Protoplasma (1997) 199: 198–207

PROTOPLASMA

Uptake of Lucifer Yellow by plant cells in the presence of endocytotic inhibitors

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Received January 30, 1997
Accepted June 24, 1997

Summary. Lucifer Yellow (LY), a membrane-impermeant anion, was able to enter *Arabidopsis thaliana* cells. LY was taken up by fluid-phase endocytosis and a plasmalemmal anionic carrier mechanism. Both mechanisms were shown to be concentration-dependent. At 0.1 mg/ml, LY was mainly taken up via fluid-phase endocytosis and concentrated in vesicular-like structures. At a ten-fold higher concentration (1 mg/ml), a plasmalemmal anionic carrier system allowed LY uptake and its accumulation in the central vacuole by a vacuolar anionic transporter. Chloroquine, cytochalasin B, monensin, and phorbol-12-myristate-13-acetate (PMA) hindered LY endocytosis. Brefeldin A did not modify LY uptake. The probenecid-sensitive carrier uptake machinery showed sensitivity to chloroquine and PMA. Therefore the probenecid-sensitive transport mechanism seems to be complex and involve both acidification of a compartment and protein kinase C activity.

Keywords: *Arabidopsis thaliana*; Endocytosis; Endocytotic inhibitors; Lucifer Yellow carbohydrazide; Probenecid-inhibitable carrier.

Abbreviations: CH carbohydrazide; DMSO dimethylsulfoxide; LY Lucifer Yellow; MES 2-[N-morpholino]-ethanesulfonic acid; MS Murashige and Skoog's medium; PMA phorbol-12-myristate-13-acetate; NAA naphthalene acetic acid.

Introduction

Endocytosis is a process which permits the uptake of exogenous material by a cell. In animal cells, it drives the entry of macromolecules which cannot diffuse across the plasma membrane. Thus, hormones, neurotransmitters, vitamins, viruses, toxins, or bacteria can enter the animal cell this way. Endocytosis can be achieved by two different pathways. The first involves clathrin-caged vesicles with integral proteic receptors which bind extracellular ligands. The formation of clathrin-caged vesicles allows the entry of the ligand into the cell. Once clathrin is released, the vesicles fuse with a lysosomal compartment where the ligand is released from its receptor. The unbound ligand can then be delivered to the cytoplasm via a mechanism which is still poorly understood (Brown et al. 1991). The second endocytotic pathway, named potocytosis, involves the presence of proteins called caveolins which are localized over regions of the plasma membrane called caveolae. These regions contain numerous phosphatidyl-inositol-anchored receptors which can bind small ligands. Formation of closed caveolae allows acidification of the compartment without fusion to lysosome. Acidification permits dissociation of the receptor-ligand complex, the latter being subsequently exported to the cytoplasm by a transporter (Rothberg et al. 1990).

Because of the occurrence of very high turgor pressures in plant cells, endocytosis was long considered impossible on thermodynamic grounds (Cram 1980). Although molecular approaches to endocytosis remained poor, ultramicroscopical studies showed that endocytosis exists in plant as well as in animal cells (Hübner et al. 1985, Galway et al. 1993). Clathrin-coated vesicles were shown to occur in plants and the biochemical characterization of plant clathrins has been reviewed (Coleman et al. 1987). On the other hand, caveolae have not yet been discovered in plants. Nevertheless, noncoated pits have been observed in plants, suggesting that caveolae vesicles might exist in plants too.

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Several markers were used to elucidate endocytotic mechanisms in plant cells. As the mesh size of the wall permits diffusion of macromolecules less than 60,000 Da (Carpita et al. 1979), only small molecules can enter the cell wall to subsequently be taken up by any endocytosis process. Endocytosis has been characterized by the use of fluorescent membrane-impermeant anions such as Lucifer Yellow (LY) (Hillmer et al. 1989), heavy metals such as lead (Hübner et al. 1985), fluorescent dextrans (FITC-dextrans; Cole et al. 1990), nutrients (biotin; Horn et al. 1990), or elicitors (Horn et al. 1989).

As there is no suitable marker with which to follow or quantify endocytosis in plants (Galway et al. 1993), we tried to determine whether the impermeant fluorescent dye LY could be transported into plant cells by any endocytotic process. In many species LY was shown to enter plant cells via a probenecid-inhibitable anionic carrier which may be located at the cytoplasm–tonoplast interface and in the cytoplasmic membrane (Cole et al. 1991, Oparka et al. 1991). According to these authors, endocytosis was not the only way for the absorption of LY.

In order to investigate whether endocytosis was implied in LY uptake, several drugs, which have been shown to inhibit endocytosis in animal cells, were used (Dash and Moore 1993). Some of them, such as brefeldin A (Misumi et al. 1986), phorbol-12-myristate-13-acetate (PMA) (Smart et al. 1994), or cytochalasin B (Dash and Moore 1993), interact with the formation of endocytotic vesicles whereas others, such as chloroquine or monensin (Maxfield 1982), prompt acidification of the lysosomal compartment. In *Arabidopsis* cells, we show that endocytosis is the main mechanism for the uptake of a low concentration of LY, while its absorption is hindered by endocytotic inhibitors. We propose that uptake of LY is carried out at the plasma membrane interface via at least two mechanisms, according to LY concentration. The first is probenecid-independent and is a classical endocytosis mechanism, whereas the second is probenecid-inhibitable but must also involve protein kinase C activity and a pH-dependent mechanism. On the other hand, uptake at the vacuolar level was driven by a probenecid-inhibitable transporter.

