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Lysophosphatidic acid is a chemoattractant for *Dictyostelium discoideum* amoebae

(amoeboid movement/chemotaxis/G protein-coupled receptor)

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ABSTRACT **The naturally occurring phospholipid lysophosphatidic acid (LPA) can induce a number of physiological responses in vertebrate cells, including platelet aggregation, smooth muscle contraction, and fibroblast proliferation. LPA is thought to activate a specific G-protein-coupled receptor, thereby triggering classic second messenger pathways such as stimulation of phospholipase C and inhibition of adenylate cyclase. Here we report that 1-oleoyl-LPA, at submicromolar concentrations, evokes a chemotactic response in amoebae of the cellular slime mold *Dictyostelium discoideum*. LPA-induced chemotaxis is specific in that other lysophospholipids, phosphatidic acid, and monoacylglycerol have no effect. We show that the response to LPA is not secondary to the accumulation of extracellular cAMP, a well-established chemoattractant for nutrient-starved *D. discoideum*. Compared with cAMP-induced chemotaxis, LPA-induced chemotaxis has a somewhat lower efficiency and is not accompanied by the characteristic cellular elongation and orientation along the gradient. These results indicate that LPA has a previously unsuspected role as a chemoattractant for *D. discoideum* and imply that its biological function as a "first messenger" is not restricted to vertebrate cells.**

Besides playing a central precursor role in lipid biosynthesis, the simple phospholipid lysophosphatidic acid (LPA; 1-acylglycerol-3-phosphate) is capable of evoking such diverse physiological responses as platelet aggregation, smooth muscle contraction, activation of Cl^- current in *Xenopus laevis* oocytes and, in fibroblasts, cell proliferation (1). LPA is rapidly produced by thrombin-activated platelets (2) and is detectable in mammalian serum (3), suggesting that LPA, which is fairly water soluble, is released from cells after its formation.

Current evidence strongly suggests that LPA activates a specific G protein-coupled receptor in its target cells, mediating stimulation of phospholipid hydrolysis and inhibition of adenylate cyclase (4, 5). Moreover, LPA induces rapid and dramatic effects on the actin cytoskeleton of mammalian cells in culture (6, 28). A putative LPA receptor of apparent molecular mass 38–40 kDa was recently identified in various LPA-responsive cell types by photoaffinity labeling (7). Thus, LPA may act as a lipid mediator serving multiple physiological functions, at least in vertebrate cells.

In search of LPA responsiveness in invertebrate cells, we examined its possible chemotactic effect on the cellular slime mold *Dictyostelium discoideum*, an easily accessible and experimentally convenient system that is gaining increasing importance for signal transduction studies. *D. discoideum* is a simple haploid eukaryotic organism that, in the vegetative phase, lives as amoebae feeding on bacteria and yeast in the soil. Upon starvation, amoebae cluster together chemotactically and aggregate to form a multicellular slug-like structure. In the next phase of the life cycle, a spore-containing fruiting body is formed. Under favorable conditions, new amoebae arise from the spores to complete the life cycle. Chemotaxis plays an essential role in various stages of the life cycle. During the vegetative phase the amoebae respond chemotactically to folic acid and pterins (8, 9), which are secreted by bacteria. Upon starvation, sensitivity to folic acid is lost and the amoebae become chemotactically responsive to cAMP, which acts through specific G protein-coupled receptors (10, 11). The receptor-mediated signaling events underlying chemotaxis are not fully understood, however. cAMP receptor occupancy triggers stimulation of phospholipase C and guanylate cyclase, with consequent formation of second messengers, as well as activation of adenylate cyclase to produce cAMP, which is partially secreted and contributes to the formation of a cAMP gradient and hence to aggregation efficiency in an autocatalytic manner. Actin polymerization and intracellular Ca^{2+} changes play an important role in *D. discoideum* chemotaxis (11–13) and are also involved in the action of LPA on vertebrate cells (6, 28).

In the present study we show that starved *D. discoideum* amoebae show positive chemotaxis toward an extracellular gradient of LPA, but not other phospholipids, in a manner that is independent of cAMP formation and secretion. These results define LPA as a chemoattractant for *D. discoideum* and show that LPA's biological role as a signaling molecule may be more widespread than previously realized.

