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Negative gravitropism in *Chara* protonemata: A model integrating the opposite gravitropic responses of protonemata and rhizoids

Dieter Hodick

Botanisches Institut, Universität Bonn, Venusbergweg 22, D-53115 Bonn, Germany; FAX: 49 (228) 732677

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Abstract. The unicellular protonema of *Chara fragilis* Desv. was investigated in order to establish a reaction chain for negative gravitropism in tip-growing cells. The time course of gravitropic bending after stimulation at angles of 45° or 90° showed three distinct phases of graviresponse. During the first hour after onset of stimulation a strong upward shift of the tip took place. This initial response was followed by an interval of almost straight growth. Complete reorientation was achieved in a third phase with very low bending rates. Gravitropic reorientation could be completely abolished by basipetal centrifugation of the cells, which lastingly removed conspicuous dark organelles from the protonema tip, thus identifying them as statoliths. Within minutes after onset of gravistimulation most or all statoliths were transported acropetally from their resting position 20–100 μm from the cell apex to the lower side of the apical dome. This transport is actin-dependent since it could be inhibited with cytochalasin B. Within minutes after arrival of the statoliths, the apical dome flattened on its lower side and bulged on the upper one. After this massive initial response the statoliths remained firmly sedimented, but the distance between this sedimented complex and the cell vertex increased from 7 μm to 22 μm during the first hour of stimulation and bending rates sharply declined. From this it is concluded that only statoliths inside the apical dome convey information about the spatial orientation of the cell in the gravitropic reaction chain. After inversion of the protonema the statoliths transiently arranged into a disk-shaped complex about 8 μm above the vertex. When this statolith complex tilted towards one side of the apical dome, growth was shifted in the opposite direction and bending started. It is argued that the statoliths intruding into the apical dome may displace a growth-organizing structure from its symmetrical position in the apex and may thus cause bending by bulging. In the positively gravitropic *Chara* rhizoids only a more stable anchorage of the growth-organizing structure is required. As a consequence, sedimented statoliths cannot dislocate this structure from the vertex. Instead they obstruct a symmetrical distribution of cell-wall-forming vesicles around the structure and thus cause bending by bowing.

Key words: *Chara* – Gravitropism – Protonema – Statolith movement – Tip growth

Introduction

Gravitropism of organs of higher plants requires specialised cells for graviperception, signal transmission, and growth response. The nature of neither the physiological signal nor the mechanism controlling growth rates in the reacting cells are unequivocally clarified. In order to reduce the complexity of the systems used for studies of gravitropism, unicellular, tip-growing cells increasingly receive attention (Sack 1993). Nevertheless a consistent gravitropic reaction chain has so far been elaborated only for the *Chara* rhizoid. In this cell, positive (downward) gravitropic curvature is achieved by a direct coupling of statolith sedimentation, deflection of Golgi vesicles from the lower to the upper cell side by sedimented statoliths, and a higher growth rate of the upper cell wall due to a higher rate of vesicle incorporation (Sievers 1967; Sievers and Schröter 1971; Sievers et al. 1979). For the negatively gravitropic protonemata of the mosses studied so far the situation is less clear since neither in *Physcomitrella* (Jenkins et al. 1986) nor in *Ceratodon* (Walker and Sack 1990; Young and Sack 1992; Schwuchow and Sack 1993) was sedimentation of statoliths observed in the apical dome, i.e. at the site of growth.

Recently, it has been shown that *Chara* nodes regenerate unicellular protonemata, which in the light microscope closely resemble rhizoids as long as blue-light-dependent photomorphogenesis is avoided (Hodick 1993). However, these protonemata perform negative gravitropism which requires a modified gravitropic reaction chain. In this paper it is shown that, in protonemata,

Abbreviation: GCSC = geometrical centre of the statolith complex

gravistimulation induces statolith movements which cannot be explained solely by sedimentation. Based on this observation a model is proposed which can account for the different gravitropic responses of rhizoid and protonema. The model takes into account the close structural similarity of the two types of cell.