Material and methods

Plant material and uptake assays

Arabidopsis thaliana C24 seeds were sterilised with 20 french chlorometric degrees sodium hypochloride : ethanol (1 : 9) solution. Seedlings were grown for three weeks on 2.5 mM MES (2-[N-morpholino]ethanesulfonic acid; Sigma Chemical Co., St. Louis, MO, U.S.A.) pH 5.8-buffered Murashige and Skoog's medium (MS; Murashige and Skoog 1962). Leaves were harvested and incubated for 24 h in MS liquid medium containing either 100 µg or 1 mg of LY (Sigma) per ml. Inhibitors were added aseptically to the autoclaved media. In order to disrupt the cells, leaves were washed three times with distilled water, dried, weighed and gently ground with a pestle and quartz in microfuges tubes. Insoluble material was removed by centrifugation at 15,000 **g** for 30 min (Sigma 2K15 centrifuge with nr. 12145 rotor) and filtration through Miracloth (Calbiochem-Novabiochem Co., La Jolla, CA, U.S.A.).

Fluorescence was measured with a fluorescence spectrophotometer Hitachi (excitation wavelength of LY 280 nm; emission wavelength of LY 540 nm).

Arabidopsis thaliana C24 suspension cells were sub-cultured every week in 2.5 mM MES, pH 5.8-buffered BS-Gamborg's medium (Gamborg et al. 1968) in the presence of 1 µM naphthalene acetic acid (NAA). Incubation of the cells was carried out by sub-culturing a 3-day-old culture in new medium containing LY. Endocytotic inhibitors were added aseptically in sterile media. After 48 h, cells were counted on a Nageotte cell, centrifuged (200 **g**; 10 min, 4 °C; Sigma 4K10 centrifuge with nr. 11140 swinging rotor) and the supernatant was removed. Cells were resuspended in cold water and three others centrifugation/resuspension steps were carried out. In order to remove the cell walls, cells were treated as described above.

Endocytotic markers and inhibitors

LY CH dipotassium salt (M.W. 521.6; Sigma) was dissolved in MS medium (2.5 mg/ml), filter sterilised, aliquoted and stored at –20 °C. For experiments using suspension cell cultures, stock solutions were in Gamborg's medium.

The endocytotic inhibitors used were brefeldin A, chloroquine, cytochalasin B, monensin, and phorbol-12-myristate-13-acetate (PMA; Sigma).

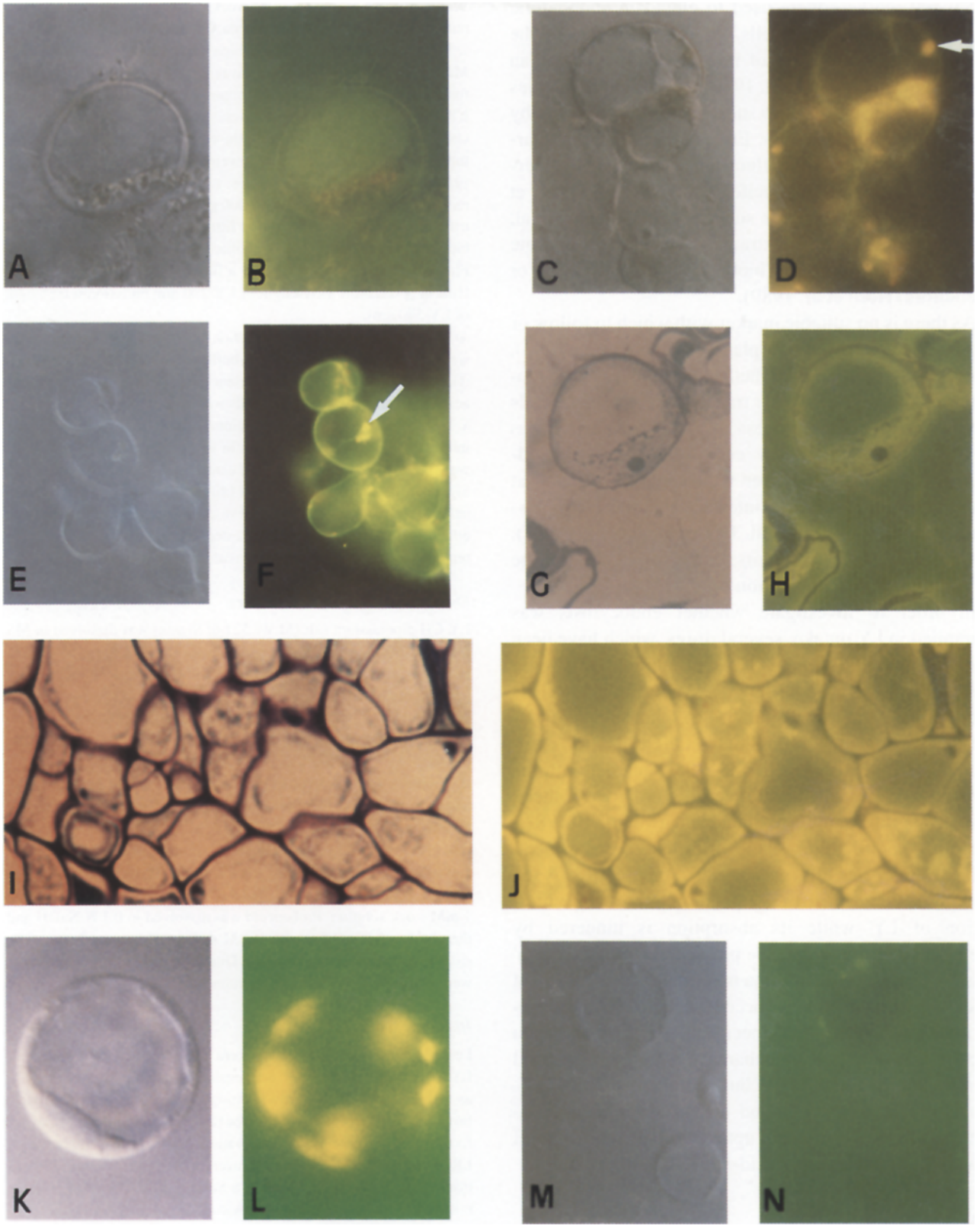
Brefeldin A was dissolved in methanol to a 1.25 mM stock solution and kept at 4 °C. Chloroquine was dissolved in MS medium to a 50 mM stock solution and filter sterilised. Cytochalasin B was dissolved in ethanol to a 20 mM stock solution. Monensin was dissolved in methanol to a 5 mM stock solution. PMA was dissolved in DMSO (Merck, Darmstadt, Federal Republic of Germany) to a 1 mM stock solution. Probenecid was dissolved in 0.1 N NaOH and then buffered to pH 5.8 with 0.1 M sodium phosphate buffer. The stock concentration was 100 mM. Dilutions of all of these inhibitors were performed in sterile MS medium.