MATERIALS AND METHODS

Materials. L-α-Phosphatidic acid (dioleoyl), LPA (1-oleoyl), monoacylglycerol (1-oleoyl), lysophosphatidylcholine (1-palmitoyl), lysophosphatidylinositol (1-oleoyl), platelet-activating factor (PAF), lyso-PAF, and cAMP were obtained from Sigma.

Cell Culture Conditions. *D. discoideum* NC-4(H) cells were grown in association with *Escherichia coli* 281 on a solid medium containing 3.3 g of peptone, 3.3 g of glucose, 4.5 g of KH_2PO_4, 1.4 g of $Na_2HPO_4 \cdot 2H_2O$, 15 g of agar in 1 liter of H_2O at 22°C. Before clearing of the bacterial lawn occurred (after about 40 hr) the cells were harvested and washed free of bacteria by repeated washes with cold 10 mM sodium/potassium phosphate buffer at pH 6.5 (PB, KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$ in water) and centrifugation at 150 × *g* for 2 min. Cells used for experiments were starved by plating them on nonnutrient agar (1.5% agar in H_2O) at a density of about 1.5×10^6 cells per cm^2 for 5–8 hr at 22°C or overnight at 5°C.

Chemotaxis and Random Movement Assays. Quantitative measurements of chemotaxis and random movement were

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Abbreviation: LPA, lysophosphatidic acid.

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performed as described before (14). In short, starved cells were deposited at a density of about 5×10^4 cells per cm^2 on a glass coverslip that was mounted on the bottom of an open Teflon culture dish (15). After the culture dish had been filled with 2 ml of PB, cells were observed with phase-contrast microscopy (40× objective). Chemotaxis was tested by placing the tip of a glass micropipette (tip diameter 1–2 μm) filled with PB containing the test substance close to the glass coverslip. Due to diffusion from the tip, a gradient of the test substance is formed. Chemotaxis is defined as net movement toward the tip of the microcapillary (chemoattractant source).

Models of three-dimensional diffusion from a pipette tip show a several-order-of-magnitude dilution of the compound just outside the tip, which depends on diffusion constant, effective pipette opening, temperature, and time (K. Inouye, personal communication). It is, however, difficult to calculate local concentrations of LPA accurately because some of these parameters are poorly defined. In concordance with the theoretical calculations, we observed that a micropipette containing 200 μM LPA, placed near the bottom of a culture dish with serum-deprived N1E-115 neuroblastoma cells, induced morphological alterations in cells near the tip, with small effects still being detectable in cells up to ≈70 μm away from the tip (K.J., unpublished data). Since these cells respond to LPA concentrations as low as 10–20 nM, we estimate that local concentrations present near the tip drop to the nanomolar range in our experimental setup.

Amoeboid movements were recorded with a video recorder (JVC type HB-D250e), and experiments were stopped either as soon as amoebae reached the tip or after 10 min. Thereafter, the tracks of individual cells were digitized and

