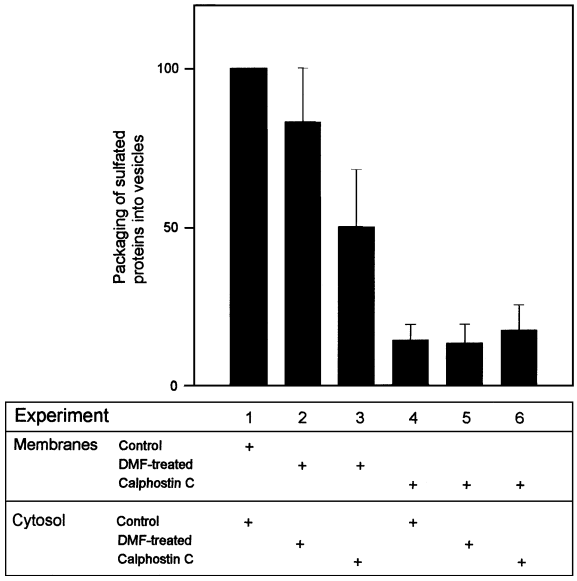

Figure 1 Inhibition of Golgi-bound PKC by calphostin C strongly impairs the formation of constitutive secretory vesicles

Membranes were isolated by centrifugation of PNS at 14000 ***g***. They were exposed to light without additions (control), or with the addition of DMF or calphostin C in DMF, then pelleted and assayed in budding assays *in vitro* as described. Cytosolic proteins, obtained by the centrifugation of PNS at 100000 ***g***, were treated as above and then, without removing the additions, included in the assays. Vesicles formed during incubation were pelleted and proteins were separated by polyacrylamide gel electrophoresis. HSPG labelling of the 100–200 kDa range was corrected by subtracting background labelling obtained by incubation at 0 °C. Corrected values of control experiments were normalized to 100; mean values of three independent experiments are given.

activities 80–100-fold higher than in the PNS. Contamination by endoplasmic-reticulum membranes, endosomes or plasma membrane could be excluded because antibodies against cytochrome *c* reductase, Rab5 or transferrin receptor failed to detect the corresponding proteins by Western blotting (results not shown).

PKC activities associated with the Golgi apparatus

PKC activities of purified Golgi preparations were measured with H1 histone or the pseudosubstrate peptide as substrates. Both assays gave similar results and the results of experiments with H1 histone as substrate are shown in Table 2. Treatment of HepG2 cells with 100 nM PMA for 10 min and for 16 h increased PKC activities in comparison with control cells (Table 2, column 1). Stimulation of PKC activities *in vitro* by the addition of PMA (Table 2, column 2) was significant in cells treated for 16 h with PMA, indicating an additional expression of PKC isoforms of the conventional group or the new group [27]. The proportion of PKC isoforms belonging to the calcium-dependent conventional group could be determined by the addition of EGTA. About half of the PKC activities were inhibited in Golgi preparations of control cells and those exposed briefly to PMA (Table 2, column 3). In contrast, PKC isoforms associated with the Golgi after 16 h of PMA treatment were not inhibited by EGTA (Table 2, column 3), indicating a decrease in conventional PKC isoform(s). The addition of calphostin C, a specific inhibitor of PKC, resulted in an approx. 85% inhibition of PKC activities, irrespective of the length of PMA treatment of cells (Table 2, column 4). Thus most of the total kinase activities measured can be attributed to PKC isoenzymes.

The formation of constitutive secretory vesicles in a cell-free system depends on PKC

The PNS of $^{35}SO_4$-labelled HepG2 cells was incubated with ATP, GTP and an ATP-regenerating system as described in [12] and transport vesicles formed were isolated by differential centrifugation. Vesicle formation was quantified by measuring the amount of $^{35}SO_4$-labelled proteins co-sedimenting with the newly formed vesicle fraction. HSPG packaging calculated from the ratio of the vesicle-bound fraction to total HSPG amounted to 15–20% under standard conditions. PMA, which stimulates HSPG secretion by intact HepG2 cells, also increased the packaging of HSPGs into transport vesicles *in vitro* (Table 3, row 2). The addition of calphostin C, which selectively inhibits PKC *in vivo* and *in vitro* [20,28], decreased the packaging of HSPGs independently of added PMA (Table 3, rows 3 and 4). Because calphostin C inhibits phorbol ester binding to PKC [28], other

