Ca$^{2+}$ chemotaxis in *Dictyostelium discoideum*

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Summary
Using a newly developed microfluidic chamber, we have demonstrated in vitro that Ca$^{2+}$ functions as a chemoattractant of aggregation-competent *Dictyostelium discoideum* amoebae, that parallel spatial gradients of cAMP and Ca$^{2+}$ are more effective than either alone, and that cAMP functions as a stronger chemoattractant than Ca$^{2+}$. Effective Ca$^{2+}$ gradients are extremely steep compared with effective cAMP gradients. This presents a paradox because there is no indication to date that steep Ca$^{2+}$ gradients are generated in aggregation territories. However, given that Ca$^{2+}$ chemotaxis is co-acquired with cAMP chemotaxis during development, we speculate on the role that Ca$^{2+}$ chemotaxis might have and the possibility that steep, transient Ca$^{2+}$ gradients are generated during natural aggregation in the interstitial regions between cells.

Key words: Chemotactic synergy, Microfluidic chamber, Parallel chemoattractant gradients, Calcium

Introduction
Calcium (Ca$^{2+}$) is ubiquitous in the environment of free-living cells as well as in cells in the tissues of plants and animals (Petersen et al., 2005; Case et al., 2007). The soluble concentration of Ca$^{2+}$ reaches 20–40 mM in osteoporotic fragments in the human body and 40 mM in endosteal niches of bone marrow (Silver et al., 1988). The soluble Ca$^{2+}$ concentration is approximately 1.1–1.3 mM in the extracellular fluids of tissues (Breitweiser, 2008) and 2.5 mM in blood (Bronner, 1997). Evidence suggests that free extracellular Ca$^{2+}$ might play a role as a chemoattractant in addition to cell-type-specific chemoattractants in a variety of developmental processes, including embryonic development (Adams et al., 2006; Chattopadhyay et al., 2007; Reitz et al., 1987), wound healing (Menon et al., 1985; Morris and Chan, 2007), angiogenesis (Aguirre et al., 2010), the immune response (Olszak et al., 2000) and tissue maintenance (Quarles et al., 1997). For example, mammalian gonadotropin-releasing hormone (GnRH) neurons undergo chemotaxis in Ca$^{2+}$ gradients generated in vitro, which has led to the hypothesis that Ca$^{2+}$ gradients in combination with gradients of the monocyte chemoattractant protein (MCP-1) regulate GnRH neuron migration during development (Chattopadhyay et al., 2007; Bandyopadhyay et al., 2007). It has also been shown that hematopoietic stem cells from antenatal mice deficient in the calcium receptor (CaR) fail to migrate from the liver to the endosteal niche of bone marrow (Adams et al., 2006), which, as noted, contains high concentrations of soluble Ca$^{2+}$ (Silver et al., 1988). Morris and coworkers (Morris and Chan, 2007) demonstrated that keratinocytes undergo chemotaxis in a Ca$^{2+}$ gradient in vitro and proposed that, in vivo, calcium and epidermal growth factor (EGF) might function together to promote wound healing, both presumably through chemotaxis. Osteoblasts have been shown to undergo chemotaxis in Ca$^{2+}$ gradients in vitro as well as in platelet-derived growth factor (PDGF) gradients in vitro (Sugimoto et al., 1993; Godwin and Soltoff, 1997), and monocytes have been shown to undergo chemotaxis in Ca$^{2+}$ as well as in chemokine gradients in vitro (Olszak et al., 2000). In addition, macrophages and macrophage-derived foam cells, which are involved in generating atherosclerotic lesions (Shi et al., 1996), and prostate and breast cancer cells also undergo chemotaxis in spatial gradients of Ca$^{2+}$ in vitro (Liao et al., 2006; Saidak et al., 2009). Finally, Brokaw (Brokaw, 1974) demonstrated that bracken fern spermatozoids undergo chemotaxis in combinatorial gradients of Ca$^{2+}$ and bimalate. Together, these observations suggest that chemotaxis in spatial gradients of Ca$^{2+}$ might be a general phenomenon conserved throughout the eukaryotic world in cells that have, during evolution, acquired chemotaxis to more specialized cell-type-specific attractants.

In *Dictyostelium discoideum*, a model system that has been highly effective in elucidating the underlying molecular mechanisms regulating animal cell chemotaxis (Van Haastert et al., 2007; Kay et al., 2008; Janetopoulos and Firtel, 2008; Kolsch et al., 2008; Iglesias and Devreotes, 2008; King and Insall, 2009; Jin et al., 2009), some interesting relationships have been reported between the extracellular cAMP signal and the extracellular concentration of Ca$^{2+}$. During *D. discoideum* aggregation, cells move in a directed fashion into an aggregation center through chemotaxis in gradients of extracellular cAMP that increase and decrease in the front and back, respectively, of relayed cAMP waves (Shaffer, 1966; Gerisch et al., 1966; Gerisch et al., 1975). In relaying a cAMP wave, precocious cells in a starved population release pulses of the chemoattractant cAMP that stimulate adjacent cells to release, in turn, a pulse of cAMP (Gerisch et al., 1975; Devreotes and Steck, 1979). Through the activity of extracellular phosphodiesterase, cAMP is then degraded (Chang, 1968; Riedel and Gerisch, 1971; Dinauer et al., 1980), which results in a cAMP signal that is relayed outwardly through the cell population as a non-dissipating, symmetrical wave (Tomchik and Devreotes, 1981; Soll et al., 2002). The spatial and temporal gradients of cAMP in the front and back of each relayed wave, which pass across cells on average every 7 minutes, are then assessed by each cell in the aggregating population, resulting in directed movement towards the aggregation center in the front of each wave of cAMP (Soll et al., 2002).

When aggregation-competent cells in buffer are challenged globally by the rapid addition of cAMP, they in turn release cAMP, which is believed to represent the fundamental component of the
relaxing cells suspended in buffer with a global CAMP signal also causes the rapid uptake of Ca\(^{2+}\), followed by release, causing fluctuations in the extracellular concentration of Ca\(^{2+}\) (Bumann et al., 1984; Wick et al., 1978; Bohme et al., 1987; Milne and Coukell, 1988; Moniakis et al., 1999). Moreover, suspended populations of aggregation-competent cells spontaneously and synchronously release pulses of CAMP, generating CAMP oscillations in the supporting medium that are more effective than an optimum Ca\(^{2+}\) gradient in directing the developmental program, that an optimum CAMP gradient is approximately the same time as CAMP chemotaxis is acquired in C. Mellonii, but are limited to speculating on the possibility that steep gradients form and on the role that Ca\(^{2+}\) chemotaxis might play during aggregation.

