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Localized endocytosis in tobacco pollen tubes: visualisation and dynamics of membrane retrieval by a fluorescent phospholipid

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Abstract Two modes of endocytosis are known to occur in eucaryotic cells: fluid phase and receptor-mediated endocytosis. Fluid-phase endocytosis in plant cells resembles the retrieval of excess plasma membrane material previously incorporated by exocytosis. Pollen tubes need to carry out strong membrane retrieval due to their fast polar tip growth. Plasma membrane labelling of pollen tubes, grown in suspension, was achieved by the incorporation of a fluorescently modified phospholipid, 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine (20 μM) and measured with a confocal laser-scanning microscope. Time course experiments revealed a highly localised and relatively fast plasma membrane retrieval below the tip within the first 5 min after phospholipid application. The retrieved fluorescent plasma membrane was quickly re-integrated into parts of the endomembrane pool and then redistributed to the pollen tube base and very tip of the apex, with the exception of the cortical endoplasmic reticulum (ER) and the mitochondria even after 1-h incubation period. Low temperature (10°C) and the actin filament depolymerizing cytochalasin D (2 μM) completely abolished plasma membrane retrieval, whereas the microtubule destabilizing herbicide oryzalin (1 μM) had no effect. Our results provide strong support for a highly localised endocytotic pathway in tobacco pollen tubes. Passive uptake of bis-Bodipy FL C_{11}-phosphocholine by mere penetration can be excluded. It is a valuable alternative to the styryl dyes often used in endocytotic studies, and may also be used to follow lipid turnover because membrane flow of labelled membranes occurs apparently not in a default manner as ascertained by its fast distribution.

Keywords Endocytosis · Bis-Bodipy FL C_{11}-phosphocholine · Pollen tube · Phospholipid

Abbreviations

bis-Bodipy FL C_{11}-phosphocholine	(1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine
β-Bodipy 581/591C_5 HPC	2-(4,4-difluoro-5-(4-phenyl 1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine
$DiOC_6(3)$	3,3-dihexyloxacarbocyanine iodine
FM4-64	*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridium dibromide
mitotracker RedCMXRos	8-(4′-chloromethyl)phenyl-2,3,5,6,11, 12, 14,15-octahydro-1H, 4H, 10H13H-diquinolizino-8H-xanthylium chloride

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Introduction

Two modes of uptake of extracellular material occur in eucaryotic cells, fluid phase and receptor-mediated endocytosis (Robinson 1996; Holstein 2002; Samaj et al. 2004). Receptor-mediated internalisation of extracellular substances into plant cells is still looked at with scepticism although recent studies indicate its occurrence in plant cells (Bahaji et al. 2001; Baluska et al. 2002; Ortiz-Zapater et al. 2006). Fluid-phase endocytosis or membrane retrieval seems to be an indispensable consequence in fast-growing systems such as the tip-growing pollen tubes, root hairs, or moss and fern caulonema tip cells in order to balance the rapid plasma membrane expansion due to the high rate of secretion (Hawes et al. 1995, Battey et al. 1999). A physiologically important example of endocytosis in plants is the finding that auxin transport is regulated by the balance of endo- and exocytosis of the PIN proteins (Friml et al. 2002; Paciorek et al. 2005).

Endocytosis is characterized by a typical sequence of events, basically the molecular preparation of the site, its invagination, followed by budding-off of clathrin-coated vesicles, and their incorporation into the intracellular membrane transport (Marsh and McMahon 1999; Holstein 2002). The first strong ultrastructural support for the occurrence of clathrin-involved endocytosis in plant cells came from studies employing the dry cleaving technique to visualise coated pits at the cytosolic plasma membrane face and the appearance of coated vesicles in its vicinity (Emons and Traas 1986). Several proteins involved in the process of clathrin-mediated endocytosis, previously well characterised in animal and yeast cells (Brodski et al. 2001) are also active in plant cells, such as clathrin heavy and light chain (Blackbourn and Jackson 1996; Scheele and Holstein 2002), adaptins (Barth and Holstein et al. 2004), and small proteins of the GTPase family (Ueda et al. 2001).