Histological analysis

Leaves from 3-week-old plants were harvested and incubated with LY as previously described. Suspension cells were incubated in LY as well and embedded in low melting point agarose. A 3.7% paraformaldehyde fixation was preferred to a glutaraldehyde one to avoid fixative fluorescence. After dehydration, leaves were infiltrated with LR-White resin and polymerised overnight at 60 °C. Semi-thin sections (250 nm) were made using a Sorval MT-1 PorterBlum ultramicrotome and contrasted with low concentration of azure blue II.

Protoplast extraction

Leaves from 3-week-old plants were harvested and infiltrated with a solution containing 1% Onozuka R-10 Macerozyme (Kinki Yakult,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Ltd., Nishinomiya, Japan), 1% Onozuka R-10 cellulase (Kinki Yakult), 0.5 M mannitol, and MS. After a 12-h incubation time, leaves were shaked at 80 rpm during 30 min. The released protoplasts were filtered through a 50 μm mesh, concentrated by centrifugation (100 g, 10 min, 18 °C; Sigma 4K10 centrifuge with nr. 11140 swinging rotor) and incubated for 6 h with LY. Visualisation was performed on an BH-5 Olympus microscope (filter excitation wavelengths: 435 and 490 nm) after three washes with a pH 5.8 buffered 0.5 M mannitol MS solution.

Some leaves were harvested, assayed for LY uptake for 2 days, incubated in a solution of 1% Onozuka R-10 Macerozyme made up in MS medium. This treatment released isolated cells which could be readily observed in the microscope.

Results

Uptake of Lucifer Yellow

After a 24-h incubation in the presence of 1 mg LY per ml, the concentration generally used, both cells isolated from leaves and suspension culture cells, contained LY which accumulated in the central vacuole (Fig. 1 A, B). In the presence of 1 mM probenecid vacuolar accumulation was inhibited whilst fluorescence was detectable in the cytoplasm (Fig. 1 C, D). Thus, as in other plants, accumulation of LY in the vacuole was probably achieved by a tonoplastic probenecid-inhibitable anion transporter. Conversely, accumulation of LY in the cytoplasm seemed to be probenecid-insensitive.

In order to ascertain whether cytoplasmic LY uptake in *Arabidopsis* cells was probenecid-insensitive, attempts were made to disconnect the cytoplasmic LY uptake mechanism from the LY vacuolar uptake system which is probenecid-sensitive. Suspension cells of *Arabidopsis* were incubated in the presence of 0.1, 0.25, 0.5, 1 and 2 mg of LY per ml, with or without probenecid. Up to 0.25 mg/ml, uptake of LY was only slightly probenecid-sensitive (Fig. 2). At these concentrations, LY was localised in the cytoplasm but was not detectable in the vacuole (Fig. 1 E, F). Semi-

