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Distribution of clathrin-coated pits is related to cortical microtubules in growing cells but not in non-growing cells

H. M. P. KENGEN and J. DERKSEN*

Department of Experimental Botany, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

SUMMARY

The distances of clathrin-coated pits to the nearest cortical microtubules were measured in four different cell types of *Equisetum hyemale*, and these were compared to the distances of artificially generated random markers to the same microtubules. Statistical analysis of the data showed young growing cells to possess non-random distributions of coated pits, while in full-grown cells coated pits seemed to be randomly distributed in relation to the microtubules.

Key-words: clathrin, coated pits, *Equisetum hyemale*, microtubules.

INTRODUCTION

Many aspects of the relationships between different cytoskeletal elements and other structures in plant cells have been investigated over the past decade. Co-distributions of microtubules and actin filaments have been seen in a great number of cells using various procedures (Tiwari *et al.* 1984; Lancelle *et al.* 1987; Goodbody *et al.* 1989; Pierson *et al.* 1989; Kengen & Derksen 1991; Kengen & De Graaf 1991); for co-orientation of cellulose microfibrils and microtubules see Emons *et al.* (1992). Relationships between intermediate filaments and microtubules have been described by Dawson *et al.* (1985). Coated pits have been observed to be connected to microfilaments (Kengen & Derksen 1991) and in human lymphoblastoid cells there is evidence of an active role of actin microfilaments in the formation of coated vesicles (Salisbury *et al.* 1980). Not only have biochemical associations between coated vesicles and microtubules in the mammalian brain been described (Imhof *et al.* 1983; Walker & Agoston 1987), but physical connections between coated pits and microtubules have also been observed in deep-etched plant suspension cells (Hawes & Martin 1986) and in dry-cleaved preparations of protoplasts (Kengen & Derksen 1991).

Coated pits and coated vesicles of plant plasma membranes are believed to play a role in retrieving excess membrane from the plasma membrane (review: Morré 1990; for collection of reviews: Hawes *et al.* 1991). Microtubules might serve as tracks for coated vesicles in transport processes, at least in animal cells (Imhof *et al.* 1983).

*To whom correspondence should be addressed.

This paper is dedicated to Professor Dr M. M. A. Sassen on the occasion of his retirement.

Table 1. Statistical analysis of the distribution of clathrin-coated pits in relation to the nearest microtubule

Plant	Cell type	Significance*
Equisetum	Young root hairs	$P<0{\cdot}001$
Equisetum	Full-grown hairs	NS
Equisetum	Meristematic cells	$P<0{\cdot}025$
Equisetum	Cortical cells	NS
Nicotiana	Protoplasts	$P<0{\cdot}001$

*Distances between microtubules and clathrin-coated pits were compared to distances between microtubules and artificially generated markers. Markers were randomly distributed. Chi-square test was used to test for significance (df=5). NS, not significant.

As the distribution of the coated pits appeared to depend on the microtubular distribution of plasma membranes of protoplasts (Kengen & Derksen 1991), an attempt was made to determine a similar relationship in meristematic (root meristem) and differentiated cells (root hairs and cortical cells) of *Equisetum hyemale*.

MATERIALS AND METHODS

Stem cuttings of *E. hyemale* L., containing several nodes were grown on an aqueous soil extract (Meekes 1985) under greenhouse conditions. After 6 days, roots emerged at the nodes and produced extensive root hair populations. Roots 3 cm long were fixed for 3–4 h in a combination of 2% glutaraldehyde and 2% formaldehyde in 100 mM 1,4-piperazine diethane-sulphonic acid (PIPES) buffer, pH 6·8, containing 10 mM ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 5 mM $MgSO_4$ and 1% tannic acid (Mallinckrodt, USA). The roots were subsequently rinsed in buffer and treated for 10 min in 5–10% cellulysin (Calbiochem) in buffer to enhance cell separation. After one rinse in buffer, rootlets were kept for 15 h in buffer. They were then transferred to distilled water where the tips were cut from the roots in order to obtain meristematic cells and cortex cells in separate preparations. Root hairs were processed for cleaving as described by Traas *et al.* (1985). Preparations were made from the tubular part only.