Materials and methods

Chara fragilis Desv. (syn. *C. globularis* Thuill.) was grown and prepared for regeneration of single-celled protonemata in darkness as described earlier (Hodick 1993). To allow observation of the specimen in an upright position a light microscope (Axiophot; Zeiss, Oberkochen, Germany) was laid on its backplate. The stage of the microscope could be fully rotated to allow any angular displacement of the protonemata from the vertical. A green filter combination with cut-on at 510 nm (OG 530+BG 18; Schott, Mainz, Germany) was inserted into the illumination light path to avoid blue-light-induced photomorphogenesis. Contrast of the specimen was achieved solely by closing the aperture diaphragm. Micrographs were taken on Agfaortho 25 film (Agfa-Gevaert AG, Leverkusen, Germany) using green light. The time courses of gravitropic curvatures were determined from series of micrographs taken at given intervals of time.

For the presentation of the overall movement of statoliths under gravistimulation a geometrical center of the statolith complex (GCSC) was calculated from serial micrographs as follows. Pictures with a final magnification of ×725 were overlayed with a Cartesian coordinate system with its origin at the vertex and the cell-axis as the x-axis (Fig. 1). The position of each statolith could thus be registered as a pair of coordinate values with the x value giving its distance from the cell vertex and the y value its position either above or below the median line of the cell. For each point of time x and y values were averaged, thus giving the GCSC. The spread of statoliths around this mean position is shown by bars delineating the area containing 68% of the x or y values (Fig. 1). For normally distributed statoliths the bars approximate one standard deviation (SD), which by definition presents a probability interval comprising 68% of single values. However, unlike the SD, they also allow for the highly asymmetrical distribution of sedimented statoliths. Cells with 20–30 statoliths were used for the determinations of the GCSC.

The movements of single statoliths were tracked using video recordings made with a high-resolution camera (ACE system; Zeiss) on VHS or U-matic tape. For the recordings, an objective with low magnification (×20) and an additional video-eyepiece (×4) were used to achieve both a large depth of focus and a high overall magnification. An orange filter combination with cut-on at 580 nm (BG 38+OG 590; Schott) was inserted into the illuminating light path. The tapes were replayed and still pictures were taken every 30 s with the video frame store of a confocal microscope (CLSM; Zeiss). The changes in statolith position were traced with a pen on overhead foils attached to the monitor screen.

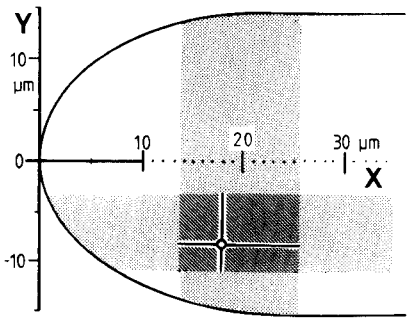


Fig. 1. Scheme for the determination of the geometrical centre of a statolith complex (GCSC) in a *Chara* protonema. In a Cartesian coordinate system with its origin at the vertex of the protonema the x and y coordinates for each statolith were determined and averaged, thus yielding the GCSC (*open circle*). The horizontal and vertical spreads of statolith positions around this point are indicated by bars indicating two zones (*shaded*) which contain 68% of all statolith positions either in the horizontal or the vertical direction. The bars approximate the standard deviation for normally distributed statoliths

For centrifugation experiments, nodes bearing protonemata were embedded with agar in a sturdy perspex chamber, which then was placed inside a reaction tube filled with culture medium (Forsberg 1965; Hodick 1993). Centrifugation was performed on a table-top centrifuge with swing-out rotor (Universal 2S; Hettich, Tuttlingen, Germany). After removal from the reaction tube, cells could be observed inside the chamber on the microscope through the transparent walls. The protonemata were perfused with cytochalasin B (ICN Biomedicals, Meckenheim, Germany) in another type of perspex chamber with an inlet and an outlet. The chamber had a volume of 0.3 ml and the perfusion rate was approx. 50 ml·h^{-1}. Small agar slabs with nodes carrying a protonema were cut out from the agar gels and perfused as complete minigel inside the chamber. Cytochalasin B was dissolved at 10 mg·ml^{-1} in dimethylsulfoxide and diluted to 10 µg·ml^{-1} with culture medium. Controls were perfused with the solution containing only 0.1% dimethylsulfoxide.