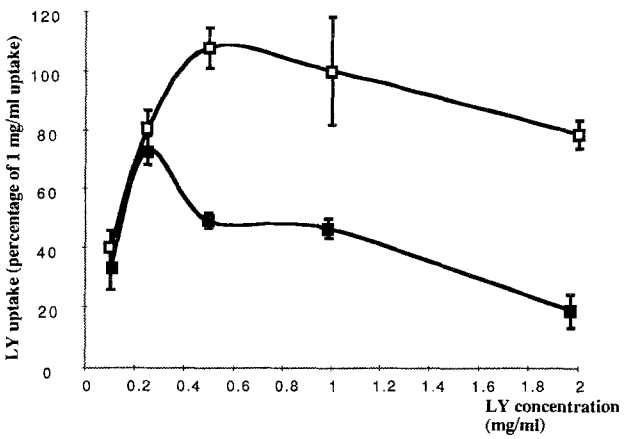

Fig. 2. Dose-dependent uptake of LY in the presence or absence of 1 mM probenecid. LY was provided at concentrations ranging from 0.1–2 mg/ml in the presence (■) or absence (□) of 1 mM probenecid. Values are means with SD of 3 independent assays

thin sections of cells incubated with 0.1 mg of LY per ml confirmed this (Fig. 1 G, H). Furthermore LY was concentrated in small vesicular-like structures which showed no detectable communications with the central vacuole (Fig. 1 I, J). Because a concentration of 0.1 mg of LY per ml permitted discrimination between the cytoplasmic and probenecid-inhibited uptake mechanisms, it was retained in further experiments.

LY was shown to accumulate in vesicular and tubular-like structures in protoplasts but not in cells of *Daucus carota* (Hilmer et al. 1989). Similarly LY was concentrated in vesicles in *Arabidopsis thaliana* protoplasts as well as in suspension cells. The presence of an extracellular proteinic component was required for uptake of LY at 0.1 mg/ml. In the presence of 100 ng of proteinase K per ml in the incubation medium, uptake of LY by the protoplasts was prevented (Fig. 1 M, N).

Fig. 1. A–N. Uptake of LY by *Arabidopsis thaliana* cells. Uptake experiments were carried out as described in Material and methods. A, C, E, K, and M are light micrographs of live cells observed with Nomarski interference contrast. G and I are light micrographs of 250 nm sections of fixed suspension-cells (G) or actively growing calluses contrasted with Azure blue II (I). B, D, F, H, J, L, and N are fluorescence light micrographs corresponding to A, C, E, G, I, K, and M respectively. **A** and **B** Uptake of LY at 1 mg/ml by suspension cells. The dye is concentrated in the central vacuole (× 640). **C** and **D** Uptake of LY at 1 mg/ml by actively growing suspension cells in the presence of 1 mM probenecid. Lucifer Yellow accumulation occurs only in the cytoplasm (× 640). **E** and **F** Uptake of LY at 0.1 mg/ml by actively growing suspension cells. The dye accumulates in the cytoplasm and is not detectable in the central vacuole (× 128). **G** and **H** Semi-thin section suspension cells incubated with 0.1 mg of LY per ml showing cytoplasmic dye accumulation (× 640). **I** and **J** Semi-thin section of actively growing calluses incubated with 0.1 mg of LY per ml. Dye accumulates in some vesicular structures other than the central one (× 1250). **K** and **L** Uptake of LY by *Arabidopsis* protoplasts. LY was not detectable in the central vacuole (× 1250). **M** and **N** Uptake of LY by *Arabidopsis* protoplasts in the presence of 100 ng proteinase K per ml (× 640)

