Membrane ruffling and macropinocytosis in A431 cells require cholesterol

Stine Grimmer[1], Bo van Deurs[2] and Kirsten Sandvig[1,*]

[1]Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway
[2]Structural Cell Biology Unit, Department of Medical Anatomy, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark
*Author for correspondence (e-mail: ksandvig@radium.uio.no)

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Summary

Cholesterol is important for the formation of caveolea and deeply invaginated clathrin-coated pits. We have now investigated whether formation of macropinosomes is dependent on the presence of cholesterol in the plasma membrane. Macropinocytosis in A431 cells was induced by the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate, a potent activator of protein kinase C (PKC). When cells were pretreated with methyl-β-cyclodextrin to extract cholesterol, the phorbol ester was unable to induce the increased endocytosis of ricin otherwise seen, although PKC could still be activated. Electron microscopy revealed that extraction of cholesterol inhibited the formation of membrane ruffles and macropinosomes at the plasma membrane. Furthermore, cholesterol depletion inhibited the phorbol ester-induced reorganization of filamentous actin at the cell periphery, a prerequisite for the formation of membrane ruffles that close into macropinosomes. Under normal conditions the small GTPase Rac1 is activated by the phorbol ester and subsequently localized to the plasma membrane, where it induces the reorganization of actin filaments required for formation of membrane ruffles. Cholesterol depletion did not inhibit the activation of Rac1. However, confocal microscopy showed that extraction of cholesterol prevented the phorbol ester-stimulated localization of Rac1 to the plasma membrane. Thus, our results demonstrate that cholesterol is required for the membrane localization of activated Rac1, actin reorganization, membrane ruffling and macropinocytosis.

Key words: Ruffling, Macropinocytosis, Rac1, Cholesterol, Ricin

Introduction

The role of specific proteins in endocytosis has been studied extensively, and lately the contribution of lipids to this process has gained increasing interest. It has been known for some years that cholesterol is important for the invaginated structure of caveolae (Rothberg et al., 1990; Schnitzer et al., 1994; Hailstones et al., 1998), and it was recently found that extraction of cholesterol from the plasma membrane with the use of methyl-β-cyclodextrin (mβCD) inhibits the invagination of clathrin-coated pits (Rodal et al., 1999; Subtil et al., 1999). By contrast, clathrin-independent endocytosis was not much affected in a number of cell lines (Rodal et al., 1999). Lately, Huttner and co-workers showed that the biogenesis of synaptic-like microvesicles (SLMVs) from the plasma membrane was cholesterol dependent (Thiele et al., 2000). They proposed that the ability of cholesterol to associate with oligomerizing membrane proteins contributes to the segregation of SLMV membrane constituents, such as synaptophysin, and the formation of synaptic-vesicle curvature. Whether cholesterol has a similar role in clathrin-dependent endocytosis is not known.

In the present study we have investigated the importance of cholesterol for macropinocytosis in A431 cells. Macropinocytosis can be induced by growth factors such as the epidermal growth factor (EGF) or by the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA). A prerequisite for macropinocytosis is the formation of plasma membrane ruffles that subsequently close to form macropinosomes. This process requires reorganization of the actin filament network to the cell periphery (Swanson and Watts, 1995; Ridley, 1994). TPA, known as a potent activator of protein kinase C (PKC) (Keller, 1990), induces macropinocytosis via Rac1, a Ras-related GTP-binding protein (Ridley et al., 1992). Activated Rac1 localizes to the plasma membrane where it stimulates actin filament reorganization and membrane ruffling (Swanson and Watts, 1995; Ridley, 1994; Ridley et al., 1992; Kraynov et al., 2000).

To study the effect of decreased and increased cellular cholesterol content on macropinocytosis, mβCD was used to extract cholesterol from the plasma membrane and a complex of mβCD and cholesterol (mβCD/chol) was used to insert cholesterol. Here we demonstrate that membrane ruffling and macropinocytosis are sensitive to a decreased cholesterol content of the plasma membrane.

Materials and Methods

Cells, antibodies and reagents

The A431 cell line was maintained in DMEM (Flow Laboratories, Irvine, UK) supplemented with 10% FCS (Life Technologies, Paisley, UK), 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine (Life Technologies).

Rhodamine-labeled phalloidin was obtained from Molecular Probes (Eugene, OR). A mouse anti-human Rac1 from Transduction Laboratories (Lexington, KY) was used for immunofluorescence localization of endogenous Rac1. For localization of Rac1 constructs we used a mouse antibody against the six amino acid epitope tag EYMPME (termed Glu-Glu) from Covance (Princeton, NJ). Detection of ARF6 for immunofluorescence was performed using a rabbit polyclonal antibody, which was a generous gift from J. G.

Donaldson (National Institutes of Health, Bethesda, MD). Fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse, FITC-labeled goat anti-rabbit and rhodamine-labeled goat anti-mouse were from Jackson Immunoresearch (West Grove, PA). Methyl-β-cyclodextrin (average degree of substitution: 10.5-14.7 methyl groups per molecule), cholesterol, Hepes, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), Pipes (dipotassium salt), saponin, horseradish peroxidase (HRP), and ricin were obtained from Sigma (St Louis, MO). Na[^{125}I] was purchased from DuPont (Brussels, Belgium). Ricin was ^{125}I-labeled as descibed (Fraker and Speck, 1978) to a specific activity of (2-6)×10^4 cpm/ng. When protease inhibitors were required, Complete^{TM} EDTA-free from Roche Diagnostics (GmbH) was used. The TPA concentrations used in this study to stimulate ruffling and macropinocytosis range from 0.1 to 1 μM. Different concentrations were used because the response at a given concentration seemed to vary. It is not clear whether this is due to changes in the cells (perhaps due to the serum batch) or due to differences in the TPA-batches.

Preparation of mβCD saturated with cholesterol

The saturated complex was prepared mainly as previously described (Klein et al., 1995). 30 mg cholesterol was added to 1 g of mβCD dissolved in 20 ml H_2O. The mixture was rotated overnight at 37°C, and the resulting clear solution freeze-dried. The complex was stored at room temperature.

Cholesterol determination

Cell monolayers were washed carefully with PBS, lysed in a buffer containing 0.1% SDS, 1 mM Na_2EDTA and 0.1 M Tris-HCl, pH 7.4, and homogenised using a 19 gauge needle attached to a 1 ml syringe. The cholesterol content was determined enzymatically by the use of a cholesterol assay kit (Sigma).

Protein determination

The protein content of the homogenised cells was measured using the micro bicinchoninic acid method (Pierce, Rockford, IL) according to the manufacturer's instructions.

Measurement of TPA-induced macropinocytosis

TPA-induced macropinocytosis was measured as the amount of ^{125}I-labeled ricin endocytosed during 15 minutes after 4 hours of serum-starvation. The cells were incubated with 1 μM TPA for 10 minutes at 37°C before addition of ^{125}I-labeled ricin, and endocytosed ricin was measured after 15 minutes at 37°C as the amount of toxin that could not be removed with lactose as previously described (Sandvig and Olsnes, 1979). To look at the effect of changes in the cholesterol level, the cells were incubated with 5 mM mβCD, mβCD/chol or a 1:1 mixture of mβCD and mβCD/chol for 30 minutes at 37°C prior to addition of TPA, and then together with TPA for the duration of the experiment.