Results

Time course of gravitropic curvature. During the first hour of a gravistimulation at 90° the protonema tips bent rapidly upward (Fig. 2A). Thereafter, curvature slowed down drastically with 20–30 h elapsing before complete reorientation. A plot of the rate of curvature shows that the gravitropic reaction proceeded in three clearly distinguishable phases (Fig. 2B). The very fast initial response

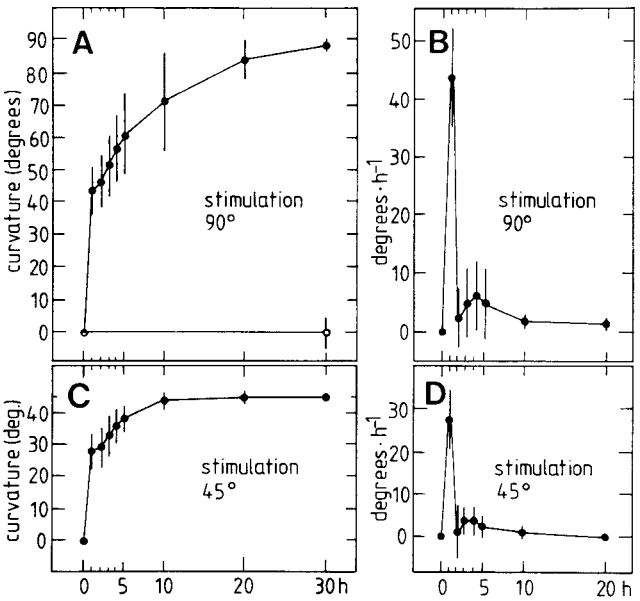
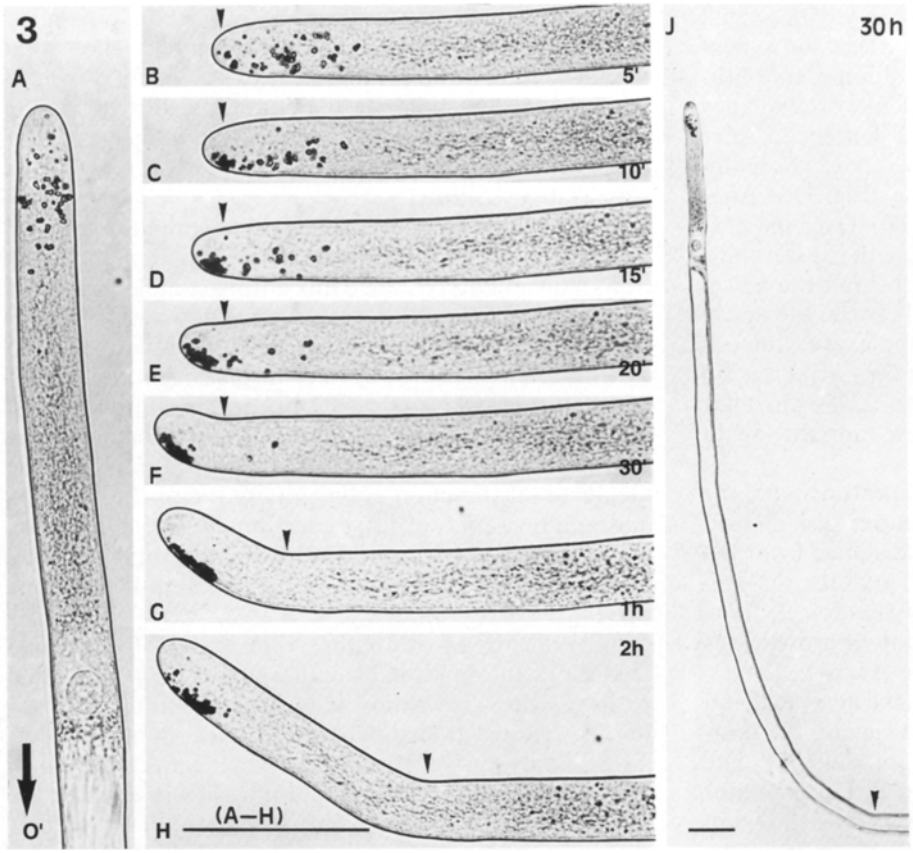


Fig. 2A–D. Time courses of gravitropic bending (**A**, C) of *Chara* protonema tips (±SD, $n=10$) and plots of the corresponding rates of bending (**B**, **D**) after stimulation at 90° (**A**, **B**) and 45° (**C**, **D**). Note that in both cases the curvatures develop discontinuously. *Open circles* in **A** represent the responses of ten protonemata with their statoliths permanently dislocated by basipetal centrifugation