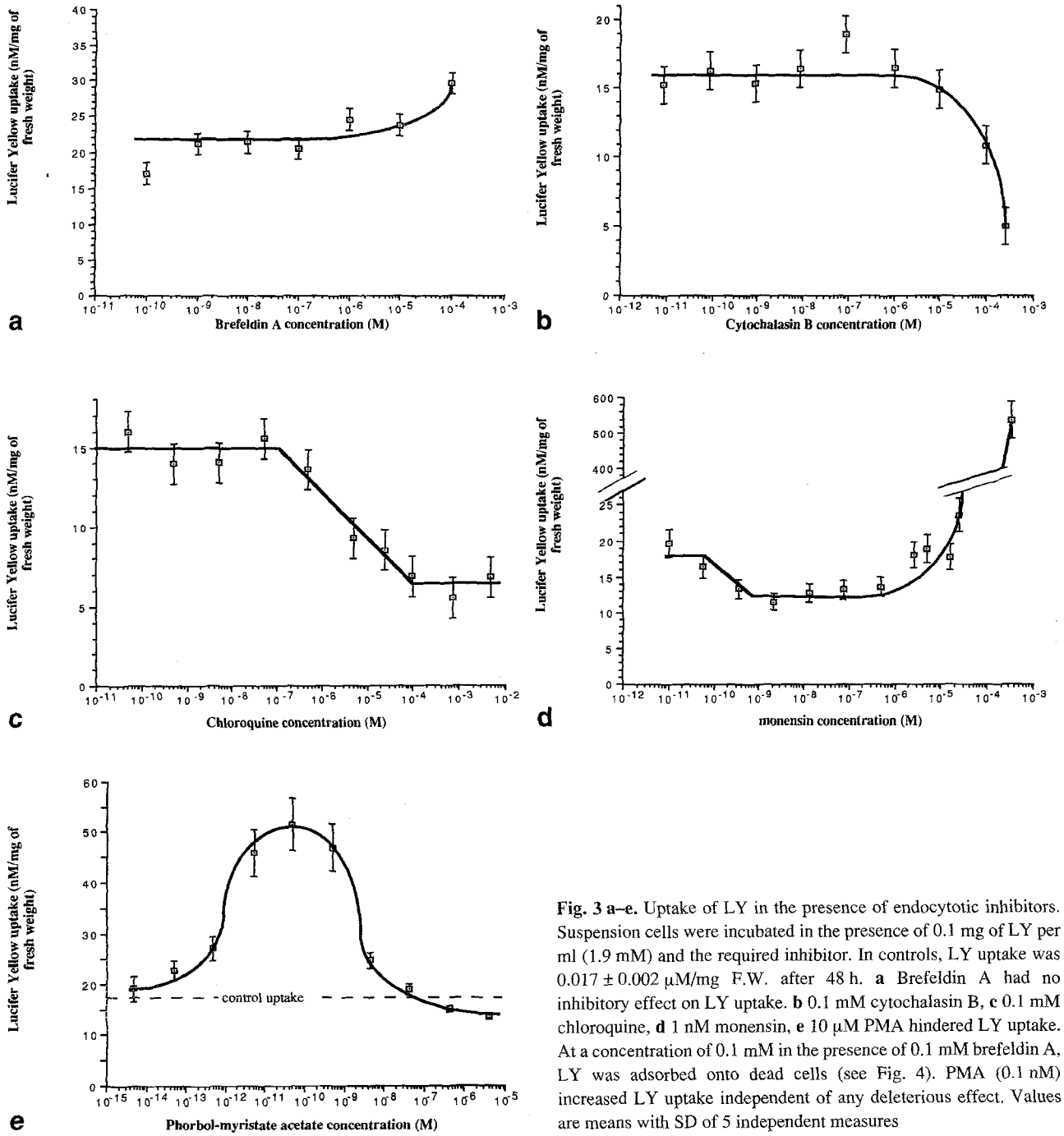

Fig. 3 a–e. Uptake of LY in the presence of endocytotic inhibitors. Suspension cells were incubated in the presence of 0.1 mg of LY per ml (1.9 mM) and the required inhibitor. In controls, LY uptake was 0.017 ± 0.002 μM/mg F.W. after 48 h. **a** Brefeldin A had no inhibitory effect on LY uptake. **b** 0.1 mM cytochalasin B, **c** 0.1 mM chloroquine, **d** 1 nM monensin, **e** 10 μM PMA hindered LY uptake. At a concentration of 0.1 mM in the presence of 0.1 mM brefeldin A, LY was adsorbed onto dead cells (see Fig. 4). PMA (0.1 nM) increased LY uptake independent of any deleterious effect. Values are means with SD of 5 independent measures

Effect of endocytotic inhibitor concentration on LY uptake

In order to evaluate to what extent endocytosis and an anion carrier were involved in the uptake of LY, endocytotic inhibitors were assayed in experiments using 0.1 mg of LY per ml (Fig. 3). As mentioned above, this concentration was chosen to focus on the uptake mechanism at the plasmalemma level.

All the inhibitors were assayed over a large concentration range with either foliar tissues (data not shown) or suspension cell cultures. Because these compounds might exert deleterious effects on cell viability, cellular growth rates were measured in their presence before LY uptake experiments were performed (Fig. 4). After an incubation time of 48 h, uptake of LY was measured according to Material and methods (Fig. 3).

