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Propagating chemoattractant waves coordinate periodic cell movement in *Dictyostelium* slugs

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SUMMARY

Migration and behaviour of *Dictyostelium* slugs results from coordinated movement of its constituent cells. It has been proposed that cell movement is controlled by propagating waves of cAMP as during aggregation and in the mound. We report the existence of optical density waves in slugs; they are initiated in the tip and propagate backwards. The waves reflect periodic cell movement and are mediated by cAMP, as injection of cAMP or cAMP phosphodiesterase disrupts wave propagation and results in effects on cell movement and, therefore, slug migration. Inhibiting the function of the cAMP receptor cAR1 blocks wave propagation, showing that the signal is mediated by cAR1. Wave initiation is strictly dependent on the tip; in decapitated slugs no new waves are initiated and slug movement stops until a new tip regenerates. Isolated tips continue to migrate while producing waves. We conclude from these observations that the tip acts as a pacemaker for cAMP waves that coordinate cell movement in slugs.

Movies available on-line

Key words: cAMP signalling, Wave propagation, Chemotaxis, Cell movement, Morphogenesis, *Dictyostelium*

INTRODUCTION

The development of multicellular organisms is highly dependent on differential cell movement. This is especially clear during gastrulation and formation of the nervous system. Gastrulation in vertebrates is controlled by special signalling regions, organisers. The best known is the Spemann organiser in the amphibian embryo. It has been shown that these regions can exert a long-range effect on the movement behaviour of cells, but relatively little is known about the signals that guide the movement of the cells and the actual mechanism of directed cell movement (Joubin and Stern, 2001). One possible mechanism for controlling these movements is chemotaxis of individual cells up gradients of signalling molecules secreted by these organising centres. Chemotaxis towards an organiser region is an essential part of the mechanism by which individual cells of the social amoebae *Dictyostelium discoideum* aggregate to form a fruiting body when they encounter a shortage of food (Loomis, 1982). Aggregation centres act as organisers by emitting periodic pulses of the chemoattractant cAMP, which is actively propagated over large distances by the surrounding cells. The periodic chemotactic movement of the cells towards the signalling centre results in the accumulation of cells in a hemispherical mound. During aggregation, the cells start to differentiate into prestalk and prespore cells, the precursors for the stalk and spore cells of the fruiting body. The prestalk cells in the mound sort out to form a distinct nipple-shaped structure called the tip, which controls further morphogenesis. The tip still has organiser-like properties, in the sense that when it is transplanted in the side of another slug it can control the behaviour of the host cells and induce a secondary axis (Raper, 1940; Rubin and Robertson, 1975). The mound elongates, falls over and forms a cylindrically shaped slug. Guided by photo- and thermotaxis, the slug migrates to the surface of the soil where it forms a fruiting body (Bonner et al., 1950).

The propagated cAMP waves during aggregation can be visualised macroscopically under darkfield conditions, as chemotactically moving cells have different light scattering properties than resting cells (Gross et al., 1976; Tomchik and Devreotes, 1981). Optical density waves are also present in mounds and we have shown recently that these waves are dependent on the activity of the aggregation stage adenylate cyclase and therefore reflect waves of cAMP (Patel et al., 2000). There is evidence that cAMP is produced by the slug tip as well. Isolated slug tips attract aggregation-competent cells that can move chemotactically towards a cAMP source (Rubin and Robertson, 1975). Furthermore, phosphodiesterase in the substratum decreases the range of this cAMP signal (Rubin, 1976). It has also been demonstrated for several *Dictyostelium* species that cells moved out of multicellular structures when placed on agar containing cAMP, which was interpreted as chemotaxis in response to an external cAMP stimulus (George, 1977; Schaap and Wang, 1984). More recently it was shown that a subpopulation of cells in the slug the so-called anterior-like cells accumulated around a micro-

pipette after the periodic injection of cAMP in the prespore region of slugs (Rietdorf et al., 1998). Furthermore, light seems to induce cAMP secretion by the slug tip, which could control the cell movement required for phototaxis of the slug (Miura and Siegert, 2000). Though partly circumstantial, these data suggest that cAMP is at least one of the signals that control cell movement in the slug stage.

Until now, it has been impossible to visualise propagating optical density waves in slugs; we have, however, studied the patterns of cell movement in slugs of axenic strains in considerable detail (Abe et al., 1994; Siegert and Weijer, 1992). The prestalk cells that comprise the anterior fifth of the slug usually show a very strong rotational movement perpendicular to the direction of slug movement (Dormann et al., 1997; Siegert and Weijer, 1992). However, the prespore cells and the anterior-like cells in the posterior part of the slug move in straight trajectories and forwards in a periodic fashion (Durston and Vork, 1979; Siegert and Weijer, 1992). We proposed that the cells in the tip responded to a scroll wave of cAMP, which propagated in the back as a twisted scroll or planar waves (Bretschneider et al., 1995; Steinbock et al., 1993). However, until now we have not been able to visualise these waves, with one exception: the flat posterior part of *Dictyostelium mucoroides* slugs (Dormann et al., 1997). In this study we have analysed several different *Dictyostelium discoideum* strains in order to establish whether waves exist in slugs of these strains and to investigate the role of the tip in the process of wave initiation.

MATERIALS AND METHODS

Cell culture and strains

The following strains were used: NC4, XP55, NP377 (Ross and Newell, 1981), Ax2, AX3, DH1, cAR3/RI9 and cAR1/RI9 (Insall et al., 1994). The cells were grown according to standard conditions in either axenic media or on SB plates in association with Klebsiella aerogenes at 22°C (Sussman, 1987). To induce development, cells were washed once (bacterially growing cells five times) in KK2 phosphate buffer (20 mM KH_2PO_4/K_2HPO_4, pH 6.8). After a final wash in de-ionised water, the cell density was adjusted to ~2×10^8 cells/ml and small drops of 5 to 10 μl were deposited of 1% water agar plates (1% Difco Bacto agar in de-ionised water). After 30 minutes the supernatant was removed and the plates incubated in the dark at 22°C for 18 to 48 hours. For cell tracking experiments, 0.5% GFP-labelled AX2 cells were mixed with NP377 cells. NP377 cells were also stained with Cell Tracker (Molecular Probes) or Neutral Red as previously described (Dormann et al., 1997).