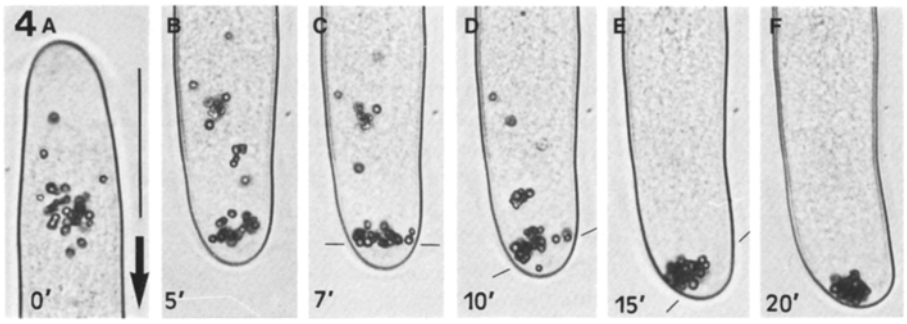


Fig. 3A–J. Serial micrographs of the gravireponse of the *Chara* protonema during stimulation at 90°. While during upright growth (**A**) only one statolith approached the cell vertex, a vigorous acropetal transport within 20 min after gravistimulation led to the accumulation of most of the statoliths on the lower side of the apical dome (**B–E**). After 20 min the shape of the apical dome changed with the upper side bulging (**E**). After 30 min (**F**) the apex pointed in a new direction and a period of straight growth followed despite continuing sedimentation of the statoliths (**G, H**). *Arrowheads* mark the position of the apex at the beginning of gravistimulation. After 30 h, reorientation was complete (**J**). *Arrow* direction of gravity vector. Bars = 100 μm; × 245 (**A–H**), × 63 (**J**)

Fig. 4. Serial micrographs of the tip of a *Chara* protonema before (**A**) and after inversion (**B–F**). Within 7 min the statoliths formed a disk-shaped complex 7 μm above the vertex (**B–C**). During the following minutes the plane of the complex tilted (*fine lines* in **C–E**) and the direction of growth changed in the opposite direction (**D–F**). *Arrow* direction of gravity vector. Bar = 50 μm; × 385

was followed by an interval of almost straight growth. In a third phase the rate of curvature reached a much smaller second maximum at hour 4 and then gradually declined to zero (Fig. 2B) without the protonema overshooting the vertical position.

A second set of ten protonemata was stimulated at 45° to test whether the very slow bending response in the former experiment after reaching approx. 45° resulted from a drastically lower gravitropic sensitivity for stimulation at angles smaller than 90°. But the curvature developed in the same way as in the 90° experiment. During the first hour the protonema tips curved by 28° in a fast reaction and completely reoriented after 10 h (Fig. 2C). The plot of the rates of curvature (Fig. 2D) also showed the typical sequence of very strong initial response followed by 1 h of almost straight growth and finally a gradual adjustment to the vertical.

Gravitropic sensitivity of protenomata could be completely abolished in the same way as in rhizoids by a basipetal centrifugation (Buder 1961; Sievers et al. 1991b). A centrifugation at 400 · *g* for 20 min had no lasting effect on the growth rates of about 70 μm · h^{-1}. But in about 30% of the cells it dislocated permanently all the dark organelles from the stationary apical cytoplasm to the basal portion of cytoplasm streaming around the vacuole (see Hodick 1993 for protonema structure). Even after 30 h of stimulation at 90° these cells showed no reorientation (Fig. 2A, open circles). This experiment proves that the dark organelles actually are the statoliths of the protonema.

