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Clathrin-independent endocytosis contributes to uptake of glucose into BY-2 protoplasts

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SUMMARY

In eukaryotic cells, several pathways exist for the internalization of plasma membrane proteins and extracellular cargo molecules. These endocytic pathways can be divided into clathrin-dependent and clathrin-independent pathways. While clathrin-dependent pathways are known to be involved in a variety of cellular processes in plants, clathrin-independent pathways have so far only been identified in animal and yeast cells. Here we show that internalization of fluorescent glucose into BY-2 cells leads to accumulation of the sugar in compartments of the endocytic pathway. This endocytic uptake of glucose was not blocked by ikarugamycin, an inhibitor of clathrin-dependent endocytosis, suggesting a role for clathrin-independent endocytosis in glucose uptake. Investigations of fusion and fission of single vesicles by membrane capacitance measurements revealed stimulation of endocytic activity by extracellular glucose. Glucose-stimulated fission of vesicles was not affected by addition of ikarugamycin or blocking of clathrin coat formation by transient over-expression of HUB1 (the C-terminal part of the clathrin heavy chain). These data demonstrate that clathrin-independent endocytosis does occur in plant cells. This pathway may represent a common mechanism for the uptake of external nutrients.

Keywords: clathrin-independent endocytosis, BY-2 cells, glucose uptake, capacitance measurements, ikarugamycin.

INTRODUCTION

Endocytosis is essential for many cellular processes, such as protein transport, membrane recycling, cell signalling, receptor functioning and uptake of external solutes. In yeast and animal cells, diverse endocytic mechanisms that meet this wide range of physiological requirements have evolved. Of these pathways, clathrin-dependent endocytosis is the best characterized, and depends on coat formation mediated by the clathrin lattice. In addition, a variety of endocytic pathways that do not involve clathrin have been described in animal cells (Mayor and Pagano, 2007). In recent years, these clathrin-independent pathways have attracted increasing attention as their roles in cellular processes such as polarization, motility, regulation of signalling and normal cell growth became apparent (Sandvig *et al.*, 2011). Although there are some indications for clathrin-independent endocytosis in plants (Moscatelli *et al.*, 2007; Onelli *et al.*, 2008), direct evidence for such a pathway is still missing, and its molecular characteristics and physiological importance are still unknown.

A candidate for a physiological process that relies on clathrin-independent endocytosis is fluid-phase endocytosis, which is involved in the uptake of assimilates (Baluska *et al.*, 2004; Etxeberria *et al.*, 2005a) but also occurs constitutively in plant cells (Gall *et al.*, 2010). In animal cells, the uptake of external solutes, including nutrients, via fluid-phase endocytosis is associated with clathrin-independent endocytosis (Damke *et al.*, 1995; Sandvig and van Deurs, 2005, Hansen and Nichols, 2009). However, there is no information about the mechanism of fluid-phase endocytosis in plants.

To fill this gap, we have analysed endocytic uptake of glucose in BY-2 cells using fluorescent microscopy and membrane capacitance measurements. Uptake of a fluorescent glucose derivative into BY-2 cells was not affected by treatment of cells with ikarugamycin (IKA), a potent inhibitor of clathrin-mediated endocytosis. This suggests a role for clathrin-independent endocytosis in the internalization of glucose. The results of membrane capacitance measurements revealed up-regulation of the formation of endocytic vesicles in the presence of glucose. This process was not blocked by IKA or over-expression of HUB1 (the C-terminal part of the clathrin heavy chain). Together, the results imply

that fluid-phase endocytosis can be stimulated by external nutrients and relies on clathrin-independent fission of vesicles.

RESULTS AND DISCUSSION

Endocytic uptake of hexose in BY-2 protoplasts

Previous studies demonstrated endocytic uptake of glucose and sucrose into heterotrophic cells (Etxeberria *et al.*, 2005a,b; Baroja-Fernandez *et al.*, 2006). This process can be defined as fluid-phase endocytosis and plays an important physiological role in sugar storage (Baroja-Fernandez *et al.*, 2006). Etxeberria *et al.* (2005b) suggested that hexose, which is required for cytosolic metabolism, is transported through plasma membrane-bound carriers into the cytosol, while reserve hexose is transported in bulk into the vacuole by endocytosis. Endocytic uptake of hexose bypasses the cytosol, and does not disrupt the highly regulated cytosolic composition by fluctuating sucrose concentrations. A similar mechanism has also been suggested for transport of NaCl to the vacuole under high salt conditions (Mazel *et al.*, 2004). Uptake of nutrients and extracellular compounds via fluid-phase endocytosis is thus most likely of general physiological importance in plant cells.