First attempts to visualise directly endocytosis or plasma membrane retrieval in plant cells were undertaken by ultrastructural means (Galway et al. 1993) or employing markers as fluorochromes which, recently, have been reviewed critically by Aniento and Robinson (2005). Fluorochromes as Lucifer yellow or styryls are favourable in contrast to fluorescently labelled dextrans or proteins because due to their molecular size they can penetrate the cell wall easier and faster. The amphiphilic styryl fluorochromes such as FM4-64 (Betz et al. 1996; Fischer-Parton et al. 2000; Kubitscheck et al. 2000; Parton et al. 2001; Atkinson et al. 2002; Parton et al. 2003; Bolte et al. 2004) or FM1-43 (Emans et al. 2002; Camacho and Malhó 2003; Bolte et al. 2004) became preferred tools for endocytotic studies. Both styryl fluorochromes attach to the outer bilayer of the plasma membrane, which becomes retrieved in a default manner to the vacuolar membrane because the dyes are hardly metabolised (Vida and Emr 1995). Grebe et al. (2003) investigated the intracellular transport of a fluorescent lipid in *Arabidopsis* by following membrane trafficking with the sterol-binding antibiotic filipin.

Here, we report on the highly localised endocytosis in tobacco pollen tubes by employing the fluorescently-modified phospholipid, bis-BODIPY FL C_{11}-PC (1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine) as marker and show the time course of endocytotic membrane retrieval and the role of cytoskeletal elements, actin filaments or microtubules. Our results indicate that this fluorescent phospholipid obviously is not retrieved by a default pathway and it might be useful for studies of membrane turnover in early endocytotic compartments.

Materials and methods

Tobacco pollen tubes (*Nicotiana sylvestris*) are characterized by relative fast growth and, therefore, they appeared very suitable for endocytotic studies. Pollen grains were allowed to germinate for 2 h in the growth medium as described by Kristen and Kappler (1995) before the fluorescent membrane marker bis-Bodipy FL C_{11}-PC (20 μM, excitation 488 nm, Mobitec, Göttingen, Germany) was administered from a stock solution (2 mM dissolved in dimethylsulfoxide). When appropriate, we used the closely related phospholipid *β*-Bodipy 581/591C_5 HPC (20 μM, excitation 548 nm) as red fluorescent marker phospholipid. The experimental concentration of the solvent was kept less than 1% shown to be without effect on tobacco pollen tube growth (Kristen and Kappler 1995). For quantification, the fluorescence of the plasma membrane and its internalised parts were measured with a confocal laser-scanning microscope equipped with an Argon/Krypton laser (TCS 4D, Leica Microsystems, Bensheim, Germany) by taking optical sections (about 0.75 μm thick) through the apex region of a pollen tube. The stack of sections of the appropriate regions was processed to a single image and the relative fluorescence intensity was calculated by the built-in analytic software of the TCS 4D microscope. The actin filament inhibitor cytochalasin D (2 μM, Sigma Chemicals, Munich, Germany) or the microtubule disassembling herbicide oryzalin (1 μM, gift of Elli Lilly, Indianapolis, USA) were added to the growing pollen tubes 1.5 h after germination, followed by the application of bis-BODIPY FL C_{11}-PC (20 μM) after another 30 min.

The endocytotic plasma membrane retrieval at low (10°C) or at regular growth temperature (27°C) was measured during the recovery from a low-temperature treatment. Pollen grains germinated for 30 min at 27°C before being transferred to low temperature for about 2 h. Then the pollen tube suspension was split into two parts, part A kept at 10°C and part B transferred to 27°C. To allow a straight comparison between endocytosis at low temperature and during recovery at regular growth temperature the marker fluorochrome bis-BODIPY FL C_{11}-PC was administered to suspension A 8 min before transferring B to 27°C. Before marker fluorochrome addition suspension B was allowed to recover for 30 min. First samples were taken in both cases 30 s after fluorochrome addition. All quantitative data obtained are from triple samples of each experiment, measuring at least 30 pollen tubes per sample. The fluorescence intensities determined from bis-BODIPY FL C_{11}-PC, resembling the retrieved plasma membrane, were processed with the software Origin (Microsoft).

Mitochondria were visualized using the mitotracker fluorochrome RedCMXRos (0.2 μM) (Molecular Probes, Netherlands), and the endoplasmic reticulum (ER) with $DiOC_6(3)$ (5 μg/mL, Quader et al. 1989).