Movements of statoliths. During undisturbed, upright growth the 5–50 statoliths were dispersed in the cytoplasm up to 100 μm from the cell vertex with the apical 20–30 μm containing only very few or none at all (Figs. 3A, 4A). The mean position of the statoliths, i.e. the GCSC, was approx. 50 μm from the cell vertex (cf. Fig. 5, 0 min), and is denoted as the basal resting position

of the statoliths. After 5 min of stimulation at 90°, a small downward shift combined with acropetal movement could be recognized (Fig. 3B). Within 15 min statoliths clearly accumulated on the lower flank of the apex (Fig. 3C, D) where the apical dome flattened. After 20 min, most statoliths had reached the apex, whose upper flank clearly bulged by that time (Fig. 3E). After 30 min a sharp bend was visible (Fig. 3F). Over the next 1.5 h the tip grew straight at about 40°, with the statoliths sedimented firmly on the lower flank behind the apical dome (Fig. 3F–H). The axial distance between the apical border of the sedimented statolith complex and the cell vertex increased from 7 ± 3 µm on average ($\pm$SD, $n = 10$) after 15–20 min of gravistimulation to 22 ± 6 µm after 1 h. After 30 h the protonema grew upright again (Fig. 3J).

Within 5–10 min of inversion of the protonemata, the statoliths became arranged in conspicuous disk-shaped complexes at 8 ± 1 µm ($\pm$SD, $n = 10$) distance from the cell vertex (Fig. 4A–C). After several minutes the statolith complexes tilted to one side of the apical dome (Fig. 4D), and subsequently the direction of growth was clearly shifted in the opposite direction (Fig. 4E, F).

In order to register more accurately the acropetal and downward components of statolith movement, the positions of the GCSC were determined for different time points after stimulation at different angles. During natural upward growth the GCSC in the four cells shown in Fig. 5 (t = 0 min) was positioned around 50 µm from the vertex and in close alignment with the cell axis. Within minutes after the onset of stimulation the GCSC started to move towards the apex in all cells. The bars presenting the spatial spread of the statolith complex show that within 5–10 min the complexes became widely dispersed in the axial direction, i.e. the statoliths started moving asynchronously. After 15–20 min the bars became narrow again, indicating that the GCSC had then reached a new, stable position.

The movements of the GCSC after stimulation at 45° and 90° did not show a marked displacement in the direction of the gravity vector. Thus during stimulation at 90° the acropetal movement was deflected downward by only 10° from the horizontal line (Fig. 5B). Stimulation at 45° even resulted in a small upward displacement of the GCSC against gravity (Fig. 5A). Only during stimulations at 135° and 180°, i.e. with the tips of the protonemata facing downward, was there any pronounced movement of the GCSC in the direction of the gravity vector (Fig. 5C, D), and sedimentation at least contributed to the acropetal movement of the statoliths. However, acropetal movement of the GCSC stopped before reaching the extreme tip (Fig. 5D).

Movements of statoliths were traced from video recordings to obtain information about their individual paths. During growth in the upright position, most statoliths were positioned behind the apical dome and performed only minor translocations (Fig. 6A) and rotations around their centres (data not shown). During the 15-min interval presented, only one statolith stayed permanently within the apical dome. Another three statoliths were transported to the apex, but soon returned to a more basal position. Within 1 min of gravistimulation at 90°, rapid acropetal transport started, with some statoliths moving at 40 µm·min^{-1} (Fig. 6B). Movement took place mainly in the axial direction with four statoliths arriving on the upper side of the apex. During subsequent sedimentation they avoided a zone approx. 5 µm wide behind the vertex. The tracks of other statoliths show a succession of acropetal and downward movements.

