Biochimica et Biophysica Acta, 978 (1989) 91–96
Elsevier

BBA 74255

Monensin-induced accumulation of neosynthesized lipids and fatty acids in a Golgi fraction prepared from etiolated leek seedlings

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(Received 15 August 1988)

Key words: Lipid transport; Fatty acid transport; Golgi complex; Monensin; (Leek seed)

The effects of monensin on the intracellular distribution of neosynthesized lipids and fatty acids between different membrane fractions prepared from etiolated leek seedlings, were investigated. The penetration of the drug into the seedlings was measured after resolution of monensin by thin-layer chromatography with a solvent system consisting of hexane/diethyl ether/acetic acid (20:80:2, v/v). The concentration of monensin in the microsomal membranes as a function of the external monensin concentration was quantified. The effects of various amounts of the drug on lipid metabolism were studied to determine monensin concentrations having only slight effects on the overall lipid synthesis. Using such conditions, an accumulation of neosynthesized lipids was found in a subcellular membrane fraction enriched in Golgi membranes.

Introduction

It has been demonstrated in vitro that, in leek epidermis, saturated very long chain fatty acids, containing more than 18 carbon atoms (VLCFA), are synthesized in the endoplasmic reticulum [1]. Since these molecules are localized mainly in the plasma membrane, the hypothesis of an intracellular transport of VLCFA from the endoplasmic reticulum to the plasma membrane was proposed [2].

A method was devised to investigate intermembrane transfer of lipids and VLCFA in 7-day-old etiolated leek seedlings in vivo [3]. Using pulse-chase experiments followed by membrane subfractionation of microsomal pellets, transfer of lipid molecules between two subcellular membrane fractions was demonstrated [3,4]. The transfer sequence of lipids and VLCFA from their site of synthesis to the plasma membrane has been studied using different approaches. The phase-partitioning method provided a purified plasma membrane fraction, which is an acceptor of the transferred lipids (PC, PE, neutral lipids) and fatty acids (C_{16} to C_{24}) [4,5]. Double-labeling experiments and pulse-chase experiments using short chase times identified the initial site of lipid synthesis and early events occurring during transport [6]. These studies suggested that an ER–Golgi–plasma membrane pathway could be one of the routes used by the cell for the intracellular transfer of lipids and VLCFA to the plasma membrane. In order to study this eventuality, the involvement of the Golgi apparatus was investigated using monensin, a carboxylic ionophore which is known to disturb the intracellular transport of peripheral and secretory proteins at the level of the Golgi apparatus (reviewed in Refs. 7 and 8).

The intracellular transport of peripheral and secretory proteins and their integration in the plasma membrane, or their vectorial excretion from the cells, have been studied [9–12] and monensin has been particularly useful for establishing the obligatory passage of these proteins through the Golgi apparatus. However, to our knowledge, the internal concentration of monensin has never been measured. In order to study the penetration of monensin into leek seedlings, we first devised techniques to extract, isolate and quantify the drug. We then determined the effects of monensin on lipid metabolism in etiolated leek seedlings by [1-^{14}C]-acetate-incorporation experiments. Concentrations of monensin having no, or only slight, effects on the level

Abbreviations: ER, endoplasmic reticulum; GLC, gas-liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; VLCFA, very long chain fatty acids.

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of lipid synthesis were used to study the effects of the drug on the intracellular distribution of the acetate-labeled lipids. We show that monensin leads to an accumulation of neosynthesized lipids and fatty acids in a membrane fraction highly enriched in Golgi vesicles.

Materials and Methods

Plant material

Leek seeds stored overnight at 4°C were sterilized with sodium hypochlorite in the presence of Triton X-100 for 2 min and then washed three times with distilled H_2O. They were then grown for 7 days in the dark at 25°C, on a growth medium already described [4].

Substrates and reagents

Monensin and all other chemicals were from Sigma (St. Louis, U.S.A.). [1-^{14}C]acetate was purchased from C.E.A. (Saclay, France).

In vivo incorporation of [1-^{14}C]acetate and monensin

Batches of 20 seedlings received 5, 10 or 20 μCi (1-^{14}C)acetate (55 mCi/mmol) and were incubated in vivo for 120, 60 or 30 min, in the presence of variable amounts of monensin. The final volume of each incubation medium was 500 μl. Monensin dilutions were performed using a 5 mM stock solution of monensin in 95% ethanol. All incubations, with or without monensin, were carried out with a constant final ethanol concentration of 0.5%.