For clathrin localisation by immunogold-labelling pollen tubes were fixed with paraformaldehyde (2%) and glutaraldehyde (0.1%) on ice (4°C) in Pipes-buffer (50 mM, pH 6.8) for 2 h, after thorough rinsing with cold buffer, dehydrated in a graded ethanol series, including a postfixation step with OsO_4 (0.25%) in 30% ethanol overnight at 4°C, embedded in LR White (Plano, Marburg, Germany) after transferring the samples to room temperature, and finally polymerised at 45°C for 48 h. Sections were cut with an Ultracut E (Leica, Bensheim, Germany), incubated with blocking buffer (Pipes-buffer, 50 mM, pH 6.8) containing 1% bovine serum albumin and 0.2% acidic bovine serum albumin for 30 min followed by the first antibody dissolved in the blocking buffer (a rabbit polyclonal antibody against the heavy chain of pea clathrin, gift of D.G. Robinson, Heidelberg, Germany) for 1 h and the second gold-labelled antibody also for 1 h at room temperature. For control samples, the first antibody was replaced by rabbit pre immune serum. Specimens were observed with a LEO 906 electron microscope (Zeiss, Oberkochen, Germany) equipped with a digital camera (794 MSC Gatan, Munich, Germany).

Results

The uptake of the fluorescent phospholipid marker was rapid enough to visualise it after the application to tobacco pollen tubes already within the first minute. Measurements earlier than 30–45 s after fluorochrome application were not possible because of the necessary handling time. The first image, acquired after 50 s revealed a slight bis-BODIPY FL C_{11}-PC fluorescence within a highly confined area in the sub-apex of the growing pollen tube which steadily and strikingly increased during the early period of measurements (Fig. 1). After about 6 min this ribbon-like fluorescent area in the tube sub-apex began to extend to the tube shank (Fig. 1d). Extending the incubation with the fluorochrome to about 10 min and longer, fluorescent vesicles became recognizable, moving rapidly in the centre of the pollen tube to basipetal regions (Fig. 2), which is in agreement with earlier reports stating that vesicle movement to the base of pollen tubes occurs in its centre along actin filaments (Cai et al. 2000). The rapidly moving fluorescent vesicles were visible in an unobstructed way at best in vigorously growing pollen tubes. The very tip of the pollen tube apex, the so-called clear zone, was still not labelled even after 10 min (Figs. 2, 3).

The viability of the pollen tubes was not affected by the lipid marker even after prolonged treatment time (24 h and longer) as deduced from observations showing intracellular movement of bis-BODIPY FL C_{11}-PC-labelled compartments. A distinct, vigorously trafficking compartment became strongly labelled by the phospholipid after about 15–20 min. These structures do not resemble mitochondria as revealed by double-labelling experiments because they are not recognized by a specific mitotracker (see ESM: S1). In order to examine if bis-BODIPY FL C_{11}-PC possibly redistributes into the ER we performed double-labelling experiments, staining the ER with the cationic green fluorescent dye $DiOC_6(3)$ (Quader et al. 1989) and employing a red fluorescent bis-Bodipy FL C_{11}-phosphocholine derivative as endocytosis marker (Fig. 4; see online version for coloured image). The double-staining pattern reveals that parts of the ER in the centre of the pollen tubes harbours both the red fluorescent phospholipid and the green fluorescent $DiOC_6(3)$ resulting in yellow fluorescent ER, whereas mitochondria, amyloplasts, and the cortical ER only show the green $DiOC_6(3)$ fluorescence (Fig. 4; see online version for coloured image).

Plasma membrane internalisation involves clathrin-coated vesicles (Holstein 2002). Immunocytochemical gold-labelling experiments indicated a relatively dense clathrin distribution along the plasma membrane in the zone of early bis-BODIPY FL C_{11}-PC-labelling and also in the cytoplasm of the pollen tube (Fig. 5). Plasma membrane labelling becomes evidently diminished with increasing distance from that zone, by about 50% or more, whereas the labelling density of the cytoplasm remained similar.

Bis-BODIPY FL C_{11}-PC internalisation during low-temperature treatment and in the presence of the actin filament inhibitor cytochalasin D was measured to exclude