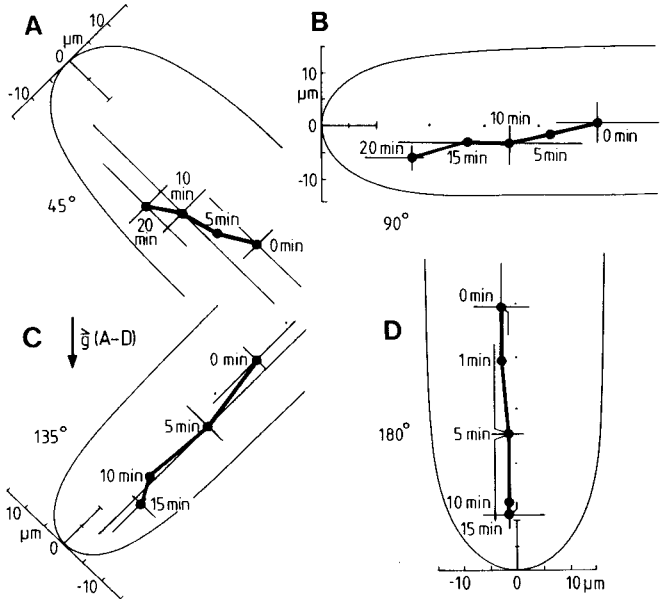


Fig. 5. Graphical representations of the movements of the geometrical centres of statolith complexes (GCSC) in *Chara* protonemata during gravistimulation at 45° (**A**), 90° (**B**), 135° (**C**), and 180° (**D**). In all cases the statolith complexes started to move acropetally and became widely dispersed. After 15–20 min they reached a new, stable position and became condensed again in a more apical position. All graphs are mounted with the vector of gravity oriented downwards. The cells contained 20–30 statoliths. The outlines of the measured cells are added for clarification

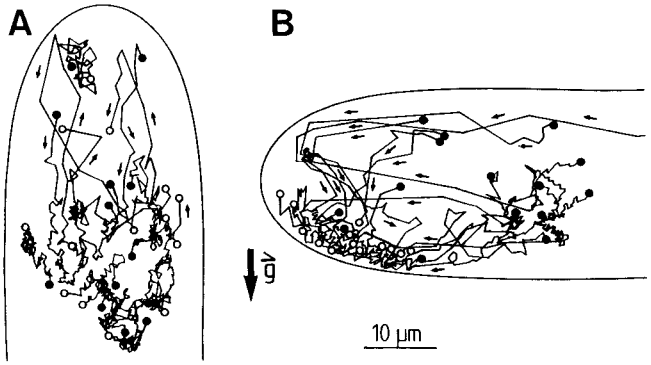


Fig. 6. Tracks of individual statoliths in a *Chara* protenema were registered for 15 min before (**A**) and during (**B**) gravistimulation at 90°. The tracks connect the positions of statoliths registered at intervals of 30 s, with the initial position (*closed circle*) and the final position (*open circle*). An interval of approx. 1 min is missing between **A** and **B** due to rotation and readjustment of the setup. Note that during upright growth (**A**), only a single statolith was permanently present in the apical dome

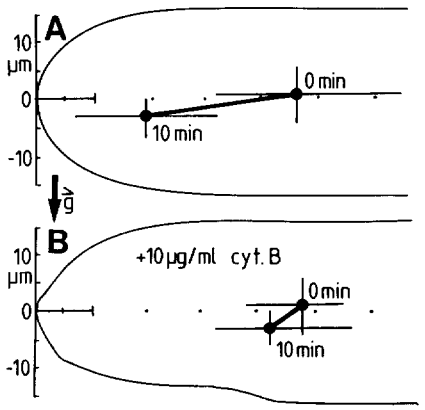


Fig. 7A, B. The movement of the GCSC in a *Chara* protonema after gravistimulation at 90° was determined (**A**) and the cell reoriented to its natural position to allow return of the statoliths to their initial positions. After an incubation with 10 µg·ml^{-1} cytochalasin B the experiment was repeated (**B**). The toxin inhibited acropetal transport of statoliths almost completely and caused irregular growth

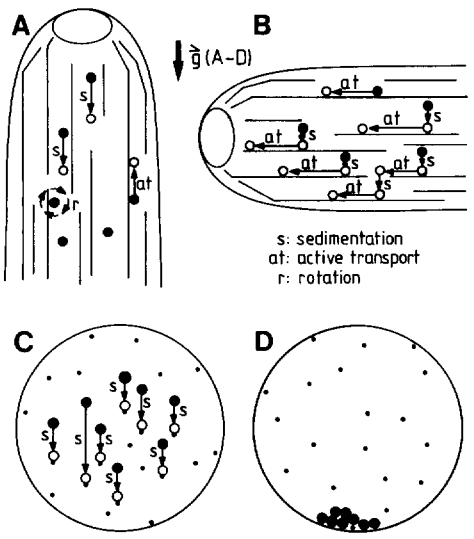


Fig. 8A–D. Schematic representation of the interactions between microfilaments and statoliths proposed for the gravitropic reaction chain of the *Chara* protonema. During upright growth (**A**) statoliths (*dark circles*, initial position; *open circles*, position after movement) can either rest in the cytoplasm, or rotate by occasional contact with microfilaments (*lines*), or be transported acropetally due to more lasting contacts. After gravistimulation (**B**) all statoliths sediment until they establish lasting contacts with microfilaments and move acropetally. A zone around the cell vertex (*ellipse*) is avoided by statoliths. The schematic cross sections of a protonema (**C, D**) demonstrate that the probability of contacting a microfilament (*dots*) is high for statoliths which are not yet sedimented (**C**), and drastically declines after complete sedimentation (**D**)