Videomicroscopy and image analysis

Darkfield waves during development were recorded as described previously (Siegert and Weijer, 1989; Siegert and Weijer, 1995). An Axiovert 135 microscope (Zeiss; objectives: FLUAR 10×/NA 0.5, Plan NEOFLUAR 10×/NA 0.3) equipped with a cooled CCD camera (Hamamatsu, C4880-82) was used to record wave propagation and cell movement in slugs. Usually the slugs were submerged under silicon oil (Dow Corning 200/20cs) to reduce light scattering on the slug surface (Siegert and Weijer, 1992). To obtain a side view, a piece of agar with a slug on it was cut out of the agar and turned on its side (Dormann et al., 1996). Microinjection experiments were performed as described (Rietdorf et al., 1998). The camera, a mechanical shutter for the brightfield illumination and a monochromator for the excitation of fluorescently labelled cells (TILL Photonics), were all controlled by the Openlab software (Improvision, version 1.7) running on a Macintosh PowerPC. Images were saved as TIFFs and transferred to a PC for analysis with the Optimas software (MediaCybernetics, version 6.1). Standard image processing techniques like image subtraction were applied to improve the visibility of the optical density waves in the slug stage (Siegert and Weijer, 1995). To determine wave propagation speed and periodicity time-space-plots were generated and analysed as described previously (Siegert and Weijer, 1989; Siegert and Weijer, 1995). Cells in the prespore region generally moved in straight lines parallel to the long axis of the slug, the waves propagated in parallel but opposite direction. For cell tracking experiments we analysed only cells that remained in the focal plane for prolonged periods of time, to avoid inaccuracies in the measurement of cell movement velocities owing to movement in the *z*-direction. Cell and wave velocities were measured with respect to the substrate; they were not corrected for slug movement.

RESULTS

Optical density waves in slugs

When we were analysing the movement of GFP-labelled cells in slugs of the *Dictyostelium discoideum* strain AX2 we

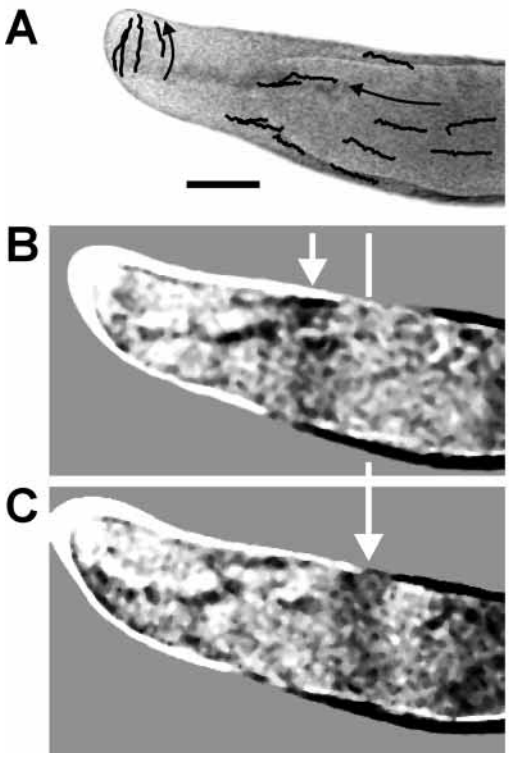

Fig. 1. Wave propagation in a slug of *Dictyostelium discoideum*. The slug shown is from a mutant AX2 strain that has been transformed with different variants of GFP expressed under the control of prestalk-specific promotors to enable cell movement analysis (see movie on CD 'GFP in Motion' *Trends Cell Biol.*). (A) Movement of cells inside the slug. GFP-labelled cells were tracked in both the prestalk and in the prespore region over 20 images (400 seconds). The tracks are superimposed on the brightfield image of the slug, the direction of cell movement is indicated by black arrows. The slug is migrating towards the left. (B) Image subtraction of the corresponding brightfield images taken at 100 second intervals reveals the wave front as dark band in the middle of the slug indicated by a white arrow. (C) The same slug 200 seconds later. The wave (white arrow) has moved backwards. Scale bar: 100 μm.

noticed in some cases the presence of travelling wave fronts in the brightfield images that had been recorded together with the fluorescence images. Fig. 1A shows the brightfield image of a slug, filmed from underneath, together with the tracks of individual GFP-labelled cells over a period of 6.6 minutes. In the tip, cells moved almost perpendicular to the direction of slug movement, they rotated around the long axis of the slug (Dormann et al., 1996; Siegert and Weijer, 1992), while cells in the prespore region were moving forwards. Though several propagated wave fronts were clearly visible when the brightfield image sequence was played back, individual frames did not reveal the wave bands satisfactorily. This was overcome by using image subtraction, which enhances the differences between two images. After this procedure, the wave fronts were easily visible as dark bands indicated by the white arrows in Fig. 1B,C. In Fig. 1B the wave appears in the anterior part of the prespore region; 200 seconds later it is located at a more posterior position (Fig. 1C). This shows that the wave is propagated backwards, opposite to the direction of cell movement in the prespore zone (Fig. 1A).