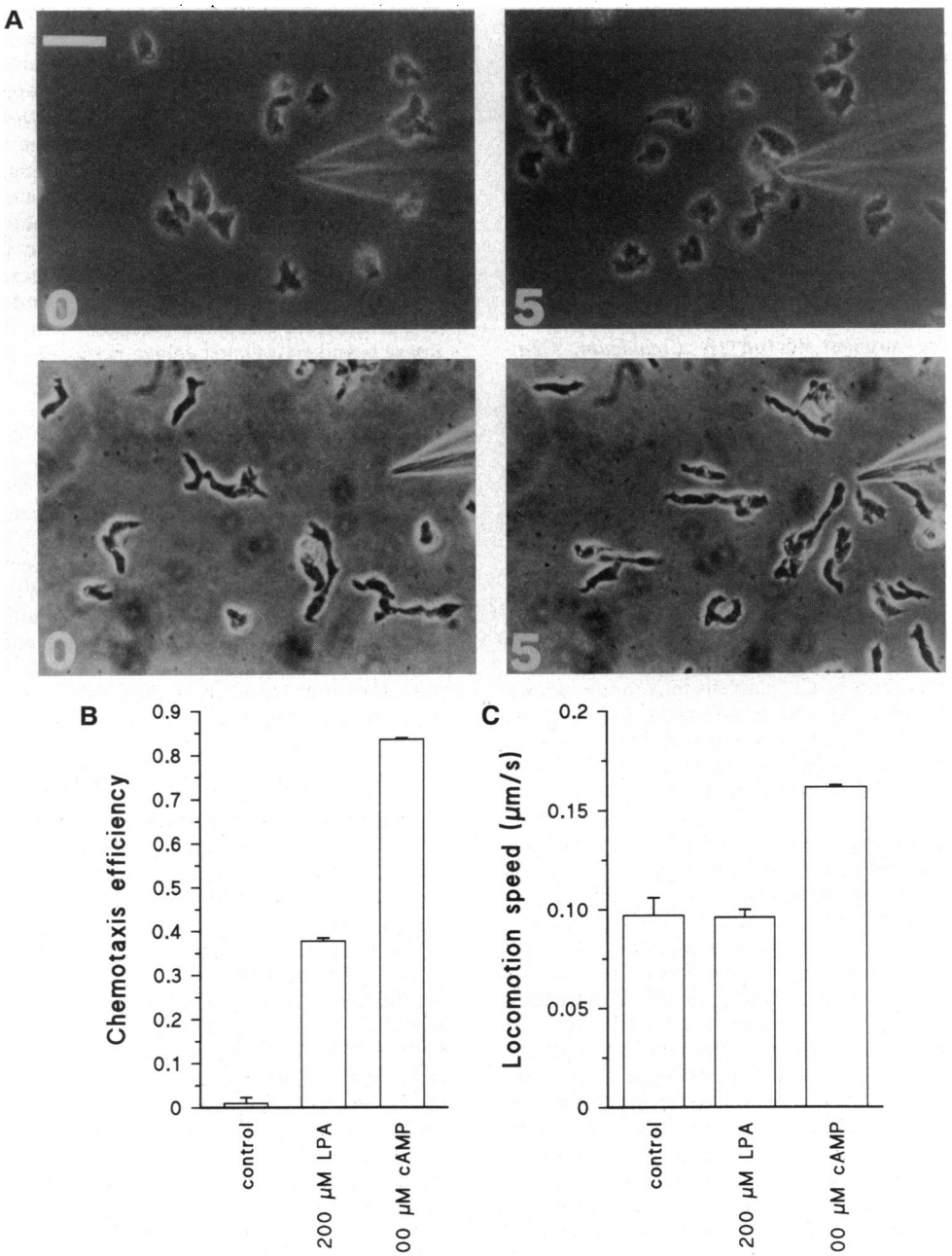

FIG. 1. Effects of LPA and cAMP on *D. discoideum* locomotion. (*A*) Migration toward a microcapillary source of LPA (200 μM; upper panels) or cAMP (100 μM; lower panels), amoebae photographed at 0 and 5 min as indicated. (Bar = 25 μm.) (*B*) Efficiency of chemotaxis (*TG/TT*) toward micropipettes containing LPA, cAMP, or buffer (control). (*C*) Effect of chemoattractant gradients on amoebae locomotion speed (*TT*), calculated for the experiments in *B*. Data are presented as mean + SEM.

analyzed on a personal computer. Cellular locomotion speed was calculated by dividing the total track length (*TT*) by time. After calculating net movement along and perpendicular to the gradient (*TG* and *TS*, respectively), the ratio *TG/TT* was determined and used as a measure for chemotaxis efficiency (14). A major experimental problem was obstruction of the tip of the microcapillary by LPA precipitates. In the presence of Ca^{2+} and other divalent cations originating from the capillary glass, LPA forms a precipitate (4) that can block the tip opening of the microcapillary, thus preventing the formation of a LPA gradient. Therefore, before and after each experiment we checked the tip opening visually while applying an air pressure pulse to the lumen. Experiments in which the tip was plugged or where precipitates were visible in the lumen were not taken into account.

Statistics. Values are expressed as means ± SEM; *n* equals the number of cells measured. Statistical significance of differences between mean values was assessed with the Student *t* test. Each data point represents the mean of at least three independent experiments.