For the preparation of meristematic (rib-meristem) and fully expanded cortical cells, the epidermal layer and stele were removed using tweezers, and the remaining parts were allowed to settle on formvar/carbon and poly-L-lysine coated grids. After the material had been attached to the grids, it was post-fixed with a 0·5% solution of osmium tetroxide in distilled water for 30 min, rinsed, transferred to a 0·5% solution of uranyl acetate in distilled water for 30 min and subsequently dehydrated in a water/ethanol series. The cell strips were critical point dried and then cleaved as described by Traas (1984). The material was examined with a Philips EM 201 or JEOL JEM 100 CX II transmission electron microscope.

The exact magnification of the micrographs of cleaved cells was established with a grating replica. Microtubules and coated pits were traced from the micrographs onto plastic sheets. Distances between microtubules and coated pits from edge to edge were measured using a Kontron Videoplan computer. Measurements were carried out on 25 cells that were derived from 7 independent experiments. To test whether the distribution

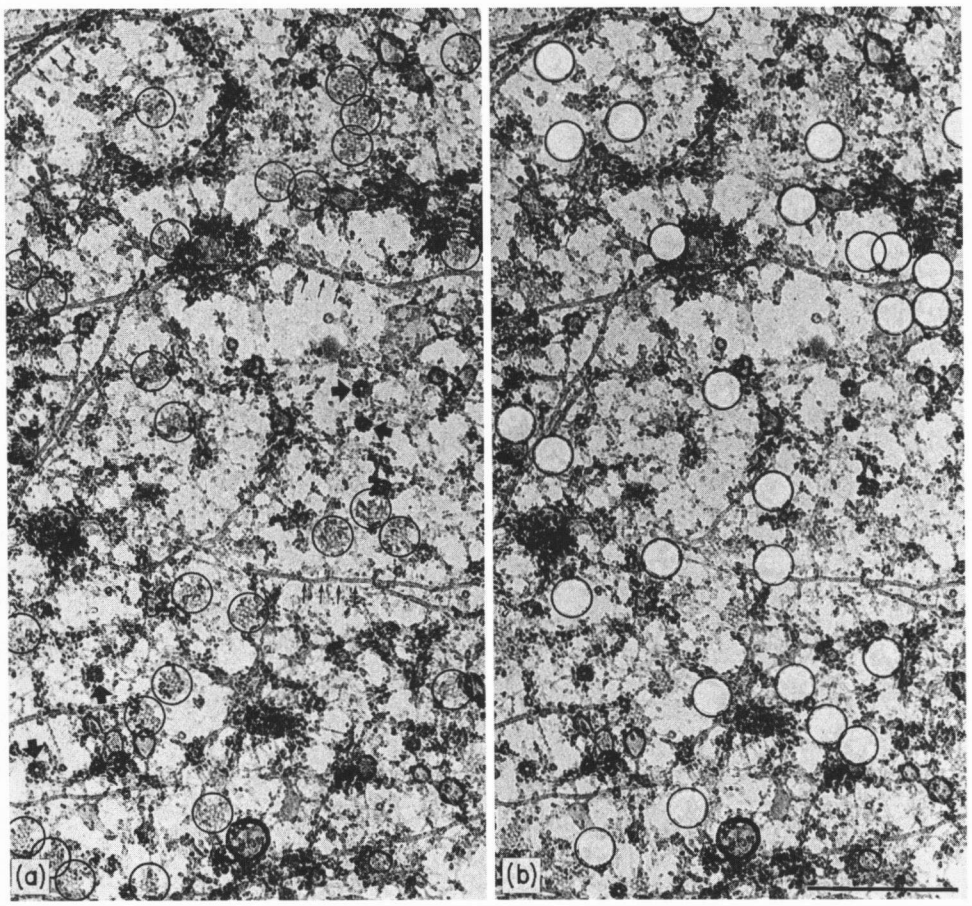
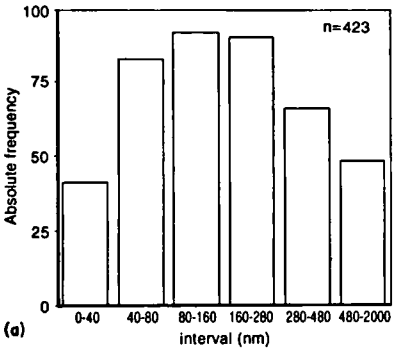


Fig. 1. Dry-cleaved preparation of a young root hair of *Equisetum hyemale*. Bar: 0·5 μm. (a) Microtubules (small arrows) and coated pits (circles) can be observed at the plasma membrane. Distances between coated pits and the nearest microtubules were measured and compared to distances of artificially generated, randomly distributed markers (b). Coated vesicles (thick arrows) were not included in the measurements.

of microtubule-coated pit distances has a structural basis or results from randomly distributed elements, we used the SAS statistical programme to generate a number of sheets displaying randomly distributed markers. These sheets were enlarged to give a number of markers per area similar to the number of coated pits on each original micrograph and projected over the traces of the microtubules; the distances between the microtubules and markers from edge to edge were then measured. The assumed diameter was the average, i.e. 80 nm for coated pits.