**Results**

**The microfluidic chamber**

A microfluidic chamber (Fig. 1A–D) was designed in collaboration with Translume (Ann Arbor, MI) that could generate stable spatial gradients of both low and high molecular weight molecules (unpublished results from this laboratory). Fluid flow was initiated through two reservoir pumps (Fig. 1A, a and b), and the fluids from the two flowed side by side into a square tube. To generate a gradient, one reservoir was filled with buffer alone and the other with buffer plus chemoattractant. The two fluids were pumped through ports a and b, respectively, meeting at an interface (Fig. 1B) where the adjacent fluids were directed through a single square channel containing a sequence of chevrons etched into the ceiling of the square tube (Fig. 1C); these chevrons acted as micromixers mediating rapid and controlled mixing in a short path perpendicular to flow (Kaplan et al., 2004; Golden et al., 2007). Cells were injected through an injection port onto the floor of the square tube of the chamber in the area of observation (Fig. 1D) downstream of the cell port. The stability of the gradient was demonstrated by measuring fluorescence in images of the channel at 1, 10 and 20 minutes.

![Diagram of microfluidic chamber](image)

### Fig. 1. Customized microfluidic chamber manufactured by Translume from optically clear, fused silica according to our specifications.

(A) Diagram of microfluidic chamber. The channel is a square tube with sides of 300 \(\mu\)m and a length of 28 mm, shown in blue Two programmable pumps control the flow rates from reservoirs a and b. (B) The interface of solutions a and b can be visualized by excitation of fluorescein (released by reservoir a) at the intersection of the inlet ports with the channel. (C) As solutions a and b flow through the chamber, they encounter a series of chevron micromixers. The gradient is generated perpendicular to the direction of flow. (D) The area of observation in the channel is adjacent to the cell injection port, the latter is where the cell suspension is introduced into the channel. Efflux occurs through tubing connected to the outlet port. (E–H) Gradient steepness is controlled by the flow rate of the pumps and plotted at different flow rates from data obtained when reservoir a contained fluorescein. The steepness of the concentration gradient of this fluorophore across the width of the chamber was determined using 2D-DIAS software (Soll, 1995) and provided in each plot in nM/\(\mu\)m. (I) The stability of the gradient was demonstrated by measuring fluorescence in images of the channel at 1, 10 and 20 minutes.
the chevrons, and imaged with an inverted microscope. The series of chevrons machined into the silica glass ceiling of the square tube (Fig. 1C) created controlled, flow-based eddies for mixing. A stable chemotactic gradient was thus generated perpendicular to the direction of flow in the area of observation, which lacked chevrons (Fig. 1D). The shapes and stability of the gradients were demonstrated by fluorophores with molecular weights ranging from 332 to 10,000 Da, with the dyes fluorescein (332 Da), Alexa Fluor 488, (570 Da), and Alexa Fluor 488 conjugated to dextran (10,000 Da). As demonstrated in Fig. 1E–H for fluorescein, the steepness of near-linear gradients of fluorescein could be controlled by flow rate. Gradients across the cells became stable within 10 seconds and remained stable thereafter, as shown in Fig. 1. The flow rate generated a stable gradient of fluorescein with a steepness of 0.833 nM/μm over a 10 minute period. Gradient steepness could be changed or the gradient reversed in less than 30 seconds (data not shown). The gradients of the three tested fluorescent molecules gave similarly near-linear gradients (data not shown), suggesting that diffusion was not a major factor in determining the shape of the gradient in this chamber, at least in the tested molecular weight range and rate of flow employed. Because no fluorescent molecules were available in the molecular weight range of Ca²⁺ (40 Da), we could only assume that the shape of the gradient was linear, but diffusion due to the low molecular weight of Ca²⁺ might occur and cause a nonlinear gradient.

**Behavior in the absence of a chemoattractant**

To characterize behavior in the chamber in the absence of chemoattractant, chemotactically responsive cells were first examined in the absence of a gradient in a buffered solution (see Materials and Methods for details) in which the concentration of either the K⁺ or Ca²⁺ (at facilitating concentrations of 40 mM and 10 mM, respectively) was uniform across the area of observation. In both cases, reservoirs a and b (Fig. 1A) contained the same cationic solution. In each experiment, motility parameters were measured for 5–10 cells in parallel during sequential 10 minute periods, beginning 10 minutes after the cells were injected into the chamber (i.e. 10–20, 20–30 and 30–40 minutes). During each period, a different set of cells was analyzed. Each parameter was averaged over the 10 minute period for each cell. Combined data for two or more independent experiments are presented as the mean ± s.d. for all cells analyzed between 10 and 40 minutes after inoculation. When perfused uniformly with either 40 mM K⁺ or 10 mM Ca²⁺ solution, cells moved on average in the direction of flow (Fig. 2A,B, respectively). Movement was directionally more persistent in the Ca²⁺ solution (supplementary material Movie S1).
The majority of cells were firmly attached to the substratum (i.e. did not peel off from the surface during the experiment) and polarized on average in the general direction of flow. Cellular translocation occurred through pseudopod extension. In order to verify that in the absence of chemoattractant, there was no bias towards the left or right of the chamber, a ‘bias index’ (BI) was computed as the net distance traveled towards the a-flow side (Fig. 1A) divided by the total distance traveled. The a-side was picked arbitrarily. Unbiased movement would result in an average BI close to 0.00 (±0.14 to –0.14); biased movement towards the a-flow side would result in a BI of between +0.15 and +1.00; and biased movement towards the b-flow side would result in an average BI of between –0.15 and –1.00. Unbiased movement would result in in approximately 50% (41–59%) of cells with a positive BI (i.e. in the direction of the a-flow side); biased movement toward the a-flow side would result in a value of approximately 60–100%; and biased movement toward the b-flow side would result in a value of 0–40%.

