Microtubules are required in amoeba chemotaxis for preferential stabilization of appropriate pseudopods

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SUMMARY

Amoebae of Physarum polycephalum exhibit chemotactic responses to glucose and to cAMP. The chemotaxing amoebae exhibit alternating locomotive movements: relatively linear locomotion and movements that change the direction of the locomotion. Such locomotive activity is tightly coupled with the changes in the number and the positions of the pseudopods; cells have one pseudopod at the leading edge during their linear locomotion, while they have multiple pseudopods when they are changing the direction of locomotion. Treatment of cells with microtubule-disrupting reagents inhibited the chemotaxis of the cells. To characterize the role of the microtubule system in chemotaxis, we quantitatively analyzed the relationship between the positions of multiple pseudopods of the amoebae and the relative stability of the pseudopods during reorientation. No significant differences were observed in the pseudopod dynamics between the untreated and the treated amoebae. In both cases, one pseudopod at the leading edge continued to expand during linear locomotion. It then split into two to three pseudopods in the reorientation phase, and the positions of the multiple pseudopods were random. Among multiple pseudopods, however, the pseudopods closer to the microneedle tip were selectively stabilized more often than those distant from the tip in the presence of the microtubule system. By contrast, such preferential stabilization of the appropriate pseudopods was completely abolished by microtubule inhibitors. The microtubule-dependent selection of appropriately located pseudopods enables amoebae to turn correctly at the reorientation step.

Key words: Physarum polycephalum, amoeba chemotaxis, microtubule, pseudopod formation

INTRODUCTION

Chemotaxis of amoeboid cells is a phenomenon that depends on amoeboid movements. Pseudopods are essential for such amoeboid movements (Taylor and Condeelis, 1979), and investigations of cellular aspects of chemotaxis in amoebae have focused on the behavior of pseudopods (Gerisch et al., 1974; Zigmond, 1974, 1978; Swanson and Taylor, 1982; Varnum-Finney et al., 1987a,b; Stossel, 1989). To explain the mechanism whereby cells sense a gradient of a chemoattractant, two general models have been proposed. In one model, the temporal sensing model, amoebae assess the gradient by sampling the concentrations of an attractant at different times, via different pilot pseudopods, during the extension of pseudopods (Gerisch et al., 1974). Among the pilot pseudopods, only those that keep receiving a positive input are stimulated to continue extension. Varnum-Finney et al. (1987b) showed that Dictyostelium amoebae extend their pseudopods towards the source of a chemoattractant as frequently as they extend them in the opposite direction. Those pseudopods formed in the direction of the source more often become a new leading edge than those extending away from the source. In the second model, the spatial sensing model, amoebae sense the gradient simultaneously at different points over their entire cell surface. As a result of integration of the sensed information, one pseudopod at the leading edge continued to expand during linear locomotion. It then split into two to three pseudopods in the reorientation phase, and the positions of the multiple pseudopods were random. Among multiple pseudopods, however, the pseudopods closer to the microneedle tip were selectively stabilized more often than those distant from the tip in the presence of the microtubule system. By contrast, such preferential stabilization of the appropriate pseudopods was completely abolished by microtubule inhibitors. The microtubule-dependent selection of appropriately located pseudopods enables amoebae to turn correctly at the reorientation step.
We report here details of the chemotaxis of the amoebae of *Physarum polycephalum*. We focused our attention on changes in the positions and numbers of the pseudopods. As a consequence, we found that microtubules were necessary for the preferential stabilization of the pseudopods that were closer than others to higher concentrations of the chemotaxant. The role of microtubules appeared to be essential for chemotaxis of *Physarum* amoebae.