Membrane subfractionation

Leek seedlings were homogenized in a grinding buffer consisting of 0.5 M sorbitol and 10 mM KH_2PO_4 (pH 7.8). The homogenate was centrifuged for 5 min at $1000 \times g$. The supernatant was centrifuged for 15 min at $12000 \times g$ and the resulting supernatant was centrifuged again for 60 min at $150000 \times g$. The resulting microsomal pellet was loaded onto a linear sucrose gradient (1.07–1.20 g/cm^3) and centrifuged for 22 h at $130000 \times g$. The gradient was then collected in 45 fractions of 250 μl [6]. Proteins were estimated according to Bradford [13], using bovine serum albumin as the standard.

Lipid extraction and analysis

Lipids of the whole seedlings and membrane fractions were extracted by chloroform/methanol (2:1, v/v) as previously described [3]. The radioactivity incorporated into lipids was determined by liquid scintillation counting. After lipid hydrolysis, the radioactivity of the fatty acids was analyzed by radio-GLC as previously described [3].

Monensin quantitation

The resolution of lipids and monensin by TLC was carried out on Merck 60F-254 HPTLC silica gel plates using a solvent system consisting of hexane/diethyl ether/acetic acid (20:80:2, v/v). Lipid and monensin bands were visualized by copper acetate/phoshoric acid charring [14] and scanned at 355 nm using a photodensitometer (CAMAG), operating in the reflectance mode, coupled to a Spectra Physics 4100 computing integrator.

Expression of results for subfractionation experiments

The study of the effects of monensin on a fraction enriched in Golgi membranes led us to compare the specific radioactivities of fractions obtained by density-gradient subfractionation of the microsomal pellets from seedlings incubated with labeled acetate, in the presence or in the absence of monensin.

After incubation, the seedlings of different batches were homogenized for subcellular fractionation. The specific radioactivity of the total lipid and fatty acid labels of the different fractions was then determined. The ratios

$$\frac{\text{spec. act. (cpm/mg protein) of fraction}}{\text{spec. act. (cpm/mg protein)of gradient}}$$

were calculated for each fraction and these values, termed relative specific radioactivities, were compared. This calculation eliminates the variations resulting from differences in the levels of [1-^{14}C]acetate incorporation from one experiment to another. Hence, we can observe the variations of the distribution of lipid radioactivities for each fraction due to the presence of monensin.

Results

Quantitation and penetration of monensin

The best separation of monensin (Fig. 1, inset) was obtained with hexane/diethyl ether/acetic acid (20:80:2, v/v), with which the drug migrates with an hF_f of 27.8 ± 0.7 (mean value ± S.D., $n = 35$). This TLC/densitometric technique allows the quantitation of as little as 50 pmol of monensin (Fig. 1).

In order to test the solubility of monensin in the solvent mixture employed for extracting lipids from biological material (chloroform/methanol; 2:1, v/v), variable amounts of monensin, solubilized in this mixture after evaporation of the ethanol of the stock solution, were spotted onto HPTLC plates. After TLC and densitometry, the curve of the densitometric peak surface area vs. the amount of monensin was compared to that obtained using similar amounts of monensin taken directly from the stock solution (95% ethanol) (Fig. 1). The two curves were identical, demonstrating the complete solubility of monensin in the solvent mixture. Using the above extraction and quantitation techniques, it was possible to determine the amount of monensin

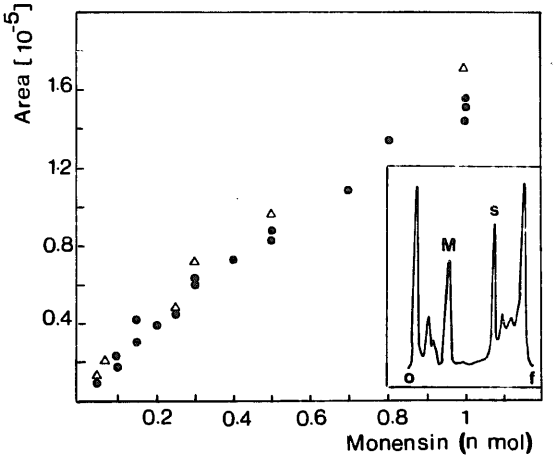

Fig. 1. Monensin quantitation. Standard curves realized by spotting variable amounts of a stock solution of monensin in ethanol 95% either directly (●), or after evaporation of ethanol and solubilization in $CHCl_3/CH_3OH$ (2:1) (△) onto HPTLC plates. The separation, revelation and densitometry were carried out as described in Materials and Methods. Inset: TLC separation of monensin (O, origin; F, front; M, monensin; S, sterols).

that actually penetrated into the seedlings under various conditions of drug concentrations (10–350 μM) and periods of incubation (1–60 min). Whatever the conditions employed, the amount of monensin taken up by the seedlings was always 1.5- to 2-times higher for the sliced seedlings than for the intact ones.