The average BI in uniform 40 mM K+ or uniform 10 mM Ca2+ was +0.02±0.30 and +0.05±0.21, respectively (Fig. 2C). The percentage of cells with a positive BI was 54 and 60%, respectively. The average instantaneous velocity was relatively high in both cationic solutions, but the average directional persistence parameter was higher in 10 mM Ca2+ than in 40 mM K+ (Fig. 2C), which had been suggested by the respective cell tracks in Fig. 2A and 2B, respectively. These results demonstrated strong flow-directed translocation, but no bias towards either side of the microfluidic chamber in the absence of a chemoattractant gradient. These results also suggested that Ca2+ was more effective in facilitating persistent flow-directed translocation than K+, which is consistent with observations by Fache and coworkers (Fache et al., 2005).

Behavior in a cAMP gradient
To demonstrate the efficacy of the microfluidic chamber for chemotaxis, a gradient of the known chemoattractant cAMP was generated either in a uniform solution of 40 mM K+ or in a uniform solution of 10 mM Ca2+ at a flow rate of 13 μl/minute. As described later in this report, this flow rate was empirically ascertained to generate an optimum Ca2+ gradient, not an optimum cAMP gradient, and was used here for comparative reasons. The concentration of cAMP in flow a was 0.1 μM, and flow b contained buffer alone. The steepness of the gradient was approximately 0.33 nM/μm, a change of 3.3% per 10 μm, which was close to the value of 0.1 nM/μm that Song and coworkers (Song et al., 2006) found previously discussed) that the Ca2+ gradient was linear. The average BI in uniform 40 mM K+ or uniform 10 mM Ca2+ was +0.31±0.24 and positive chemotaxis of 92% (Fig. 2F). In a cAMP gradient generated in a uniform 10 mM Ca2+ solution, the majority of cells also moved in the direction of the cAMP gradient (Fig. 2E) with a similar average CI of +0.34±0.27 and a similar positive chemotaxis of 89% (Fig. 2F). In cAMP gradients in both 40 mM K+ and 10 mM Ca2+ solutions, cells moved on average in the general direction of the increasing cAMP gradient, but at an angle between that of the gradient and that of flow (Fig. 2D,E), indicating that the direction of cellular translocation was affected by both the mechanical forces of flow and the cAMP gradient. The instantaneous velocities achieved in cAMP gradients (Fig. 2F) were lower than those achieved in the absence of cAMP, when the same flow rate was used in a homogeneous Ca2+ solution (Fig. 2C).

To assess the effectiveness of the chamber for a cAMP gradient with a more optimum steepness (Song et al., 2006; Fuller et al., 2010), the flow rate from each reservoir was halved to 7 μl/minute, which resulted in a stable gradient with a steepness of approximately 0.19 nM/μm, a decrease of approximately one third. These experiments were performed in a uniform 10 mM Ca2+ solution. The CI under these conditions for the first 20 minutes was +0.46±0.03 (n=45). The cells moved so persistently and efficiently up the cAMP gradient that they rapidly accumulated at the a-flow side of the chamber wall and, therefore, could not be monitored after 20 minutes. These results validated the effectiveness of the chamber we developed for assessing chemotaxis.

Chemotaxis in a Ca2+ gradient
To test for Ca2+ chemotaxis, aggregation-competent cells were treated with stable spatial gradients of Ca2+, employing a concentration of 10 mM, the optimum concentration for facilitating polarity and motility (Lusche et al., 2009), in reservoir a. Reservoir b contained buffer alone (i.e. lacking added Ca2+). The concentration in the source channel was in the millimolar range, in agreement with the concentration found necessary in the source reservoir of Boyden chambers to elicit chemotaxis of a variety of animal cells. Reported concentrations included 5 mM for osteoblasts (Godwin and Soltoff, 1997), 3.5 mM for neurons (Chattopadhyay et al., 2007), 4.5 mM for monocytes (Olszak et al., 2000) and 3–10 mM for bone-marrow-derived Fkli-1 CD34+ progenitor cells (Aguirre et al., 2010). Preliminary qualitative analyses of chemotaxis based on the orientation of cellular translocation suggested that flow rates of 13–15 μl/minute per reservoir resulted in optimum movement in the direction of increasing Ca2+. This established an estimated gradient of 33 μM/μm, a change of 3.3% per 10 μm, based on the assumption (as previously discussed) that the Ca2+ gradient was linear.

Cells in a Ca2+ gradient moved on average in the direction of increasing Ca2+, and did so at an angle between the direction of the gradient and the direction of flow (Fig. 3A; supplementary material Movie S2), as was the case for cAMP chemotaxis under similar flow conditions (Fig. 2D,E). The average CI for the pooled cells (n=112) of six experiments was +0.20±0.22, and the proportion of cells with a positive CI was 84% (Fig. 3D). When the gradient was generated in the microfluidic chamber in the reverse direction (i.e. 10 mM Ca2+ solution in reservoir b and buffer alone in reservoir a), the direction of translocation reversed (Fig. 3B), again excluding the possibility of directional bias owing to a chamber artifact. The
translocation of these cells in a Ca\(^{2+}\) gradient over a 10 minute period is presented in Fig. 3C. These results demonstrate that Ca\(^{2+}\) functions as a chemoattractant for aggregation-competent D. discoideum amoebae. It should be noted, however, that even in an optimum Ca\(^{2+}\) gradient, the average CI, a reflection of chemotactic efficiency, appeared to be lower than in an optimum or even suboptimum gradient of cAMP (+0.20±0.22 versus +0.34±0.27 or +0.31±0.24, respectively) and the percentage of cells undergoing positive chemotaxis was slightly lower (84 versus 92 or 89%, respectively).

A dose-response analysis was performed in which the Ca\(^{2+}\) concentration in the a-flow channel (the Ca\(^{2+}\) source for the gradient) was varied, but the flow rate was maintained at 15 \(\mu\)l/minute. The concentration that gave a 100% response was 10 mM; a 50% response was obtained at 1 mM and a 10% response, estimated from the curve (Fig. 3E), at 20 \(\mu\)M. The estimated steepness of the gradient, based on the assumption that the gradients were linear, was 33 \(\mu\)M/\(\mu\)m for maximum response, 3.3 \(\mu\)M/\(\mu\)m for 50% response and approximately 66 nM/\(\mu\)m for a 10% response.