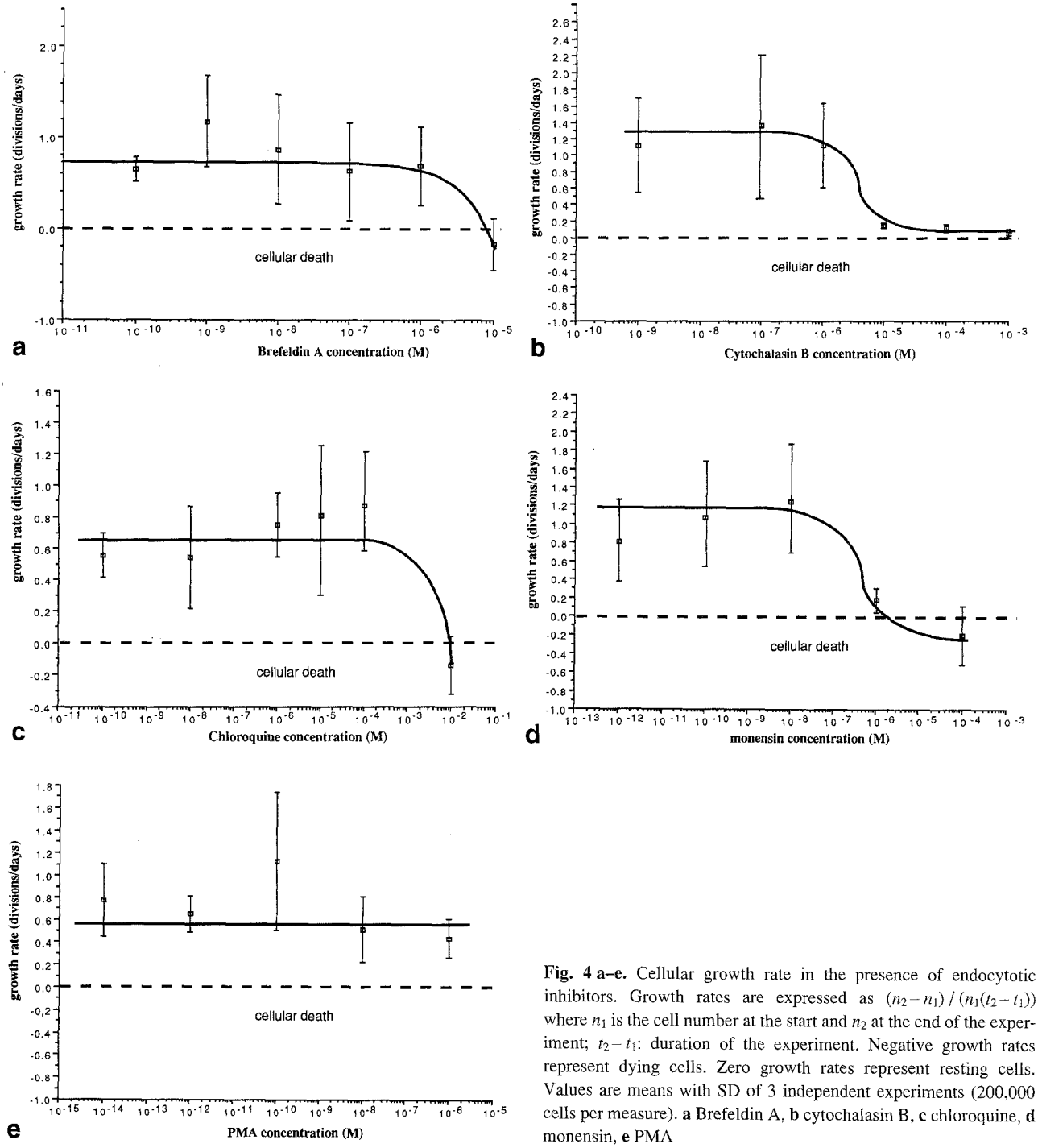

Fig. 4 a–e. Cellular growth rate in the presence of endocytotic inhibitors. Growth rates are expressed as $(n_2-n_1)/(n_1(t_2-t_1))$ where n_1 is the cell number at the start and n_2 at the end of the experiment; t_2-t_1: duration of the experiment. Negative growth rates represent dying cells. Zero growth rates represent resting cells. Values are means with SD of 3 independent experiments (200,000 cells per measure). **a** Brefeldin A, **b** cytochalasin B, **c** chloroquine, **d** monensin, **e** PMA

Up to 10 μM brefeldin A, which induces clathrin dissociation, did not have any significant effect on the uptake of LY, although LY was slightly increased at concentrations above 100 μM. However at such concentrations cell growth was also inhibited and cells rapidly died (Fig. 4 a). Enhanced fixation of LY probably resulted from adsorption of the dye on dead cells.

Monensin is a cationic monovalent ionophore which prompts dissipation of pH gradients and neutralization of membrane compartments, preventing lysosome acidification or Golgi trafficking. At concentrations as low as 1 nM to 100 nM, it inhibited LY uptake in *Arabidopsis* cells by about 30% (Fig. 3 d). Thus a monovalent anion-dependent mechanism

could be implicated in LY uptake. When concentrations up to 1 mM were used, the uptake of LY was enhanced 60 times because of a loss in cellular viability resulting in breaks in the plasma membrane and adsorption of LY onto several cellular structures (Fig. 4 d). In order to assess more accurately whether a pH-dependent mechanism was involved in the uptake of LY, chloroquine which modifies endosomic pH, was assayed.

Chloroquine is a weak base which prevents ATPase-directed acidification of the endocytotic compartments. Acidification is required to transfer materials from endocytotic vesicles to the cytoplasm. Inhibiting acidification would result in the inhibition of endocytosis. In *Arabidopsis*, 1 mM chloroquine reduced the uptake of LY (Fig. 3 c). No deleterious effects on cellular viability were noticed at concentrations below 10 mM.

Uptake of LY was also inhibited by cytochalasin B, which inhibits the polymerisation of actin monomers. Such inhibition occurred at concentrations above 10 μM. Therefore an actin-dependent mechanism was probably involved in LY uptake. 10 μM cytochalasin B reduced the growth rate of the cells to zero, but, although they were unable to divide, their viability was retained and adsorption of LY on cellular components of such resting cells was not detectable (Fig. 4 b). As actin is necessary to form vesicles, we propose that vesicles are also implied in the uptake of LY.

All the endocytotic inhibitors were also assayed against the transport of LY at a concentration of 1 mg/ml. Only chloroquine exerted an effect on the uptake of LY, the other inhibitors exerted no significant effect (Fig. 5 a).

PMA is a phorbol ester activating protein kinase C, which activates phosphorylation of numerous proteins (e.g., kinases, phosphatases) which are either activated or inhibited. In plants, no effect of PMA on endocytosis has yet been described. In *Arabidopsis* cells, supplying PMA did not modify the transport of LY at a concentration of 1 mg/ml (Fig. 5 a). Conversely when the LY concentration was 0.1 mg/ml its uptake was double that in controls in the presence of PMA concentrations as low as 0.1 nM (Figs. 3 and 5 b), whereas it was reduced by about 40% by 1 μM PMA (Fig. 3 e). Enhanced uptake could not be ascribed to possible toxic effects of the drug, since PMA-treated cells divided and grew at least as rapidly as control cells (Fig. 4 e). Moreover, although its uptake was enhanced by 0.1 nM PMA, LY remained within the cytoplasm (data not shown).