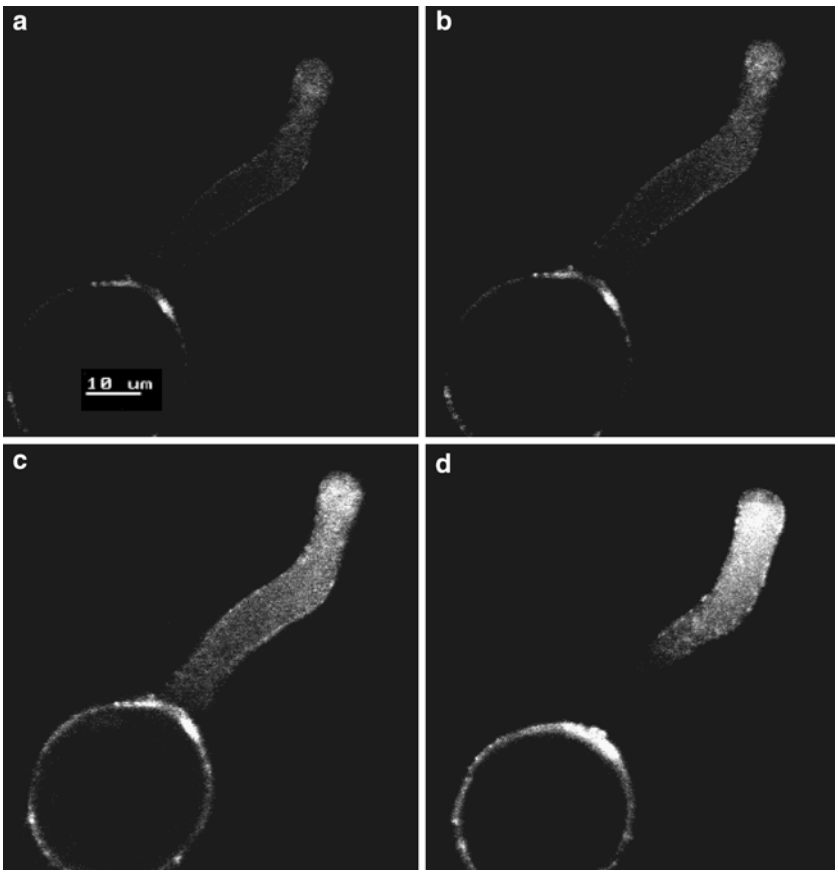

Fig. 1 Time course of the endocytotic uptake of fluorescent bis-BODIPY FL C_{11}-PC into a tobacco pollen tube visualised by confocal laser-scanning microscopy. Images were taken in the centre of a pollen tube apex by a single scan after **a** 50 s, **b** 120 s, **c** 210 s, and **d** 330 s. Note the highly localised uptake of the fluorescent lipid in the apex of the tube shortly below its clear zone. The autofluorescence of sporopollenin marks the edge of the pollen grain

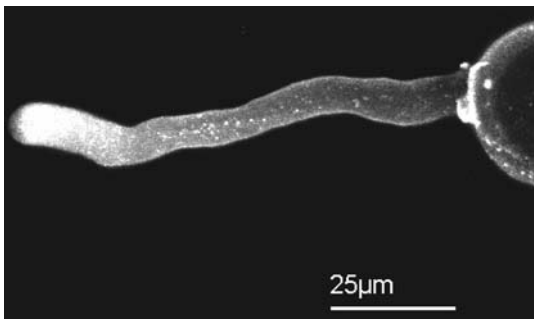

Fig. 2 Uptake of the fluorescent lipid bis-BODIPY FL C_{11}-PC into a tobacco pollen tube visualized by confocal laser-scanning microscopy. The image was taken in the centre of a pollen tube by a single scan 12 min after fluorochrome application. Note the distinct region of membrane retrieval, strongly fluorescent compartments in the centre of the pollen tube reflecting their transport to the tube base, and that the very apex of the tip region, the clear zone, is yet only weakly labelled by the fluorescent marker

passive uptake. Lowering the growth temperature from 27 to 10°C drastically reduces, at least by 90%, the growth rate of tobacco pollen tubes and thus secretion, but pollen tubes regained normal growth rates, about 1 µm/min as estimated by the pollen tube growth test, after transferring the suspension back to the regular growth temperature when the low temperature treatment did not extend a period of 6 h (Kristen and Kappler 1995). The observed internalisation of the fluorochrome bis-BODIPY FL C_{11}-PC at low temperature perfectly corresponds with these growth parameters. A relatively low background level remained at 10°C, which recovers after transferring the pollen tubes back to the regular growth temperature. In Fig. 6, the recovery of phospholipid endocytosis is shown after pollen tubes were allowed to adapt for 30 min to the growth temperature of 27°C prior to the addition of the fluorochrome. Within 8 min, the fluorescence in the pollen tube apex increased by about twofold (Fig. 6). The observed uptake pattern during recovery resembles that of untreated pollen tubes (Fig. 7).