Measurement of PKC activity

Cells were seeded onto 10 cm petri dishes (5×10^5 cells per dish) 2 days in advance. They were then washed twice with DMEM medium, serum-starved for 4 hours and preincubated with or without 5 mM mβCD or mβCD/chol for 30 minutes at 37°C before addition of 1 μM TPA. After incubating further for 10 minutes, the PKC activity was measured using the Protein Kinase C Assay System from Life Technologies according to the manufacturer's instructions.

Electron microscopy

The cells were washed twice with Hepes medium, serum-starved for 4 hours and preincubated with or without 5 mM mβCD, mβCD/chol or a 1:1 mixture of mβCD and mβCD/chol for 30 minutes at 37°C before addition of 1 μM TPA and HRP (10 mg/ml). After incubating the cells further for 15 minutes at 37°C, they were washed with PBS and fixed in monolayer with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 60 minutes at room temperature. The cells were then carefully washed with PBS (five times) and incubated in PBS containing 0.5 mg/ml diaminobenzidine and 0.5 μl/ml of a 30% H_2O_2 solution for 60 minutes at room temperature. The cells were then washed, scraped off the flasks, pelleted, post-fixed with OsO_4, contrasted en block with 1% uranyl acetate, dehydrated in a graded series of ethanols and embedded in Epon. Sections were further contrasted with lead citrate and uranyl acetate and examined in a Phillips CM 100 electron microscope (Phillips, Eindhoven, The Netherlands).

Immunofluorescence

Cells grown on glass coverslips were serum-starved for 4 hours before incubation with or without 5 mM mβCD, mβCD/chol or a 1:1 mixture of mβCD and mβCD/chol for 30 minutes at 37°C, followed by incubation with TPA (concentrations as indicated in figure legends) in the absence or presence of mβCD, mβCD/chol or a 1:1 mixture of mβCD and mβCD/chol for the indicated times at 37°C. After a brief wash with PBS, the cells were processed accordingly.

For vizualisation of actin the cells were fixed and permeabilized with 3% paraformaldehyde in PBS for 1 hour at room temperature, and then incubated with 50 mM NH_4Cl in PBS for 10 minutes. The cells were labeled with rhodamine-labeled phalloidin diluted in PBS containing 0.5% FCS for 30 minutes at room temperature.

For localization of endogenous Rac1 or epitope-tagged Rac1, the cells were permeabilized with 0.05% saponin in Pipes buffer (80 mM Pipes (dipotassium salt), 5 mM EGTA, 1 mM $MgCl_2$, pH 6.8) for 5 minutes at room temperature both before and after fixation with 3% paraformaldehyde for 15 minutes, and then incubated with 50 mM NH_4Cl in PBS for 10 minutes. The cells were labeled with primary and secondary antibodies diluted in PBS containing 0.05% saponin for 30 minutes at room temperature.

After staining, the coverslips were mounted in Mowiol (Calbiochem, San Diego, CA). Confocal microscopy was performed by using a Leica (Wetzlar, Germany) confocal microscope. Images were taken at ×63 magnification and captured as images at 1024×1024 pixels. Montages of images were prepared with the use of PhotoShop 4.0 (Adobe, Mountain View, CA).

Transient transfection of cells

The wild-type and T17N mutant Rac1 constructs containing an N-terminal epitope tag (MEYMPMEHM; termed EE) in the modified pCDL-SRα expression vector (pXS) (Takebe et al., 1988) and untagged wild-type and T27N mutant ARF6 constructs also in the pXS expression vector were a generous gift from J.G. Donaldson (National Institutes of Health, Bethesda, MD).

Cells grown on glass coverslips were transfected using Fugene 6 (Roche) according to the manufacturer's instructions. 40 hours after transfection the cells were serum-starved for 4 hours before being stimulated with 0.1 μM TPA for 10 minutes and processed for indirect immunofluorescence.

Determination of Rac1 activation state

Cells grown to confluence in 3 cm dishes were washed twice with phosphate-free DMEM (Life Technologies), and serum-starved for 4 hours at 37°C with CO_2 in the same medium supplemented with 0.5 mCi/ml [^{32}P]orthophosphate. The cells were then incubated with or without 5 mM mβCD for 30 minutes at 37°C with CO_2 before stimulation with 0.5 μM TPA for 10 minutes. After being washed three times with ice-cold PBS, the cells were lysed in the lysis buffer

(50 mM Hepes, pH 7.4, 1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM GTP, 1 mM ATP, 10 mM Na-phosphate, protease inhibitors) for 5 minutes on ice. Nuclei and cell debris were removed by centrifugation at 15,000 ***g*** for 2 minutes at 4°C, and the soluble fraction was subjected to immunoprecipitation for 1.5 hours at 4°C with 5 μg/ml of anti-Rac1 coupled to protein A-sepharose per sample. The beads were then washed three times with buffer (50 mM Hepes, pH 7.4, 500 mM NaCl, 5 $MgCl_2$) containing 1% Triton X-100, and three times with the same buffer containing 0.1% Triton X-100 and 0.005% SDS. The bound nucleotides were eluted in 8 μl elution buffer (2 mM EDTA, 2 mM DTT, 0.2% SDS, 5 mM GDP, 5 mM GTP) for 15 minutes at 70°C, spotted onto a 0.1 mm PEI-cellulose TLC plate (Aldrich, Milwaukee, WI), and developed for 40 minutes in 0.6 M Na-phosphate, pH 3.4 for separation. Separated [^{32}P]GDP and [^{32}P]GTP were quantified with the PhosphorImager (Applied Biosystems, Foster City, CA).

Binding of Rac1 to liposomes

Liposomes were prepared as earlier described (Patki et al., 1997) by mixing 50% phosphatidylserine and 50% phosphatidylethanolamine or 50% phosphatidylserine, 45% phosphatidylethanolamine and 5% cholesterol. The mixtures were dried under nitrogen, and resuspended to a final concentration of 1 mg/ml of total phospholipid in a Hepes buffer (50 mM Hepes, pH 7.2, 100 mM NaCl, 0.5 mM EDTA). The resuspended lipids were sonicated on ice for 5 minutes to obtain a homogeneous suspension. Liposomes were collected by centrifugation at 140,000 ***g*** for 10 minutes and resuspended in lysis buffer (20 mM Hepes, pH 7.2, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, protease inhibitors) to 3.75 mg/ml of total lipid.

To measure the binding of Rac1, a cell lysate was prepared of cells grown to confluence in 10 cm dishes, washed briefly with ice-cold PBS and scraped into 0.5 ml of lysis buffer (see above) on ice. The cells were lysed by passage through a 27-gauge needle attached to a 1 ml syringe five times at 4°C, and nuclei and cell debris were removed from the homogenates by spinning at 2500 ***g*** for 5 minutes at 4°C. Aliquots of lysate were mixed with 200 μl of each liposome mixture, vortexed once, and after 15 minutes at room temperature centrifuged at 140,000 ***g*** for 10 minutes. The supernatants and liposome pellets were separated on 12% SDS-PAGE and transferred to a PVDF membrane (Millipore Corporation, Bedford, MA) for detection of Rac1.