The visibility of waves in slugs in strain dependent

In the initial experiments using AX2 strains we found that only a fraction of the slugs recorded under the same optical conditions showed wave propagation (Table 1). As earlier accounts of waves of periodic cell movement in slugs were largely based on observations made in the wild-type strain NC4 (Durston and Vork, 1979; Durston et al., 1979), we re-examined this strain and, indeed, waves were visible in about 88% of the slugs (Table 1). Given that the optical density waves reflect subtle cell shape changes, we reasoned that strains like the streamer mutant NP377, which shows prolonged cell polarisation after stimulation with cAMP (Newell and Liu, 1992), might show more pronounced darkfield waves, as is the case during aggregation. Waves were recorded in almost all instances in this strain and their amplitude was enhanced (Table 1). Several experiments were carried out on the widely used strains AX3 and DH1, because they are the parent strains for many mutants in the cAMP signalling system, such as adenylate cyclases, G proteins and cAMP receptors, which could be used to elucidate the role of cAMP signalling in the slug. Unfortunately, we could never detect optical density waves in the strain Ax3 and only in two out of 20 DH1 slugs. The best waves were found in the bacterially growing strains (NC4, XP55, NP377). To assess whether the growth conditions (axenic versus growth on bacteria) affected the visibility of waves, we performed a series of experiments in which DH1 cells were grown in association with bacteria. However, no waves could be observed (Table 1). There seem to be inherent, as yet uncharacterised, differences in the strains analysed, which govern the visibility of optical density waves and are independent from the tested growth conditions.

Table 1. Frequency of optical density waves in slugs of different strains

Strain	Number of slugs with waves	Total number of slugs	Percentage of slugs with waves
NC4	32	36	88
XP55	5	5	100
NP377	45	47	95
AX2	7	12	58
AX3	0	13	0
DH1	2	20	10
DH1(bact.)	0	10	0
CAR1/RI9	0	19	0
CAR3/RI9	9	15	60
Total	100	177	

The visibility of waves in slugs is strongly strain dependent. NC4 is the original wild-type *D. discoideum* strain. XP55 is another wild-type strain, which is the parental strain for the streamer mutant NP377. All other strains grow axenically. DH1(bact.), bacterially grown DH1 cells.

Wave velocity and period during development

We quantified wave propagation speed and wave periodicity in slugs using time-space plots and compared their properties with the dynamics of the waves observed at earlier stages of development (Table 2). The data shown are those obtained for strain NP377, but they are representative of all the other *D. discoideum* strains that we have studied (data not shown). During the early stages of aggregation the velocity of darkfield waves was very high, reaching up more than 200 μm/minute. Wave speed declined dramatically in mounds, in agreement with previous studies (Rietdorf et al., 1996; Siegert and Weijer, 1989). In slugs the wave propagation speed decreased even further to ~32 μm/minute. The wave period decreased strongly from aggregation up to the mound stage, but in slugs we found an unexpectedly long periodicity of ~7.7 minutes. This suggests that there is another dramatic change in the signal relay properties of the cells during or after the transition from the mound to the slug stage. This may depend on the expression of different cAMP receptors in the slug stage or possibly the expression of new adenylate cyclases and/or cAMP phosphodiesterases (Kim et al., 1998a; Kim et al., 1998b; Shaulsky et al., 1998; Thomason et al., 1999). We cannot investigate this at the moment, as we are not able to visualise wave propagation in Ax3, the strain in which mutants of these molecules are available (with one exception).

Correlation between wave propagation and cell movement

During aggregation and the mound stage of development, the optical density waves are caused by the coordinated periodic movement of the cells in response to the passing cAMP waves. To establish that this is also the case in slugs, we investigated the relationship between wave propagation and cell movement in the streamer mutant NP377. We mixed 0.5% GFP-labelled AX2 cells with unlabelled NP377 cells, and recorded wave propagation and cell movement under brightfield and fluorescence illumination. We tracked labelled cells in the fluorescence images and, in order to detect the passing wave

Table 2. Comparison of wave propagation speed and wave period during development of strain NP377

	Darkfield waves (early aggregation)	Mound	Slug
Velocity (μm/minutes)	200±43 (20 centres)	49.5±7.7 (eight mounds)	32.29±10.5 (21 slugs)
Period (minutes)	4.18±0.8 (20 centres)	2.34±2.7 (eight mounds)	7.69±1.91 (21 slugs)

Mean values and standard deviations are shown.