However, the molecular components of this endocytic pathway have not yet been determined. To test whether BY-2 protoplasts provide a suitable model system for studying the mechanisms of fluid-phase endocytosis, we analysed the uptake of hexose using the non-metabolizable glucose derivative 2-NBDG [2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose] in BY-2 protoplasts. 2-NBDG is a fluorescent form of deoxyglycose; its non-metabolizable nature allows determination of the dynamics of uptake and accumulation inside cells.

Uptake of 2-NBDG into BY-2 protoplasts was monitored either in protoplasts isolated from cells kept in culture medium or cells incubated in glucose-free medium for at least 8 h. Under both conditions, 2-NBDG accumulated inside protoplasts; there was no appreciable difference between glucose-starved (Figure 1a) and normal protoplasts (Figure 1b,c). In all cells, the fluorescent signal was visible inside the cytosol in form of bright spots (Figure 1a–c). In addition, most cells also showed a diffuse distribution of 2-NBDG in the cytosol (Figure 1a,b) and inside the vacuole (Figure 1c).

Uptake of hexose and deoxyglucose can in principle occur either via plasma membrane-bound carriers (Büttner and Sauer, 2000; Oliveira *et al.*, 2002) or endocytosis (Etxeberria *et al.*, 2005c). Carrier-mediated internalization of glucose results in distribution of glucose in the cytosol, but endocytic uptake of hexose results in accumulation of the sugar in endocytic vesicles and compartments of the endocytic pathway. The diffuse fluorescence found in the cytosol of BY-2 cells incubated in 2-NBDG most likely results from the presence of cytosolic 2-NBDG that has been transported across the plasma membrane by a carrier-mediated mechanism. In addition, 2-NBDG-containing endocytic vesicles, which are too small to be resolved by confocal microscopy, may also contribute to the diffuse background labelling. These vesicles follow the endocytic pathway, leading to accumulation of fluorescence in endosomal compartments such as the early endosome, the trans-Golgi network (TGN), multivesicular bodies and vacuoles. To determine whether the bright fluorescent spots observed in BY-2 protoplasts correspond to compartments of the endocytic pathway, we transiently transfected protoplasts with SYP61–RFP, a marker of the TGN (Cai *et al.*, 2010). Transfected protoplasts showed numerous spots labelled by SYP61–RFP (Figure 1e). After incubation of these protoplasts in 2-NBDG, we found a clear co-localization of compartments labelled by 2-NBDG and SYP61–RFP (Figure 1f). The apparent co-localization was confirmed by correlation of the peaks of a fluorescent intensity profile (Figure 1g). The results of these