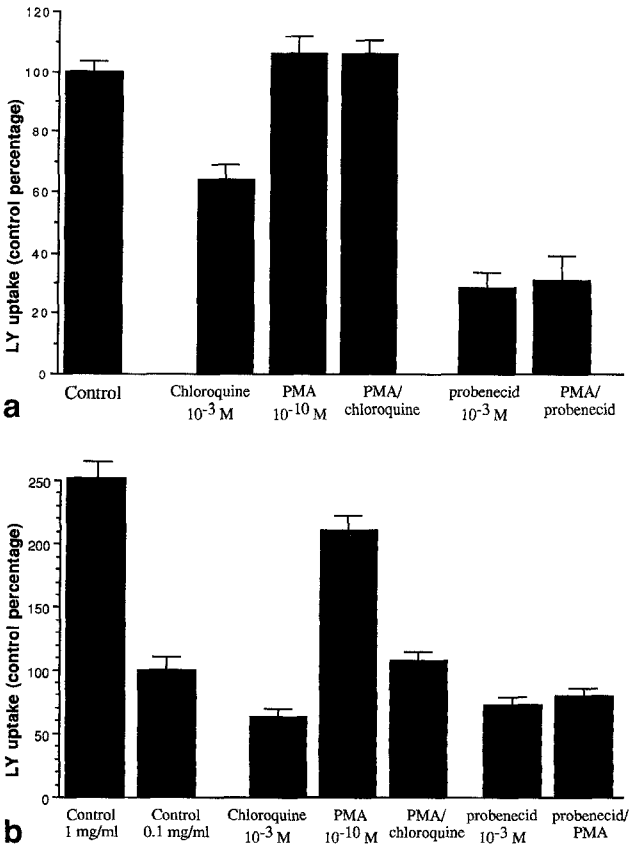

Fig. 5 a, b. Effect of inhibitor combinations upon Lucifer Yellow uptake by suspension cells. LY uptake in the presence of inhibitors was expressed as a percentage of the control. Values are means with SD of 3 independent assays. **a** Uptake assays were performed using 1 mg of LY per ml. Chloroquine, probenecid, and PMA probenecid 1 mM hindered LY uptake. Conversely, PMA and PMA chloroquine did not significantly modify LY uptake. **b** Uptake experiments were performed using 0.1 mg of LY per ml. Chloroquine and probenecid reduced LY uptake slightly. Conversely, compared to controls at an LY concentration of 0.1 mg/ml PMA doubled its uptake, reaching approximately 80% of the level of controls at an LY concentration of 1 mg/ml. This PMA-dependent activation was hindered by both chloroquine and probenecid

On the other hand, 1 mM probenecid, which is known to inhibit anion carriers (Oparka et al. 1991), decreased LY transport by about 70% when the LY concentration was 1 mg/ml (Fig. 5 a). Therefore under these experimental conditions anionic carrier uptake should represent about 70% of LY uptake. However, when the LY concentration was 0.1 mg/ml, its transport was only slightly modified (Fig. 5 b).

Effect of combination of two inhibitors on LY uptake

In order to elucidate simultaneously the PMA-induced uptake mechanism and the probenecid-

induced effects, PMA was assayed in association either with other endocytotic inhibitors or with probenecid. Applying chloroquine with PMA did not change the transport of LY at a concentration of 1 mg/ml (Fig. 5 a), whereas it nullified the PMA effect on the LY uptake at a concentration of 0.1 mg/ml (Fig. 5 b).

Interestingly, when probenecid was associated with PMA, LY was accumulated just as with probenecid alone, indicating that both chloroquine and probenecid interacted with the uptake mechanism(s) induced by PMA. As they exerted identical inhibition, they presumably act on the same LY uptake mechanism(s) which should be protein kinase C-sensitive. In addition it implies a probenecid-inhibitable carrier and the active acidification of an endocytotic compartment.

Discussion

LY, an anionic fluorochrome with a pK_a lower than 1, cannot diffuse freely across membranes (Stewart 1981). In animal cells, it enters the cell via fluid-phase endocytosis (Wilcox and Franceschini 1984, Oka and Weigel 1989). It has been shown to enter *Daucus carota* cells and accumulate in the central vacuole (Hillmer et al. 1989). According to the latter, LY might enter plant cells via pinocytosis, as it does in animal cells. A probenecid-sensitive anionic carrier has been shown to be present on the tonoplast, allowing vacuolar accumulation of LY (Cole et al. 1991). These authors also hypothesized the presence of an anionic carrier on the plasmalemma permitting the uptake of LY into the cytoplasm. In cells of *Daucus carota* and *Zea mays*, LY uptake was performed by an anion carrier as well as by pinocytosis (Cole et al. 1991).