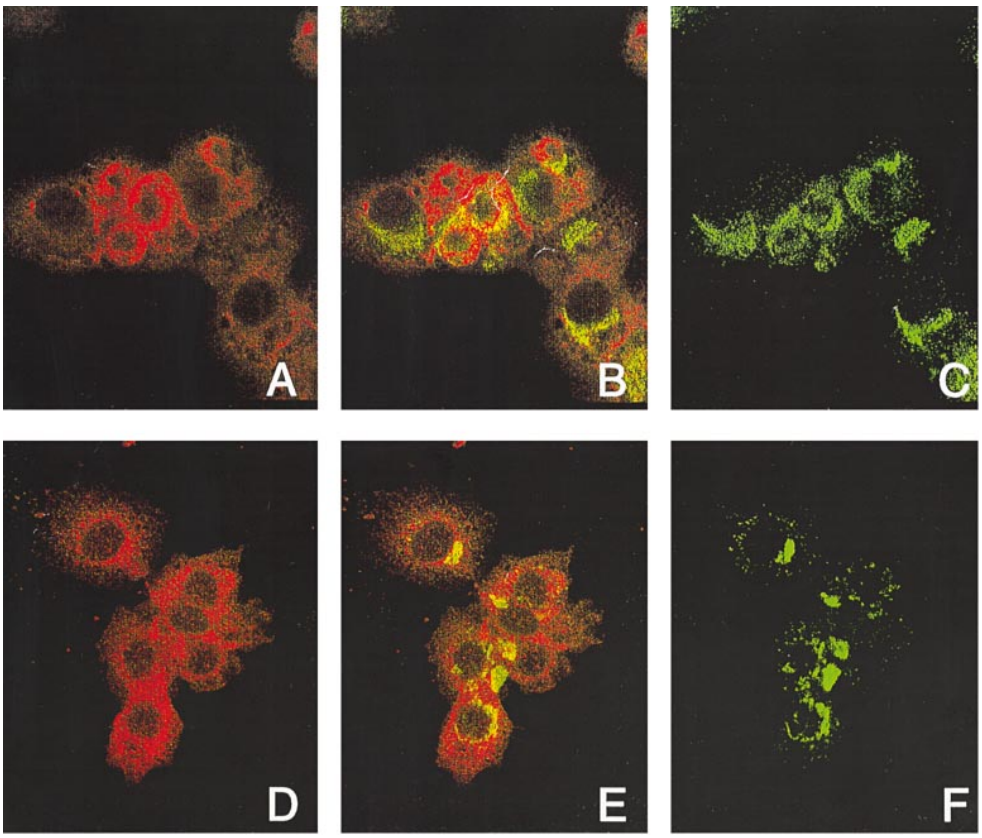

Figure 2 PKC-α and PKC-ζ bind to the Golgi apparatus of HepG2 cells, as shown by co-localization with TGN38

PKC-α (**A**) and PKC-ζ (**D**) were detected by confocal microscopy by using monoclonal subtype-specific antibodies and, as a second antibody, anti-(mouse IgG)–Cy3 conjugate (shown in red). To identify Golgi membranes, TGN38, a Golgi-specific marker, was labelled by an antibody directed against its C-terminus, which in turn was stained with anti-(rabbit IgG)–FITC conjugate [green in (**C**) and (**F**)]. Superposition of TGN38 staining of the corresponding specimens with those of PKC-α or PKC-ζ, shown in (**B**) and (**E**) respectively, clearly demonstrates overlapping in the Golgi area (orange/yellow).

PMA-binding protein(s), as suggested in [29], could also become inhibited. To exclude the effects of a PMA-binding protein and to confirm an involvement of PKC, an inhibitor that binds to the kinase domain of PKC was applied. The staurosporine derivative Ro 31-8220 binds specifically to PKC and inhibits the enzyme by competing with ATP [30]. Therefore the Ro 31-8220-mediated inhibition of PKC is reversed at high ATP concentrations. As expected, vesicle formation *in vitro* was marginally inhibited at 1 mM ATP and the inhibition was increased on decreasing the ATP concentration to 0.1 mM or below. The highest inhibition was observed at 20 μM ATP, the lowest ATP concentration that still supports vesicle budding.