**MATERIALS AND METHODS**

**Cell culture**

Haploid cells of *Physarum polycephalum*, strain J, were obtained from a single spore (Wakasugi and Ohita, 1973). They were maintained in culture, with *Aerobacter aerogenes*, on nutrient-agar plates that contained 5.00 g/l glucose, 0.50 g/l yeast extract, 5.00 g/l bacto-tryptone, 2 mM MgSO4, and 25 mM potassium phosphate buffer (pH 6.5; Wakasugi and Ohita, 1973). Under these culture conditions, cells mostly formed cysts after amoebic growth. To obtain amoeboid cells, the cysts were harvested and washed with several successive centrifugations (800 g, 2 min) in PDF buffer, which contained 20 mM KCl, 40 mM NaPO4, 0.7 mM CaCl2, and 2 mM MgSO4, pH 6.3, at 22°C. This buffer was originally developed for experiments with the amoebae of *Dictyostelium discoideum* (Bennett and Condeelis, 1984).

Washed cysts were repleted at high density (3 × 10^6 to 5 × 10^6 cells/9 cm plate) with *A. aerogenes* on the same nutrient-agar plates as used for the stock culture. After incubation for 24-48 hours at 22°C, cells were harvested at the exponential phase of growth and suspended in PDF buffer at 22°C. Cells cultured and harvested in this way were 100% amoeboid, and there were no cysts or flagellates.

To prepare amoebae for microscopy, cells suspended in PDF buffer were allowed to settle on glass coverslips that had been coated with 1.5% agar or with 0.01% polyethyleneimine. After starvation in a moist chamber at 22°C for 16-24 hours, cells were used in subsequent experiments.

**Video recording of live cells**

The amoebae were observed with Nomarski optics under an Olympus inverted microscope (IM-2) or a Nikon microscope (Optiphot2). Images of locomoting cells were monitored and recorded with a TV camera (WV-1550; Panasonic, Osaka, Japan) and a videocassette recorder (NV-FS5; Panasonic). Images on the recorded tapes were captured with a personal computer (Macintosh Quadra 950; Apple Computer, Cupertino, CA, USA) equipped with an analog-digital converter for the video images (Radius Inc., San José, CA, USA).

**Chemotactic stimulation**

Chemotactic stimulation was provided by solutions of 10 mM glucose or 1 μM CAMP in PDF buffer, which were allowed to diffuse from the tip of a glass microneedle (Gerisch et al., 1975; Swanson and Taylor, 1982). The microneedle, which was connected to a micromixer (IM-6; Narishige, Tokyo, Japan), was manipulated by use of a micromanipulator (MO-202; Narishige, Tokyo, Japan). There were no significant differences in the chemotactic behavior stimulated with glucose and that with CAMP, as described below. Formation of a stable gradient of a chemotactic attractant was confirmed by examination of fluorescence, from a medium that contained 6 mM CaCl2, around the tip of a microneedle that contained a calcium-sensitive fluorescent dye, fura-2 (Dojindo Laboratories, Kumamoto, Japan) at 1 μM (Gryniewicz et al., 1985). The fluorescence image was captured by a personal computer as described above, and the fluorescence intensities were quantitated using NIH Image 1.53. The concentration gradient of the fluorescent dye was stable for at least 10 minutes.

**Quantitative analysis of chemotactic tracks**

The positions of the centroids of individual amoebae were determined with the image-processor, Σ-III (Nippon Avionics Co., Tokyo, Japan). In brief, a cell’s behavior was recorded by video microscopy as described above. A frozen image of cells was displayed via the image-processor on a video screen (PVM-1442Q; SONY, Tokyo, Japan). The screen had 480 × 640 pixels and each pixel was assigned x, y coordinates according to its position on the screen. For accuracy, we used differential-interference contrast optics and a manual procedure for the determination of cell contours. The contour of a cell was bounded and fitted by a rectangle that was placed horizontally on the monitor screen. The centroid of the rectangle was regarded as the apparent centroid of the cell. This estimation of the location of the centroid contains unavoidable errors. The apparent centroid occupies different positions in a cell if the rectangle is placed at different orientations on the screen. The distance between the apparent centroid and the true centroid, which was obtained by calculation of the mean x and mean y coordinates of the pixels located along the edge of the cell (Varnum et al., 1986), was computed and found to have a mean value of 5.6 ± 2.4 pixels (n=66) on the screen. This value is equivalent to 1.1 ± 0.46 μm, which is about 6% of the mean length of 10 amoebae with various shapes; each cell had an area of approximately 5500-8000 pixels. Based on these values of the errors, the symbols in Fig. 2 are expressed so that their sizes correspond to those that incorporate 60% of the maximum value of the error.