Surprisingly, if the steepness of the Ca\(^{2+}\) gradient was dampened by generating a gradient in which flow a contained 10 mM Ca\(^{2+}\) and flow b contained 1 mM Ca\(^{2+}\), the average CI was close to 0.0 and the positive chemotaxis close to 50%. If diffusion were not a factor, this would amount to only a 10% decrease in steepness in comparison to a gradient generated with 0 mM Ca\(^{2+}\) in flow b. The result could be explained if diffusion represented an effector of steepness at a flow rate of 15 \(\mu\)l/minute. Diffusion from flow a partially countered by diffusion from flow b would result in a sigmoidal gradient. Lowering the flow rate to 7 \(\mu\)l/minute, but maintaining 10 mM Ca\(^{2+}\) in flow a and buffer alone in flow b, had an effect on chemotaxis similar to adding 1 mM Ca\(^{2+}\) to flow b. At a flow rate of 7 \(\mu\)l/minute, the CI was close to zero and at 11 \(\mu\)l/minute it was close to 0.09. At 13 \(\mu\)l/minute, the CI reached a maximum greater than +0.20, which was maintained through a flow rate of 15 \(\mu\)l/minute. The change in steepness between flow rates of 7 and 15 \(\mu\)l/minute, based on the assumption that gradients of Ca\(^{2+}\) are linear and diffusion is not a factor, would have resulted in a decrease in steepness of 28%, which should have only a minor effect on chemotactic efficiency. Halving the flow rate, however, could have had a dramatic effect on Ca\(^{2+}\) diffusion which, in turn, would have had a dramatic effect on the shape of the gradient.

**Reversing the gradient during Ca\(^{2+}\) chemotaxis**

Because the microfluidic chamber we developed allowed rapid reversal of a spatial gradient, we tested the effects of changing the direction of a Ca\(^{2+}\) gradient on cellular behavior. Cells undergoing chemotaxis in a spatial gradient of Ca\(^{2+}\) changed direction within 1 minute of gradient reversal, as demonstrated in the tracks of the two representative cells in Fig. 4A,B. Difference pictures of cells were generated during reversal, in which the perimeter image of a cell at each time point was superimposed upon the perimeter image that had been generated 10 seconds earlier (Soll, 1995). The regions in the later image not overlapping the earlier image were considered ‘expansion zones’ and color-coded green, and the regions in the earlier image not overlapping the later image considered ‘contraction zones’ and color-coded red (Fig. 4C). Expansion zones have been shown to correlate with the position of extending new lateral pseudopods (Soll, 1995) or expansion of a portion of a prior
anterior pseudopod at an angle to the direction of prior translocation, a process that has been referred to as ‘splitting’ (Andrew and Insall, 2007). For simplicity, we will refer to both types of expansion as ‘lateral pseudopod formation’. During the period immediately following gradient reversal, cells extended lateral pseudopods both in the new direction of increasing Ca\(^{2+}\) concentration and in that of decreasing Ca\(^{2+}\) concentration, and then moved in the direction of the lateral pseudopod that had extended in the direction of increasing concentration (Fig. 4C). By tracking the position of the uropod of each cell, which possessed a visible cluster of tail fibers under the microscopic conditions employed (Heid et al., 2005) (not shown here), it was clear that the uropod retained its integrity and followed the direction of the lateral pseudopod that extended in the correct direction, a turn similar to that described for cells turning in the absence of attractant (Soll et al., 2009). Five additional cells experiencing gradient reversal exhibited similar pseudopod dynamics to the one represented in Fig. 4C (data not shown).

**Responses to a global Ca\(^{2+}\) signal and temporal Ca\(^{2+}\) gradients**

When cAMP, at a concentration equal to that at the peak of a natural wave, is rapidly added to *D. discoideum* cells translocating in buffer, they ‘cringe’ (Futrelle et al., 1982; Futrelle, 1982; Wessels et al., 1989). In the first 30 seconds, the cringe includes a dramatic decrease in velocity, rounding up, blebbing, the relocalization of myosin from the cytoplasm to cortex and a spike in F-actin concentration (Hall et al., 1988; Yumura and Fukui, 1985; Condeelis et al., 1990; Wessels et al., 1989; Postma et al., 2003). When 10 mM Ca\(^{2+}\) solution was added to cells translocating in 40 mM K\(^{+}\) solution, there was a similar immediate decrease in velocity from 11.9±1.9 μm/min to 6.8±1.0 μm/min (n=20), but cells did not round up. Instead, they became highly active in lateral pseudopod formation. This response shared at least one aspect of a cAMP cringe, a dramatic decrease in velocity, but differed otherwise.

*D. discoideum* cells can also be challenged in a perfusion chamber with temporal waves of cAMP at 7 minute intervals, which roughly mimic the temporal dynamics of an average natural cAMP wave in the absence of established spatial gradients of cAMP. Under these conditions, cells undergo behavioral and morphological changes in the increasing temporal gradient in the front of each wave, and decreasing temporal gradient in the back, similar to the changes in the front and back of natural waves (Varnum et al., 1985; Wessels et al., 1992). When challenged with similar temporal gradients of Ca\(^{2+}\) at 7 minute intervals, with peak concentrations of 20 mM and troughs below 0.20 mM, similar behavioral changes did not occur (data not shown), providing no indication that *D. discoideum* amoebae could assess temporal waves of Ca\(^{2+}\) as they do temporal waves of cAMP.