The quantity of monensin taken up by the seedlings increases with the length of the incubation period up to 30 min, after which time it reaches a plateau. The ratios of the amount of internalized monensin to the total quantity of monensin with sliced seedlings ranged from 0.04 to 0.08, and appeared to be independent of the concentration of the drug. In order to determine the localization of the internal monensin, 100 seedlings were incubated for 30 min with 100 and 350 μM monensin, i.e., 250 or 875 nmol of externally applied monensin. After subcellular fractionation, we found 0.2 and 0.4 nmol of monensin, respectively, in the microsomal pellets (1.5 mg of proteins). Thus, assuming that the microsomal membranes from etiolated leek seedlings have a lipid-to-protein ratio of 0.5 (w/w) [4], these values correspond to a monensin-to-lipid molar ratio of $1 \cdot 10^{-4}$ and $2 \cdot 10^{-4}$, respectively, in the microsomal pellet, whereas 70% of the drug which penetrated into the seedlings remained in the supernatant. The binding of monensin to microsomal membranes was further examined in vitro. Microsomal membranes (1.5 mg) were incubated for 30 min with 25, 40 or 50 nmol monensin (50, 80 and 100 μM). After sedimenting the membranes, the drug present in the microsomal pellet was extracted as indicated above and quantified by densitometry. Under these conditions, 75 pmol of monensin were found to be associated with the membranes, whatever the external monensin concentration, corresponding to a monensin-to-lipid molar ratio of 10^{-4} for these membranes. This result obtained in vitro confirmed that there was only weak binding of the drug to the intracellular membranes.

In addition, in vivo and in vitro experiments showed that the drug was not degraded within the cells for incubation times of up to 120 min.

Effect of monensin on lipid neosynthesis

After incubation of the seedlings with [1-^{14}C]acetate and various concentrations of monensin for 30, 60 and 120 min, the lipids were extracted and analyzed. For a 30 min incubation, monensin concentrations of up to 10 μM had no effect on the global label incorporation into the lipids (Fig. 2), but for 60 or 120 min incubations, a dose-dependent inhibition of lipid synthesis by monensin was observed. Furthermore, for experiments carried out with a given concentration of monensin, the inhibition increased with time (Fig. 2). The quantitative analysis by TLC of the phospholipids and neutral lipids did not show marked changes in the label distribution between the various lipids.

The effects of the drug on fatty acid synthesis was further investigated by incubating the seedlings with monensin for 30 and 120 min. Variations of the monensin concentration from 0.1 to 10 μM had no effect on the total label incorporation into fatty acids after 30 min incubation, but modified the distribution of this label between the different fatty acids (Table I). Hence, the ratios of the radioactivity of C_{20} to C_{18} and VLCFA to C_{18} fatty acids decreased as a function of the monensin concentration and reaching 16.5 and 15% of their initial values, respectively (for 10 μM monen-

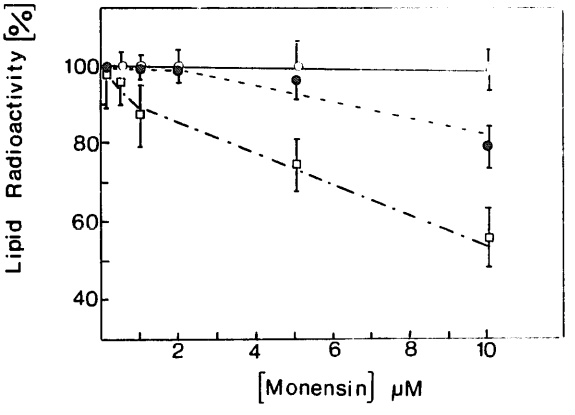

Fig. 2. Effect of monensin on overall lipid synthesis. After incubation of the seedlings with [1-^{14}C]acetate and monensin at various concentrations for 30 (○), 60 (●) and 120 min (□), the lipids of the whole seedlings were extracted and analysed as described in Materials and Methods. The relative values 100% were given to the assays carried out in the absence of monensin (10^5, $7.5 \cdot 10^5$ and $3.5 \cdot 10^6$ dpm/20 seedlings, respectively for 30, 60 and 120 min incubation). The results are given as the percentage of lipid biosynthesis from acetate as a function of the monensin concentration, and are the mean values ± S.D. of five experiments.