To obtain chemotactic tracks, centroids of chemotactically moving amoebae were plotted at intervals of 5 seconds. These sampling intervals were short enough to allow detection of the changes in the locomotive activity of the amoeba.

To quantitate chemotactic orientation of cells towards the source of the attractant, the centripetal components of the net movements were calculated from the locomoting tracks of cells. The centripetal components were the vectors, directed towards the tip of a microneedle that contained the attractant, of the net movements of the amoebae. Briefly, first, the net movements were obtained by determining the change in the x, y coordinates of the centroid of one cell over the course of one minute. Second, the net movements were divided geometrically into two vectorial components, i.e. the centripetal components, directed towards the tip of the microneedle, and the vertical components perpendicular to the centripetal components. The values of the centripetal components were plotted against the distance from the tip of the microneedle. When an amoeba moved away from the tip of the microneedle, the value of the centripetal component was negative. In these cases, the movement was centrifugal.

**Calculation of cell area and pseudopod area**

The original images of the amoebae on the recorded tapes were processed for calculation of cell areas and pseudopod areas. Images were captured at 5 second intervals with a personal computer (Macintosh IICI; Apple Computer, Cupertino, CA, USA) equipped with an analog-digital converter (RasterOps 364; Santa Clara, CA, USA). The contours of cells and pseudopods were traced manually on the digital images with a commercially available software system (Studio8; Electronic Arts, San Mateo, CA, USA). We defined pseudopods as hyaline sheet-like extensions that were distinguishable from the particulate regions of the cytoplasm in the main body of the cell on the DIC images (Taylor and Condeelis, 1979). The areas of both cells and pseudopods were computed by counting the numbers of pixels contained within the traces.

**Quantification of pseudopod position with respect to the tip of a microneedle**

The positions of pseudopods were determined as explained in the scheme shown in Fig. 1. In brief, both the cell centroids and the pseudopod centroids were obtained by calculation of the mean x and mean y coordinates of the pixels located along the traced edges of the cells and pseudopods (Varnum et al., 1986). The coordinates were set for individual amoebae as shown in Fig. 1, such that the cell centroid and the tip of the microneedle became the origin (0,0) and a site on
the microneedle (+) and the cell centroid (C1 and C2) fall on the lateral region of the cells. The centroids of pseudopods (P1, P2, and P3) in terms of these coordinates are shown in the inset. Pseudopods are illustrated by shading. Inset: the centroids of pseudopods found in the proximal region (heavy stippling), the distal region (without stippling), and lateral region (light stippling) were scored. Proximal and distal regions of cells were defined as fanlike areas making an angle of 120° ahead of and behind the cell centroid with respect to the tip of the microneedle. The remaining areas were regarded as the lateral region of the cells.

the y axis (y>0), respectively. Positions of pseudopod centroids were then assigned by reference to these coordinates (inset). With few exceptions, pseudopod centroids having positive values on the y axis were located in the region of the cell that faced a higher concentration of attractant and those having negative values faced a lower concentration.

**Immunofluorescence procedure**

To determine whether or not microtubules were disrupted by nocodazole, we used indirect immunofluorescence to observe the subcellular distribution of tubulin in amoebae that had been pretreated with 5 µM nocodazole or 10 µM mebendazole (Sigma, St Louis, MO, USA) for 10 minutes at 22°C. Fixation and staining of cells were performed by the method of Curioni and Condeelis (1985) with some modifications. A mouse monoclonal antibody against chicken tubulin (Amersham, Buckinghamshire, England) was used at 10-20 µg/ml, with subsequent staining with FITC-labeled sheep antibodies against mouse IgG at a dilution of 1:100 (Amersham). Immunofluorescent images of cells were observed with a Nikon Optiphot 2 microscope equipped with a X100 objective lens NA 1.25. Photographs were taken on Kodak T-Max 3200 film.