In *Arabidopsis thaliana* C24, LY at a concentration of 1 mg/ml, the concentration used in most endocytosis research, accumulated within the vacuole. This accumulation was probenecid-sensitive suggesting that an anion carrier was at least involved in transport into the vacuole. Moreover supplying endocytotic inhibitors other than chloroquine did not decrease the uptake of LY, indicating that anionic carrier activity probably constituted the main uptake mechanism.

Conversely, LY at a concentration of 0.1 mg/ml was transported and stored in the cytoplasm, but was not detectable in the vacuole. These data favour the assumption that an anion carrier at the tonoplast was inefficient when LY was added at a low concentration, indicating that this putative carrier has a low affinity for the dye.

To highlight the mechanisms involved in the uptake of low concentrations of LY at the plasma membrane, drugs known to inhibit endocytosis in animal cells were used. At concentrations which did not affect the survival of the cells of *A. thaliana*, monensin, chloroquine, and cytochalasin B exerted an inhibitory effect on LY accumulation in the cytoplasm. Conversely, brefeldin A did not inhibit the uptake of the dye, indicating that coated-pit endocytosis was not involved. On the whole, these data support the assumption that LY at a concentration of 0.1 mg/ml was mainly transported into *Arabidopsis* cells by endocytosis. However, addition of brefeldin A did not impair the uptake of LY indicating that the endocytotic mechanism did not involve clathrins unless brefeldin A does not act on plant clathrins. Coleman et al. (1987) showed that animal and plant clathrins were similar but not totally identical. However, Driouich et al. (1993) showed that brefeldin A hindered *Acer pseudoplatanus* Golgi trafficking, suggesting that this action was clathrin-dependent.

PMA exerted opposite effects on the transport of LY at a concentration of 0.1 mg/ml to *Arabidopsis* cells. Uptake was enhanced by nanomolar concentrations and inhibited by micromolar concentrations of PMA, a dual effect previously reported in mammals. At micromolar concentrations caveolae-mediated endocytosis was decreased as a consequence of protein kinase C phosphorylation of coat proteins which were subsequently removed from the plasma membrane preventing caveolae-mediated endocytosis (Smart et al. 1994, Parton et al. 1994). However, at nanomolar concentrations PMA induced increased fluid-phase endocytosis. The mechanism of this increase remains unknown, the only available data showing that this increase was not linked to coated pits (Sato et al. 1996).

Thus the PMA effect in plants resembles that in mammal cells. However, data obtained from PMA associated with probenecid are intriguing because the PMA-induced uptake of LY was counteracted by probenecid which inhibits anion carrier activities. Slow anion channels were shown to be strongly activated by phosphorylation events in broad bean guard cells (Schmidt et al. 1995). Thus it can be postulated that PMA activated the LY uptake by signalling pathway(s) involving phosphorylation-dephosphorylation reactions. Thus, on the basis of the data obtained with probenecid and probenecid plus PMA, anion carrier activities probably operate at the plasmalemma as well. Such activities might be low

when LY concentration was 0.1 mg/ml, but increased when LY concentration was 1 mg/ml. It is not known whether the plasma membrane and the tonoplast systems are composed of different or the same unique carrier, or whether one transporter changes its properties (its affinity) depending on environmental conditions. However, the PMA-induced effect is also inhibited by chloroquine, a weak base impairing pH decrease in endocytotic vesicles. Acidification usually occurs in endocytotic vesicles originating from coated pits after fusion with the lysosome, whereas vesicles originating from caveolae do not fuse with any lysosomal compartment but are acidified. This acidification is mediated by ATPases. Inhibition of vesicle acidification by chloroquine impairs the subsequent endocytotic steps (Rothberg et al. 1990).

Consequently the inhibitory effects of both chloroquine and probenecid on PMA-enhanced uptake of LY seem mutually exclusive, unless endocytotic vesicles originating from the plasma membrane comprised regions with anionic carriers, regulated by phosphorylation events. In the absence of PMA, plasmalemma-derived anionic carrier activities might function at the vesicular-like structures. This would imply that the probenecid-inhibitable anion carrier activities acted with the acidification machinery of the endocytotic vesicles.

In conclusion, according to the experimental methodology we used, we propound that the uptake of LY in *Arabidopsis thaliana* was achieved via two mechanisms. As in other plants, both endocytosis and anionic carrier(s) seemingly prompted LY uptake according to its concentration. At 1 mg/ml, 70% of LY was taken up by the way of anionic transporter(s). At 0.1 mg/ml, LY was mainly taken up by an endocytotic mechanism which was not relayed to coated-pits, anion carrier activities, if operating, would be low. Work is presently under way to determine whether (i) channel currents are modified at the plasma membrane and/or the tonoplast by either probenecid or PMA; (ii) phosphorylation-dephosphorylation reactions modify the uptake of LY.

Acknowledgements

We are grateful to Dr. C. Slomiani (CNRS URA 42, Lille, France) for assistance with microscopy, and to Drs. C. Bergougnioux (IBP, Orsay, France) and D. Inzé (INRA-University of Gent, Belgium) for providing the *Arabidopsis* suspension cell culture. This work was supported by the Région Nord-Pas-de-Calais (France). Robert Roszak is indebted to the Région Nord-Pas-de-Calais and Région Picardie (France) for a grant.

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