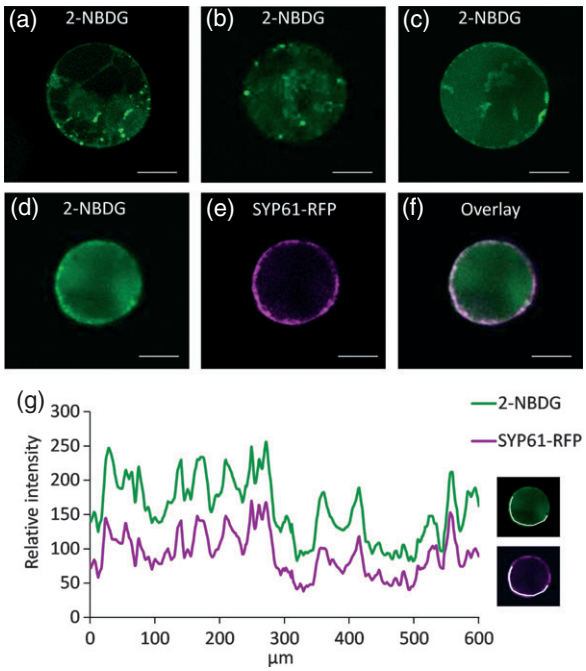

Figure 1. 2-NBDG localizes to endosomal compartments in BY-2 protoplasts.
(a–c) Fluorescent images of BY-2 cells 1 h after incubation in 5 mM 2-NBDG. 2-NBDG accumulated in bright spots in the cytosol and inside the vacuole in protoplasts from glucose-starved cells (a) and protoplasts isolated from normal cultured cells (b, c).
(d–f) 2-NBDG uptake in BY-2 cells expressing the TGN marker SYP61–RFP. 2-NBDG accumulated in bright spots in the cytosol and inside the vacuole (d). SYP61–RFP was visible in small compartments in the cytosol (e). The 2-NBDG signal (green) partially co-localized with the TGN marker SYP61–RFP (magenta) (f). Co-localization is indicated by the white colour.
(g) Intensity profile of 2-NBDG (green) and SYP61–RFP (magenta) taken along the white line indicated in the protoplast images on the right.
Images represent single optical confocal sections through equatorial plane of the protoplasts. Scale bars = 10 µm.

experiments imply that internalized 2-NBDG accumulates partly in the TGN. The accumulation of 2-NBDG in compartments of the endocytic pathway confirms that endocytosis is indeed involved in the uptake of hexose into BY-2 cells. BY-2 cells thus provide a suitable model system to study the mechanisms of fluid-phase endocytosis.

Inhibition of clathrin does not affect endocytic uptake of hexose

Studies on endocytosis in animal cells suggest that uptake of solutes via fluid-phase endocytosis occurs via a clathrin-independent mechanism (Doherty and McMahon 2009). To investigate the role of clathrin in the endocytic uptake of hexose into BY-2 cells, we used ikarugamycin (IKA), a strong and specific inhibitor of clathrin-mediated endocytosis in plant and animal cells (Luo *et al.*, 2001; Moscatelli *et al.*, 2007; Onelli *et al.*, 2008). We first analysed the effect of IKA on endocytosis of the plasma membrane marker FM4-64. Previous investigations on FM4-64 demonstrated that internalization of the dye is mainly mediated by clathrin-dependent endocytosis (Dhonukshe *et al.*, 2007). Uptake of FM4-64 was observed in IKA-treated and control BY-2 protoplasts over a period of 1 h. In control protoplasts, strong labelling of the endomembrane system by FM4-64 was visible after 10 min (Figure 2a, upper row). In contrast, IKA-treated cells showed almost no FM4-64 uptake. Even after 60 min, only weak FM4-64 labelling was visible inside the cell (Figure 2a, lower row).

For quantification of endocytosis, we estimated the ratio of intracellular (membrane) fluorescence to whole-cell fluorescence over time. The ratio at time zero was set to 1. The rapid increase of the relative fluorescence in control protoplasts reflects rapid endocytosis of the dye (Figure 2b). However, in protoplasts incubated in 10 μM IKA for 30 min, the relative fluorescence barely changed over time, demonstrating that endocytosis of FM4-64 is strongly inhibited by IKA. Our results thus confirm the inhibitory effect of IKA on clathrin-dependent endocytosis in BY-2 cells. Next we analysed the effect of IKA on internalization of the glucose derivative 2-NBDG. Figure 2(c) shows a representative example of a BY-2 protoplast treated with IKA for 30 min before incubation in 2-NBDG. The fluorescent glucose derivative still accumulated inside the cell and in endosomal compartments. We did not find any difference in the uptake of 2-NBDG between IKA-treated and non-treated cells, demonstrating that endocytic uptake of hexose is not inhibited by IKA. In addition, analysis of the localization of FM4-64 and 2-NBDG revealed that only a few spots were marked by both 2-NBDG and FM4-64 (Figure 2d). These results appear to argue against endocytotic uptake of 2-NBDG. However, studies on yeast mutants suggested that FM4-64 enters the cell by a non-fluid-phase endocytic mechanism (Vida and Emr, 1995), and in *Chara corallina* internodal cells, FM dyes have been shown to label specific plasma membrane domains (Klima and Foissner, 2008). We therefore propose that different endocytic pathways are involved in the uptake of hexose and internalization of