The distribution of distances to microtubules of coated pits and complementary markers was tested for significance using the chi-square test (see Table 1). To be able to compare the results obtained on *E. hyemale* with the results on protoplasts of *Nicotiana plumbaginifolia*, described earlier by the same authors (Kengen & Derksen 1991), we put data from the latter into the same interval groups as those used for *E. hyemale*, including the measurements in the lower interval of 0–40 nm (see Kengen & Derksen 1991).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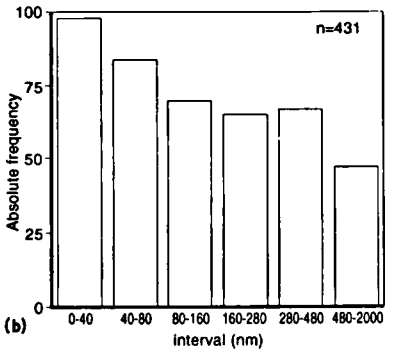
50
25
0
0-40
40-80
80-160
160-280
280-480
480-2000
interval (nm)
(a)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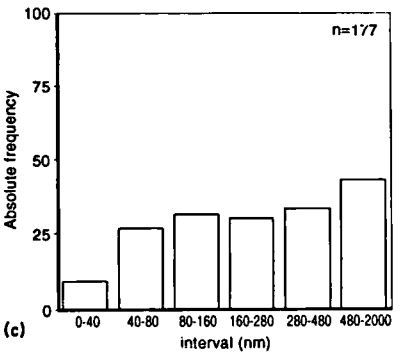
(b)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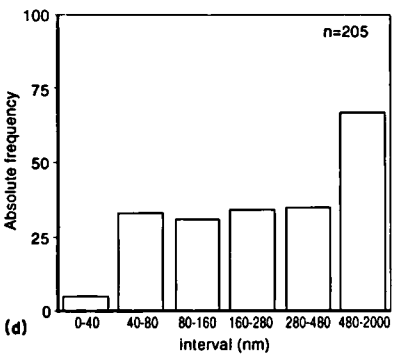
(c)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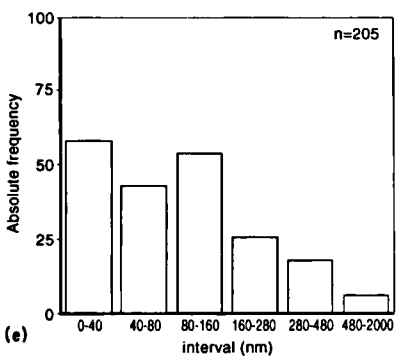
(d)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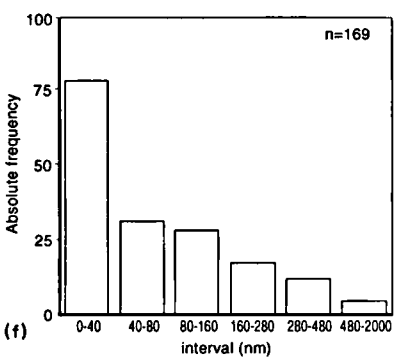
(e)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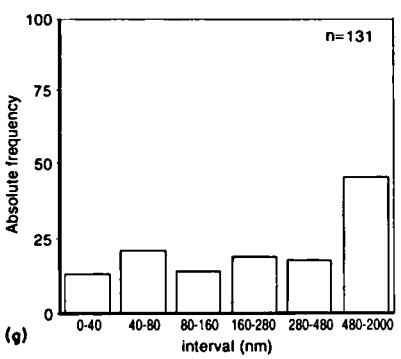
(f)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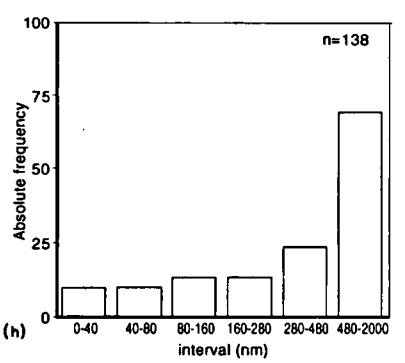
(g)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(h)