Results

Cholesterol is required for TPA-induced macropinocytosis in A431 cells

To investigate whether macropinocytosis, which can be induced in serum-starved A431 cells by addition of TPA (Sandvig and van Deurs, 1990), is dependent on cholesterol in the membrane, we first measured the ability of mβCD and a complex of mβCD and cholesterol (mβCD/chol) to affect the cholesterol level in A431 cells. After 30 minutes incubation with 5 mM mβCD the cellular cholesterol content was decreased to about 70% compared with the control level, and it decreased further to 60% after 60 minutes (Table 1). As shown earlier, this decrease in the cholesterol level does not cause any measurable changes in membrane permeability nor does it significantly affect protein synthesis in A431 cells (Rodal et al., 1999). Treatment with 5 mM mβCD/chol for 30 minutes increased the cholesterol content in the cells by 50% (Table 1), while incubation for 60 minutes had no further effect on the amount of incorporated cholesterol (Table 1).

TPA-induced macropinocytosis can be measured as increased uptake of the plant toxin ricin (Sandvig and van Deurs, 1990). We therefore investigated whether a decreased or increased cellular cholesterol level affected this stimulation of endocytosis. A431 cells were serum-starved for 4 hours and incubated with TPA for 10 minutes to stimulate macropinocytosis before the endocytosis of ricin was measured. To remove or insert cholesterol, the cells were incubated with 5 mM mβCD or mβCD/chol for 30 minutes prior to addition of TPA. As shown in Fig. 1, TPA treatment increased ricin endocytosis by nearly 60%. This stimulation of ricin endocytosis was inhibited following treatment with mβCD, while treatment with mβCD/chol had no effect on the TPA-stimulated increase in ricin endocytosis (Fig. 1). As previously shown (Rodal et al., 1999), extraction of cholesterol had essentially no effect on the basal ricin endocytosis in this cell line (Fig. 1). Control experiments showed that when a 1:1 mixture of mβCD and mβCD/chol (total concentration of 5 mM) was added, a mixture without any significant effect on the cholesterol level (98.4%±3.6, n=3), the TPA-induced endocytosis of ricin was unaffected (Fig. 1). In addition, as observed for TPA, EGF-stimulated macropinocytosis was also inhibited following cholesterol depletion (data not shown).

Table 1. The cholesterol content after incubation with mβCD or mβCD/chol

Treatment	Cholesterol after 30 minutes*	Cholesterol after 60 minutes*
Control	25.0±2.8 (100%)	24.6±2.5 (100%)
5 mM mβCD	17.3±2.9 (69%)	14.3±2.2 (58%)
5 mM mβCD/chol	37.4±4.1 (150%)	37.6±4.1 (152%)

*Cholesterol mass is expressed as μg per mg of cell protein, and the data are means±s.d. from three independent assays each performed in duplicate.

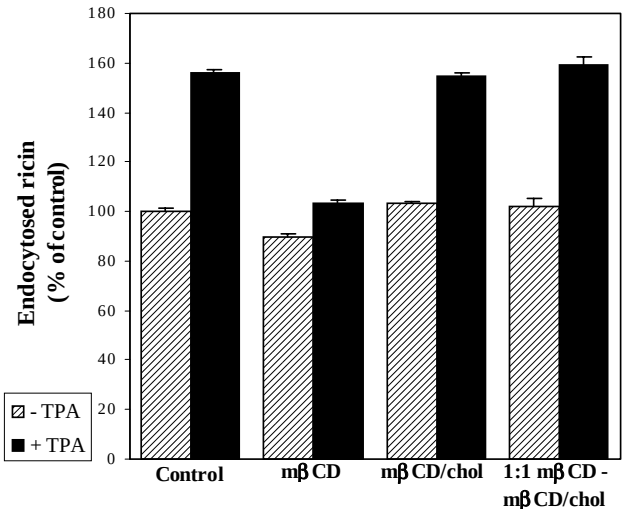

Fig. 1. Effect of mβCD, mβCD/chol and a 1:1 mixture of mβCD and mβCD/chol (which does not change the cellular cholesterol content) on ricin endocytosis in the absence and presence of TPA to stimulate macropinocytosis. A431 cells were washed twice in Hepes medium, serum-starved for 4 hours and incubated with or without 5 mM mβCD, mβCD/chol or a 1:1 mixture of mβCD and mβCD/chol for 30 minutes at 37°C prior to addition of 1 μM TPA. After 10 minutes ^{125}I-labeled ricin was added to the cells, and the amounts of endocytosed ricin were measured after 15 minutes, as described in Materials and Methods. The error bars show deviations (s.d.) between three independent experiments.

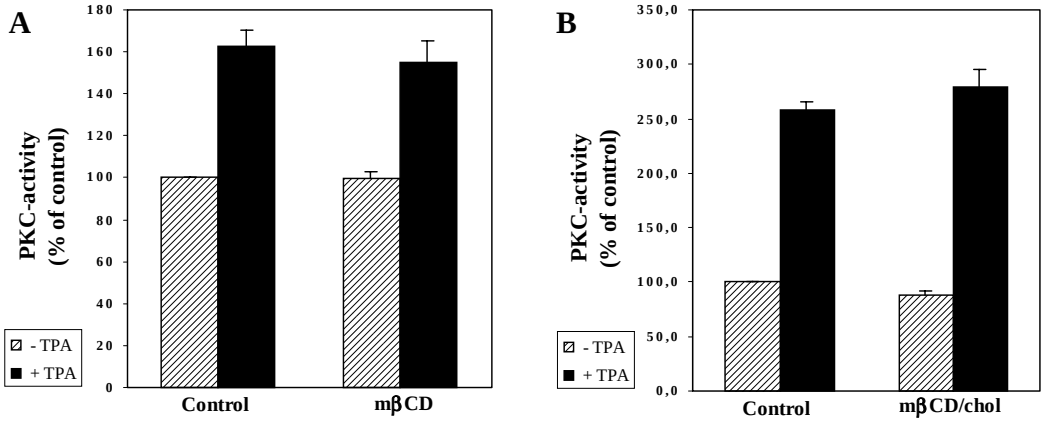

Fig. 2. Effect of TPA and mβCD (A) or mβCD/chol (B) on the activity of PKC. A431 cells were washed twice with DMEM medium, serum-starved for 4 hours and preincubated with or without 5 mM mβCD or mβCD/chol for 30 minutes at 37°C before addition of 1 μM TPA. After 10 minutes at 37°C, the PKC activity was measured using the Protein Kinase C Assay System according to the manufacturer's instructions. The error bars show deviations between duplicates of a typical experiment.