To identify the axial transport system presented in Fig. 6, the drug cytochalasin B (Hejnowicz and Sievers 1981; Cooper 1987), which very specifically inhibits transport processes by direct interaction with actin (Ohmori et al. 1992), was applied. The movements of the GCSC during gravistimulation of protonemata with either intact or poisoned microfilaments were compared. An untreated cell was recorded during 10 min of stimulation at 90°, which shifted the GCSC 26 µm acropetally from its basal resting position and 4 µm downward in the direction of gravity (Fig. 7A). Then the cell was oriented upright again to allow the return of the statoliths to their former positions. After a subsequent perfusion of the system with 10 µg ·ml^{-1} cytochalasin B for 30 min, cytoplasmic streaming slowed down and tip growth and statolith rotation ceased. Now a second stimulation at 90° for 10 min was performed. Acropetal movement of the GCSC after drug application was only 5 µm (Fig. 7B), which clearly demonstrated the actin-dependence of this component of movement. The small downward shift of 4 µm (Fig. 7B) occurred irrespective of poisoning and is therefore regarded as mere sedimentation.

Discussion

Observations of statoliths in *Chara* protonemata during undisturbed growth or gravistimulation showed three types of movements, i.e. acropetal transport, rotation around their centre, and basipetal movement. From the tracks of statoliths (Fig. 6) and the sensitivity of transport to cytochalasin B (Fig. 7) it is concluded that acropetal transport is driven by axially oriented microfilaments, which may also cause the rotations (Hejnowicz and Sievers 1981). The basipetal movement represents sedimentation of the statoliths since it does not occur in cells which are gravistimulated at 90° or larger angles, i.e. with the gravity vector not pointing in the basal direction. The three components of movement can be combined in a scheme as follows (Fig. 8):

During undisturbed, upright growth, statoliths lie in a resting position far from the apex. They occasionally come into contact with axially oriented microfilaments, which leads to rotations or, if contact is more lasting, to acropetal transport (Fig. 8A). On approaching the apex, statoliths become detached from the microfilaments and sediment back to their basal resting position in the denser cytoplasm behind the apex. Gravistimulation leads to a small, lateral displacement of the statoliths in the basal resting position by sedimentation, allowing more contacts with microfilaments and enhanced acropetal transport (Fig. 8B). During transport a statolith may lose its contact with the microfilament and sediment until it falls onto another filament and takes up acropetal transport again. As a result, within minutes, statoliths enter the apical dome mainly on its lower side (cf. Fig. 3E). This asymmetric intrusion of statoliths may displace a structure, e.g. an apical body (Spitzenkörper) as was found in *Chara* rhizoids (Sievers and Schnepf 1981), to the upper side of the apical dome, thus shifting upwards the centre of growth.

This model implies that (i) the apical dome contains a displacable structure controlling cell wall extension and (ii) that only statoliths in the apical dome convey information about the spatial orientation of the cell which leads to gravitropic bending. Inversion experiments (Figs. 4, 5D) yield circumstantial evidence for the first implication. Within a few minutes after inversion, statoliths approached within 8 µm of the vertex without advancing further and became arranged in a flat complex.

After a while this complex tilted to one side of the apex and the direction of growth changed in the opposite direction. A simple explanation for this sequence is that the statoliths sediment upon a densely structured apical body and that a slightly inhomogeneous distribution within this flat statolith complex makes it topple, thereby pushing the apical body sideways. For the *Chara* rhizoid it was shown that during normal orientation the disk-like arrangement of statoliths is stabilized by acropetally and basipetally directed shear forces (Hejnowicz and Sievers 1981; Sievers et al. 1991a; Volkmann et al. 1991), and that it takes considerable centrifugal acceleration to displace the complex further acropetally (Braun and Sievers 1993). In the protonema a basipetal force may exist, but it must be weaker than in rhizoids since it does not prevent the statoliths approaching the vertex during toppling.