**RESULTS**

**Microtubules are necessary for chemotaxis of *P. polycephalum* amoebae**

Individual amoebae of *P. polycephalum* were placed in a stable gradient of an attractant and each showed a chemotactic response. Amoebae kept moving more or less towards the tip of the microneedle during chemotactic stimulation (Fig. 2A,B). Note that it was very rare for an amoeba to arrive at the tip of the microneedle solely as a result of linear locomotion against the gradient of an attractant, as described below in detail.

The chemotactic response was demonstrated more clearly by a quantitative analysis of the locomotive tracks of individual amoebae (Fig. 3A-D). From the locomotive tracks, we calculated the centripetal components towards the tip of the microneedle (see Materials and Methods for details). The centripetal components, which are the vectorial components up the gradient of the net movements, are a measure of the chemotactic orientation of the amoebae. In the case of unstimulated amoebae, centrifugal movements (negative values on the y axis) were observed to the same extent as centripetal movements. That is to say, migration of the amoebae was totally nondirectional and random (Fig. 3A). Upon chemotactic stimulation, the amoebae showed a rapid decrease in centrifugal movements within one minute (Fig. 3B). Such dominance of centripetal components over centrifugal components was maintained during chemotactic stimulation (Fig. 3C,D). As a result, most amoebae (79%, n=84) that had been located within 80 µm of the tip of the microneedle before stim-
immunofluorescence microscopy (see Fig. 4). Nocodazole much smaller numbers of microtubules, as determined by presence of nocodazole. The treated amoebae had shorter and 10 minutes and stimulated them with an attractant in the orientation after chemotactic stimulation. Although 20% Fig. 3, the treated amoebae continued to move with random inhibited the chemotactic responses (Fig. 2C,D). As shown in chemotaxis, we pretreated amoebae with 5 \( \mu M \) nocodazole for 10 minutes and stimulated them with 1 \( \mu M \) cAMP as described in the Materials and Methods. (A and E) Centripetal components of locomotive tracks of unstimulated amoebae; (B and F) 0 to 1 minute after stimulation; (C and G) 5 to 6 minutes after stimulation; (D and H) 10 to 11 minutes after stimulation. The untreated amoebae exhibiting random locomotion before stimulation began to migrate toward the tip of the microneedle in the first minute, irrespective of their distance from the tip (A-D). By contrast, centrifugal movements and centripetal movements of the treated amoebae were observed to the same extent before and after chemotactic stimulation, showing the absence of chemotaxis by cells treated with nocodazole (E-H).

Fig. 3. Quantitative analysis of chemotactic orientation of the amoebae toward the tip of a microneedle in the absence (A-D) and in the presence (E-H) of nocodazole. Amoebae were treated with 5 \( \mu M \) nocodazole and stimulated with 1 \( \mu M \) cAMP as described in the legend to Fig. 2. Chemotactic responses of individual amoebae are expressed as centripetal movement, and details are given in Materials and Methods. (A and E) Centripetal components of locomotive tracks of unstimulated amoebae; (B and F) 0 to 1 minute after stimulation; (C and G) 5 to 6 minutes after stimulation; (D and H) 10 to 11 minutes after stimulation. The untreated amoebae exhibiting random locomotion before stimulation began to migrate toward the tip of the microneedle in the first minute, irrespective of their distance from the tip (A-D). By contrast, centrifugal movements and centripetal movements of the treated amoebae were observed to the same extent before and after chemotactic stimulation, showing the absence of chemotaxis by cells treated with nocodazole (E-H).

ulation reached the tip within 10 minutes after the start of chemotactic stimulation.