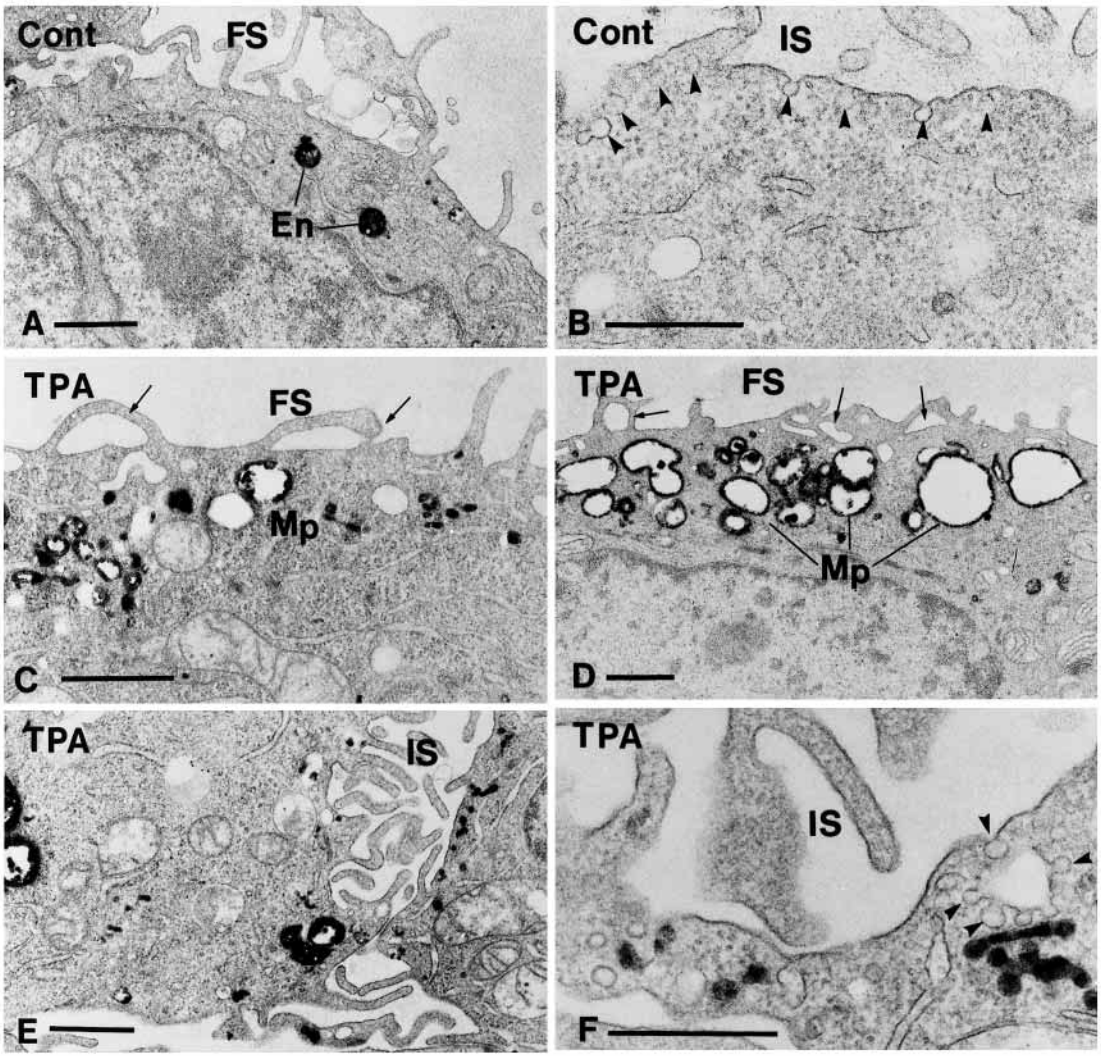

Fig. 3. TPA induces macropinocytosis in A431 cells. (A,B) EM micrographs of control A431 cells (Cont) serum-starved for 4 hours and then incubated with HRP (10 mg/ml) for 15 minutes at 37°C before processing for EM. Note the irregular membrane on the free surface (FS) of the cell in A. Moderate endocytosis of HRP into endosomes (En) has taken place. B shows the lateral membrane of a cell facing the intercellular space (IS). Here a few microvilli-like structures are seen. Arrowheads indicate the presence of caveolea. (C-F) Serum-starved A431 cells incubated with HRP as above but in the presence of 1 μM TPA. C and D show closure of membrane ruffles (arrows) and subsequent formation of large amounts of HRP-containing macropinosomes (Mp) at the free surface (FS). E and F show that TPA treatment also leads to an increased complexity of the lateral membranes at the intercellular spaces (IS) and that caveolea are present at the lateral membrane facing the intercellular space (arrowheads in F). Bars, 1 μm (A,C-E); 0.5 μm (B,F).

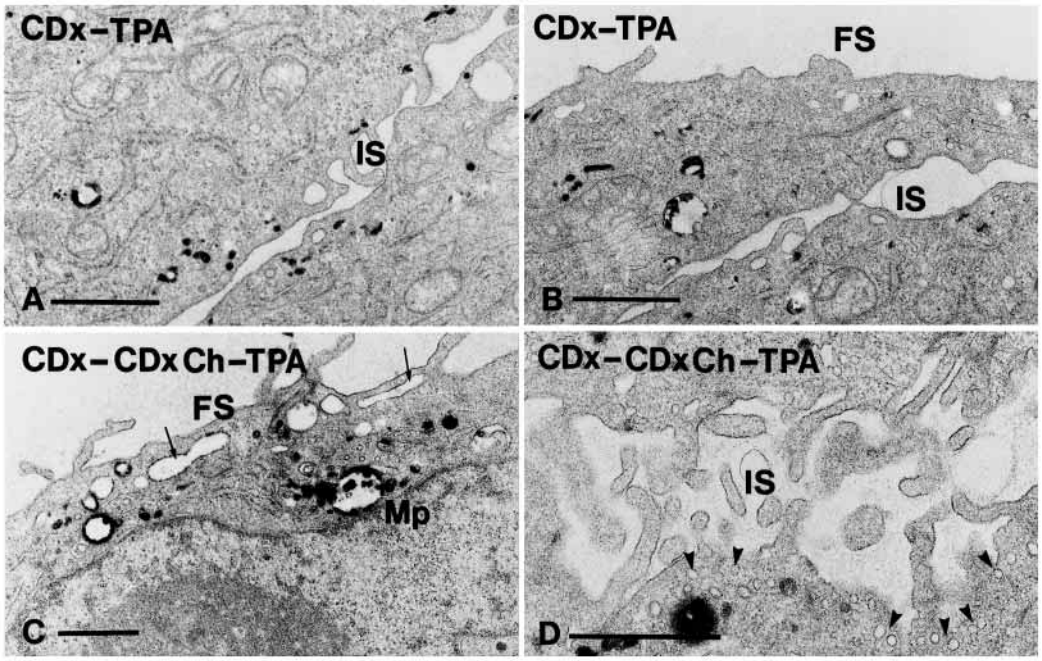

Fig. 4. Cholesterol is required for the TPA-induced macropinocytosis. (A,B). A431 cells were serum-starved for 4 hours, preincubated for 30 minutes at 37°C with 5 mM mβCD to remove cholesterol and then for 15 minutes at 37°C with HRP (10 mg/ml) in the presence of both 5 mM mβCD and 1 μM TPA (CDx-TPA). It is evident how treatment with mβCD reduces the expression of membrane ruffles at the free surface (FS in B) and prevents the TPA-induced macropinocytosis of HRP from this surface as well as formation of a more complex membrane structure at the lateral membranes facing the intercellular spaces (IS). Moreover, only few caveolea are present. In C and D, serum-starved A431 cells were preincubated for 30 minutes at 37°C with a 1:1 mixture of 2.5 mM mβCD and 2.5 mM mβCD/chol (a mixture that does not change the cellular cholesterol content) and then for 15 minutes at 37°C with HRP in the presence of both TPA and the mβCD mixture (CDx-CDxCh-TPA). Under this condition closure of ruffles (arrows) at the free surface (FS) and the formation of HRP-containing macropinosomes (Mp) take place, complex membrane structures are formed at the lateral membranes at the intercellular spaces (IS), and caveolea are also present at these membranes (arrowheads in D). Bars, 1 μm.