The PNS of cells briefly stimulated by PMA supported budding *in vitro* more efficiently than did control cells (Table 3, row 9), whereas PMA treatment for 16 h decreased the activity of the PNS (Table 3, row 11). In both cases vesicle formation was inhibited by the addition of calphostin C (Table 3, rows 10 and 12).

Membrane-bound PKC and the formation of constitutive secretory vesicles

To test the impact of membrane-bound and cytosolic PKC forms on vesicle formation, PNS was further fractionated. Golgi-enriched membranes were pelleted at 14000 ***g*** and supernatant proteins purified by a 100000 ***g*** centrifugation step as described in [21]. Assays combining both fractions were supplemented with ATP, GTP and an energy-regenerating system. The efficiency of vesicle formation was comparable with those in experiments performed with PNS (Table 3). To test the contributions of Golgi membrane-bound PKC and cytosolic PKC to vesicle formation, membrane and cytosolic fractions were treated separately with calphostin C. Major inhibitory effects of the solvent, dimethylformamide (DMF) were ruled out by treating both membranes and cytosol with DMF. Only a marginal inhibition was observed (Figure 1, column 2). Indeed, treatment of membranes with calphostin C caused severe inhibition in all cases independently of whether or not the cytosol had been treated (Figure 1, columns 4–6). Whereas, in all experiments described above, the excess of calphostin C could be removed after the illumination step by pelleting the membranes, we were unable to separate the excess of calphostin C from the cytosolic fraction without impairing budding efficiency (as indicated in the controls in the absence of calphostin C). Partial inhibition of membrane-bound PKC by excess calphostin C in the dark [20] could therefore not be prevented. Nevertheless, the inhibition did not exceed 50 %, showing a moderate, at most, contribution of cytosolic PKC isoforms (Figure 1, column 3).

PKC-α and PKC-ζ are bound to the Golgi apparatus

The impact of Golgi-bound PKC on vesicle formation at the

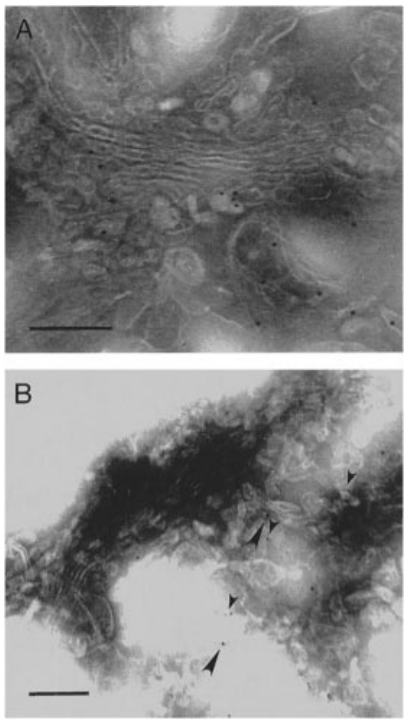

Figure 3 PKC-ζ binds to the trans-Golgi/TGN

PKC-ζ was detected on ultrathin sections of HepG2 cells by labelling with a subtype-specific antibody and anti-(mouse IgG) coupled to 12 nm gold particles (**A**, **B**). The trans-Golgi area was additionally labelled with an antibody against the C-terminus of TGN38 and subsequently with anti-(rabbit IgG) coupled to 5 nm gold particles (**B**). Gold particles (5 nm) are marked by small arrowheads and 12 nm particles by large arrowheads in (**B**). Scale bars, 0.2 μm.

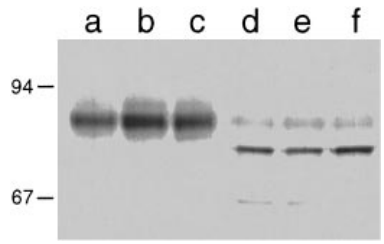

Figure 4 PKC-α and PKC-ζ are attached to highly enriched Golgi membranes

Golgi membranes of untreated HepG2 cells (lanes a and d) or HepG2 cells treated with 100 nM PMA either for 10 min (lanes b and e) or 16 h (lanes c and f) were isolated as described in the Experimental section. By analysis of Western blots with subtype-specific antibodies, PKC-α (lanes a–c) and PKC-ζ (lanes d–f) were detected, with apparent molecular masses of 81 and 77 kDa respectively. The positions of molecular-mass markers are shown in kDa at the left.