To test whether or not amoebae need microtubules for chemotaxis, we pretreated amoebae with 5 \( \mu M \) nocodazole for 10 minutes and stimulated them with an attractant in the presence of nocodazole. The treated amoebae had shorter and much smaller numbers of microtubules, as determined by immunofluorescence microscopy (see Fig. 4). Nocodazole inhibited the chemotactic responses (Fig. 2C,D). As shown in Fig. 3, the treated amoebae continued to move with random orientation after chemotactic stimulation. Although 20% (n=54) of treated amoebae were able to travel successfully to the tip of microneedle, they seemed to arrive there merely by chance, since their centripetal movements occurred to the same extent as their centrifugal movements in the presence of nocodazole (Fig. 3E-H). Therefore, it appears that the microtubule system is necessary for chemotaxis of P. polycephalum amoebae. Mebendazole at 10 \( \mu M \), which is known to inhibit the polymerization of tubulin (Laclette et al., 1980), also disrupted the microtubules, as determined by immunofluorescence microscopy, and inhibited the chemotaxis of Physarum amoebae in a similar manner (data not shown).

Microtubules have a pivotal role in the preferential maintenance of specific pseudopods

It has been well established that a migrating amoeba extends pseudopods at its leading edge and slides forward. Thus, the position of pseudopods in an amoeba is closely related to the direction of migration. As Fig. 2 shows, the Physarum amoebae exhibited two different, alternating types of locomotive movement: relatively linear locomotion and less linear movements that resulted in decreased net migration. When amoebae were moving in almost straight lines, the centroids of the amoebae moved at relatively high speeds (about 20-30 \( \mu m/minute \)) in a consistent direction. When the amoebae reduced their migration speeds (about 3-10 \( \mu m/minute \)), they often changed the direction of their locomotion. Such locomotive activity was tightly coupled with morphological changes; cells adopted a polarized shape during linear locomotion (Fig. 5E,F; unipolar cells), and they had two to three pseudopods during the reorientation phase (Fig. 5A,B,C,D,G; multipolar cells). The morphology of the amoebae during reorientation indicated that the amoebae reoriented themselves first by formation of multiple pseudopods and then by selective maintenance of one pseudopod. The selection of one pseudopod resulted in the formation of a new leading edge. Thus, the stability of the pseudopods on multipolar cells varied significantly.

Such preferential stabilization of one pseudopod was more clearly shown by analyzing the relationship between the positions of the pseudopods, relative to the gradient of the attractants, and the stability of pseudopods (Fig. 6; see Materials and Methods for details). The stabilization of pseudopods occurred irrespective of whether nocodazole was absent (Fig. 6A) or present (Fig. 6B). The number of pseudopods changed repeatedly from one (clear zone in Fig. 6) to two (shadow zone in Fig. 6); the repeats are shown for five times in Fig. 6A and seven times in Fig. 6B. Selection of pseudopods to be stabilized did not depend on the initial sizes of the pseudopods when two new pseudopods appeared by splitting one previous pseudopod. In Fig. 6A, the larger pseudopods continued to grow in the first three turns, while the smaller pseudopods were chosen in the next two turns. A similar inclination is obvious in the presence of nocodazole (Fig. 6B). The untreated amoeba, however, in all cases showed correct selection of the pseudopod to be stabilized (thick lines). The stabilized pseudopods were those formed on the cell side that corresponded to the higher concentration of the attractant. By contrast, such selection of appropriate pseudopods was abolished by microtubule inhibitors. The treated amoebae chose appropriate pseudopods in only three cases out of seven; in four cases, the amoebae stabilized those pseudopods that
were located on the cell side away from the source of the chemoattractant.

The dependence on microtubules of the preferential stabilization of appropriate pseudopods during chemotaxis was confirmed statistically by analyzing the positions and the relative stability of over one thousand pseudopods as shown in Fig. 7. In unipolar cells, the pseudopods were primarily located in the proximal regions with respect to the tip of the microneedle (Fig. 7A, \(n=82\)). Pseudopods continued to be extended preferentially at the cell margin that faced the higher concentration of attractant (\(\gamma>0\)). By contrast, the pseudopods of drug-treated amoebae were distributed almost randomly with respect to the tip of the microneedle (Fig. 7C, \(n=260\)). Though the treated unipolar cells similarly had one continuously extending pseudopod at the leading edge, the pseudopod region of the treated unipolar cells tended to be wider, spreading slightly toward the lateral sides of the cell, than the pseudopod region of untreated unipolar cells.