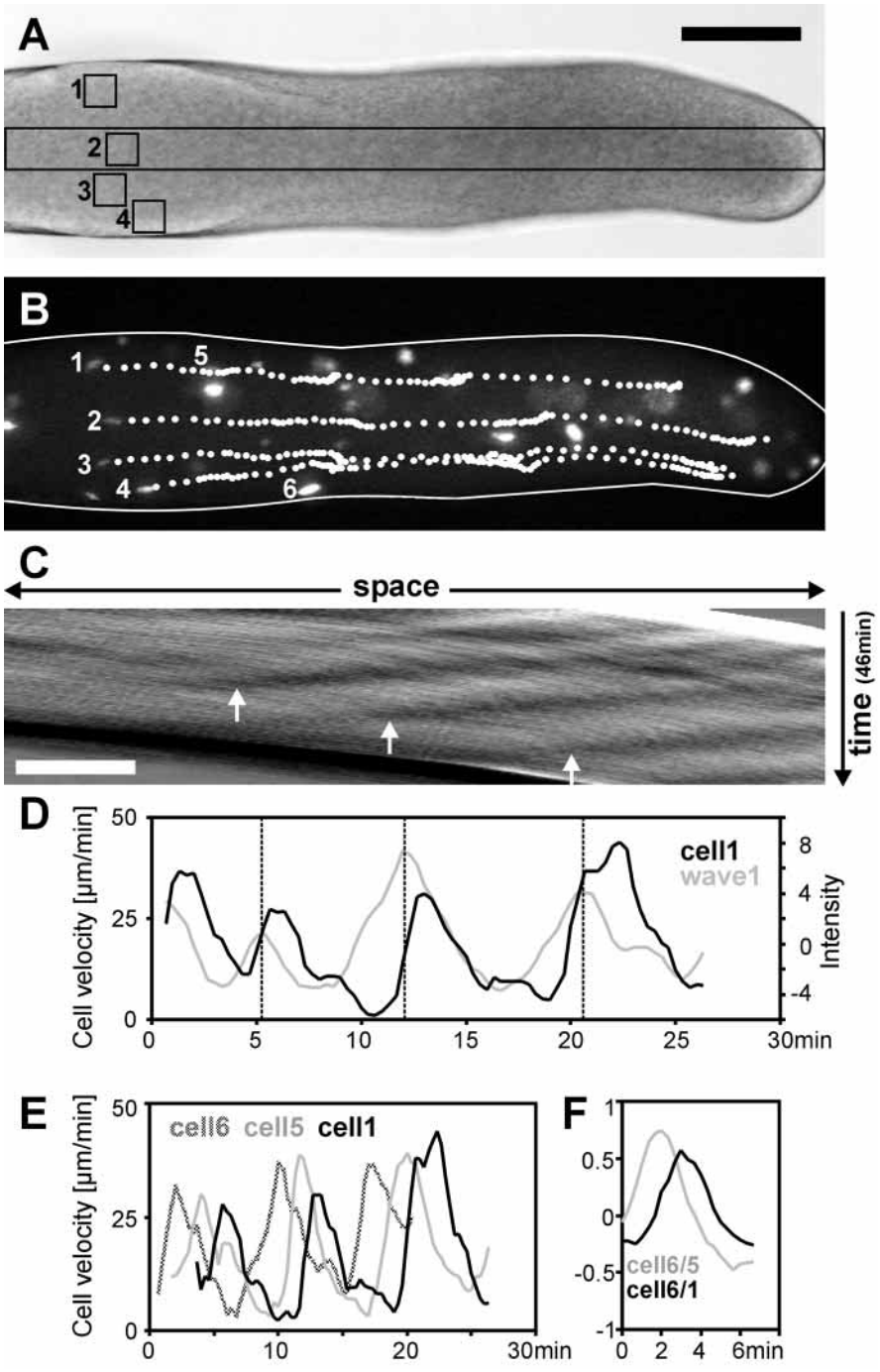

Fig. 2. Correlation of wave propagation and cell movement in streamer NP377 slugs. (A) Brightfield image of a slug shown from below. To detect the optical density waves the average brightness was measured in small squares at the position of the cells. Four squares are shown (1-4); the cells are visible in the corresponding fluorescence image (B). The long rectangle over the midline of the whole slug indicates the position of the measuring window for the time-space plot (C). (B) Fluorescence image of the slug showing the GFP-labelled cells. The positions of the four cells at different time points of the sequence are indicated by white dots. (C) The time-space plot reveals the presence of several waves propagated from the tip to the back of the slug over a period of 46 minutes. The waves are visible as dark sloping bands; the white arrows point at three waves just before they seem to disappear in the rear part of the slug. To enhance the visibility of the waves, this time-space plot was created by using a sequence of subtracted images. (D) Graph showing the changes of velocity and average brightness of cell 1. The velocity is plotted in black, the intensity in grey. Owing to a drift in the brightfield signal, the intensity data were corrected by subtracting the values of a calculated linear regression line from the original data. The curves were smoothed by a running average over three data points. Broken lines mark the intensity peaks. (E) Periodic movement of cells 1, 5 and 6 from (B). (F) Cross-correlation analysis of the cell velocities. Scale bars: 100 µm.

fronts, we measured the average brightness in little squares (25×25 pixels) at the position of the cells in the corresponding brightfield images to get a measure of the wave dynamics in the region of the tracked cell (Fig. 2A,B). Fig. 2A shows the brightfield image of a slug, together with four squares that have been placed at the positions of the four cells that are marked in the fluorescence image (Fig. 2B). The white dots mark the successive positions of these cells over time at 20 second intervals, that could be followed for more than 25 minutes. The behaviour of the cells is coordinated, phases of faster and slower movement seem to coincide. The time-space plot in Fig. 2C shows the presence of at least three optical density waves. The graphs of velocity and intensity for cell 1 in Fig. 2D show the same periodicity with a phase shift of ~70 seconds. The maxima of the optical density signal coincide with the highest change in cell velocity, providing good evidence for the fact that the waves are associated with the cell shape changes that accompany the changes in cell velocity. Whenever the cells detect the chemotactic signal, they respond by moving faster. As the chemotactic signal represented by the optical density waves travels from the front to the back of the slug, cells located at different positions along the long axis should respond to the signal at different time points. This is demonstrated for the cells 1, 5 and 6 (Fig. 2B,E). Cell 6 receives the signal first as indicated by an increase in cell velocity, followed by cell 5 and finally cell 1. From the cross-correlation analysis of the velocities for cell 6 and cell 5 and cell 6 and cell 1 (Fig. 2F), the time delays were determined as 2 minutes and 3 minutes, respectively. Together with the distance between the cells (Fig. 2B; cell6/5: 77 µm; cell6/1: 170 µm), we can calculate the signal propagation speed which averages at ~47 µm/min which matches the ~42 µm/min wave propagation speed that was deduced from the time-space plot (Fig. 2C).

Table 3. Comparison of wave propagation and cell movement parameters in slugs

	Cells	Waves
Period (minutes)	6.1±1.5 *n*=41 cells (nine slugs)	8.7±1.7 *n*=22 waves (seven slugs)
Velocity (μm/minute)	16.0±10.3 *n*=41 cells (nine slugs)	35.8±9.3 *n*=26 waves (eight slugs)
Calculated period (minutes)	6.01	

Mean values and standard deviations are shown. The 'calculated period', the period of the signal the cells experience (P_C), was calculated from the wave period (P_W) and cell and wave velocities (V_C and V_W), because the cells are moving towards the source of the signal waves. Using the formula $P_C=P_W(V_W/(V_W+V_C))$ and the measured mean values from the table, the period P_C was calculated.

Table 3 summarises the results of cells tracked in slugs in nine independent experiments. The discrepancy in the periodicity data between cells and waves is due to the fact that the cells move towards the signal wave. The wave period is derived from time-space plots and measures how often a wave passes through a fixed point. As the cells are not stationary, they detect the signal waves more often, which is reflected by the shorter period. However, one can calculate the period of the signal the cells actually experience based on the measured wave period and the velocities of cells and waves (formula in the footnotes of Table 3). The calculated period and the measured period of cell movement are identical, suggesting that the differences are indeed the result of a Doppler effect resulting in shorter periods seen by the cells moving in the direction of the signal.