RESULTS AND DISCUSSION

Chemotaxis of *D. discoideum* Amoebae Up an LPA Gradient. Within minutes of the placement of an LPA-filled micropipette close to the bottom of the culture dish, individual *D. discoideum* amoebae that have been induced to proceed into the aggregation-competent phase by starvation for 5–8 hr start to migrate toward the tip of the pipette (Fig. 1*A*). LPA-induced migration was statistically highly significant and well reproducible between many independent experiments involving different cultures of starved *D. discoideum*. Chemotaxis efficiency (*TG/TT*, see *Materials and Methods*) toward an LPA source was about half that observed with the classic chemoattractant cAMP (Fig. 1*B*). Noteworthy, LPA did not induce the cellular elongation and orientation along the gradient that are characteristic for migration in a cAMP gradient (Fig. 1*A*). Whereas the locomotion speed of cells moving in a gradient of cAMP roughly doubled (13, 14), LPA did not accelerate the amoebae (Fig. 1*C*). Thus, migration toward LPA and that toward cAMP differ both quantitatively and qualitatively in a number of aspects. This observation supports the idea that locomotion speed and cellular orientation are independently regulated parameters (13).

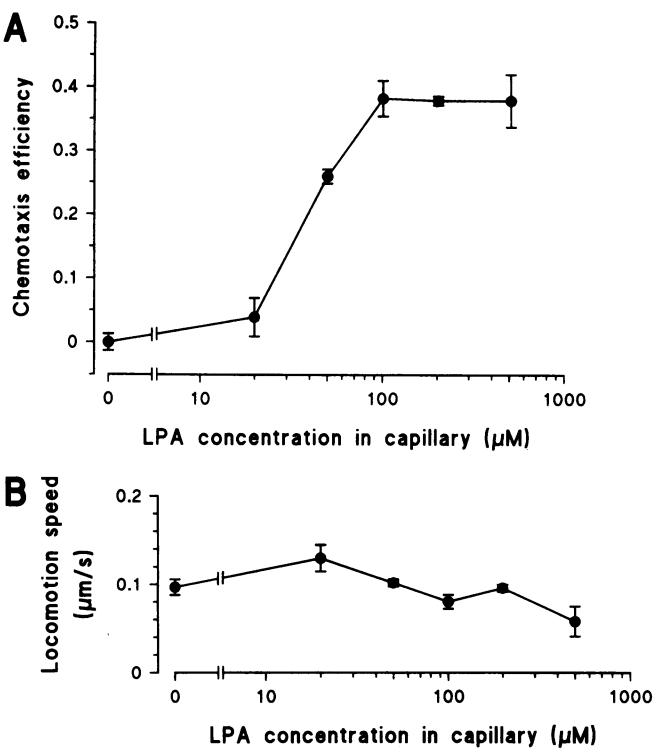

FIG. 2. Dependence of locomotion parameters on capillary LPA concentration. (*A*) Efficiency of chemotaxis, determined for different concentrations of LPA. Data (mean ± SEM) are from 3–11 independent experiments. (*B*) Cellular locomotion speed, calculated from the experiments presented in *A*.

Concentration Dependence. The dose–response relationship for LPA-induced chemotaxis is shown in Fig. 2*A*. Maximal chemotactic response was observed at an LPA concentration of 100 μM in the capillary, whereas chemotaxis was not observed at doses below 20 μM. Locomotion speed hardly varied with LPA concentration (Fig. 2*B*) and did not differ from that of randomly moving cells.

Local LPA concentrations at some distance from the pipette tip, although hard to calculate exactly, can deviate by several orders of magnitude from the concentration inside the micropipette, as explained in *Materials and Methods*. In an alternative approach to the chemotaxis experiments, we measured migration toward a pipette filled with 200 μM LPA while increasing concentrations of LPA were present in the incubation medium. Chemotactic efficiency dropped to control values as concentration of LPA in the medium was increased (Fig. 3). Chemotaxis was absent at ≈2 μM, implying that LPA-induced chemotaxis occurs over the concentration range 0–2 μM. This dose–response relationship corresponds roughly with that for early responses in vertebrate cells (4, 6).

