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CHEMOTACTIC RESPONSES OF *DICTYOSTELIUM DISCOIDEUM* AMOEBAE TO A CYCLIC AMP CONCENTRATION GRADIENT: EVIDENCE TO SUPPORT A SPATIAL MECHANISM FOR SENSING CYCLIC AMP

TOMOMI TANI[1,*] AND YUTAKA NAITOH[2]

[1]*Department of Biology, Graduate College of Arts and Sciences, University of Tokyo, Komaba, Meguro-ku, Tokyo 153, Japan* and [2]*Pacific Biomedical Research Center, University of Hawaii at Manoa, Snyder Hall 306, 2538 The Mall, Honolulu, HI 96822, USA*

*Present address: Pacific Biomedical Research Center, University of Hawaii at Manoa, Snyder Hall 306, 2538 The Mall, Honolulu, HI 96822, USA (e-mail: tani@pbrc.hawaii.edu)

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Summary

The motile responses of *Dictyostelium discoideum* amoebae to a cyclic AMP (cAMP) concentration gradient were examined using a novel assay system. In this system, a cAMP concentration gradient was generated, while the overall cAMP concentration could be either increased or decreased in a chamber containing amoebae. The chemotactic responses of amoebae were examined immediately after they had been subjected to the cAMP concentration gradient. Amoebae moving in random directions in a reference solution ascended a cAMP concentration gradient after they had been exposed to the gradient irrespective of whether there was an increase or a decrease in the overall cAMP concentration. This strongly supports the idea that *D. discoideum* amoebae can sense a spatial cAMP gradient around them and that this causes their chemoaccumulation behavior. Ascending locomotion became less conspicuous when the amoebae were treated with a homogeneous cAMP solution for approximately 8 min before exposure to a cAMP gradient. This cAMP pretreatment reduced the sensitivity of the amoeba to a cAMP concentration gradient. The cAMP concentration gradient could be reversed in less than 30 s in this assay system, allowing the generation of a cAMP wave by accumulating amoebae to be mimicked. The ascending amoebae continued to move in the same direction for 1–2 min after the gradient had been reversed. This is consistent with the well-known observation that reversal of a cAMP concentration gradient experienced by the amoebae passing through a cAMP wave does not negate their chemotactic movement towards the accumulation center.

Key words: *Dictyostelium discoideum*, amoeba, chemotaxis, cyclic AMP sensing, cyclic AMP concentration gradient, spatial mechanism, temporal mechanism.

Introduction

Amoebae of the cellular slime mold *Dictyostelium discoideum* accumulate to form a pseudoplasmodium when they are starved (Bonner, 1947). This accumulation is mediated by the chemotactic responses of the amoebae to adenosine-3′,5′-cyclic monophosphate (cAMP) (Konijn et al., 1967). Many investigators have examined chemotactic responses with particular reference to cAMP-sensing by the cell and to the subsequent intracellular signaling that controls the motile activity of the amoebae and leads to their accumulation (Gerisch, 1982; Konijn and Van Haastert, 1987; Caterina and Devreotes, 1991; Fukui, 1993; Maniak et al., 1995; Xiao et al., 1997). However, the mechanism(s) by which the amoebae detect the direction of the center of the cAMP pulse is not yet fully understood.

There are two main ways in which differences in concentration of a chemical stimulant could be sensed by a cell: as a spatial concentration difference along the surface of the cell (spatial gradient; Mato et al., 1975) or as a temporal concentration difference at some specific site on the cell surface (temporal gradient; Gerisch et al., 1975). Zigmond (1974, 1977) demonstrated that horse and human leukocytes could detect a spatial gradient of γ-globulin and extended their pseudopods to move towards the source of γ-globulin. Several investigators have examined the chemotactic responses of *D. discoideum* amoebae in a variety of assay systems, in which both spatial and temporal cAMP concentration gradients could be controlled (Konijn, 1970; Futrelle, 1982; Van Haastert, 1983; Vicker et al., 1984; Varnum et al., 1985; Fisher et al., 1989). Most of their results support the idea that *D. discoideum* amoebae sense a temporal cAMP gradient during chemoaccumulation. However, the involvement of spatial gradient-sensing in the mechanism leading to

chemoaccumulation of the amoebae could not be excluded entirely.