TPA is a potent activator of PKC (Keller, 1990), and formation of ruffles at the plasma membrane can in some cells be stimulated by PKC (Swanson and Watts, 1995; Miyata et al., 1989). Therefore, we wanted to investigate whether the PKC activity was affected by changes in the cellular cholesterol content. However, neither treatment with mβCD nor mβCD/chol had any effect on the TPA-stimulated PKC activity (Fig. 2A,B). This was supported by the finding that mβCD inhibited the stimulated increase in ricin endocytosis even when added after TPA (data not shown), while mβCD/chol still had no effect on the TPA-induced increase in endocytosed ricin. Thus, cholesterol depletion inhibits the TPA-stimulated increase in ricin endocytosis without affecting PKC-activity.

Cholesterol depletion inhibits TPA-stimulated macropinocytosis at the cell membrane

A prerequisite for macropinocytosis is formation of plasma membrane ruffles and closure of these to form macropinosomes. To investigate whether this process was affected by changes in the cholesterol content, serum-starved cells incubated in the absence or presence of 5 mM mβCD or mβCD/chol prior to and along with TPA treatment were studied by electron microscopy. In addition, to check that mβCD in itself did not affect the processes studied, the effect of a 1:1 mixture of mβCD and mβCD/chol (total concentration of 5 mM) was investigated. As described above, the 1:1 mixture of mβCD and mβCD/chol has no significant effect on the cholesterol level nor on the TPA-induced increase of ricin endocytosis (Fig. 1).

Control cells had irregular membrne protrusions at the free (‘apical’) surface and a few microvilli-like structures at the intercellular space (Fig. 3A,B). There was no macropinocytosis; only moderate endocytosis of HRP into endosomes had taken place (Fig. 3A). In addition, caveolea were present at the lateral membrane facing the intercellular space (Fig. 3B, arrowheads). Stimulation with TPA resulted in closure of ruffles (Fig. 3C,D, arrows), and subsequent formation of a large number of HRP-containing macropinosomes at the free surface (Fig. 3C,D). Furthermore, TPA treatment led to an increased complexity of lateral membranes at the intercellular space (Fig. 3E,F). As in control cells, caveolea were present along the intercellular space (Fig. 3F, arrowheads). Following treatment with mβCD, TPA-stimulated macropinocytosis of HRP at the free surface and formation of complex membrane structures at the lateral membranes facing the intercellular space were strongly inhibited (Fig. 4A,B). Moreover, the number of caveolea at the lateral membrane facing the intercellular space were strongly reduced (Fig. 4B).

In contrast to the observations described above, treatment with a 1:1 mixture of mβCD and mβCD/chol did not affect the TPA-induced macropinocytic activity at the free surface (Fig. 4C, arrows). In addition, complex membrane structures were formed at the lateral membranes at the intercellular space, and caveolea were also present at these membranes (Fig. 4D). Also, increasing the cholesterol content by treatment with mβCD/chol had no effect on the TPA-induced formation of macropinosomes at the free surface nor on the presence of caveolea and complex membrane structures at the intracellular space. An overview of the findings obtained by electron microscopy is presented in Table 2.

Table 2. The effect of TPA and changes in cholesterol level on the morphology of A431 cells

Experiment	Cholesterol level	Ruffles, closure of ruffles	Macropinocytosis of HRP	Caveolae
1. Control	Unchanged	A few ruffles and microvilli-like structures	–	Numerous
2. TPA	Unchanged	Ruffles and complex membrane structures, closure of ruffles	+	Numerous
3. mβCD	Decreased	Like 1, but cell surface was more smooth (fewer ruffles and complex membrane structures)	–	Largely lacking
4. mβCD+TPA	Decreased	Like 3	–	Largely lacking
5. mβCD+ mβCD/chol	Unchanged	Like 1	–	Numerous
6. mβCD+ mβCD/chol+TPA	Unchanged	Like 2	+	Numerous
7. mβCD/chol	Increased	Like 1	–	Numerous
8. mβCD/chol+TPA	Increased	Like 2	+	Numerous

For a detailed description of the experimental conditions, see legends to Figs 3 and 4, and Table 1.

TPA-induced actin reorganization at the cell periphery is inhibited following extraction of cholesterol

Since formation of membrane ruffles is dependent on the reorganization of filamentous actin at the plasma membrane (Swanson and Watts, 1995; Ridley, 1994), we investigated whether any changes in the TPA-stimulated actin reorganization were discernible following changes in the cholesterol level. Serum-starved cells were incubated in the absence or presence of 5 mM mβCD, mβCD/chol or a 1:1 mixture of mβCD and mβCD/chol prior to and along with TPA treatment and studied in the confocal microscope using rhodamine-labeled phalloidin to visualize filamentous actin.

In control cells the actin cytoskeleton was seen mainly as distinct filament bundles at the free surface of the cell, running both parallel and perpendicular to the surface (Fig. 5A-C). The latter orientation probably corresponds to the irregular protrusions seen at the free surface in control cells by EM. The actin staining along the intercellular space showed great variation, from hardly visible to quite pronounced (Fig. 5A-C). Treatment with TPA primarily caused a marked reorganization of the actin cytoskeleton at the cell periphery (Fig. 5D-I). Within 2-4 minutes ruffling activity was observed at the free surface (Fig. 5E-G). After 8-10 minutes this activity was somewhat reduced (Fig. 5H,I). Also, TPA treatment led within minutes to an increased and consistent actin staining at the lateral borders of the cells (Fig. 5D-I), presumably corresponding to the increased frequency of complex membrane structures at the lateral intercellular space seen by EM after TPA treatment. Importantly, the actin reorganization at the free surface observed after TPA treatment was prevented by cholesterol depletion (Fig. 6A,B). Neither an increased cholesterol level nor treatment with a 1:1 mixture of mβCD and mβCD/chol that does not change the cholesterol content had any effect on the TPA-induced reorganization of the actin filaments to the cell periphery (Fig. 6C,D). Thus, cholesterol depletion seems to inhibit TPA-stimulated ruffling at the plasma membrane by inhibiting the reorganization of the filamentous actin network at the cell periphery.