**Parallel gradients of cAMP and Ca\(^{2+}\)**

We next analyzed the behavior of cells in parallel gradients of cAMP and Ca\(^{2+}\), generated simultaneously in the same direction. The flow conditions we used were optimum for generating the most efficient Ca\(^{2+}\) gradient. In parallel spatial gradients of Ca\(^{2+}\) and cAMP, in which a solution of 10 mM Ca\(^{2+}\) plus 0.1 μM cAMP flowed from reservoir a and buffer alone from reservoir b, cells moved up the parallel gradients of Ca\(^{2+}\) and cAMP in a highly persistent and directional manner (Fig. 5). The CI for cells analyzed between 10 and 40 minutes after inoculation was +0.42±0.28 (Fig. 5C), which was higher than that for cells in a cAMP gradient alone under similar flow conditions, which averaged +0.34±0.27 (Fig. 2F), and higher than that for cells in a Ca\(^{2+}\) gradient alone, which averaged +0.20±0.22 (Fig. 3D). The CI in parallel gradients was on average 24% higher than the CI in a cAMP gradient alone, and 110% higher than a Ca\(^{2+}\) gradient alone; the differences were reproducible. The differences in the mean CI for cells in parallel gradients versus that of cells in either a cAMP gradient alone or a Ca\(^{2+}\) gradient alone were significant, with P-values of 4×10\(^{-3}\) and 6×10\(^{-5}\), respectively. The percentage of cells undergoing positive chemotaxis in parallel gradients (90%), however, was similar to that of cells in a cAMP gradient alone (89%) or a Ca\(^{2+}\) gradient alone (84%), indicating that parallel gradients caused a greater increase in chemotactic efficiency than in positive chemotaxis. Similar results were obtained when the Ca\(^{2+}\) gradient alone or the parallel Ca\(^{2+}\) plus cAMP gradients were generated in TB supplemented with 40 mM K\(^{+}\) (data not shown). The CI in a Ca\(^{2+}\) gradient, a cAMP gradient and combined gradients exhibited a hierarchy of Ca\(^{2+}\) + cAMP > cAMP > Ca\(^{2+}\), as was the case with gradients generated in TB alone.

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**Fig. 4. Cells undergoing chemotaxis in a spatial gradient of Ca\(^{2+}\) change direction upon gradient reversal through lateral pseudopod extension.**

(A,B) The behavior of representative cells upon gradient reversal at 11 minutes (vertical dashed line). The average CI is presented for each cell following the initial and reversed gradient.

(C) Difference pictures of the cell in A before and after reversal. Green areas are expansion zones and red areas are contraction zones. Arrows denote direction of translocation. Green arrows denote direction of gradients.
Opposing gradients of cAMP and Ca²⁺

We next tested the response of cells to opposing gradients of cAMP and Ca²⁺. Because it appeared that even under conditions optimum for a Ca²⁺ gradient, but not for a cAMP gradient, the latter elicited a higher average CI, we predicted that cells in opposing cAMP and Ca²⁺ gradients would on average chemotax towards cAMP, but less efficiently. That was the result obtained. Cell behavior was analyzed in the chamber in which the a-channel contained 0.1 μM cAMP solution and the b-channel contained 10 mM Ca²⁺ solution. The majority of cells in opposing gradients underwent chemotaxis up the cAMP gradient, but at a reduced positive chemotaxis of 77%. The efficiency of chemotaxis was also reduced, as indicated by a CI of +0.19±0.24 (Fig. 5C), roughly two thirds the value obtained in a cAMP gradient alone (Fig. 2F).

Time comparison of chemotactic efficiency in parallel gradients

Because we had noticed that chemotaxis measurements during the first and last 10 minute intervals of a 60 minute period could vary under select conditions, we tested whether there were temporal differences involved in the assessment of individual, parallel and opposing cAMP and Ca²⁺ gradients. We computed CIs at 10 minute intervals between 10 and 40 minutes after inoculation for cells in individual, parallel and opposing gradients. The average CI was then plotted for the time intervals 10–20, 20–30 and 30–40 minutes. The data were pooled from two or more experiments for each 10 minute interval under each gradient condition. Experiments were performed at flow rates optimum for a Ca²⁺ gradient, for reasons explained earlier. The pooled data at the bottom of each panel in Fig. 6 show that the CI was higher in the cAMP gradient alone than in the Ca²⁺ gradient alone through the three 10 minute time intervals (Fig. 6A,B, respectively). In parallel gradients of cAMP and Ca²⁺ generated in the same direction, the CI was higher in each of the 10 minute intervals than that of the cAMP or Ca²⁺ gradient alone (Fig. 6A–C). Finally, in opposing gradients of cAMP and Ca²⁺, the majority of cells moved in the direction of the cAMP gradient, not the Ca²⁺ gradient, through each 10 minute time interval, but the chemotactic efficiency in each interval was below that observed in a cAMP gradient alone (Fig. 6A,D). This suggested that an optimum Ca²⁺ gradient represented a weaker signal than a cAMP gradient, but still functioned when opposed to the cAMP gradient in a competitive fashion, causing a decrease in the efficiency of cAMP chemotaxis.

Vegetative cells do not chemotax in the Ca²⁺ gradient optimum for aggregation-competent cells

We next tested whether vegetative cells also underwent Ca²⁺ chemotaxis. As previously reported (Varnum et al., 1986), cells grown axenically in liquid nutrient medium do not polarize well (i.e. remain relatively round) and translocate at reduced velocity, unless starved for several hours. It was, therefore, not possible to assess the chemotactic responsiveness of freshly grown axenic cells to a Ca²⁺ gradient. By marked contrast, bacterially grown cells of strain Ax2 polarized immediately on a glass surface and were highly motile, as previously reported (Varnum et al., 1986). These cells failed to undergo chemotaxis in a spatial gradient of Ca²⁺ in which flow a contained 10 mM Ca²⁺ solution or 40 mM K⁺ solution in a microfluidic chamber (data not shown). The incapacity of vegetative cells to respond to spatial gradients of cAMP has been previously reported using different chemotactic assays (Pan et al., 1972; Devreotes and Steck, 1979; Varnum and Soll, 1981). Vegetative cells also exhibited no chemotactic response to a gradient of Ca²⁺ in which flow a contained 10 mM Ca²⁺ solution and flow b contained buffer, the optimum conditions for Ca²⁺ chemotaxis of aggregation-competent cells. The average instantaneous velocity for pooled data of four independent experiments, which included a combined 45 cells, was relatively high (10.3±3.5 μm/minute), but the average CI was close to zero (−0.04±0.34). Vegetative cells also moved on average in the direction of flow, but with less orientation than aggregation-competent cells. The standard deviation of the mean CI was high for vegetative cells because directionality up and down the gradient was more random. The percentage of cells undergoing positive chemotaxis for the combined 45 cells was 55%, indicating no positive chemotaxis.
analysis of cells in a Ca\(^{2+}\) gradient generated in TB supplemented with 40 mM K\(^+\) also resulted in a CI close to 0.0 (data not shown). These results indicate that although vegetative cells grown in the presence of bacteria are highly motile, they do not exhibit a chemotactic response to the Ca\(^{2+}\) gradient effective for aggregation-competent cells. This does not rule out the possibility that a gradient of different steepness would be effective, although qualitative experiments performed at lower and higher Ca\(^{2+}\) concentrations in flow a failed to reveal chemotaxis (data not shown).