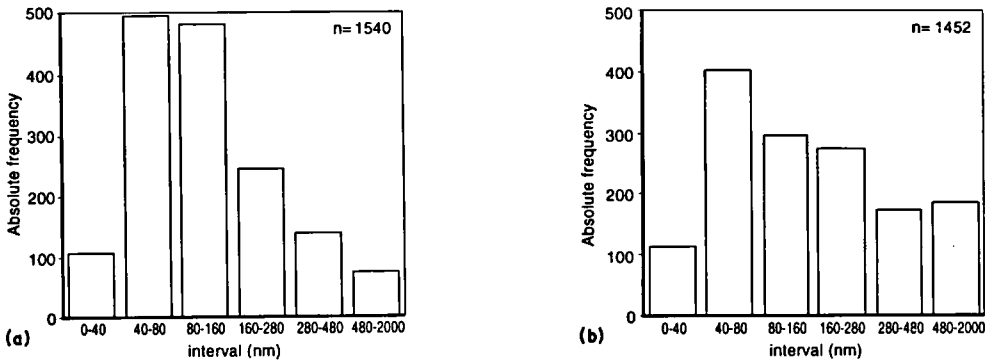

Fig. 3. *Nicotiana plumbaginifolia* (protoplasts). Distances between coated pits and the nearest microtubules (a) compared to distances between the artificially generated, randomly distributed markers and their nearest microtubule (b).

RESULTS AND DISCUSSION

Measurements of the distances between microtubules and randomly generated markers (Fig. 1) are given in Figs 2 and 3. The density of coated pits per unit area was high in protoplasts of *N. plumbaginifolia* (Figs 3a and b), growing root hairs (Figs 1, 2a and b) and meristematic cells (Figs 2e and f) of *E. hyemale*, and relatively low in older (full-grown) root hairs (Figs 2c and d) and cortical cells (Figs 2g and h) of *E. hyemale* (see also Emons & Traas 1986).

In the study presented here, the distribution of coated pits appeared to relate to the distribution of microtubules, as has been shown previously for protoplasts of *N. plumbaginifolia* (Kengen & Derksen 1991). We used different cell types of *E. hyemale* to determine whether a similar relationship was true in other cell types. Our results clearly indicate that in both cortical cells and full-grown root hairs the distribution of coated pits does not differ from a random one (Figs 2c, d, g and h). However, in both growing root hairs and meristematic cells (Figs 1, 2a and b), their distribution is non-random and relates to the distribution of the cortical microtubules. The distribution of coated pits in these cells indicates the coated pits to be located at certain distances from the cortical microtubules. The nature of the relationship between microtubules and coated pits remains uncertain and confounding cannot be excluded. Taking into consideration the large and variable distances between coated pits and microtubules, a direct interaction between microtubules and coated pit proteins is excluded. However, as microtubules and actin filaments show strong interrelationships and often co-distribute, the relationship between microtubules and coated pits may be caused by actin filaments, especially as microfilaments have been observed connecting both coated pits and coated vesicles to microtubules (Kengen & Derksen 1991). Unfortunately, actin filaments are not well-preserved in dry-cleaved preparations (Kengen & Derksen 1991). Coated pits are believed to play a role in retrieving excess membrane during cell growth (review: Morré

Fig. 2. *Equisetum hyemale*. Measurements of the distances of microtubules to the edges of coated pits (a, c, e, g) and microtubules to the edges of artificially generated, randomly distributed markers (b, d, f, h). Identical intervals were compared in both groups. (a and b) Young growing root hairs. (c and d) Full-grown root hairs. (e and f) Meristematic cells. (g and h) Cortical cells. The statistical method allows the intervals to be freely chosen, provided that the same intervals are compared in each experiment. The absolute frequency is the actual amount measured.

1990) and a higher density of coated pits in actively growing cell types compared to full-grown cells might be expected (Emons & Traas 1986). Therefore, if microtubules relate to, or even determine the formation site of coated pits, as was suggested previously by Kengen & Derksen (1991), a more pronounced relationship between coated pits and microtubules would be expected to exist in cells more active in retrieving excess membrane, and hence in actively growing cells.

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