TGN raises the question of which PKC isoforms are attached to the Golgi apparatus. To study the distribution of PKC isoenzymes in HepG2 cells, subtype-specific antibodies were applied. Antibodies specific for PKC-α react predominantly with membranous structures in the perinuclear region (Figure 2A), but also stain peripheral vesicles and nuclei (result not shown). PKC-ζ specific antibodies reacted with vesicular structures in the perinuclear region (Figure 2D). Both antibodies bound to PKC-α or PKC-ζ were labelled with anti-mouse IgG conjugated with Cy3 as the secondary antibody. Golgi membranes were localized in the same specimens (Figures 2C and 2F) by subsequent incubation with an antibody that reacts specifically with the C-terminus of TGN38, a trans-membrane protein localized predominantly at the TGN [31]. The anti-TGN38 antibody was revealed by incubation with anti-(rabbit IgG)–FITC conjugate. As shown by superposition of PKC and TGN38 staining, both PKC-α and PKC-ζ are also bound to the Golgi membranes (Figures 2B and 2E).

To study the intra-Golgi localization of PKC isoforms, immunoelectron microscopy of ultrathin cryosections of HepG2 cells was used. PKC-α-specific antibodies binds to cis and trans regions of the Golgi apparatus (results not shown), whereas most PKC-ζ-specific signals are restricted to trans-Golgi and TGN area (Figure 3A). TGN and trans-Golgi were identified by their morphology and by double-staining with the TGN38-specific antibody (Figure 3B).

Both PKC isoenzymes, PKC-α and PKC-ζ, were identified in Golgi-enriched preparations from HepG2 cells by Western blotting (Figures 4a and 4d), with apparent molecular masses of 81 and 76 kDa respectively. The intensity of the PKC-α band increased on stimulation of cells for 10 min with PMA (Figure 4b), whereas the signal of PKC-ζ did not change (Figure 4e). Long-term PMA treatment, which is known to down-regulate PKC-α and PKC-ζ in fibroblasts [32], did not significantly reduce the amount of Golgi-bound forms in HepG2 cells (Figures 4c and 4f).

DISCUSSION

Membrane-bound PKC supports the formation of constitutive post-Golgi vesicles

Protein kinases are indispensable mediators of intracellular protein transport, as confirmed by studies on endocytosis [33,34], transcytosis [35], transport from endoplasmic reticulum to Golgi [36,37] and exocytosis (reviewed in [38]). Recent findings support the notion that the post-Golgi transport is also regulated by protein kinases. For instance, transport of VSV G protein to the basolateral membrane of polarized MDCK cells was stimulated by both cAMP and phorbol dibutyrate [39]. Post-Golgi transport of VSV G protein was inhibited by calphostin C [29]. AlF_4^--mediated inhibition of vesicle formation at the TGN in PC12 cell extracts was reversed by the addition of cAMP or PMA [21]. Finally, packaging and proteolytic processing of amyloid precursor protein was stimulated by PMA *in vivo* and by the addition of PKC *in vitro* [40,41].

Constitutive transport of cellular proteins can be measured by HSPG secretion [42]. In both RBL and MDCK cells, the addition of PMA stimulated the secretion of HSPGs [9,10], implicating the formation of vesicles at the TGN, the transport of vesicles, or PKC-stimulated exocytosis. To study the impact of PKC on the formation of transport vesicles at the TGN, we used the human hepatoma HepG2 cell. The formation of constitutive vesicles *in vitro* at the TGN was measured by using systems adapted from Tooze and Huttner [12] and Ohashi and Huttner [21]. The efficiency of budding *in vitro*, derived from the ratio between vesicle- and Golgi-bound labelled HSPG, is 15–20%. This value is similar to that reported in studies with PC12 cells and rat liver [42,43]. PNS from HepG2 cells is as active as a system combining Golgi-enriched membranes and cytosolic proteins. Vesicle formation in either case is stimulated by raising the concentration of cytosolic proteins (results not shown) and stops after 10–15 min of incubation, which is in agreement with experiments performed with rat liver Golgi [43].