**Discussion**

**Ca\(^{2+}\) facilitation of cell motility**

The extracellular concentration of Ca\(^{2+}\) has been shown to facilitate motility in cells from bacteria to vertebrates. For instance, the nonflagellated marine cyanobacterium *Synechococcus* requires a minimum extracellular Ca\(^{2+}\) concentration of 2 mM (Pitta et al., 1997), and flagellated sea urchin sperm require an extracellular Ca\(^{2+}\) concentration of 9 mM (Young and Nelson, 1974) to attain maximum velocity. These facilitating concentrations are close to the concentration of soluble Ca\(^{2+}\) in seawater, which is approximately 10 mM (Goldberg et al., 1971). It might therefore be no coincidence that the extracellular concentration of Ca\(^{2+}\) necessary to attain optimum motility of *D. discoideum* amoebae in vitro is 10 mM (Lusche et al., 2009). Amoebae are found at high densities in soil (Raper, 1935), in which the soluble concentration of Ca\(^{2+}\) ranges from 3 to 12 mM (Bangerth, 1979; McLaughlin and Wimmer, 1999), and in manure (Gilbert et al., 2009), in which the Ca\(^{2+}\) concentration is usually far higher than 10 mM (Sager, 2007; Qian et al., 2005). Maeda (Maeda, 1970) demonstrated over 40 years ago that *D. discoideum* amoebae aggregate relatively normally in very high concentrations of Ca\(^{2+}\) (approaching 100 mM). In a variety of niches in the vertebrate body, the concentration of soluble Ca\(^{2+}\) has also been found to be, as previously noted, in the millimolar range (Menon et al., 1985; Silver et al., 1988; Adams et al., 2006; Breitwieser, 2008; Olzsak et al., 2000). Consistent with these findings, there is evidence that extracellular concentrations of Ca\(^{2+}\) between 1 and 10 mM are optimum for vertebrate cell motility (Nemeth, 1995; Dvorak et al., 2004; Morris and Chan, 2007).

**Ca\(^{2+}\) chemotaxis in general**

In addition to facilitating basic cell motility, it has been shown that spatial gradients of extracellular Ca\(^{2+}\) induce chemotaxis of a surprisingly wide variety of cell types in vitro. Brokaw (Brokaw, 1974) demonstrated that bracken fern spermatozoids undergo chemotaxis up combinatorial gradients of Ca\(^{2+}\) and bimaleate. Ca\(^{2+}\) alone has been demonstrated as a chemoattractant for an ever-increasing number of cell types, including gonadotropin-releasing hormone (GnRH) neurons (Chattopadhyay et al., 2007), hematopoietic stem cells (Lapidot and Kollet, 2002; Adams et al., 2006), keratinocytes (Menon et al., 1985), osteoblasts (Godwin and Soltoff, 1977) and monocytes (Olzsak et al., 2000). However, chemotaxis has been demonstrated in most of these cases in vitro, primarily using Boyden chambers (Boyden, 1962), without...
quantitative comparisons to cell-type-specific attractants and without testing parallel or opposing gradients. Here, we have performed such an analysis and demonstrated that aggregation-competent *D. discoideum* amoebae undergo positive chemotaxis up spatial gradients of Ca\(^{2+}\) generated in a microfluidic chamber that have been estimated to be relatively steep in comparison to effective cAMP gradients in the same chamber; that chemotaxis in an optimum Ca\(^{2+}\) gradient is not as efficient as that in a cAMP gradient; that a parallel spatial gradient of Ca\(^{2+}\) enhances chemotaxis up a spatial gradient of cAMP; and that Ca\(^{2+}\) chemotaxis appears to be acquired during the preaggregation period of the developmental program, when cAMP chemotaxis is acquired (Klein, 1988).

The microfluidic chamber and flow-induced motility

In order to test for Ca\(^{2+}\) chemotaxis in *D. discoideum* amoebae, we developed with Translume, a chamber with a number of assets. The chamber was built of fused silica glass, which provides a high level of optical clarity and can be reutilized indefinitely. It can be used at most natural and physiological temperatures, and with varying rates of flow. It provides stable gradients that form within 10 seconds and that can be changed or reversed within 30 seconds. Using the response of *D. discoideum* cells to cAMP to verify the efficacy of the chamber, we demonstrated that the proportion of cells in the area of observation undergoing positive chemotaxis approached 100% in most experiments. In both cAMP and Ca\(^{2+}\) gradients, we have consistently obtained 85–100% of the entire cell population undergoing chemotaxis. Using available fluorescent molecules through a range of molecular weights (332, 570 and 10,000 Da), we demonstrated that, at least in this range, the generated gradients were nearly linear. By extrapolation, we have assumed that the same was true for Ca\(^{2+}\), which has a molecular weight of 40 Da. However, because low molecular weight fluorescent molecules were unattainable, this remains an assumption. Diffusion could play a role in the gradients generated in the chamber for very low molecular weight molecules like Ca\(^{2+}\). In such cases, the gradients might not be linear across the area of observation in the chamber. The results of experiments in which steepness was flattened by adding 10 mM Ca\(^{2+}\) to flow a and 1 mM Ca\(^{2+}\) to flow b, combined with the data obtained from experiments in which flow rate was reduced, suggest that diffusion indeed influences the shape of optimum Ca\(^{2+}\) gradients.