TABLE I

Effect of monensin on the fatty acid metabolism

After incubation of the seedlings with [1-^{14}C]acetate, as indicated in Materials and Methods, the lipids of the whole seedlings were extracted and the label of the different fatty acids was analysed by radio-GLC as previously described [3].

[Monensin] (μM)	Incubation time (min)	Radioactivity (dpm·10^{-5})			
		C_{16}	C_{18}	VLCFA	total FA
0	30	5.7	3.9	4.4	14.1
0	120	7.0	5.0	1.4	13.5
0.1	30	5.2	5.1	2.7	13.0
0.1	120	5.0	3.9	1.2	10.2
1	30	5.8	6.0	1.9	13.7
1	120	3.4	2.7	1.0	7.2
10	30	4.9	6.9	1.2	13.0
50	120	1.2	1.1	0.5	2.9

sin). These results suggested a decrease of the elongase activities with a related accumulation of the C_{18} precursor fatty acids (Table II)

The same experiment carried out for 120 min showed a marked decrease of the total fatty acid label (Table I). In addition, the ratio of the label of C_{22} to C_{20} fatty acids also decreased (1.84 and 1.11, respectively, for 0 and 50 μM monensin) showing a possible perturbation of the C_{20} to C_{22} elongation step. It has been demonstrated that this elongation step is carried out mainly by the C_{20}-CoA elongase in membranes associated with the Golgi complex, using C_{20} fatty acyl-CoA as substrate [15]. The results presented in Table I are in good agreement with the localization of the C_{20}-CoA elongase in the Golgi apparatus.

Although the elongase activities seem to be modified in the presence of monensin for both 30 and 120 min incubation periods, the total acetate labeling was not modified for the 30 min incubation period.

Effect of monensin on the intracellular lipid distribution

After incubation with [1-^{14}C]acetate for 30 or 120 min, with or without monensin, the seedlings were homogenized for subcellular fractionation. The total lipid and fatty acid labels in the different membrane fractions were then determined and the effects of the drug on the intracellular distribution of the acetate-labeled lipids and fatty acids were examined. Various membrane fractions were obtained by centrifugation of the microsomal pellet on a linear sucrose gradient according to Refs. 6 and 16.

The previous characterization of the different membrane fractions [16] showed that 60% of the latent inosine-5'-diphosphatase activity (a marker of Golgi structures [8,17]) of the whole gradient was found in the 1.13–1.14 g/cm^2 fraction. The assays of other markers (CDP-choline diacylglycerol choline phosphotransferase and NADPH–cytochrome *c* reductase, for the ER, and glucane synthetase II for the plasmalemma) showed that this fraction is only weakly contaminated by other membranes [16].

TABLE II

Effect of monensin on the distribution of the labeled lipids in the different subcellular membrane fractions after a 120 min incubation

Two batches of seedlings were incubated with [1-^{14}C]acetate for 120 min, with or without monensin 1 μM. After subcellular fractionation, the total lipid and fatty acid labels of the different fractions were determined. The specific radioactivities of the lipids, or fatty acids, of the different fractions are given in cpm/mg of proteins. The values in brackets (relative specific radioactivities) are the ratios of the specific radioactivities of the fraction to that of the whole gradient. PM, plasma membrane; mon, monensin.