Wave propagation and cell movement after tip removal

In order to confirm that the waves are generated in the slug tip, we conducted a series of experiments in which the tip was removed during the course of the recording. We observed intact slugs for 20 to 30 minutes, after which we cut off the tip and continued to record the posterior part of the slug. After the tip had been removed, the wave fronts that were present in the prespore zone continued to propagate towards the back; however, no new waves were initiated during the first 20 minutes after the tip had been cut off (data not shown). This suggests that the pacemaker responsible for the generation of the signal waves resides in the prestalk region of the slug. When we looked at the behaviour of isolated slug tips we found that they always continued to produce waves and to display forward migration (Fig. 3A,B). Waves were still present in nine out of ten cases with a period of 6.0±1.0 minutes (five slugs; Fig. 3C). As the position of the wave front in Fig. 3B indicates, the waves are initiated in the anterior part of the slug tip and seem to propagate as planar waves.

The loss of the tip immediately affects the movement of the cells in the posterior part of slug. In the intact slug, cells moved periodically in response to the signal waves as can be seen in the cell tracks and the corresponding velocity plots (Fig. 4A,B). After removal of the tip, cell movement was much less organised (Fig. 4C,D). There were no more signs of periodic movement, although cell 3 seemed to show another peak in velocity about 11 minutes after the tip was cut off. Most cells simply slowed down (e.g. cells 2 and 4). The velocity of cells close to the point of the cut (Fig. 4D, cell 1) decreased dramatically within the first 5 minutes after the operation to about 1/3 of the pre-cut velocity (average cell velocity before the cut: 18.4±10.8 μm/min, *n*=52 cells; after cut: 5.8±3.5 μm/min, *n*=10 cells). The further away the cells were from the point of the cut, the longer it took them to slow down, but after about 20 minutes most of the cells moved at around 5 μm/min. These data suggest that the tip is required for the coordinated periodic movement of cells.

The effect of cAMP microinjection of wave propagation

It had been reported previously that the periodic injection of cAMP in slugs leads to the chemotactic attraction of anterior-like cells to the tip of the micropipette (Durston et al., 1979; Rietdorf et al., 1998). We investigated the effect of cAMP injection on the wave system. The micropipette was usually positioned in the anterior part of the prespore region. However, periodic injection from a pipette filled with 10^{-4} M cAMP at 6 minute intervals did not show any effect (*n*=8 slugs). The 6 minute period was chosen because this was the period of cell movement as described above. The slugs continued to migrate and wave propagation was undisturbed (data not shown). We then increased the concentration to 10^{-2} M cAMP, which had a very strong effect on slug migration. Injection of cAMP resulted in a loss of wave propagation beyond the site of injection (15 out of 15 slugs). The prespore cells posterior and partly anterior to the site of injection stopped moving, as can be seen from the broken black line in the time-space plot (Fig. 5C; 21 out of 23 slugs), while the prestalk region of the slug

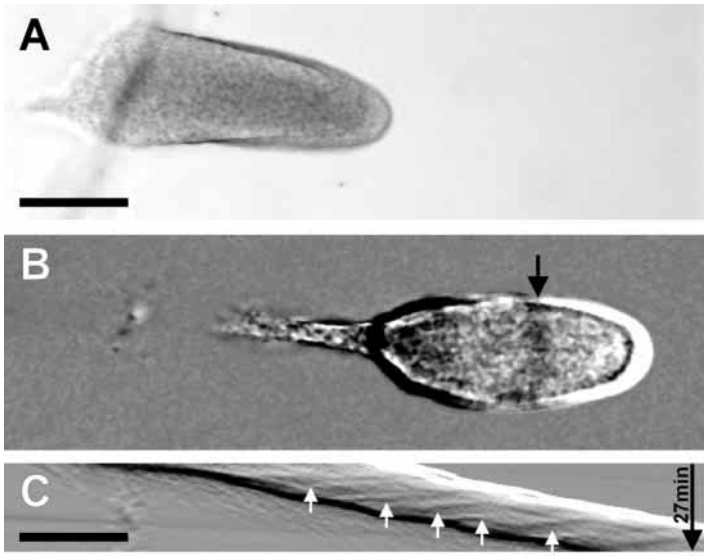

Fig. 3. Wave propagation in isolated slug tips. (A) Brightfield image of an isolated tip from a slug of strain NP377. The tip was separated from the prespore region with a fine syringe needle. (B) Subtracted image showing the same tip 16 minutes later. The black arrow points at the dark appearing wave front. (C) Time-space plot over the whole sequence of subtracted images. The white arrows mark five propagated waves. Scale bars: 100 μm.

usually continued to migrate resulting in splitting of the slug (data not shown). Anterior-like cells in the prespore region moved rapidly in the direction of the pipette tip, but continued to form a tip only after the micropipette had been removed. This suggests that the amount of cAMP emitted by the pipette tip was such that it blocked the cell autonomous periodic cAMP production. The effect of cAMP injection on wave propagation is illustrated in Fig. 5A,B. After the cAMP pulse, the wave fronts disappear immediately, also visible in the time-space plot in accordance with the notion that high levels of cAMP will wipe out the endogenous cAMP relay (Fig. 5C). We also locally depleted the cAMP signal by local injection of high amounts of bovine heart cAMP phosphodiesterase (PDE). In about half of the cases the waves were not propagated beyond the site of PDE injection as shown in Fig. 5D-F. Waves were initiated in the tip (Fig. 5E) but they disappeared at around the position where PDE had been injected, as can be seen in the time-space plot (Fig. 5F). This also resulted in a noticeable slowdown of the local movement speed, as indicated by the broken black lines in the time space plot (Fig. 5F). This suggests that the optical density waves are the result of propagated cAMP waves that coordinate chemotactic cell movement. The inhibition of cell movement is not complete, which we attribute to the PDE activity injected not being able to wipe out cAMP signalling completely for longer periods of time.