The one drawback to our chamber is the rate of flow necessary to establish a gradient of a low molecular weight molecule such as Ca\(^{2+}\). Shear has been shown to induce *D. discoideum* motility in the direction of flow (Decave et al., 2002a; Decave et al., 2002b; Decave et al., 2003; Fache et al., 2005), which appears to involve stretch-activated calcium channels (Lombardi et al., 2008). We found that the high flow rates necessary for generating a Ca\(^{2+}\) gradient in our chamber similarly caused flow-directed motility. When subjected either to a cAMP or Ca\(^{2+}\) gradient at these high flow rates, the flow signal, combined with the chemotactic gradient caused movement in a direction between the flow vector and the gradient vector. This did not, however, hamper our capacity to assess the chemotactic responsiveness of cells to cAMP and Ca\(^{2+}\) gradients.

The steepness of the Ca\(^{2+}\) gradient

We found the effective concentration of Ca\(^{2+}\) in the source flow (a flow) to be extremely high compared with the effective concentration of cAMP. As noted, we estimated the optimum Ca\(^{2+}\) gradient in the microfluidic chamber, assuming linearity, to be approximately 33 μM/μm, which is 10\(^5\) times that of an effective cAMP gradient in the same chamber. This apparently large difference is consistent with the difference in the binding constants of the cAMP receptor of *D. discoideum* and the Ca\(^{2+}\) receptors analyzed in animal cells. The binding constant of the cAMP receptor is 25±8 nM (Johnson et al., 1992), whereas that of the most carefully analyzed animal cell Ca\(^{2+}\)-sensing receptor is 4.2–18.6 mM (Huang et al., 2007), again a difference of approximately 10\(^5\). Because of this difference, the concentration differential of Ca\(^{2+}\) that actually binds along the length of a cell might be similar to that for cAMP in gradients differing in steepness by 10\(^5\).

Chemosensitivity in parallel cAMP and Ca\(^{2+}\) gradients

The microfluidic chamber and flow-induced motility

Three lines of evidence suggested that, on average, cells undergo chemotaxis more robustly up a cAMP gradient than up an optimum Ca\(^{2+}\) gradient. First, when analyzed over time, cells in cAMP gradients attained higher average chemotactic indices than cells in Ca\(^{2+}\) gradients, even when both gradients were generated at a flow rate found to be optimum for Ca\(^{2+}\) chemotaxis, not for cAMP chemotaxis. Second, when chemotaxis was analyzed in optimal cAMP gradients, chemotactic efficiency was significantly higher than that achieved in optimum Ca\(^{2+}\) gradients. Third, when opposing cAMP and Ca\(^{2+}\) gradients were generated at flow rates optimum for the latter, the majority (77%) of cells still made net progress in the direction of increasing cAMP, not in the direction of increasing Ca\(^{2+}\), but the mean CI, which reflects the efficiency of chemotaxis, was far below that of cells in a cAMP gradient alone. The results suggested that cells still responded to the opposing, but less effective Ca\(^{2+}\) signal, which decreased the efficiency of chemotaxis in the more effective cAMP gradient. Perhaps most relevant to the possible role that Ca\(^{2+}\) might play in aggregation territories, we found that in parallel cAMP and Ca\(^{2+}\) gradients generated in the same direction, the average CIs were higher than in a cAMP gradient alone or in a Ca\(^{2+}\) gradient alone, suggesting additivity.

Speculation on the establishment of steep Ca\(^{2+}\) gradients and the role of Ca\(^{2+}\) chemotaxis

Our data indicate that the capacity to undergo chemotaxis in apparently steep gradients of Ca\(^{2+}\) is acquired during early development, in association with the acquisition of cAMP chemotaxis. But how plausible is it that steep gradients are generated in naturally aggregating cell population and, if generated, what role would Ca\(^{2+}\) chemotaxis play, given that cAMP has been considered (for a host of reasons) the lone player? In relation to steepness, we have assumed that the Ca\(^{2+}\) gradient generated in vitro is linear. This assumption is made on the basis of gradients measured with fluorescent molecules in the 300–10,000 Da range, where diffusion does not appear to be an issue. But, as noted, we could not measure gradients of low molecular weight due to the unavailability of markers, and therefore could only assume that low molecular weight molecules also form linear gradients. Several observations suggest that diffusion by Ca\(^{2+}\) might change the shape of an effective gradient. But, even if that were the case, there is no observation that would suggest that cAMP can induce the release of a high-enough concentration of Ca\(^{2+}\) to result in the steep gradients found to be effective in vitro. The absence of supporting data, however, does not disprove the existence of such gradients. For instance, if Ca\(^{2+}\) were released in a pulse at a very high concentration from a localized portion of the cell, such as the
uropod, a suggestion put forward by Parent and coworkers for cAMP (Kriebel et al., 2008; García et al., 2009), a steep, highly transient gradient of Ca^{2+} might form in the interstitial area between two closely associated cells. Until a method is devised for obtaining a measurement, or a ‘snapshot’, of the spatial distribution of Ca^{2+} in a natural population of aggregating cells, such as achieved for the distribution of cAMP using an isotope dilution–fluorography technique (Tomchik and Devreotes, 1981), we can only speculate on the existence of such Ca^{2+} gradients.

If we speculate that such gradients do exist, what role might they play? Given that we found that cells do not assess the distribution of cAMP using an isotope dilution–fluorography technique, we can only speculate. Therefore, we have discovered a very real Ca^{2+} port (Fig. 1A). The complete volume of the microfluidic device could be flushed of suspensions were introduced into the viewing area (Fig. 1D) through the cell injection port (Fig. 1D). The port was capped shut and cells were allowed to adhere to the bottom coverslip for 5 minutes. One inlet port of the chamber was connected to a pump-driven reservoir containing the 40 mM K^{+} solution and the other inlet port to a pump-driven reservoir containing the 40 mM Ca^{2+} solution. Following insertion of the outlet tube, the pump connected to the reservoir containing 40 mM K^{+} solution was turned on and this buffer solution was perfused at a rate of 4 μl/minute for 10 minutes. The solution was then immediately switched to 40 mM K^{+} containing 10 mM Ca^{2+}. This procedure allowed for a rapid turnover of solutions within the chamber. Images were acquired and analyzed as described below.