Endocytosis involves a selective cytoskeletal-dependent retrograde vesicle transport necessary to incorporate the original plasma membrane lipids and proteins retrieved via clathrin-coated pits and coated vesicles into the intracellular membrane pool (Robinson 1996; Holstein 2002). Any interference by highly selective inhibitors of the cytoskeletal-dependent vesicle transport should distinguish if the fluorescent phospholipid marker is taken up in a passive or in an active manner (Fig. 7). In these experiments pollen grains germinated for 2 h prior to the inhibitor treatment for

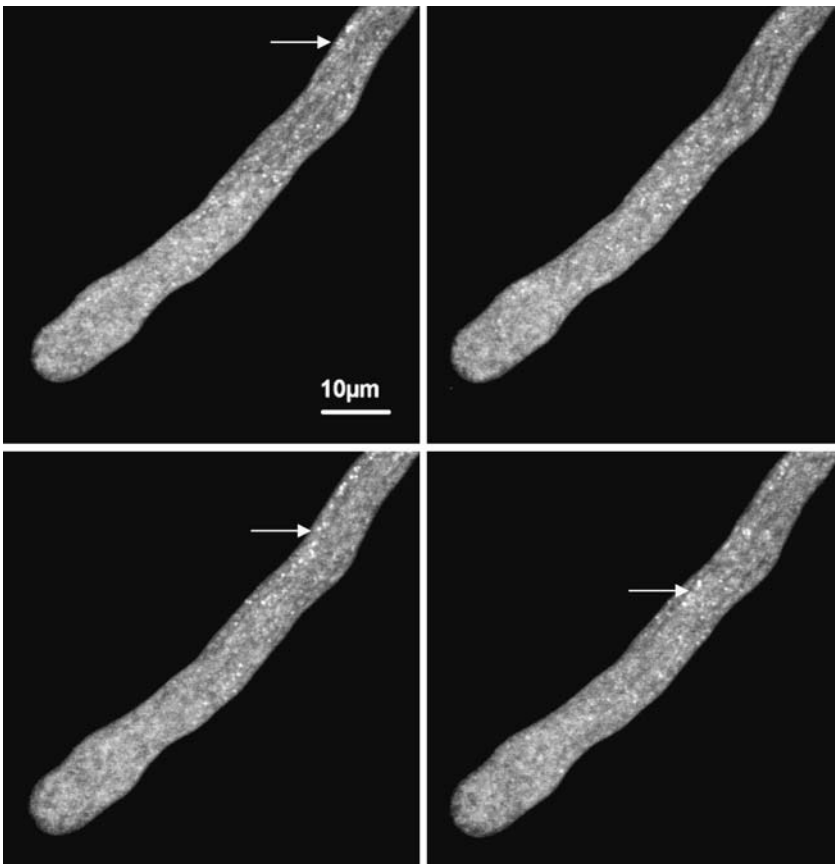

Fig. 3 Endocytotic plasma membrane retrieval: distribution of the fluorescent marker phospholipid bis-Bodipy FL C_{11}-phosphocholine into endomembranes after 30 min. Images 1, 5, 10, and 15 are displayed of a time sequence of 15 images taken with a confocal scanning laser microscope without moving the microscopical stage neither in z- nor in xy-direction throughout the acquisition time (3 min). Note the changes in the distribution of brightly fluorescent large vesicular compartments (*arrows*), which could represent endosomes or lipid bodies, whereas the dense, but less strongly fluorescent endomembranes represent, most likely, the endoplasmic reticulum. Mitochondria are not distinguishable in the *black* and *white images* but are visible as small fast moving compartments in the supplemented movie (see ESM: S1)

30 min and then the marker fluorochrome was applied. Bis-BODIPY FL C_{11}-PC internalisation was not inhibited in the presence of the microtubule destabilizing herbicide oryzalin (5 µM) but became completely inhibited by the actin filament-depolymerizing agent cytochalasin D (2 µM) (Fig. 7).

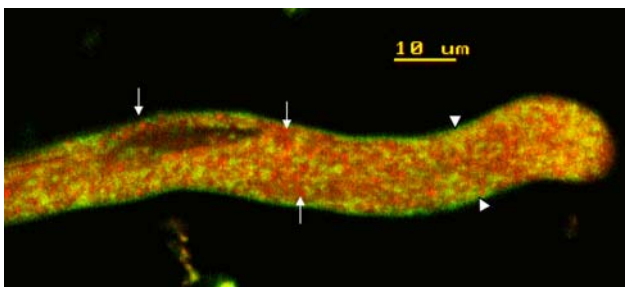

Fig. 4 Staining of the endocytotic pathway with the phospholipid *β*-Bodipy 581/591C_5 HPC (*red fluorescence*) for 30 min and the endoplasmic reticulum with $DiOC_6$ for 3 min. Partly the *red fluorescent* phospholipid and $DiOC_6$(3) co-localize (*yellow fluorescence*) indicating that the phospholipid became incorporated into parts of the endoplasmic reticulum. No co-localisation of the fluorescent phospholipid and $DiOC_6$ is detectable in the cortical endoplasmic reticulum (green $DiOC_6$ fluorescence, *arrow heads*) and the strong fluorescent compartments (red phospholipid fluorescence, *arrows*). (Coloured image: see online version)