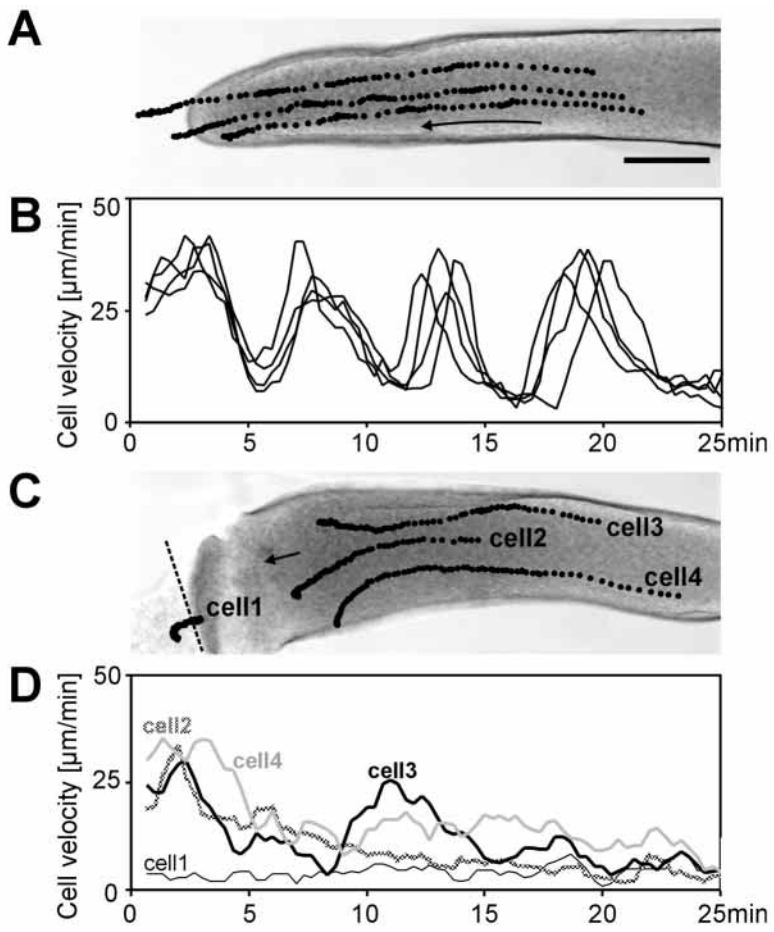

Fig. 4. Effect of tip removal on cell movement in slugs. (A) The tracks of three cells are indicated by black dots and superimposed on the brightfield image of the first time point. The arrow indicates the direction of cell movement. The pattern of dots in each track shows that cells go through phases of fast and slow movement. (B) Velocity plot of four cells, including the three cells from A. The periodic movement changes are obvious. (C) Tracks of four cells from the same slug after the tip was removed. The broken line marks the position where the tip was cut off. (D) Velocity profiles of the four cells shown in C. The coordinated behaviour of the cells is lost. Scale bar: 100 μm.

The role of cAMP receptors in wave propagation

In another approach we tried to determine which cAMP receptors are involved in the cAMP signalling. *Dictyostelium* slugs express four different cAMP receptors. Prespore cells express the high-affinity receptors cAR1 and cAR3, while the prestalk cells express both cAR1 and the low-affinity receptors cAR2 and cAR4. During aggregation, the high-affinity cAR1 receptor is mainly responsible for cell-cell communication. In slugs, however, it is not clear which receptor carries the cell-cell signalling process. Prespore cells express the high-affinity receptors cAR1 and cAR3, while prestalk cells express the high-affinity receptors cAR1 and the low-affinity receptors cAR2 and cAR4 (Saxe et al., 1996; Yu and Saxe, 1996). In order to test the role of the cAR1 receptor in wave propagation in slugs, we used the adenosine analogue IPA (2′,3′-*o*-isopropylidene adenosine). Adenosine has been reported to inhibit cAMP binding to cAR1, but not to cAR2 or cAR3, and both adenosine and IPA have been shown to inhibit cAMP-dependent prespore gene expression in cell lines expressing only cAR1, but not in strains only expressing cAR3 or cAR2 (VerkerkeVanWijk et al., 1998). Furthermore, IPA blocks cAMP relay at the aggregation stage (H. Patel and C. J. Weijer, unpublished) and counteracts the cAMP-induced nuclear translocation of STATa in prespore cells of slugs (Dormann et al., 2001a). When we transferred NP377 slugs to agar containing 100 μM IPA we found seven out of 10 slugs with waves, but increasing the IPA concentration up to 3 mM abolished wave propagation completely in all slugs tested (1 mM IPA: 5/13 slugs; 2 mM: 2/8 slugs; 3 mM: 0/8 slugs), suggesting that the detection of the periodic chemotactic signal is mainly mediated by cAR1 in vivo. Slug movement was not blocked completely, but slugs treated with high IPA concentrations tended to culminate immediately, indicating that culmination may involve a change in the dynamics of cell-cell signalling (data not shown).

We went on to study two mutant strains that overexpressed either cAR1 or cAR3 in a $cAR1^{null}/cAR3^{null}$ background, to look for the presence of waves in slugs, although these strains were derived from AX3. There was no sign of waves in the strain expressing cAR1 (cAR1/RI9, Table1). But surprisingly a significant number of slugs of cAR3/RI9 (Table 1) formed waves with high frequency (period: 3.8±0.9 minutes), yet with velocities comparable to NP377 or NC4. This shows that Ax3 derived strains are able to generate visible optical density waves. It also shows that although in the wild-type situation the waves in the prespore zone are mostly propagated through the dominant cAR1 receptor (see above), the waves in the