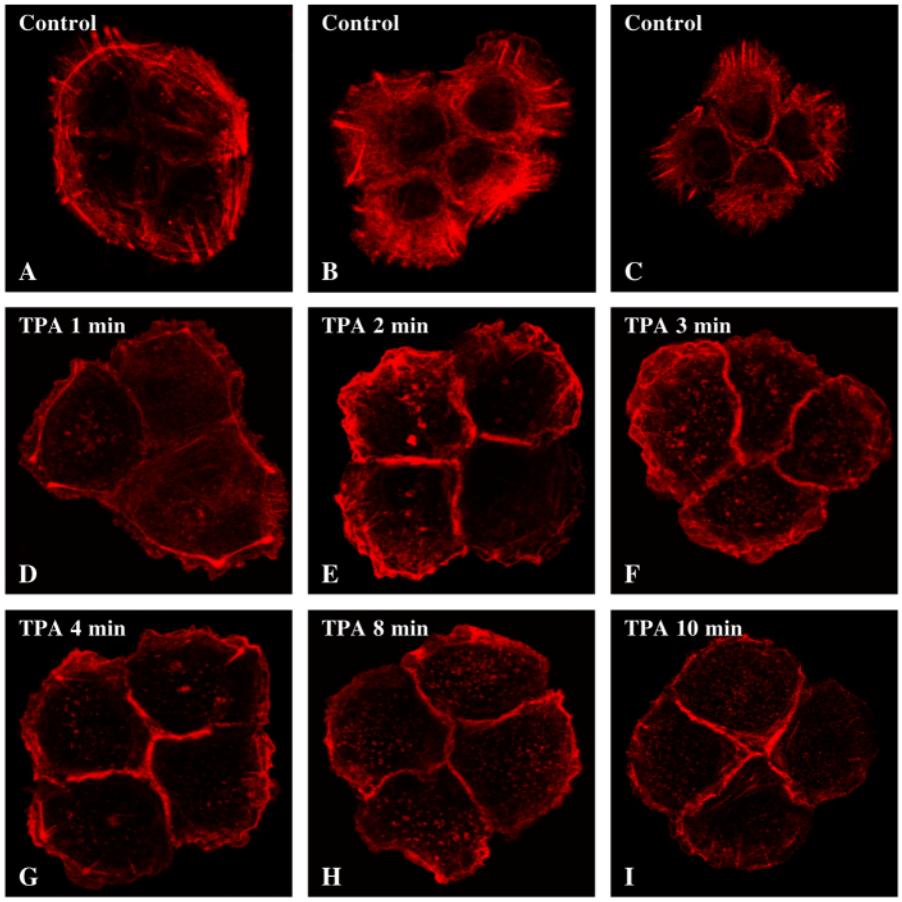

Fig. 5. TPA-induced reorganization of actin filaments at the cell periphery. (A-C) Serum-starved A431 cells (control) fixed, permeabilized and stained with rhodamine-labeled phalloidin to visualize filamentous actin. (D-I) Serum-starved A431 cells incubated with 0.5 μM TPA for 1, 2, 3, 4, 8 or 10 minutes at 37°C and then processed as the control cells to visualize filamentous actin. Note that distinct stress fibers at the free surface of the cells rapidly disappear and are replaced by actin associated with ruffles as well as an apparent increase in actin staining at the lateral membranes (D-I).

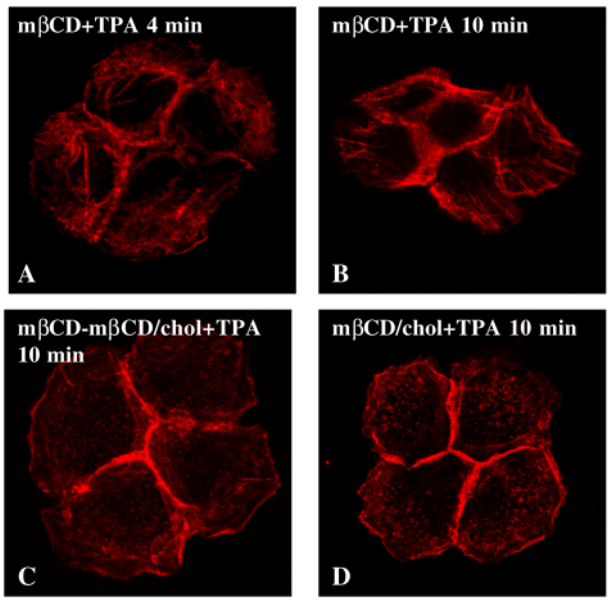

Fig. 6. Cholesterol is required for the TPA-induced ruffling. (A,B) Serum-starved A431 cells were incubated with 5 mM mβCD for 30 minutes prior to addition of 0.5 μM TPA. After 4 or 10 minutes at 37°C the cells were fixed, permeabilized and stained with rhodamine-labeled phalloidin to visualize filamentous actin. In C and D, serum-starved A431 cells were incubated with a 1:1 mixture of mβCD and mβCD/chol (which does not change the cellular cholesterol content) and mβCD/chol, respectively, for 30 minutes prior to addition of 0.1 μM TPA. After 10 minutes at 37°C the cells were fixed, permeabilized and stained with rhodamine-labeled phalloidin to visualize filamentous actin.

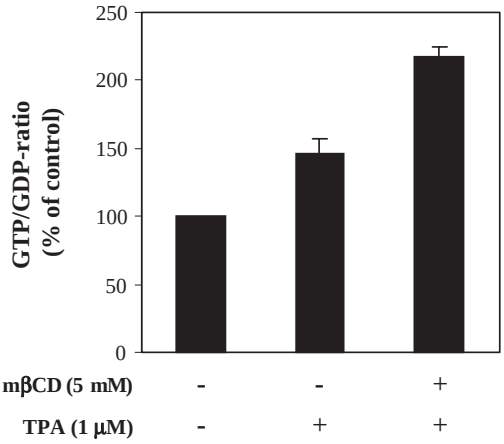

Fig. 7. Effect of TPA and mβCD on the activation state of Rac1. A431 cells were serum-starved in the presence of 0.5 mCi/ml [^{32}P]orthophosphate to label endogenous pools of GTP and GDP, and then incubated with or without 5 mM mβCD for 30 minutes at 37°C prior to addition of 0.5 μM TPA. After lysis of the cells with detergent, Rac1 was immunoprecipitated and ^{32}P-labelled nucleotides bound to Rac1 analyzed by TLC as described in Materials and Methods. The error bars show deviations (s.d.) between three independent experiments.

The localization of Rac1 to the plasma membrane is dependent on cholesterol

Earlier studies have shown that TPA-stimulation can localize the activated GTP-binding protein Rac1 to the plasma membrane where it induces actin reorganization necessary for formation of membrane ruffles (Ridley, 1994; Ridley et al., 1992). Thus, lack of actin filament reorganization and formation of ruffles following cholesterol depletion might be due to prevention of Rac1 activation or localization of activated Rac1 to the plasma membrane. We first investigated whether the TPA-induced activation of Rac1 was affected by cholesterol depletion by determining the GTP:GDP ratio of Rac1. For this purpose the cells were incubated with $^{32}PO_4^{3-}$ during serum-starvation to label endogenous pools of GTP and GDP. The cells were then lysed, Rac1 was immunoprecipitated and ^{32}P-labelled nucleotides bound to the immunoprecipitated protein were analyzed by thin-layer chromatography. As shown in Fig. 7, treatment with TPA increased the GTP:GDP ratio of Rac1 to about 150%. Interestingly, treatment with mβCD prior to TPA-stimulation increased the GTP:GDP ratio of Rac1 even further, to about 218% of control levels. Thus, cholesterol depletion does not prevent the TPA-induced Rac1 activation, but rather increases it.

We then investigated whether the membrane localization of activated Rac1 was affected by decreased cellular cholesterol levels. Serum-starved cells incubated in the absence or presence of 5 mM mβCD prior to and along with TPA treatment were labeled with antibody against Rac1 and studied in the confocal microscope. As shown in Fig. 8Aa, Rac1 could not be observed at the plasma membrane in control cells. Following TPA-stimulation Rac1 was clearly visible at the free surface of the cells where membrane ruffling and macropinocytosis take place (Fig. 8Ab). However, after treatment with mβCD, Rac1 was no longer localized to the cell periphery following TPA-stimulation (Fig. 8Ac). Also, there was no longer staining of the membrane between the cells. Furthermore, when TPA-stimulated cells were subjected to a 5 minutes pulse with 15 mM mβCD to remove cholesterol, Rac1 was (in contrast to cells not exposed to mβCD) no longer detected at the cell periphery (Fig. 8Ad). Thus, Rac1 already localized to the plasma membrane is also sensitive to cholesterol depletion. By contrast, treatment with mβCD/chol did not affect the TPA-stimulated localization of Rac1 to the cell periphery (data not shown). To verify that Rac1 is important for TPA-induced ruffling and macropinocytosis in A431 cells, the effect of TPA-stimulation on cells transiently transfected with wild-type or a dominant-negative mutant form of Rac1 was investigated. Following TPA-stimulation wtRac1 was able to localize to the free surface of the cells and also actin reorganization was induced (Fig. 8Ba,b). In contrast, the dominant-negative Rac1T17N did not localize to the free surface and the TPA-induced actin reorganization otherwise seen was inhibited (Fig. 8Bc,d). Consequently, Rac1 is required for ruffling and macropinocytosis in A431 cells.