Analysis of multipolar cells indicated a random orientation of the pseudopods irrespective of whether microtubules were present or absent (Fig. 7B, D). The positions of pseudopods with respect to the tip of the microneedle were scored by the method illustrated in Fig. 1. In the absence of nocodazole, the distribution of pseudopods in the multipolar cells was 39% proximal, 29% lateral and 32% distal (Fig. 7B, \(n=517\)). In the presence of nocodazole, the distribution was not very different, being 38% proximal, 26% lateral and 36% distal (Fig. 7D, \(n=519\)). Thus, disruption of microtubules did not disturb the randomly oriented nature of pseudopod formation in multipolar cells.

The most stabilized pseudopods among the multiple pseudopods are represented as filled circles in Fig. 7B. The percentages of pseudopods in the lateral and the distal regions of the cells were 31% and 2%, respectively. Thus, the pseudopods on the side of the cell where there was a higher concentration of an attractant were preferentially stabilized. These results are consistent with observations of *Dictyostelium* amoebae made previously by Varnum-Finney et al. (1987b). In marked contrast, no such asymmetrical distribution of the selected pseudopods was seen in amoebae treated with nocodazole (Fig. 7D). The distribution of the most stabilized pseudopods was random, being 29% proximal, 32% lateral and 39% distal. Thus, microtubules obviously have a pivotal role in the selection of the chemotactically important pseudopods.

**DISCUSSION**

**Microtubules are necessary for the appropriate selection of pseudopods during chemotaxis**

The chemotactic response of *Physarum* amoebae was achieved by repeated bouts of linear locomotion and changes in direction. Irrespective of whether microtubule inhibitors were present or absent, the amoebae exhibited reorientation behavior (Fig. 2). The pseudopod dynamics were not affected significantly by inhibitors (Figs 6, 7). However, the treated amoebae did not exhibit any chemotactic responses (Figs 2, 3). These results indicate that the microtubule system enabled the amoebae to select the appropriate direction during the reorientation process. Such an ability can be attributed to the appropriate selection of pseudopods during the reorientation process because the changes in the direction of locomotion occurred in the multipolar phase of the cells (Figs 5, 6, 7). Fig. 7C implies that unipolar cells were forced to migrate mostly in incorrect directions as a result of the loss of ability to select the appropriate pseudopods, caused by microtubule inhibitors. These results show that microtubules are necessary to stabilize selectively the pseudopod on the side of the cell that is closest to the chemoattractant more effectively than the other pseudopods. Such preferential stabilization leads to selection...
of the correct direction of locomotion as a consequence. We speculate that a chemoattractant provides information for the microtubule system, which uses the information to bring about preferential stabilization of the appropriate pseudopods.

There are previous reports that microtubules are not obligatory for chemotaxis in amoeboid cells. For example, cytokine-plasts, which are motile fragments produced from neutrophils, lack microtubules and still exhibit chemotaxis (Malawista and de Boisfleury, 1982). Directed migration of neutrophils is barely affected by microtubule-disrupting reagents (Allan and Wilkinson, 1978; Devreotes and Zigmond, 1988). However, disruption of microtubules renders the chemotactic tracks more multi-directional than those of control cells (Allan and Wilkinson, 1978). Devreotes and Zigmond (1988) interpreted this observation as an effect of microtubules on the cell via inhibition of the extension of pseudopods from the rear and the sides, such that the pseudopod facing the higher concentration of an attractant is preferentially maintained. Microtubules seem to be functioning in exactly this way in *Physarum* amoebae in the unipolar phase. The view of Devreotes and Zigmond may explain the lateral spreading of the pseudopod region in unipolar cells treated with nocodazole. Also, the unipolar cells resemble neutrophils in terms of shape (Fig. 5E,F), the preferential maintenance of one pseudopod in the direction of the tip of the microneedle (Fig. 7A), and the relatively linear locomotion (Fig. 2).