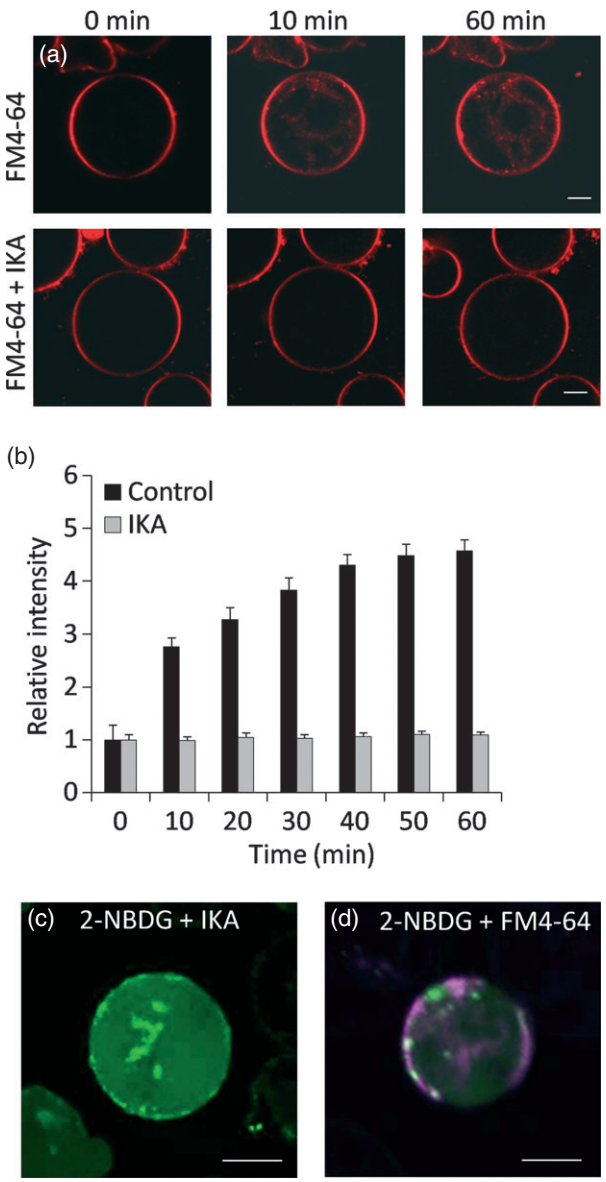

Figure 2. Inhibition of clathrin-mediated endocytosis by IKA blocks internalization of FM4-64 but not endocytic uptake of 2-NBDG.
(a) Time series of BY-2 protoplasts incubated in 10 μM FM4-64. Under control conditions, FM4-64 uptake was visible within 10 min (top row). No FM4-64 uptake was detectable in protoplasts incubated in 10 μM IKA for 30 min before addition of the dye (bottom row).
(b) Quantification of FM4-64 uptake. The relative fluorescence intensity is given as the ratio of intracellular fluorescence to whole-cell fluorescence. The value at time zero was set to 1.
(c) Protoplasts treated with 10 μM IKA for 30 min before incubation in 2-NBDG. 2-NBDG still accumulated in bright spots within the cell.
(d) Protoplasts incubated in 5mM 2-NBDG and 10 μM FM4-64 for 1 h. The 2-NBDG signal (green) and the FM4-64 signal (magenta) show only partial co-localization.
Scale bars = 10 μm.

Table 1 Summary of recorded fusion or fission events

	Total recording time (h)	Measurements ± events	Transient fusion/h	Permanent fusion/h	Transient fission/h	Permanent fission/h
Control	21.13	32/58	10.48 ± 5.75	9.17 ± 2.78	6.83 ± 4.22	5.80 ± 1.38
Glucose	3.29	14/5	3.46 ± 2.42	2.43 ± 0.57	0.84 ± 0.58	17.81 ± 6.51
IKA	5.36	9/3	3.67 ± 2.04	7.46 ± 1.76	6.50 ± 4.26	1.75 ± 0.78
Glucose + IKA	2.68	9/1	3.00 ± 2.10	2.40 ± 0.87	3.27 ± 1.86	10.77 ± 2.91
Glucose + HUB1	2.91	8/5	1.45 ± 0.93	0.92 ± 0.71	0.00	6.82 ± 2.25

The numbers of fusion and fission events per hour represent the mean frequency of events for each measurement. For control measurements, the calculation is based on recordings published previously (Bandmann *et al.*, 2011). Values are means ± SEM.