Discussion

Endocytotic studies involving a fluorescent marker face three major problems; first that the marker substance may simply penetrate the plasma membrane, second that it might be toxic, and third that its internalisation is rather slow. These drawbacks do not apply to endocytotic membrane retrieval in tip growing tobacco pollen tubes employing the fluorescently modified phospholipid, bis-BODIPY FL C_{11}-PC.

Pollen tube growth is characterised by an excessive directional flow of secretory vesicles which deliver cell wall material to the tube apex and cause a strong expansion of the plasma membrane surface area due to the oversupply of fusing secretory vesicles (Geitman and Emons 2000; Hepler et al. 2001). Membrane internalisation is one possibility to balance the excessive plasma membrane expansion. Derksen et al. (1995a) estimated for tobacco pollen tubes that the delivery of secretory vesicles mounts to about 430-μm^2 new plasma membrane area per minute but only about 50 μm^2 are necessary for a tube elongation rate of 2 µm/min. Their calculation regarding the area of the plasma membrane occupied by coated pits indicates that the major part of the excess membrane is retrieved by a coated pit-mediated mechanism. Coated pits were mainly

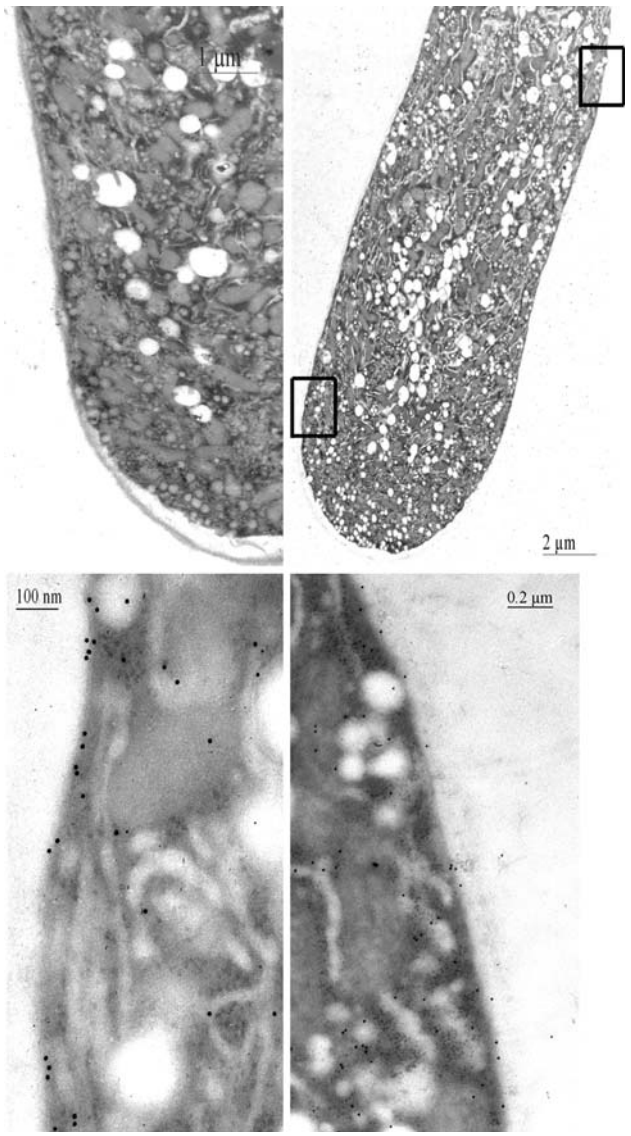

Fig. 5 Clathrin localisation in growing tobacco pollen tubes by the immunogold-labelling technique. *Upper left* control, pre immune serum instead first antibody, *Upper right* overview of an apical zone and the upper shank, *Lower left*: detail of the cortex area following the clear zone (*lower rectangle*), distinctly labelled plasma membrane, but also the cytoplasm. *Lower right*: detail of a cortex area (*upper rectangle*), about 20 µm from the tip. Reduced labelling density at the plasma membrane by about 50%