Membrane fractions (g·cm^{-3})	Characterization according to Ref. 6 & 16	Lipid label		Fatty acid label	
		−mon	+mon	−mon	+mon
1.08–1.09	intermediary membranes ER and Golgi markers	429 (2.50)	402 (1.95)	298 (2.38)	302 (1.85)
1.12	main site of lipid synthesis ER markers	370 (2.11)	404 (1.95)	276 (2.20)	322 (1.97)
1.13–1.14	Golgi-enriched fraction Golgi marker	136 (0.81)	208 (1.01)	80 (0.66)	171 (1.04)
1.16–1.18	contaminated PM PM, ER and Golgi markers	132 (0.77)	133 (0.73)	92 (0.65)	100 (0.61)
Whole gradient	microsomes	172 (1.00)	205 (1.00)	125 (1.00)	162 (1.00)

In one experiment, using an incubation period of 120 min, the specific radioactivity (cpm/mg of proteins) of the Golgi-enriched fraction (1.13–1.14 g/cm^3) increased by 53% in the presence of 1 μM monensin. At the same time, the specific radioactivities in the other fractions undergo only small modifications, −6.3, +9.2 and 0% for the light (1.08–1.09 g/cm^3), ER (1.12 g/cm^3) and contaminated plasma membrane (1.16–1.18 g/cm^3) fractions, respectively. The specific radioactivity of the whole gradient increases by 19.4% in the presence of monensin. These results have been standardized by dividing the specific radioactivities of the different fractions by that of the whole gradient; this standardization gave rise to relative specific radioactivities (given in parentheses in Table II). After lipid hydrolysis and radio-GLC analysis of the fatty acid-methyl esters, we determined the fatty-acid-specific radioactivities of the different fractions. The increase by 114% of the specific radioactivities of the fatty acids in the Golgi-enriched fraction (Table II) indicates that the accumulation observed for the total lipid label was due mostly to the fatty acid label.

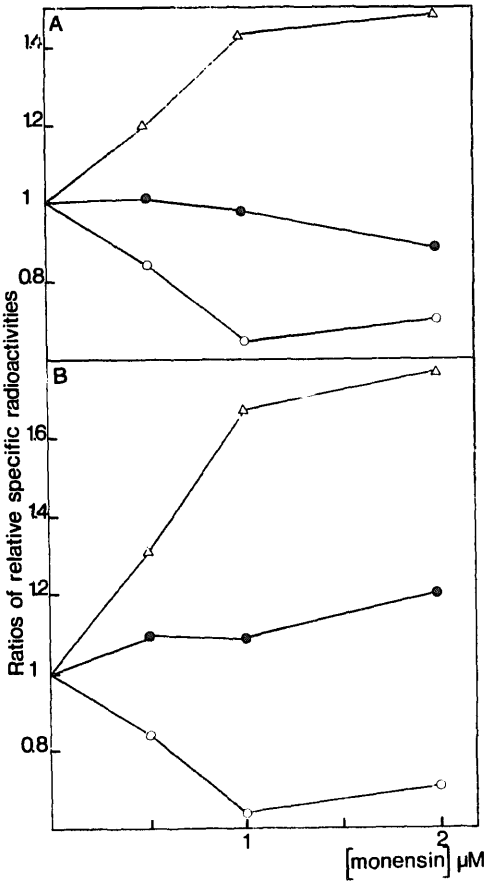

Fig. 3. Effect of monensin on the distribution of labeled lipids in the different subcellular membrane fractions after a 30 min incubation. Four batches of seedlings were incubated with [1-^{14}C]acetate for 120 min with 0, 0.5, 1 and 2 μM monensin. After subcellular fractionation, the total lipid and fatty acid labels of the different fractions were determined. The results represent the evolution, as a function of monensin concentration, of the relative specific radioactivities of the lipids (A), or fatty acids (B) for each fraction (1.08–1.09 (○); 1.12 (●); 1.13–1.14 g/cm^3 (△)).

Similar experiments were carried out using three different concentrations of monensin (0.5, 1 and 2 μM) and an incubation period of 30 min. Under these conditions, the total incorporation of radioactivity into lipids is unaffected by the monensin (Fig. 2 and Table I). We observed a dose-dependent increase of the relative specific radioactivity of the lipids in the Golgi-enriched fraction (1.13–1.14 g/cm^3), while it decreased in the light fraction (1.08–1.09 g/cm^3) in the presence of monensin (−27% at 1 μM) and remained more or less constant at the site of synthesis (1.12 g/cm^3 fraction) for concentrations of up to 2 μM monensin (−11%, Fig. 3A). As already observed for the 120 min incubation, the accumulation of the label within the Golgi-enriched fraction was enhanced for the fatty acids (Fig. 3B) (+67% at 1 μM monensin), as compared to the total lipids. Moreover, for both the 30 and 120 min experiments, the accumulation of labeled lipids is essentially due to the C_{18}-fatty acids, while the VLCFA are not (30 min), or only slightly (120 min) accumulated. These results demonstrate that monensin induces an intracellular redistribution of the acetate-labeled lipids and in particular their accumulation in the 1.13–1.14 g/cm^3 fraction enriched in Golgi membranes.