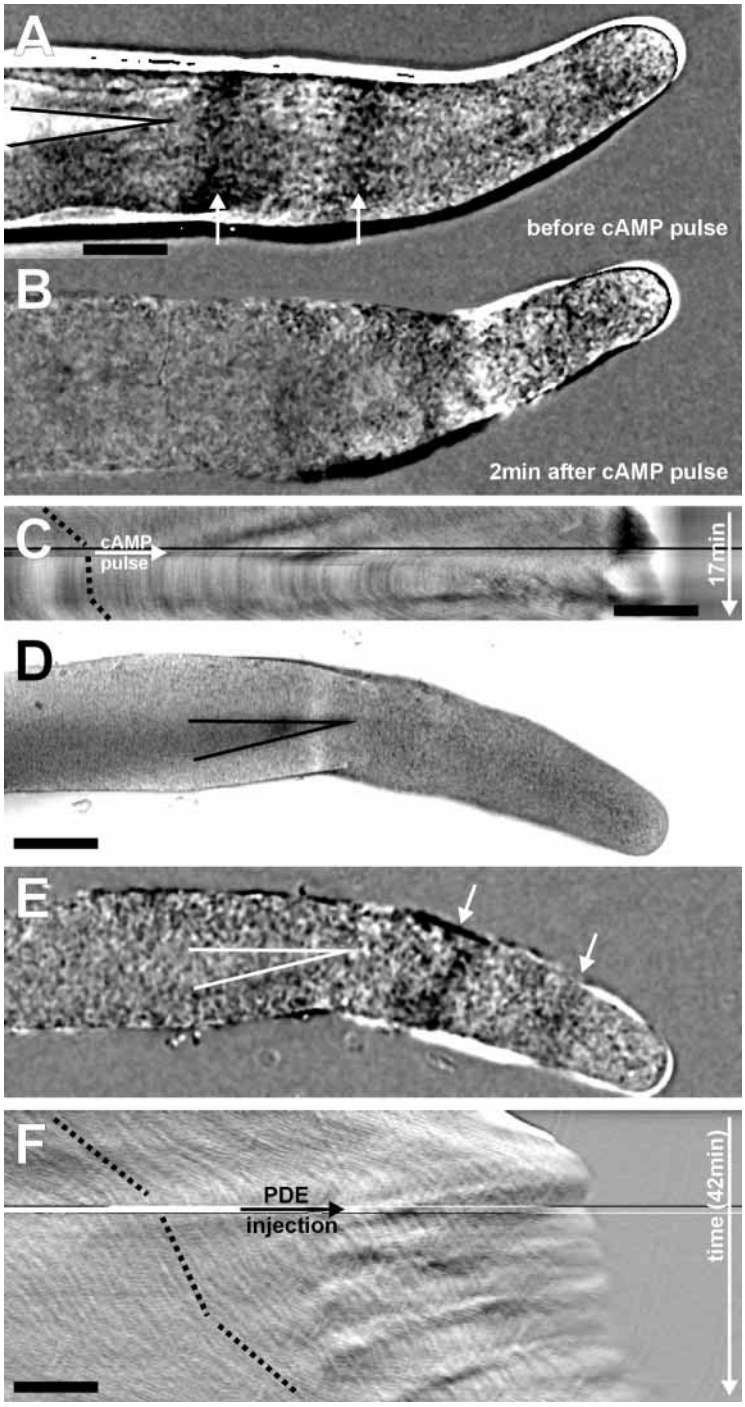

Fig. 5. Microinjection in slugs. (A) Subtracted image of a slug showing two wave fronts (arrows), the position of the microelectrode containing 10^{-2} M cAMP is indicated. (B) The waves have disappeared after the cAMP pulse, the microelectrode had been removed after the injection. (C) Time-space plot showing the disappearance of the waves after cAMP injection. The arrow indicates the position of micro-pipette and the time of the injection. (D) Brightfield image of a slug with an inserted micro-electrode (outline) containing phosphodiesterase (5 mg/ml). (E) Subtracted image of the same slug. The arrows point at two wave fronts coming from the slug tip. (F) Time-space plot. The waves stop around the injection site. The arrow indicates where and when the micro-pipette was inserted. Scale bars: 100 μm.

prespore zone can also be propagated by the cAR3 receptor if expressed at high enough levels, as this is the only receptor expressed in prespore cells of cAR3/RI9 slugs. It was noticeable that the frequency of the waves in the strain was much higher than in the non axenic strains (NC4, XP55). Mixing experiments between NC4 and cAR3/RI9 showed that with just 5% NC4 cells, the frequency went down from 3.8±0.9 minutes to 5±1.0 minutes (n=19) and at 20% NC4 cells decreased further to 8.4±0.7 minutes (n=10), the same as in 100% NC4 slugs (n=17).

DISCUSSION

Our results demonstrate the existence of optical density waves in *Dicyostelium discoideum* slugs (Fig. 1, Fig. 2). The waves are initiated periodically in the anterior part of the slug tip and propagated backwards, often over the entire length of the slug (Fig. 2, Fig. 3). The waves reflect the coordinated periodic movement behaviour of the cells in the slugs as shown by the correlation between local optical density and instantaneous cell movement speed (Fig. 2). The tip is essential for the coordination of cell movement. Once removed, the periodic movement behaviour of the cells in the back of the slug ceases (Fig. 4), suggesting that the tip is indeed acting as an organiser by acting as a pacemaker for cAMP waves.

There were noticeable differences in the visibility of the optical density waves between wild-type strains and axenic cell lines. They were easily detectable in NC4 and its derivatives XP55 and NP377; however, they were difficult to detect in the axenic strains AX2 and DH1. We detected them only in a few DH1 slugs and even if we could detect them they were very faint; however, we have never seen them in slugs of the parent strain Ax3. The fact that we were able to observe OD waves in the Ax3-derived strain DH1, but in not in Ax3 itself, shows that 'small' differences determine whether we can or cannot detect waves. This is further supported by the differences in visibility of OD waves in slugs of cAR3/RI9 and cAR1/RI9. We can easily detect waves in slugs of the DH1-derived cAR3/RI9 strain, but have not been able to observe them in slugs of the closely related cAR1/RI9 strain, strains that are believed to differ genetically only in the type of cAMP receptor they express. As cAMP receptors are involved in controlling both the dynamics of cAMP signalling and the chemotactic movement response of the cells, it remains to be decided which of these responses ultimately controls visibility of the waves. It is possible that the magnitude of the cringe response, the initial rounding of a cell after cAMP stimulation (Futrelle et al., 1982), will turn out to be an important determinant. The dynamics of signalling is very strain dependent, as indicated by the mixing experiments between cAR3/RI9 and NC4 cells, which showed that the period of the waves increased very rapidly with increasing numbers of NC4 cells; at 20%NC4 cells the periods of the waves are already as long as in 100% NC4 slugs.