To examine whether the spatial mechanism or the temporal mechanism is primarily responsible for cAMP-sensing by *D. discoideum* amoebae, we examined the motile responses of individual amoebae to cAMP using a novel assay system. We obtained positive evidence in support of the spatial mechanism. Some portions of this paper have been presented verbally elsewhere (Tani and Naitoh, 1992, 1993).

Materials and methods

Cell preparation

Amoebae of *Dictyostelium discoideum* (strain, NC-4; obtained from Dr K. Yanagisawa, The University of Tsukuba) were grown on an agar plate (5.0 g l^{-1} glucose, 0.5 g l^{-1} yeast extract, 7.5 g l^{-1} proteose peptone, 22.5 g l^{-1} KH_2PO_4, 6.8 g l^{-1} K_2HPO_4, 5.0 g l^{-1} $MgSO_4{\cdot}7H_2O$ and 15 g l^{-1} agar) at 20 °C with *Klebsiella aerogenes* provided as their food (Sussman, 1966). Approximately 72 h after inoculation, the amoebae formed into many small aggregates on the agar plate. The aggregates were harvested and suspended in a Ca^{2+}-free saline solution containing (final concentration in mmol l^{-1}) 10 KCl, 10 NaCl, 1.0 EGTA and 3.0 Hepes (pH 7.0) for 1–2 min to remove the intercellular adherent material. The suspension was then gently shaken to break the aggregates into individual cells.

Experimental solutions

The reference solution for the assay of the chemotactic behavior of *D. discoideum* amoebae in response to cAMP was as follows (final concentration in mmol l^{-1}): 10 KCl, 10 NaCl, 2.7 $CaCl_2$, 3.0 Hepes (pH 7.0). Test solutions containing various concentrations of cAMP (final concentration 10^{-8} to 10^{-6} mol l^{-1}) were prepared by adding cAMP·Na to the reference solution. A Mant-cAMP (2′-methylanthraniloyl derivative of cAMP)-containing solution used to make fluorometric measurement of the concentration gradient in the assay chamber was also prepared by adding Mant-cAMP·Na (final concentration 10^{-4} mol l^{-1}) to the reference solution. All chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Assay system

The design of our assay system was based on a system used to study the chemotactic behavior of *Paramecium caudatum* (Nakazato and Naitoh, 1993). Two pieces of coverslip (0.17 mm thick) of similar size (50 mm×6 mm) (a, a′ in Fig. 1A) were glued in parallel 12 mm apart on a glass slide (76 mm×52 mm×1.3 mm) (s in Fig. 1A) to make a trough. Two other pieces of coverslip (12 mm×18 mm) were placed over the right and left portions of the trough approximately 10 mm apart (b, b′ in Fig. 1A) to create two separate spaces in the trough under them, one for the reference solution and the other for a cAMP-containing test solution. A thin (0.4 mm×18 mm) strip of coverslip was placed over the central portion of the trough to make a thin space for the suspension of amoebae (c in Fig. 1A). Hereafter, the space for the reference solution will be termed the ‘reference chamber’, that for the test solution the ‘test chamber’ and that for the suspension of amoebae the ‘cell chamber’.