Since membrane cholesterol is important for localization of Rac1 to the free surface, we investigated whether one could measure a cholesterol-dependent binding of Rac1 to liposomes. However, this was not the case (data not shown). Hence, although cholesterol is required for the localization of activated Rac1 to the plasma membrane, additional components may be necessary for this localization. It has previously been shown that the ARF proteins function as regulators of membrane traffic (Moss and Vaughan, 1998), and it has been suggested

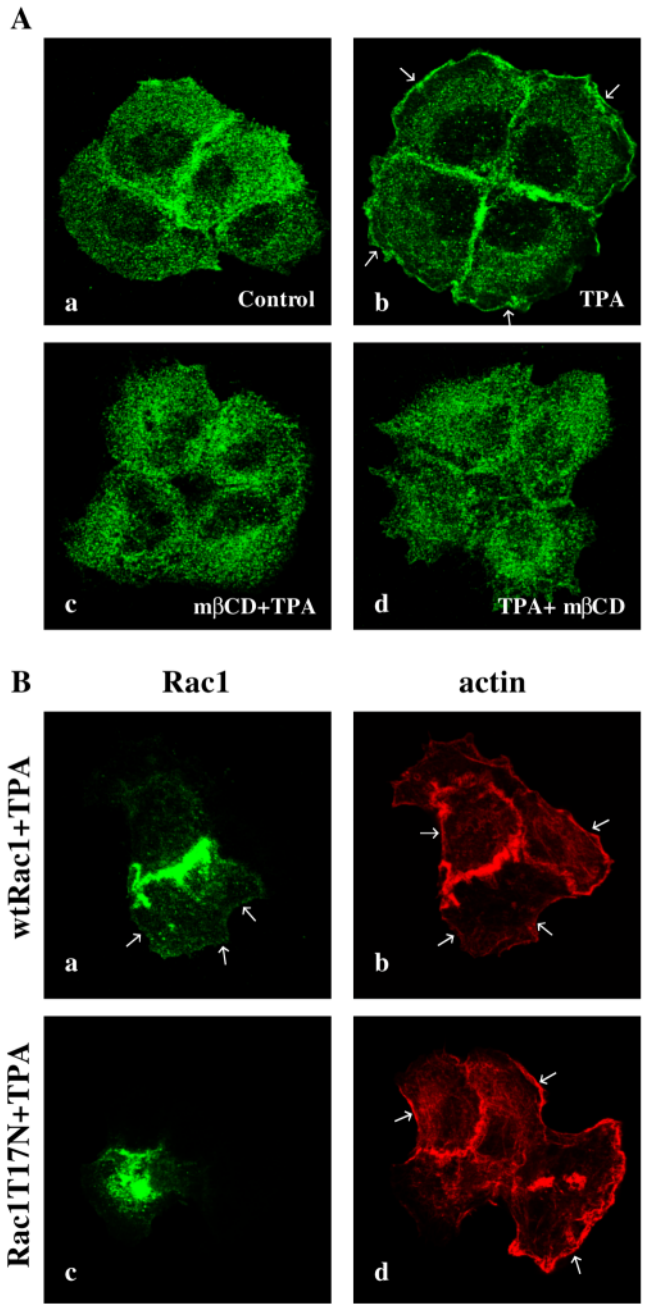

Fig. 8. (A) Cholesterol is required for the TPA-induced localization of Rac1 to the free surface of the cells. (a-c) Serum-starved cells were untreated or incubated with or without 5 mM mβCD for 30 minutes at 37°C prior to addition of 0.1 μM TPA. After 10 minutes at 37°C the cells were fixed, permeabilized and stained with an antibody against Rac1. Arrows in b indicate localization of Rac1 to the free surface, where ruffling and macropinocytosis takes place. In d the cells were first incubated with 0.1 μM TPA for 10 minutes to localize Rac1 to the cell periphery, followed by a 5 minute pulse with 15 mM mβCD to remove cholesterol. The cells were then processed to visualize endogenous Rac1. (B) Dominant-negative Rac1T17N inhibits TPA-induced actin reorganization required for membrane ruffling. Serum-starved A431 cells transiently transfected with wild-type Rac1 (a,b) or Rac1T17N (c,d) were incubated with 0.1 μM TPA for 10 minutes and processed for indirect immunofluorescence. Rac1 was labeled with a mouse antibody against six amino acids in the EE-epitope, and F-actin was labeled with rhodamine-phalloidin. Arrows in 'a' indicate localization of Rac1 to the free surface (compare with c), while arrows in b indicate actin associated with ruffles in both transfected and untransfected cells. It should be noted that in d peripheral actin staining (arrows) is observed only in the cells not transfected with the dominant-negative mutant.

that ARF6 is required for localization of activated Rac1 to the plasma membrane (Radhakrishna et al., 1999; Zhang et al., 1999). Thus, the possibility that ARF6 colocalized with Rac1 at the cell periphery in TPA-stimulated cells was investigated. As shown (Fig. 9), TPA treatment changed the distribution also of ARF6, and ARF6 localized to Rac1-containing regions at the free surface in TPA-stimulated cells. This TPA-induced localization was inhibited following treatment with mβCD to remove cholesterol (Fig. 9). Consequently, cholesterol depletion may affect the ability of ARF6 to localize activated Rac1 to the plasma membrane. We also transfected A431 cells with a dominant-negative ARF6 mutant to investigate whether this would affect the TPA-induced Rac1 localization and actin distribution. However, these experiments were not conclusive: in some transfected cells mutant ARF6 seemed to inhibit downstream effects whereas in others this was not clear, a finding that may be due to the high endogenous level of ARF6 in A431 cells (J. G. Donaldson, personal communication) (Donaldson and Radhakrishna, 2001).

Discussion

The results presented here indicate that cholesterol is necessary for plasma membrane localization of activated Rac1, actin reorganization and membrane ruffling, all prerequisites for macropinocytosis. Macropinocytosis can be induced in A431 cells by treatment with the phorbol ester TPA or the growth factor EGF, and can be quantified as an increased uptake of ricin (Sandvig and van Deurs, 1990). As shown here, this stimulated increase in ricin endocytosis was inhibited following extraction of cholesterol from the plasma membrane. The possibility existed that cholesterol was essential for the TPA-induced stimulation of PKC. However, cholesterol depletion prior to TPA-stimulation did not affect the activity of PKC. Importantly, electron microscopy showed that extraction of cholesterol inhibited the TPA-stimulated macropinocytosis of HRP at the plasma membrane. Furthermore, confocal microscopy showed that actin reorganization at the free surface following TPA-stimulation, a prerequisite for membrane ruffling (Swanson and Watts, 1995; Ridley, 1994), was perturbed in cholesterol-depleted cells. The inhibition was not due to side effects of mβCD as neither mβCD/chol nor a 1:1 mixture of mβCD and mβCD/chol (which does not affect the cellular cholesterol content) inhibited the TPA-stimulated actin reorganization, formation of membrane ruffles and increased uptake of ricin. Thus, cholesterol is essential for the reorganization of actin filaments at the cell periphery normally seen after addition of TPA.