That the slug tip is a source of acrasin (cAMP) was first

established by Bonner, who showed that aggregation competent cells are attracted by the slug, especially by the tip (Bonner, 1949). This has been supported by recent studies. Light stimulates the tip to release cAMP, which probably plays a role in the modulation of cell behaviour required for slug phototaxis (Miura and Siegert, 2000). Furthermore, the nuclear localisation of the transcription factor STATa in prestalk cells in the slug tip and its absence in the prespore zone reflects a quantitative difference in average extracellular cAMP concentration between the tip and the prespore zone. It seems likely that the tip shows on average a high concentration of cAMP, while the prespore zone shows a lower average concentration of extracellular cAMP (Dormann et al., 2001a). Our data show that there is a chemotactic signal propagating from the tip of the slug towards the back. The cAMP and phosphodiesterase injection experiments suggest that the in vivo signal is cAMP, as is the case during aggregation and in the mound stage of development (Patel et al., 2000). We have shown that the cAR1-specific inhibitor IPA blocks wave propagation in slugs in a dose-dependent manner. This suggests that the cAMP receptor cAR1 carries the cAMP relay signal in the wild-type strain NC4. IPA does not result in a complete block of slug migration, indicating that the signal can be carried in part by other cAMP receptors in the absence of functional cAR1 molecules, in agreement with the finding that waves are clearly visible in strain cAR3/RI9 during the aggregation and mound stages of development (Dormann et al., 2001a) and in slugs (this paper). However as millimolar concentrations of IPA in agar were required for full inhibition of optical density wave propagation, while in suspension 10-100 μm of IPA is sufficient to block prespore-specific gene expression completely, we cannot rule out nonspecific effects of IPA on targets other than cAR1, which could alter cell behaviour.

The results we describe are most consistent with the idea of cAMP being the propagating signal molecule. Nevertheless, we cannot rule out the possibility that other chemoattractants carry the propagated signal. Although microinjection of cAMP effectively blocked wave propagation and resulted in an immediate cessation of cell movement, we did not succeed in initiating waves by microinjection of cAMP as was we did in mounds (Rietdorf et al., 1998). It could be that it is just difficult to find the exact experimental conditions (amplitude, frequency) to initiate new waves or, alternatively, it could be that the primary signal is not cAMP. Similarly, the incomplete inhibition of cell movement in slugs by injection of bovine heart PDE could be interpreted as a failure to breakdown completely all the cAMP during a wave, we cannot, however, rule out the possibility that other chemoattractants may exist. We described before the directed movement of a specialised group of prestalk-like cells in slugs, involved in initiating culmination and destined to form the basal disk of the fruiting body (Dormann et al., 1996; Jermyn et al., 1996). They move independently from the majority of cells in the slugs, suggesting that their behaviour could be regulated by a separate chemoattractant.

In summary, we have now provided experimental evidence for the existence of propagating waves of a chemoattractant, most probably cAMP, in slugs. We have measured their dynamics and shown that they are important in controlling cell and, therefore, slug movement. These observations of wave propagation in slugs taken together with the earlier

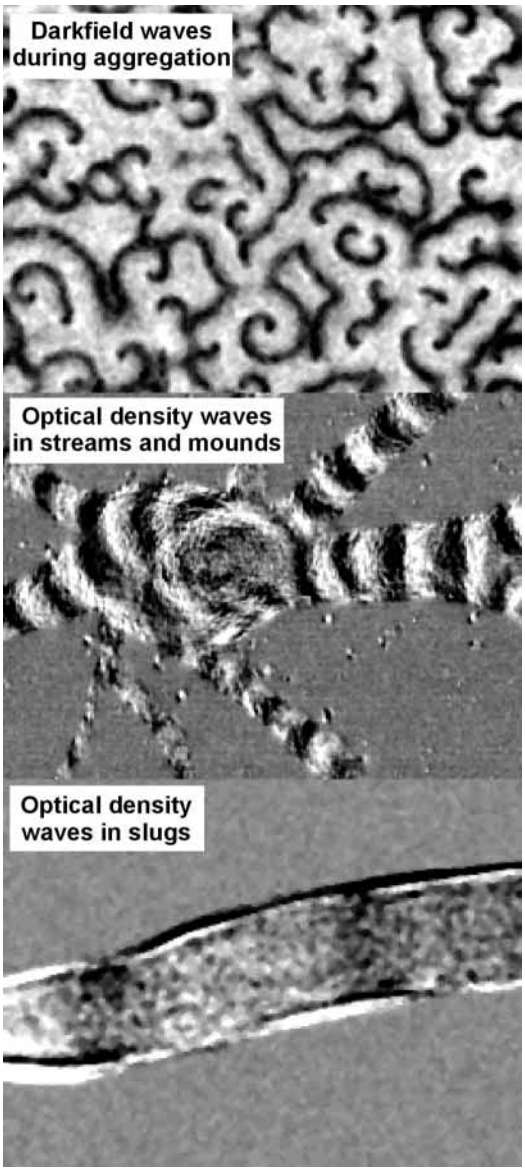

Fig. 6. Optical density waves in strain NP377 during different stages of development.

demonstration of cAMP wave propagation during the aggregation and mound stages of development (Fig. 6) now give good experimental support for our hypothesis: that *Dictyostelium* morphogenesis is controlled by the interplay between the dynamics and geometry of cAMP wave propagation and the chemotactic cell movement of the cells in response to these waves (Dormann et al., 2000). Further studies will elucidate whether the formation of the fruiting body follows the same principles. This seems likely as a periodic upwards movement of the rising fruiting body has already been described (Durston et al., 1976) and the ability of cells from culminates to respond chemotactically to cAMP signals has also been demonstrated (Kitami, 1984).

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