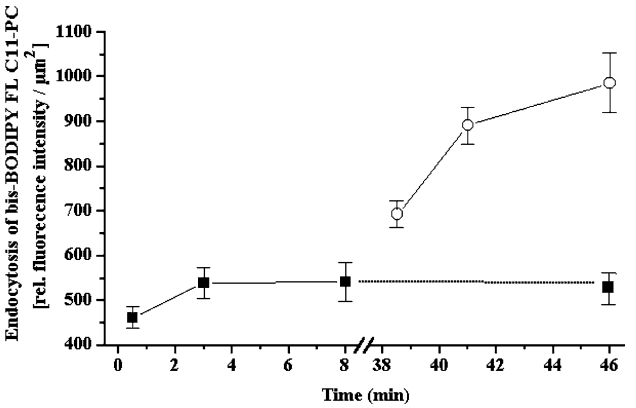

Fig. 6 Temperature dependence of endocytotic plasma membrane retrieval into tobacco pollen tubes. Pollen grains germinated for 30 min at 27°C before transference to low temperature (10°C). The internalisation of bis-BODIPY FL C_{11}-PC by tobacco pollen tubes was quantified by confocal fluorescence microscopy. Low temperature (10°C, *squares*): measurements were started 8 min (t = 0) before transferring one part of the suspension back to regular growth temperature (27°C); the first sample was taken after 30 s, the last one after 2 h 46 min at 10°C (t = 46). Regular growth temperature (27°C, *circles*): the other part of pollen tube suspension was transferred to 27°C for 30 min (after culturing for 2 h at 10°C), and then the fluorochrome was administered (t = 38) and the first sample was again taken after 30 s. Fluorochrome internalisation is shown after application for the first 8 min

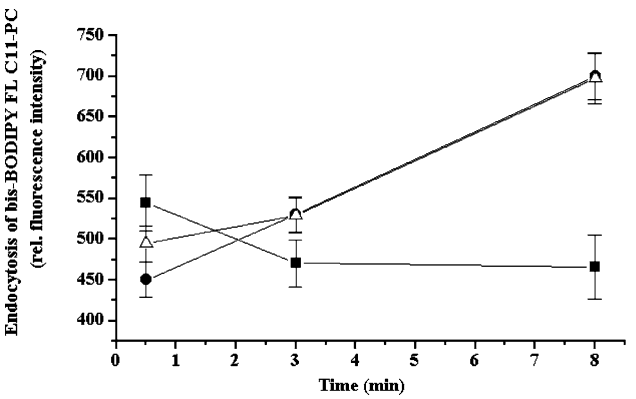

Fig. 7 Endocytotic plasma membrane retrieval into tobacco pollen tubes in the presence of cytoskeletal inhibitors. Time course of bis-BODIPY FL C_{11}-PC internalisation by untreated pollen tubes (*filled circles*), in the presence of the microtubule destabilizing herbicide oryzalin (1 µM, *open triangles*), and in the presence of the the actin filament inhibitor cytochalasin D (2 µM, *filled squares*). The inhibitor treatment started 30 min before fluorochrome application at t = 0. First samples were taken after 30 s

observed in a zone below the pollen tube apex (Emons and Traas 1986; Derksen et al. 1995b), which corresponds to the zone of highest fluorochrome uptake.

A mere penetration phenomenon regarding the uptake of the extracellularly-applied bis-BODIPY FL C_{11}-PC can be excluded because it largely enters the pollen tube in a distinct region, is inhibited by low temperature treatment, and by interference with actin filament organization. The region of uptake coincides with the site of clathrin localisation at the plasma membrane as shown by immunofluorescence technique for *L. longiflorum* (Blackbourn and Jackson 1996), or as indicated by immunogold-labelling in this study. Actin filaments are the track for organelle movement in higher plant cells and play a pivotal role in the formation and maintenance of pollen tube apex by guiding the flow of the secreting vesicles in connection with the motor protein myosin (Geitman and Emons 2000; Vidali and Hepler

2001). Bis- BODIPY FL C_{11}-PC uptake and intracellular movement is prevented by both, low-temperature treatment and the actin filament disassembling agent cytochalasin D, whereas microtubules play no role. At low temperature intracellular actomyosin-dependent organelle movement is inhibited in onion epidermal cells although the actin filaments are not affected by the low temperature but, most likely, the motor protein myosin (Quader et al. 1989). Plasma membrane internalisation in tobacco pollen tubes thus depends on an unimpaired actomyosin system as shown for *L. longiflorum* pollen tubes (Parton et al. 2001) and *Arabidospis* root epidermis cells (Grebe et al. 2003).