FM4-64, and that the parts of the membrane that form the endocytic vesicle involved in hexose uptake are not labelled by FM4-64. Together, the results of these experiments imply that endocytic uptake of hexose into BY-2 protoplasts occurs via a clathrin-independent mechanism.

Furthermore, the accumulation of 2-NBDG in the TGN shows that clathrin-independent fluid-phase endocytosis follows (at least partly) the pathway described for clathrin-mediated endocytosis. This suggests that clathrin-independent and clathrin-mediated endocytosis intersect at the TGN in plant cells as they do in animal cells (Gruenberg, 2001). The TGN thus appears to operate as a sorting station for external solutes destined to the vacuole, underlining the central role of the TGN in sorting of vesicle-mediated transport.

Endocytic events are highly up-regulated by glucose

To further analyse the mechanism of endocytic glucose uptake, we performed membrane capacitance measurements in the cell-attached mode on BY-2 protoplasts. This technique allowed us to monitor the very first step of the endocytic pathway, fission of vesicles from the plasma membrane, in real time. Previous measurements on BY-2 protoplasts revealed spontaneous upward and downward steps in membrane capacitance (C_m) that reflected exo- and endocytosis, respectively, of single vesicles (Bandmann *et al.*, 2011). The recorded changes in C_m could be grouped into four categories according to their different kinetics: (i) transient fusion of exocytotic vesicles, (ii) transient fission of an endocytotic vesicle, (iii) permanent fusion, and (iv) permanent fission of a single vesicle. Under control conditions, the number of fusion events was slightly higher than the number of fission events (Table 1). In the presence of glucose, the picture changed completely. With 244 mM glucose in the patch pipette, the number of fusion events and the number of transient fission events decreased considerably, and the frequency of permanent endocytic events increased fourfold (Table 1).

This glucose-stimulated increase in permanent endocytic activity became even more obvious when the frequency of this event was normalized to the overall number of events (Figure 3). Under control conditions, the relative frequency of permanent endocytic events was only 18%. In the presence of glucose, the relative frequency of permanent endocytic events increased to 73% (Figure 3).

Figure 4 shows a representative example of a capacitance measurement in the presence of glucose. Often several downward steps reflecting successive fission of single vesicles were observed. The capacitance measurements thus demonstrate that glucose is able to stimulate endocytic activity in BY-2 protoplasts. It is not possible to draw conclusions regarding the vesicular cargo from capacitance measurements alone. However, taking into account that treatment of cells with fluorescently labelled hexose leads to accumulation of hexose in compartments of the endomembrane system (see above), the observed endocytic activity is most likely responsible for the endocytic uptake of hexose. This implies the existence of an extracellular hexose sensor involved in the up-regulation of endocytosis. Furthermore, as glucose was only added to the pipette solution, the

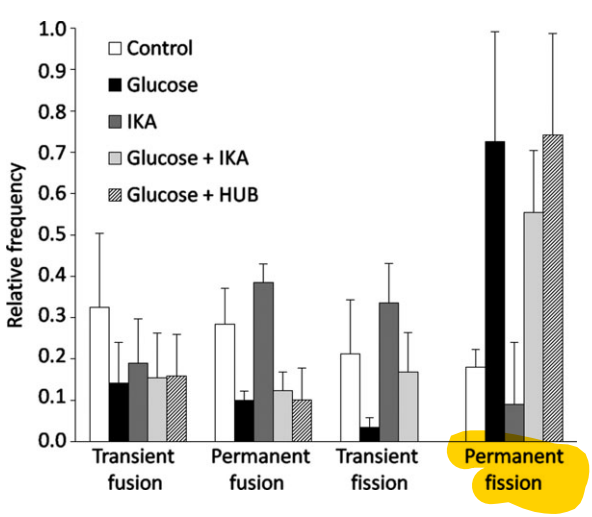

Figure 3. Permanent endocytosis is highly up-regulated in the presence of glucose.
Relative frequency of the various kinetic modes of exo- and endocytic events given as the number of events per hour recording time relative to the total number of events per hour (set to 100%). Measurements were performed under various conditions as indicated. Values are means ± SEM.