Effects of Other Lipids. For a number of early responses in vertebrate cells, LPA action is specific in that other phospholipids tested have no effect (4, 6, 7). We therefore investigated whether the chemoattractant activity on *D. discoideum* is specific for LPA. Mean efficiencies of chemotaxis were determined for related phospholipids, for monoacylglycerol, the major metabolite of LPA in fibroblasts (16), and for lyso-platelet-activating factor, an ether lysolipid that is endogenous in *D. discoideum* (17) (Fig. 4). Of the compounds tested, only LPA was capable to induce a chemotactic response. Thus, LPA shows specificity in inducing chemotaxis in *D. discoideum* amoebae.

Chemotactic Mechanism. Classically, a chemoattractant imposes direction onto randomly moving organisms. However, if LPA were immobilizing or lytic to the cells, net accumulation of amoebae near the tip would also occur, since cells approaching the tip through random walk would be unable to move away. Indeed, prolonged (20-min) incubation with LPA at 200 μM was observed to be lytic for various cell

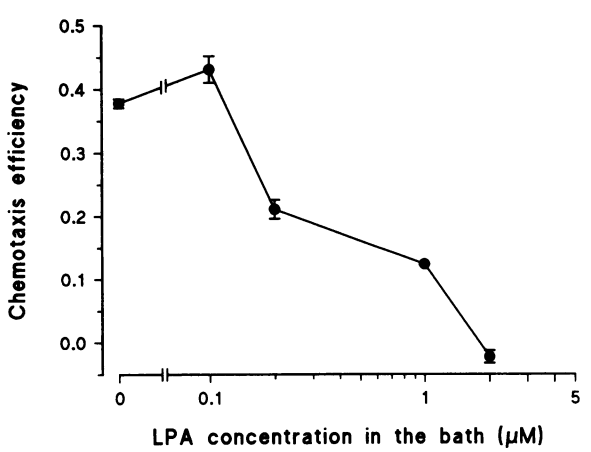

FIG. 3. Modulation of LPA-induced chemotaxis by added LPA. Efficiency of chemotaxis toward a microcapillary filled with 200 μM LPA was determined for different concentrations of LPA present in the medium.

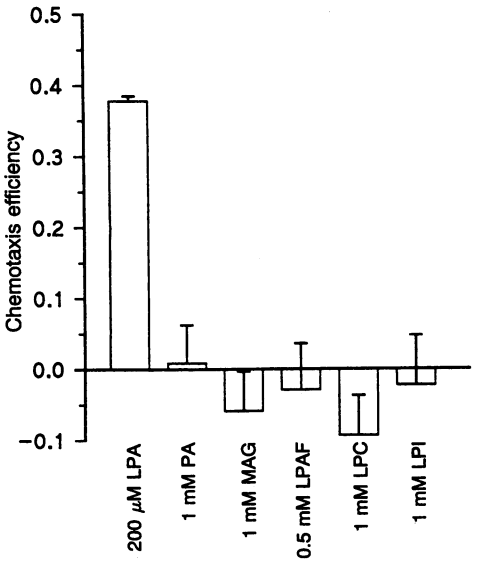

FIG. 4. Induction of chemotaxis by various lipids. Micropipettes were filled with PB containing the test compounds at the concentrations indicated, and efficiency of chemotaxis was determined. PA, phosphatidic acid; LPI, lysophosphatidylinositol; LPC, lysophosphatidylcholine; MAG, monoacylglycerol; LPAF, lyso-platelet-activating factor.

types, including *D. discoideum* amoeba (but only in nominally Ca^{2+}- and Mg^{2+}-free media; K.J., unpublished data). Although local concentrations of LPA inducing chemotaxis (see above) were estimated to be less than 1/100 of the lytic concentration, we thought it important to investigate this possible artefact in detail. To this end, we measured the migration speed of randomly moving amoebae exposed to various concentrations of LPA (Table 1). For concentrations of LPA up to 200 μM there is no significant effect on amoebae velocity. These results are supported by the relatively constant speed of locomotion during chemotaxis towards micropipettes filled with different concentrations of LPA (Fig. 2*B*) and by the lack of accumulation of amoebae near the tip of micropipettes filled with lysophosphatidylcholine (Fig. 4), which is lytic to cells both in the presence and in the absence of divalent cations. Hence, concentrations of LPA present near the cells in our experimental setup do not immobilize or slow down the amoebae, and we conclude that the LPA source attracts amoebae by directing their movement.