Discussion

The assay of monensin has allowed, for the first time, the determination of the amount of this drug that really enters into the seedlings and the proportion of the drug that is incorporated into the microsomal membranes. It has been demonstrated that only a very small amount of the monensin was associated with the endomembrane system, the majority being recovered in the supernatant. Monensin, considered as a small amphipathic molecule, may partition between the aqueous phase and the membranes. The partition coefficient is

$$K_p = (D_M/D_{Aq}) \cdot (vol_{Aq}/vol_M)$$

where D is the amount of the drug in the membranes (M), or the aqueous medium (Aq), and vol_{Aq} is the volume of the aqueous medium and vol_M is the volume of the membranes. Applied to our results, this calculation leads to K_p values of 0.46 and 0.23 for monensin concentrations of 50 and 100 μM, respectively. The weak binding of the drug to microsomal membranes (observed in vitro and in vivo) were compared to the results obtained by Conrad and Singer [18] for two small amphipathic molecules, and we conclude that monensin behaves as a classical amphipathic molecule with a low solubility in the microsomal membranes. Studies of the effects of the drug on lipid metabolism have shown that, using short incubation periods (30

min) and monensin concentrations of up to 2 μM, the overall fatty acid synthesis from acetate is unchanged, thus allowing the study of the effects of the drug on the intracelluiar distribution of the labeled lipids. Under these conditions, a dose-dependent accumulation of neosynthesized lipids was observed in a fraction enriched in Golgi membranes. The analysis by radio-GLC of the fatty acyl moieties of the lipids in this fraction leads to the following two questions:

(1) Since fatty-acid-containing lipids and sterols are both labeled after in vivo [1-^{14}C]acetate incorporation by leek seedlings, does the difference between the label accumulation into the total lipids and the fatty acids indicate that the intracellular transport of the sterols and of the glycerolipids could be distinct? This would be in good agreement with a number of studies realized on animal cells [19–25] and yeast [26], which have shown that the mechanisms of lipid transfer depend on the lipid class (for example, PE and sphingolipids [19,20], and PC and cholesterol [21,22]) and, for a given lipid, on the biological material used (for example, the glucocerebrosides in Chinese hamster lung fibroblasts [20] and brain cells [25]).

(2) Most of the label accumulated in the fatty acyl moieties of the Golgi membranes is found in C_{18}, whatever the incubation period, while the VLCFA's are not (30 min), or only slightly (120 min), accumulated. Does a direct perturbation of the elongation by monensin prevent further transport of fatty acid, or does a perturbation of the transport prevent the C_{18}-fatty acids from reaching their elongation site(s)?

As a first attempt to answer this question, in vitro experiments have been carried out to determine a possible direct inhibitory effect of monensin on the elongation activities. It was found that, for monensin concentrations of between 0.1 and 10 μM, all the elongation activities (C_{18}-CoA elongase, C_{20}-CoA elongase and the ATP-dependent elongase (for review, see Ref. 15) were unaffected in vitro. These data favour the hypothesis that the main effect of monensin concerns the lipid transfer; monensin could prevent the access of the fatty acid precursors to the elongation site(s).

Whatever the mechanisms by which monensin acts on the various elongating activities in vivo, the results presented in this paper clearly show that the presence of monensin leads to the accumulation of neosynthesized fatty acids in a subcellular fraction highly enriched in Golgi membranes and, consequently, suggest that the Golgi complex could be partly involved in the intracellular transport of lipids in leek seedlings. The maintenance of the lipid composition of intracellular membranes is most likely regulated by several transport mechanisms and the identification of these mechanisms in living cells has proven to be a difficult problem [27]. Using pulse-chase experiments, some authors made the assumption of an intracellular lipid transport by membrane flow, but they did not answer the question of the implication of the Golgi apparatus [6,28,29]. The work presented in this paper strongly suggests that some lipids could be routed through the Golgi apparatus prior to their subsequent distribution throughout the cell.

Acknowledgements

This work was supported by the C.N.R.S. and the University of Bordeaux II, France. The authors wish to thank M.A. Heape for rereading the manuscript.

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