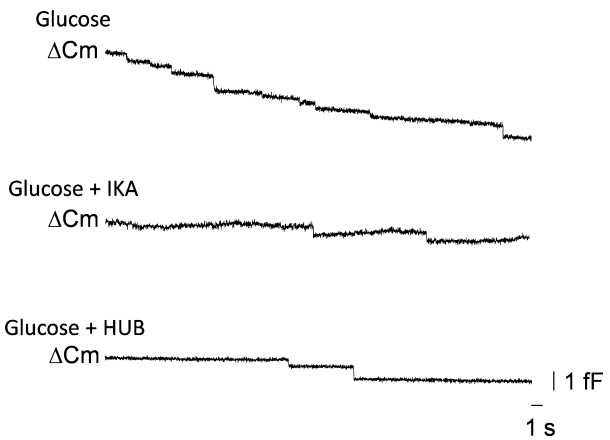

Figure 4. Representative capacitance measurements.
Capacitance recordings showing successive downward deflections corresponding to permanent fission of vesicles. Measurements were performed under various conditions as indicated on the left.

sensing of glucose was restricted to the small area of the plasma membrane under the patch pipette (approximately 1 μm^2). The ability of the cell to respond to this local increase in glucose suggests that sensing of glucose and stimulation of endocytosis are locally coupled. Members of the plant sugar transporter family have been proposed to play a direct role in the signal transduction responsible for regulation of sugar transport (Lalonde *et al.*, 1999). Whether these transporters are also involved in the regulation of endocytic sugar uptake remains to be shown.

As demonstrated above, uptake of hexose into BY-2 cells was clathrin-independent. Consequently, glucose-stimulated endocytosis should also not depend on clathrin coat formation. To test this hypothesis, we analysed the effect of IKA on the frequency of fusion and fission events in BY-2 protoplasts in the absence and presence of glucose. Incubation of protoplasts in IKA led to small changes in the relative frequency of both fusion and fission events (Figure 3). The number of permanent fusion events and transient fission events increased slightly, whereas the relative frequency of transient exocytosis and permanent endocytosis decreased in IKA-treated cells. We next analysed whether stimulation of endocytosis by glucose is influenced by IKA. Figure 3 shows that addition of IKA slightly reduced the stimulatory effect of glucose. However, the relative frequency of permanent endocytic events was still three times higher compared to measurements without glucose (Figure 3). A representative capacitance trace recorded in the presence of IKA and glucose is shown in Figure 4. Again, successive downward steps reflecting fission of single vesicles were frequently observed. This demonstrates that inhibition of clathrin coat formation by IKA does not prevent the stimulatory effect of glucose on endocytosis.

To confirm the function of clathrin-independent vesicle formation in glucose-stimulated endocytosis, we performed capacitance measurements on BY-2 protoplasts transiently over-expressing the C-terminal part of the clathrin heavy chain (termed HUB1) fused to GFP. HUB1 binds to the clathrin light chains, making them unavailable for clathrin cage formation. It thus acts as a strong inhibitor of clathrin-dependent endocytosis (Dhonukshe *et al.*, 2007). In HUB1–GFP-transfected cells, the glucose-stimulated increase in the relative frequency of permanent endocytic events did not differ from that in untransfected cells (Figure 3). The representative capacitance trace shown in Figure 4 shows sequential downward steps resulting from endocytic events. The lack of inhibition of glucose-stimulated endocytosis by incubation in IKA or transient expression of HUB1–GFP supports the hypothesis that endocytic uptake of hexose into BY-2 protoplasts occurs via a clathrin-independent mechanism.

Capacitance measurements also provide an estimation of the diameter of the vesicles (for details, see Experimental Procedures). The distribution of the size of the endocytic vesicles recorded in the presence of glucose is shown in Figure 5. Most vesicles (88%) had a diameter between 80 and 220 nm (median diameter 133 nm). A similar distribution was found in cells treated with IKA or transfected with HUB1–GFP (Figure 5). The diameter of these vesicles is in the same range as the diameter estimated for vesicles internalized by fluid-phase endocytosis in turgid guard cells (Gall *et al.*, 2010). This suggests that the absence of turgor in protoplasts does not affect the size of endocytic vesicles. For most plant clathrin vesicles, a diameter between 70 and 100 nm has been determined from electron micrographs. This is similar to the diameter of endocytic vesicles in this study recorded upon blocking of clathrin coat formation.