Lack of cAMP Involvement. It is conceivable that LPA-induced chemotaxis is secondary to cAMP formation and release. However, when cAMP (100 μM) was present in the bathing medium, a condition that effectively blocks chemotaxis toward a cAMP source (18), LPA-induced chemotaxis was unaffected (mean chemotaxis efficiency = 0.449 ± 0.020, $n = 50$). Conversely, in the continuous presence of LPA (1–20 μM), cells showed normal chemotaxis up a cAMP gradient (not shown). This conclusion is confirmed by experiments using the mutant SYNAG-7. SYNAG-7 was originally isolated by Frantz (19) by selection for the absence of an aggregation response, and it was found that it is defective in G protein-mediated activation of adenylate cyclase. SYNAG-7 shows normal chemotaxis toward a source of cAMP but is unable to add to the formation of a gradient during the aggregation response. Yet migration of SYNAG-7 up a gradient of LPA did not differ from that of wild-type cells (mean chemotaxis efficiency = 0.345 ± 0.055, $n = 26$). Taken together, these data indicate that LPA-induced chemotaxis of *D. discoideum* is not secondary to cAMP liberation.

Table 1. Random locomotion speed of starved *D. discoideum* cells incubated in PB with different concentrations of LPA

LPA conc. μM	Random locomotion speed, μm/s	*n*
0	0.102 ± 0.048	40
5	0.101 ± 0.040	55
10	0.114 ± 0.028	36
200*	0.119 ± 0.036	21

*Lytic upon prolonged incubation.

Concluding Remarks. We have shown that LPA, at submicromolar concentrations, acts as a chemoattractant for amoebae of *D. discoideum* and that this action is not mimicked by other (lyso)lipids. Furthermore, aggregation of amoebae is not attributable to LPA-induced immobilization of the cells, nor is it secondary to cAMP liberation.

In vertebrate cells, it appears that exogenous LPA triggers its multiple physiological effects by activation of (a) specific G protein-coupled receptor(s). By inference, the simplest hypothesis explaining LPA's chemotactic effects reported here is that *D. discoideum* amoebae express a specific receptor for LPA. Verification of this hypothesis awaits experiments aimed at identifying an LPA-binding protein in *D. discoideum*. Among the intracellular events involved in cAMP-induced chemotaxis are a transient increase in inositol 1,4,5-trisphosphate (20), a rise in intracellular Ca^{2+} (21), a transient increase in cGMP (22, 23), a rise in intracellular pH (e.g., refs. 14 and 24), and rearrangements of actin filaments (25, 26). LPA stimulates inositolphospholipid hydrolysis with subsequent Ca^{2+} mobilization in a wide variety of vertebrate cell types (4, 27), and it is also a potent inducer of actin polymerization and microfilament rearrangement (6, 28). Future research addressing these issues should reveal whether similar mechanisms are involved in the chemotactic responses triggered by these compounds.

What might be the function of a chemotactic response to LPA? One could envision that LPA is produced by *D. discoideum* cells and serves as an additional clustering signal to the amoebae at a certain stage in the life cycle. Alternatively, LPA may be secreted by bacteria or released by damaged cells. In this respect, it is noteworthy that LPA is a normal precursor in *de novo* lipid biosynthesis in all prokaryotic and eukaryotic cells. If extracellular LPA is present at concentrations high enough to be detected by the amoebae, it should be feasible to trace the source by using standard biochemical techniques. Regardless of the physiological function of LPA in the life cycle of *D. discoideum*, we anticipate that the molecular genetics techniques available in *D. discoideum* will help to increase our knowledge about the molecular mechanism by which LPA exerts its effects.

Our first observation on LPA-induced chemotaxis was made at the Department of Botany of Kyoto University (Kyoto, Japan) in cooperation with K. Inouye. We thank the Cell Biology and Genetics Unit, Institute of Molecular Plant Sciences, Leiden University (Prof. Dr. T. M. Konijn) for providing *D. discoideum* amoebae and cell culture facilities. B.V.D. and K.J. were financially supported by the Netherlands Organization for Pure Research (BION and Foundation for Biophysics).

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