Cell response to uniform solutions of 40 mM K^{+} or 10 mM Ca^{2+}

To analyze the behavior of aggregation-competent amoebae in the absence of a gradient of attractant, both reservoir pumps a and b of the microfluidic chamber were operated as described above except that reservoir a was filled with a solution containing either 40 mM K^{+} solution or 10 mM Ca^{2+} solution. The tubing and inlet ports were filled with the same solution (i.e. 40 mM K^{+} or 10 mM Ca^{2+}). The tubing from each reservoir syringe was then connected to its respective inlet port via a Luer lock. The pumps were turned on and the chamber primed by flushing with 40 mM K^{+} or 10 mM Ca^{2+} solution. The exit tubing was then clamped shut and 300 μl of a cell suspension at a density of 5 × 10^{5} cells/ml was inoculated into the cell injection port (Fig. 1D). The port was then clamped shut and cells were allowed to adhere to the surface for 10 minutes. The clamp was then removed from the exit tube, the pumps simultaneously turned on to generate flow a and flow b, and image acquisition started.

Cell response to a cAMP gradient within the microfluidic chamber

The microfluidic chamber was primed as described above except that reservoir a was filled with the facilitating cation solution (40 mM K^{+} or 10 mM Ca^{2+}) plus 0.1 μM cAMP, and reservoir b with the appropriate cation solution alone. Cells were inoculated into the injection port, allowed to adhere, and the flow initiated.

Cell response to Ca^{2+} gradients and combination gradients

The microfluidic chamber was primed and operated as described above except that to generate a Ca^{2+} gradient, one reservoir was filled with a solution containing TB plus 1, 5 or 10 mM Ca^{2+} and the other reservoir with TB alone. To generate parallel Ca^{2+} and cAMP gradients, reservoir a was filled with a solution containing TB containing 10 mM Ca^{2+} plus 0.1 μM cAMP, and reservoir b with TB alone. Finally, to generate opposing Ca^{2+} and cAMP gradients, reservoir a was filled with TB containing 0.1 μM cAMP, and reservoir b with TB containing 10 mM Ca^{2+}. Cell inoculation and flow were as described above.

Cell response to a rapid shift in Ca^{2+} concentration

Cell response to a rapid shift from 40 mM K^{+} solution to 40 mM Ca^{2+} – was measured in the Sykes–Moore perfusion chamber (Bellco Glass, Vineland, NJ). The chamber is described in detail elsewhere (Varnum et al., 1985; Varnum et al., 1986). In brief, aggregation-competent amoebae were inoculated into the chamber at a low density (10–20 cells/mm²) and allowed to adhere to the surface of the bottom coverslip for 5 minutes. One inlet port of the chamber was connected to a pump-driven reservoir containing the 40 mM K^{+} solution and the other inlet port to a pump-driven reservoir containing 40 mM K^{+} plus 10 mM Ca^{2+}. Following insertion of the outlet tube, the pump connected to the reservoir containing 40 mM K^{+} solution was turned on and this buffer solution was perfused at a rate of 4 μl/minute for 10 minutes. The solution was then immediately switched to 40 mM K^{+} containing 10 mM Ca^{2+}. This procedure allowed for a rapid turnover of solutions within the chamber. Images were acquired and analyzed as described above.

Cell response to temporal waves of Ca^{2+}

Methods for treating cells with temporal waves have been described in detail elsewhere (Varnum et al., 1985). Briefly, aggregation-competent amoebae were inoculated into the Sykes–Moore perfusion chamber as described above and then treated with a series of increasing and decreasing temporal gradients of calcium in TB at a periodicity of 7 minutes using the NE-1000 Multiphase Programmable Syringe Pump (New Era Pump Systems, Wantagh, NY) (Geiger et al., 2003). The concentration of calcium ranged from 0.2 mM at the trough of each wave to 20 mM at each peak. Images were acquired and analyzed as described below.

Strain maintenance, growth and development

In order to maintain genotypic and phenotypic homogeneity, frozen stocks of *Dictyostelium discoideum* strain AX-2 were restituted every 2 weeks as described elsewhere in detail (Varnum et al., 1986). Development was initiated according to methods previously described (Soll, 1979; Varnum et al., 1986; Wessels et al., 2006). In brief, cells at the low log phase of growth (2 × 10^{5} cells/ml) in HL-5 broth (http://dictybase.org) were washed free of nutrient medium, re-suspended in buffered salt solution (BSS; 20 mM KCl, 2.5 mM MgCl_{2}, 5 mM Na_{2}HPO_{4}, pH 6.4) and distributed as a smooth carpet on filter pads (Sussman, 1987; Soll, 1979; Varnum et al., 1986; Wessels et al., 2006) saturated with BSS. Under these conditions, aggregation typically occurred at 6 hours and represented the stage at which velocity and chemotactic efficiency were maximal (Varnum et al., 1986). For experimental purposes, all cells used in assays involving Ca^{2+} were washed and incubated for 90 minutes in TB containing 10 mM CaCl_{2}. The major cation in BSS is 40 mM K^{+}, which has been shown to be the prime facilitator of polarity and motility in this solution; 10 mM Ca^{2+} has been shown to be the prime facilitator in TB + Ca^{2+} solution (Lusche et al., 2009). BSS is therefore referred to as ‘40 mM K^{+} solution’ and TB + 10 mM CaCl_{2} as ‘10 mM Ca^{2+} solution’. For analysis of the chemotactic response of vegetative cells, amoebae were grown for 24–48 hours to a density of 5 × 10^{5} cells/ml in 20 ml of SM broth (http://dictybase.org) in association with freshly diluted *Klebsiella aerogenes* (Sussman, 1987).
Analysis of vegetative cells

Cells were washed with PBS to remove unbound antibodies and then fixed in 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.1% Triton X-100 for 30 min. After blocking with 3% BSA for 1 h, cells were incubated with primary antibodies overnight at 4°C. Following extensive washing, cells were incubated with secondary antibodies for 1 h. The nuclei were stained with DAPI for 10 min. Images were captured using a confocal microscope.

Image acquisition and 2-DI AS analysis

Fluorescence images were acquired using a spectral confocal microscope. The excitation wavelength was set at 488 nm for Alexa Fluor 488 and 543 nm for Alexa Fluor 568. The emission was detected using bandpass filters. Images were analyzed using ImageJ software.

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