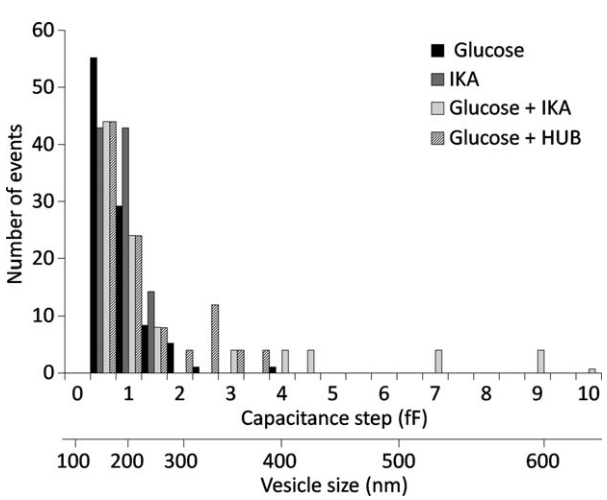

Figure 5. Distribution of endocytic vesicle size.
Size distribution of endocytic vesicles for permanent endocytic events. Data are derived from recordings under various conditions as indicated.

Thus glucose-stimulated endocytosis involves vesicles with similar size to clathrin-coated vesicles but their formation does not depend on clathrin. Together, the results directly demonstrate the existence of clathrin-independent endocytosis in plants and provide a functional role for this pathway.

Concluding remarks

Endocytosis is a key feature of all eukaryotic cells and is involved in diverse cellular processes such as regulation of cell-surface expression of membrane proteins, remodelling of the plasma membrane, recycling of vesicles and nutrient uptake (for reviews, see Conner and Schmid, 2003; Murphy *et al.*, 2005). To meet the various requirements of such processes, cells possess a variety of endocytic pathways that are regulated by diverse molecular machineries. The best studied pathway is clathrin-mediated endocytosis, and this is the only pathway so far described for plant cells. However, the results presented here show that a clathrin-independent endocytic pathway contributes to internalization of glucose into BY-2 cells. This pathway may also represent a common mechanism for the uptake of external nutrients. Endocytosed glucose was found to follow the same pathway as the cargo internalized by clathrin-mediated endocytosis. In both cases, cargo accumulated at the TGN, which functions as a central sorting station. Although speculative at this stage, clathrin-independent endocytosis is likely to play an important role not only in the uptake of hexose but also of other nutrients, and may also be involved in various other cellular processes. In animal and yeast cells, an increasing number of components that are involved in clathrin-independent formation of endocytic vesicles have been identified (Kumari *et al.*, 2010). Homologues of some of these components, such as dynamin, are also found in plants (Hong *et al.*, 2003) but have so far not been associated with clathrin-independent endocytosis. A future task will thus be to identify and characterize the molecular machinery that mediates clathrin-independent endocytic pathways in plants.

EXPERIMENTAL PROCEDURES

Cell culture and protoplast isolation

BY-2 cells were cultured in Linsmaier and Skoog medium (Duchefa, http://www.duchefa.com) supplemented with 30 g L^{-1} sucrose, 1 mg ml^{-1} 2,4-dichlorophenoxyacetic acid and 1 mg ml^{-1} thiamine. Sub-culturing was performed twice a week by adding 10 ml of the cell suspension into 100 ml of new medium. Cultures were maintained in the dark at 25°C under rapid shaking (100 rpm).

For protoplast isolation, cells were collected 2–3 days after subcultivation, and 2 ml of the cell culture suspension were incubated with 2 ml digestion medium for 3–4 h at 37°C under slow shaking (50 rpm). The digestion medium contained 3% cellulase R10 (Yakult Honsha Co. Ltd), 0.2% macerozyme (Yakult Honsha Co. Ltd, www.yakult.co.jp/ypi/en/product.html), 0.1% pectolyase (Yakult Honsha Co. Ltd), 8 mM $CaCl_2$, 25 mM MES/KOH, pH 5.6, adjusted to 540 mOsmol/kg using sucrose. After digestion, cells were collected by centrifugation at 100 ***g*** for 10 min, washed twice for 1 min in wash solution (30 mM $CaCl_2$, 30 mM KCl, 20 mM MES, pH 6.5, adjusted to 430 mOsmol/kg using sorbitol), and stored at 4°C until use.