Cholesterol has been found to be required for the membrane localization of several proteins (Oliferenko et al., 1999; Maekawa et al., 1999; Thiele et al., 2000). Interestingly, confocal microscopy showed that the TPA-stimulated localization of Rac1 to the cell periphery was inhibited in

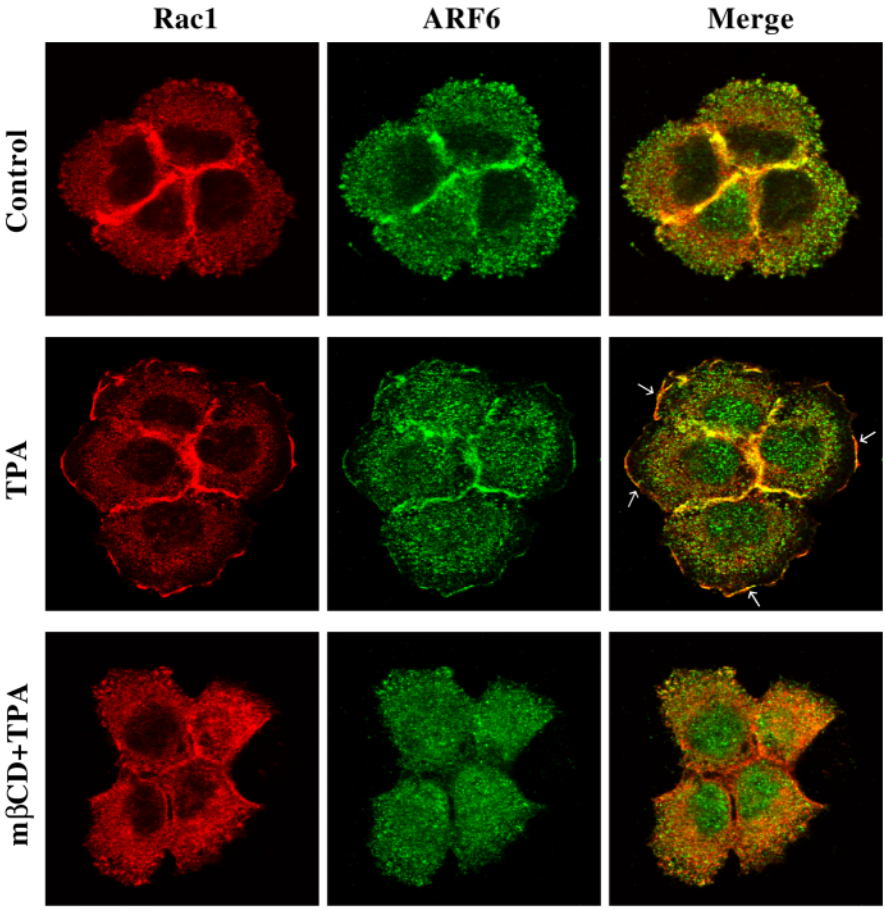

Fig. 9. Removal of cholesterol affects the TPA-induced localization of ARF6 with Rac1 at the free surface of the cells. Serum-starved cells were untreated or incubated with or without 5 mM mβCD for 30 minutes at 37°C prior to addition of 0.1 μM TPA. After 10 minutes at 37°C the cells were fixed, permeabilized and stained with antibodies to visualize endogenous Rac1 and ARF6. Arrows indicate the localization of both Rac1 and ARF6 to the free surface where ruffling and macropinocytosis take place.

cholesterol-depleted cells. Furthermore, localization of membrane-associated Rac1 to the plasma membrane (following TPA-stimulation) was also changed by cholesterol depletion. These results are in agreement with recent findings showing that Rac1 is localized in raft domains (Michaely et al., 1999; Kumanogoh et al., 2001), and can be partially solubilized following treatment with mβCD (Kumanogoh et al., 2001). Following stimulation with phorbol esters the small GTPase Rac1 is normally activated and localized to the plasma membrane where it induces actin reorganization and subsequent membrane ruffling (Ridley et al., 1992). As shown here, the TPA-stimulated activation of Rac1 was not inhibited by decreased cellular cholesterol levels. Rather, treatment with mβCD increased the TPA-stimulated activation of Rac1 by nearly 70%. The reason for this increase is not obvious, but could be due to the lack of membrane localization and a membrane-dependent or membrane-localized GTPase activity. Thus, the inhibition of membrane ruffling following cholesterol depletion was not due to prevention of Rac1 activation, but seems to be due to lack of membrane localization of GTP-bound Rac1.

Cholesterol depletion might lead to a redistribution of other lipids required for the formation of ruffles. Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] has been detected in cholesterol-enriched microdomains (Pike and Casey, 1996), and such a compartmentalization would be lost following treatment with mβCD (Pike and Miller, 1998). Accumulation of PtdIns(4,5)P_2 at the plasma membrane was found to precede the formation of ruffles prior to macropinocytosis (Tall et al., 2000), and it has been suggested that the local concentration of this lipid promotes the recruitment of proteins necessary for actin anchorage and reorganization (Tall et al., 2000; Botelho et al., 2000). However, accumulation of PtdIns(4,5)P_2 seems to occur downstream of membrane recruitment of Rac1 (Tolias et al., 2000). It was recently shown that activated Rac1 stimulates PtdIns(4,5)P_2 synthesis through binding to phosphatidylinositol-4-phosphate-5-kinase α [PtdIns(4)P5-kinase α] resulting in actin reorganization (Tolias et al., 1995; Tolias et al., 2000). As cholesterol depletion inhibits the localization of activated Rac1 to the plasma membrane this might prevent stimulation of PtdIns(4,5)P_2 synthesis required for actin reorganization and subsequent membrane ruffling at the plasma membrane. This is in agreement with our findings that actin reorganization at the cell periphery following TPA-stimulation was perturbed in cholesterol-depleted cells.

Although cholesterol-rich membrane domains might be required for the localization of activated Rac1 at the plasma membrane, such domains may not be sufficient. Cholesterol could be required for an indirect binding of Rac1 to the plasma membrane through protein-protein interactions. Several proteins have been found to bind directly to the activated Rac1 and participate in the ruffling response (Van Aelst et al., 1996; Di Cesare et al., 2000; Hansen and Nelson, 2001). One protein that might be important for Rac1 function is ARF6. ARF6 belongs to a protein family that function as regulators of membrane traffic (Moss and Vaughan, 1998). It has been suggested that ARF6 is required for the localization of activated Rac1 to the plasma membrane (Radhakrishna et al., 1999; Zhang et al., 1999). Interestingly, we found that cholesterol depletion inhibited the TPA-induced colocalization of ARF6 and Rac1 at the free surface of the cells. Although this does not demonstrate that ARF6 is involved in the response studied here, cholesterol-dependent binding of ARF6 might be required for localization of activated Rac1 to the plasma membrane. This could explain why we were unable to demonstrate a cholesterol-dependent binding of Rac1 to liposomes. Together our results suggest that cholesterol is necessary for the membrane association of the small GTPase Rac1 required for membrane ruffling and macropinocytosis.

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