The second implication, namely that only statoliths within the apical dome convey orientational information, is well supported by a pecularity in the time course of gravitropic curvature. During the first hour of stimulation at 90° the protonemata bend by 44° whereas during the second hour bending declines to only 2° on average (Fig. 2A, B). Surprisingly, this very sharp decline in curvature takes place despite a continuous and complete sedimentation of all statoliths on the lower cell wall (Fig. 3G, H). However, the distance between the sedimented statolith complex and the cell vertex increased from 7 μm on average after 15–20 min of stimulation, i.e. at the onset of the rapid initial bending, to 22 μm after 1 h of stimulation. Sedimentation at this distance from the vertex obviously cannot cause gravitropic curvature.

The scheme of statolith transport presented in Fig. 8A, B can readily explain why statoliths approach the apex en masse only during the initial phase of stimulation. Assuming an even distribution of the microfilaments across the cell diameter, the probability for a contact between a statolith and microfilaments increases with the path length of sedimentation. During upright growth, statoliths are distributed evenly (Fig. 5, $t = 0$ min), which yields an average maximal path length of half the cell diameter during stimulation at 90°. This long path makes an interaction between statoliths and microfilaments highly probable (Fig. 8C). In contrast, after 1 h of stimulation, the statoliths are completely sedimented on the lower cell wall and the probability of contacting a microfilament is minimal (Fig. 8D). Only after dispersal of the statolith complex, e.g. by Brownian motion or cytoplasmic migration (McKerracher and Heath 1987), may single statoliths reach a microfilament, be pulled into the apex, and elicit small graviresponses, which add up to the very slow second phase of bending.

The gravitropism of the *Chara* protonema has important features in common with that of the *Chara* rhizoid. In both types of cell, statolith sedimentation takes place at the site of growth, i.e. in the apical dome, with the statoliths obviously acting directly on cell wall formation (Sievers 1967; Sievers and Schröter 1971; Sievers et al. 1991b). In both cell types, statoliths can be transported acropetally by cytochalasin-sensitive interaction with actin microfilaments (Hejnowicz and Sievers 1981; Sievers et al. 1991b; Braun and Sievers 1993). However, while in the protonema most statoliths stay in a basal resting position during upright growth, in inverted rhizoids "the prevalence of acropetal saltations... brings the center of the statolith group nearer to the tip, a position reminiscent of that observed in vertically growing rhizoids" (Hejnowicz and Sievers 1981). The cause of the opposite directions of the gravitropic curvatures may lie in different anchorages of the apical body. According to Bartnicki-Garcia et al. (1989), this structure works as a vesicle-supply center (VSC), which is pulled towards the vertex while releasing vesicles evenly in all directions. In the case of the protenema the VSC and thus the point of maximal growth might be displaced from the vertex by statoliths intruding into the apical dome, which would cause bending by bulging. Assuming that in rhizoids the VSC is anchored more firmly to the vertex, e.g. by cytoskeletal elements between the apical body and plasma membrane, as in hyphal tip cells (Howard 1981), sedimented statoliths could obstruct the symmetrical distribution around the VSC without affecting the position of the point of maximal growth. This causes bending by bowing, i.e. "differential intercalar growth located basal to the very tip" (Sievers and Schnepf 1981).

The gravitropism of the *Chara* protonema differs fundamentally from that of the moss protonemata so far investigated in that sedimentation of statoliths is not detectable in *Physcomitrella* (Jenkins et al. 1986) or takes place at a considerable distance from the apical dome in *Ceratodon* (Walker and Sack 1990; Young and Sack 1992). Even during inversion the statoliths are excluded from the apex in *Ceratodon* (Schwuchow and Sack 1993). Therefore, in mosses, a direct interaction of statoliths with a growth-regulating structure in the apical dome seems to be impossible. In *Ceratodon* a concentration of microtubules occurs below sedimented amyloplasts (Schwuchow et al. 1990), which may play a role in signal transmission to the growing tip (Sack 1993). Interestingly, in the same moss, axial transport of amyloplasts was observed, which led to an accumulation of amyloplasts in the apical dome around the "center of the new direction of growth" (Young and Sack 1992). However, a role for these apical amyloplasts as statoliths in negative gravitropism could not be shown in the moss (Walker and Sack 1991; Young and Sack 1992).

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