Transfection of BY-2 protoplasts

For polyethylene glycol-mediated transfection, 100 μl of protoplast suspension (2–5 × 10^6 protoplasts) was placed in a Petri dish (35 mm). Plasmid DNA (30 μg) was diluted in distilled water to a final volume of 500 μl and added to the protoplasts. Immediately after addition of the plasmid DNA, 600 μl of polyethylene glycol solution (40% polyethylene glycol 4000, 100 mM $CaCl_2$, 200 mM sucrose, pH 6) was added to the suspension drop by drop. After 15 min incubation at room temperature, 1 ml of wash solution was added. To slowly re-adjust the osmolarity of the protoplast suspension, 1, 2 and 5 ml of wash solution were added at intervals of 15 min. Protoplasts were incubated overnight at 25°C before use for capacitance measurements or microscopic analysis.

Drug treatment

For drug treatment experiments, IKA (Biomol, http://www.biomol.de) was added from a stock solution (1 mM in DMSO) to tobacco BY-2 protoplasts to a final concentration of 10 μM. Protoplasts were incubated in IKA for 30 min before use for capacitance measurements or microscopic analysis.

Confocal imaging

BY-2 protoplasts were investigated using a Leica TCS SP spectral confocal microscope (http://www.leica.com/), either directly after protoplast isolation or approximately 24 h after transfection. Images were acquired using an HCX PL APO CS 20×/0.70 objective or an HCX PL APO 63 ×/1.2 w objective. 2-NBDG (Invitrogen, http://www.invitrogen.com/) was used as a stock solution of 500 mM in distilled water. Protoplasts were incubated for 1 h in 5 mM 2-NBDG and washed twice for 1 min with wash solution before confocal analysis. 2-NBDG was excited using the 488 nm line of a 25 mW argon laser, and fluorescence was detected at 505–580 nm. RFP was excited using the 543 nm line of a 25 mW helium/neon laser, and fluorescence was detected at 560–600 nm. For plasma membrane staining and monitoring of endocytosis, protoplasts were incubated in 10 μM FM4-64 (Invitrogen) for 10 min on ice to ensure saturation of the plasma membrane with the dye. FM4-64 was excited using the 488 nm line of a 25 mW argon laser, and fluorescence was detected at 630–700 nm. Images were analysed using ImageJ software (http://rsbweb.nih.gov/ij) and Leica confocal software 2.00. For analysis of the relative intracellular fluorescence, the outline of the cell was determined using the plasma membrane marker FM4-64. The area inside the outline of the protoplasts was taken as a measure of the intracellular fluorescence intensity. The relative intracellular fluorescence is given as the ratio of intracellular fluorescence to whole-cell fluorescence. The ratio at time point zero was set to 1. Values presented are means ± SEM.

Electrophysiology

Membrane capacitance measurements were performed as described previously (Bandmann *et al.*, 2011). Protoplasts were bathed in external solution (30 mM $CaCl_2$, 54 mM potassium gluconate, 25 mM MES/KOH, pH 5.6, adjusted to 435 mOsmol/kg using sorbitol). Patch pipettes were either filled with this external solution or with external solution containing 244 mM glucose instead of sorbitol for adjustment of the osmolarity. The signal/noise ratio was optimized on the basis of previous measurements (Bandmann *et al.*, 2011). Stepwise changes in the capacitance trace which correspond to exo- or endocytic events were analysed using the cursor option in

the software sub-routine CellAn (Celica, www.celica.si) written for MATLAB (MathWorks Inc., www.mathworks.de). Data were stored in a MySQL Community Server 5.1.49 database (Oracle, www.oracle.com), calculations were performed using the CAMMC web application (yQ-it, www.yq-it.com), and results were again stored in the database.

From the vesicle capacitance C_v, the surface area (A) and thus the diameter of vesicles could be calculated from $C_v = c \times A$, where $A = 4 \times Pi \times d^2$ and c is the specific membrane capacitance (8 mF/m^2) determined for the plasma membrane of plant cells (White *et al.*, 1999).

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