There are various reports of heterogeneous interactions of microtubules with multiple pseudopods. Asymmetric distribution of microtubules has been found in the growth cones of living neurons in *Xenopus* (Tanaka and Kirschner, 1991) and grasshopper (Sabry et al., 1991). Before any obvious asymmetry can be seen in the overall shapes of the growth cones, microtubules selectively invade the lamella that has been formed in the future direction of migration. In newt eosinophils, which also show changes in direction during chemotactic migration (Brundage et al., 1991), Fay et al. (1989) observed similar behavior of microtubules. That is, microtubules in eosinophils seemed to move close to the cell

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**Fig. 5.** Typical changes in shape of chemotactically moving *Physarum* amoebae during the reorientation process. Sequential video images (A-G) and the corresponding drawings (H-N) show an amoeba alternating between the unipolar and the multipolar shapes. The hyaline pseudopods are represented by the gray regions in H-N. The numbers near the pseudopods represent the areas in µm². The centroids of the cell and the pseudopods are indicated by the black and the white dots, respectively. The cross (+) represents the tip of the microneedle. First, the amoeba exhibited two pseudopods that kept extending in almost opposite directions, while the tail (one of the multiple pseudopods in the last reorientation) was retracted toward the cell body (A-C and H-J). Next, one pseudopod on the side of cell with a higher concentration of the attractant was maintained and the other began to be retracted (D-E and K-L). In consequence, the cell had a polarized shape with one pseudopod at the leading edge and moved toward the source of the attractant (E-F and L-M). The cell began to have two pseudopods again by splitting the pseudopod at the leading edge and resumed its multipolar shape (G and N). Note that the pseudopods in the multipolar cell were formed and extended almost equally on both surfaces of the cell facing the source and away from the source of the chemoattractant. Bar, 20 µm. Time is given as minutes:seconds.
Role of microtubules in chemotaxis

By what mechanism do microtubules control the stability of the pseudopods? Microtubules may interact directly with microfilaments and affect the dynamics of the pseudopods. Interaction of microtubules with microfilaments has been observed at the biochemical level (Nishida et al., 1981; Pollard et al., 1984; Sattilaro, 1986; Itano and Hatano, 1991), but there is little evidence for any functional interactions in vivo. Uyeda and Furuya (1989) found that thick bundles of microfilaments slide over bundles of microtubules in permeabilized flagellates of *P. polycephalum*. It is also possible that the disruption of the microtubule network has important consequences for pseudopod dynamics via motor proteins. It has been shown that the elimination of one of the myosin I genes from *Dictyostelium* amoebae results in an alteration in pseudopod dynamics (Wessels et al., 1991). Fukui et al. (1990) reported changes in the distribution of microtubules in multinucleated cells induced by disruption of the myosin II gene in *Dictyostelium* amoebae, which may suggest mediation of the interaction between microtubules and pseudopods by myosin II. Kinesin was shown to regulate pseudopodial activity in fibroblasts (Rodionov et al., 1993). Alternatively, microtubules may affect the adhesiveness of the pseudopods. Rinnerthaler et al. (1988) reported close apposition of one end of a microtubule to the site of cell-substratum contact at the leading edge of chick fibroblasts. They suggested that microtubules may select, stabilize and potentiate such contacts. It is quite possible that stable contacts are favorable for stabilization of a pseudopod. Therefore, if such contacts are formed beneath one of the multiple pseudopods in the *Physarum* amoebae, it would be maintained preferentially. There is also a possibility that the selective stabilization of pseudopods may result from the polarized transport of chemotactant receptor to the appropriate pseudopod surface. Ojakian and Schwimmer (1992) reported that the insertion of a glycoprotein into the cell’s apical membrane is dependent on microtubules.