Bis-BODIPY FL C_{11}-PC internalisation is fast enough to measure the time course of the membrane retrieval already within the first minutes after applying the fluorescent phospholipid. Uptake of Bodipy-labelled phosphatidic acid into periphal zones of the pollen tube was noticed after 5–15 min (Potocky et al. 2003), and that of Bodipy-labelled Brefeldin A even within the first minute of application (Parton et al. 2001). These Bodipy-labelled molecules may be suitable tools for studies on particular aspects of endocytosis. Styryl fluorochromes (FM-dyes) immediately stained the plasma membrane but are particularly internalised by different cell types. Parton et al. (2001) recognized FM4-64 uptake within 1–2 min by *L. longiflorum* pollen tubes and Camacho and Malhó (2003) detected in the apical dome of *Agapanthus umbellatus* pollen FM1-43 incorporation within a few minutes. In cultured cells FM4-64 uptake became visible after about 5–10 min (Emans et al. 2002; Bolte et al. 2004) and in root epidermis cells sterol internalisation only after 10 min (Grebe et al. 2003).

The staining pattern by FM dyes differs from that of bis-BODIPY FL C_{11}-PC. FM dyes. They, at first, stained central parts of the clear zone, whereas this zone only became labelled after about 10 min by bis-BODIPY FL C_{11}-PC depending on the viability (growth rate) of the pollen tubes. After this period, vesicles of the pollen tube shank were already labelled, too, except the vacuolar membrane. Although proofs are still missing, FM4-64 is, most likely, directed via Golgi apparatus to the tonoplast in a default manner (Yano et al. 2004). The FM dyes are even under suspicion to be toxic (Bolte et al. 2004; Meckel et al. 2004). They have, hitherto, not been noticed to label the ER. Bis-BODIPY FL C_{11}-PC is unequivocally not toxic to pollen tubes because movement remains very vigorous even after a treatment for 24 h. It would quickly cease if the phospholipid would perturb cell metabolism (Geitman and Emons 2000). Cultured higher plant cells also exhibit no apparent metabolic harm after taking up bis-BODIPY FL C_{11}-PC (Chandra et al. 1996; Paul et al. 1998).

After 10–20 min, strongly fluorescent compartments became detectable through bis- BODIPY FL C_{11}-PC uptake (see ESM: S1). Their movement resembles that of mitochondria but because of their shape they might be endosomes, clustered dictyosomes (Boevink et al. 1998), or lipid bodies. Lipid bodies originate from the ER, and both ER and Golgi membranes harbour enzymes of phospholipid metabolism, so re-routing of internalised vesicles to these compartments could be feasible (Battey et al. 1999). Grebe et al. (2003) showed that in *Arabidopsis* root epidermis cells the internalised filipin fluorescent sterol marker accumulated in early endosomes, but neither in the ER nor in dictyosomes as indicated by experiments employing Brefeldin A or cytochalasin D. However, filipin routing should be strongly dependent on the presence of sterols, which predominantly reside in the plasma membrane, in the tonoplast, and in the outer envelope of chloroplasts (vom Dorp et al. 1990). Double-staining experiments using $DiOC_6(3)$ to stain the ER indicate that parts of the ER in the centre of the pollen tubes probably become labelled by the red fluorescent β-Bodipy 581/591C_5 HPC, substituting bis-BODIPY FL C_{11}-PC, but obviously not the cortical ER as distinguishable by the preserved green fluorescence of $DiOC_6(3)$ (see online version for coloured image). BODIPY-labelled phopholipids may partly become metabolised by phospholipase A activity (Paul et al. 1998) and this may lead to the observed labelling pattern. Consequently, the use of the fluorochrome may render possible particular studies on membrane lipid turnover during endocytosis.

In conclusion, our results show that the fluorescent phospholipid bis-BODIPY FL C_{11}-PC is a good marker for measuring the relatively fast, active, and highly localized endocytosis in tobacco pollen tubes. This phospholipid appears to be a valuable alternative to the often-used styryl dyes or the filipin sterol procedure (Aniento and Robinson (2005). Its distribution by retrograde and antrograde membrane flow seems less restricted and it, in addition, may be a useful tool to study the role of membrane lipids in secretion and endocytosis.

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