The impact of PKC on vesicle formation at the TGN is demonstrated by three lines of evidence. First, PMA, which induces PKC binding to Golgi membranes, stimulates Golgi-bound PKC activity and increases packaging of HSPGs in transport vesicles *in vitro*, as well as transport and secretion *in vivo*. Secondly, calphostin C, a specific inhibitor of PKC reacting with the regulatory domain, inhibits HSPG secretion *in vivo* and vesicle formation *in vitro*. Thirdly, Ro 31-8220, a PKC inhibitor interacting specifically with the catalytic domain, inhibits vesicle formation *in vitro*. Experiments to down-regulate PKC isoenzymes were not successful because even 16 h of PMA treatment did not decrease PKC activity (Table 2, row 3) or signals in Western blots (Figures 4c and 4f). However, a decrease in vesicle formation by using PNS of these cells (Table 3, row 11) indicates that as well as PKC other factors, essential for vesicle formation, might become limiting.

To clarify whether Golgi-bound or cytosolic PKC isoenzymes support vesicle formation, membranes and cytosolic proteins were treated separately with calphostin C. Strong inhibition was observed in all assays containing calphostin C-treated membranes. Treatment of cytosolic proteins resulted in a 50% inhibition that might originate from two effects. Soluble PKC should become completely inhibited by covalent binding of calphostin C on illumination [20]. Moreover the untreated, membrane-bound, PKC forms might become partly inhibited by the excess of calphostin C that was involuntarily added with the cytosolic fraction. Because illumination was omitted after the addition of membranes, only a partial inhibition of membrane-bound PKC forms was observed [20], resulting in a budding activity of approx. 50%.

The strong inhibition of HSPG secretion *in vivo* and of vesicle formation *in vitro* demonstrates that PKC activity at the TGN is essential for constitutive vesicle transport. Moreover, the similar effect of PMA both on secretion and on vesicle formation supports the notion that vesicle formation at the TGN determines the velocity of HSPG secretion and that this constitutive transport process is modulated by TGN-bound PKC.

Comparing inhibitory activities of calphostin C on both budding systems *in vitro* shows that the PNS-based system is more resistant than the reconstituted system. Considering that PKC stimulates an early step in vesicle formation, like binding of ADP-ribosylation factor to the membranes [9], and assuming that later stages might not depend on PKC, the inhibition of early budding stages by calphostin C would result in a partial inhibition of vesicle formation in the PNS-based system. Owing to a loss of Golgi-bound proteins during membrane isolation (P. Westermann, unpublished work), late stages of buds may become diminished and formation of new buds would become essential for vesicle formation. As a result the calphostin C-mediated inhibition would increase in budding systems reconstituted *in vitro* from isolated Golgi membranes and cytosol.

PKC-α and PKC-ζ are bound to Golgi membranes

Evidence for a localization of PKC isoforms PKC-βI [44] and PKC-βII [45,46] in the vicinity of the Golgi apparatus was first obtained by immunoelectron microscopy of rat brain sections. Expression of cDNA species in NIH 3T3 cells revealed that other PKC isoforms can also attach to Golgi membranes. Immunofluorescence microscopy showed that PKC-δ and PKC-η [47] as well as PKC-ϵ [48] bind to the Golgi apparatus and that PKC-γ can be stimulated, by PMA treatment of cells, to move to the Golgi area [47]. To analyse the attachment of PKC isoforms under defined culture conditions, a simple and fast method for preparation of enriched Golgi membranes from cells cultivated *in vitro* was developed. Highly enriched Golgi preparations showing an 80–100-fold increase in galactosyltransferase activity over that in PNS were obtained by two centrifugation steps.

Two PKC isoforms, PKC-α and PKC-ζ, co-purify with highly enriched Golgi membranes of HepG2 cells, as shown by Western blotting. The localization of both isoforms in the Golgi apparatus was confirmed by immunofluorescence microscopy. Owing to their binding to the trans-Golgi/TGN area, as shown by immunoelecton microscopy, both PKC-α and PKC-ζ might be involved in vesicle formation. Stimulation of vesicle formation by PMA, which is accompanied by an increased binding of PKC-α to the Golgi apparatus, underlines the importance of PKC-α for modulating constitutive vesicle transport. In contrast, vesicle formation was also observed in the absence of calcium (results not shown), indicating that PKC isoenzymes, which do not belong to the calcium-dependent conventional group, might also be involved. PKC-ζ could be a kinase that maintains a calcium-independent, fundamental budding activity.

Note added in proof (received 2 October 1996)

While this paper was being proofed out, independent work was published by Simon et al. [49] showing that PKC activity is needed for packaging VSV G protein into post-Golgi vesicles.

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