Two representative models of chemotaxis by amoebae and the relevance of our observations

In our study, during chemotaxis, the amoebae of *P. polycephalum* formed multidirectional pseudopods (Figs 5, 6, 7), which are apparently inconsistent with both the temporal sensing model proposed by Gerisch et al. (1974) and the spatial sensing model proposed by Zigmond (1974, 1978). Our observation can, however, be incorporated into either of the two models if the models are slightly modified. The temporal sensing model could incorporate the multipolar cells if the putative functioning pilot pseudopods (Gerisch et al., 1974) are equivalent to the well developed lamellipodia in the amoebae in *P. polycephalum*. The spatial sensing model also seems to provide an explanation for the multipolar shape if we make the following assumptions: the spatial comparisons of the concentration of the attractant over the surface of the cell are suppressed for a time or, alternatively, an orchestrated response to the chemotactic stimulation is postponed for a while in *Physarum* amoebae. In either case, the chemotactic response of the cell would be postponed, with resultant formation of randomly directed multiple pseudopods.

Chemotaxis of the amoebae of *P. polycephalum* can be compared with that of other amoebae as follows. Reorientation of locomotion occurs during chemotaxis in a repeated manner. As a result of each correct reorientation, the *Physarum* amoebae move little by little up the gradient of the chemotactant. The locomotion of single amoebae has been reported to be rhythmic in polymorphonuclear leukocytes (Murray et
Fig. 7. Positions of pseudopods in unipolar cells (A and C) and multipolar cells (B and D). The y axis is set so that both the tip of the microneedle and the cell centroid fall on the y axis. The positions of the centroids of individual pseudopods are shown as open and filled circles. The most stabilized pseudopods (see text) among the many pseudopods are represented by filled circles (B and D). (A and B) Positions of pseudopods in the normal amoebae. Unipolar cells mainly have a pseudopod in the proximal region of the cell (A), while multipolar cells extend multiple pseudopods in random directions. The selectively stabilized pseudopods are mostly in the proximal region (B). (C and D) Positions of pseudopods in amoebae after treatment with 5 µM nocodazole or 10 µM mebendazole. Unipolar cells mostly have one pseudopod located in the lateral region (C). The stabilized pseudopods are distributed randomly around the cell centroid (D), indicating that microtubules are necessary for preferential stabilization of pseudopods. The positions of pseudopods with respect to the tip of the microneedle were scored by the method illustrated in Fig. 1. This scoring process contains some errors that arise from variation in the gradient of chemoattractants from true concentric circles as shown in Fig. 2A. To minimize error in the data interpretation, however, we narrowed the angles of both the proximal and the distal regions to 120° instead of 180°. There were no significant differences in the distribution of these plots after stimulation with glucose or with cAMP.

al., 1992) and Dictyostelium discoideum (Wessels et al., 1994). In each cycle of the rhythm, two events seem necessarily to occur successively to achieve chemotaxis of Physarum amoebae. First, the pseudopods in the multipolar cells are formed in random directions relative to the gradient of the chemoattractant. This may have relevance to the finding by Varnum-Finney et al. (1987b) that pseudopods are formed as frequently on the surface of a Dictyostelium amoeba facing the source as they are on the surface away from the source of chemoattractant, although the Dictyostelium amoebae did not assume a multipolar shape in the gradient. The ensuing and the most crucial step in the chemotaxis of Physarum amoebae is the correct selection of the appropriate pseudopod from the many pseudopods in the reorientation process. The latter event was shown here to be microtubule-dependent. During this decision-making process, the concentration gradient of the attractant could be assessed by either a spatial or a temporal mechanism, as described above. Swanson and Taylor (1982) claim that the extension of pseudopods from the cell surface towards cAMP is only a local response by the cell and that the amoeba’s cytoplasm then has to be coordinated in the cell as a whole to achieve the directed movements. They suggest that such cytoplasmic coordination plays a prominent role, at least in situations where more than two pseudopods sense the chemotactic stimulus, and they speculate that this coordination is dependent on microtubules. The conclusion of the present study is consistent with their view that Dictyostelium amoeba chemotaxis is achieved by these two sequential events, a local cell surface response followed by a microtubule-dependent global response. Although the underlying molecular mechanism for the dependence of the selective stabilization of the appropriate pseudopod on microtubules remains to be clarified, this work provides evidence that microtubules are necessary for a process related to both the establishment of directionality and the sensing mechanism in amoeba chemotaxis.

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REFERENCES


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