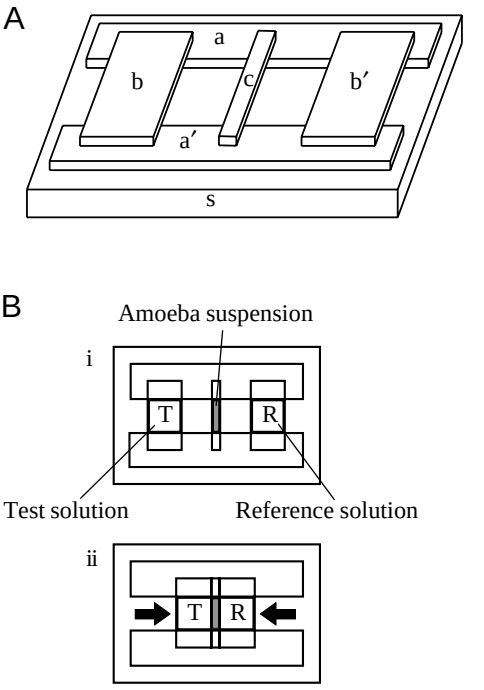

Fig. 1. Experimental chambers used to assay the chemotactic behavior of *Dictyostelium discoideum* amoebae. (A) Assay chambers. a, a′, coverslips (50 mm×6 mm) forming a trough on a glass slide (s); b, b′, coverslips (12 mm×18 mm) used to make the test chamber and the reference chamber in the trough; c, another coverslip (0.4 mm×18 mm) forming the cell chamber in the trough. (B) Procedure used to generate a cAMP concentration gradient in the cell chamber. (i) An amoeba-containing suspension is introduced into the cell chamber. Reference solution (R) and test solution (T) are introduced into the right and left chambers, respectively. (ii) The three chambers are connected by pushing the coverslips for T and R towards (arrows) the cell chamber. See text for details.

Approximately 1 µl of amoeba suspension was introduced into the cell chamber. Most of the amoebae sank, became attached to the bottom surface of the chamber within 2–3 min and were uniformly distributed on the surface. The density of the amoebae in the solution was adjusted to 10^3 to 1.5×10^3 cells mm^{-2} on the bottom surface. The Ca^{2+}-free solution in the cell chamber was then replaced with the reference solution.

As shown in Fig. 1Bi, a coverslip for the reference chamber (R) and another for the test chamber (T) were gently slid over the trough towards the amoeba-containing cell chamber by pushing the edges of both coverslips with the tips of a pair of forceps until they came into contact with the coverslip of the cell chamber (Fig. 1Bii). Thus, the test solution and the reference solution, each under its respective coverslip, were moved towards the cell chamber as the coverslips were slid together, so that the solutions in the three chambers became

continuous. A cAMP concentration gradient was generated in the cell chamber as cAMP diffused into the chamber when the solutions were joined, and the amoebae in the chamber were, therefore, exposed to both a cAMP concentration gradient and an increase in cAMP concentration.

When the reference solution in the cell chamber was replaced with a cAMP-containing test solution prior to joining the chambers, cAMP diffused out of the cell chamber towards the reference chamber, thus generating a cAMP concentration gradient in the cell chamber. The amoebae, in this case, were exposed to a cAMP concentration gradient and, concomitantly, to a decrease in cAMP concentration.

Estimation of cAMP concentration in the cell chamber

To determine the cAMP concentration gradient and its change with time in the cell chamber after a reference solution and a cAMP-containing test solution had been connected, we first simulated the cAMP concentration profile in the assay chambers on the basis of the diffusion constant of cAMP (approximately $10^{-7}\,m\,s^{-1}$) using Mathematica 3.0 (Wolfram Research Inc., Illinois, USA). Fig. 2 shows the simulated relative cAMP concentration in a chamber containing the reference solution (R) and in a chamber containing a cAMP test solution (T) 1, 2 and 4 min after the chambers had been connected as a function of the distance from the border between the two chambers. The concentration gradient of cAMP tends to decrease with time after the two chambers are connected. The overall cAMP concentration increases with time in the R chamber, while it decreases in the T chamber. The cAMP concentration profiles in an area outlined by a broken rectangle at the right of the figure (R portion) correspond to those predicted in the area of the actual cell chamber in which the chemotactic behavior of the amoebae is examined after the reference solution in the cell chamber is connected with a cAMP-containing test solution in the test chamber. The cAMP concentration profiles in a broken rectangle at the left of the figure (T portion) correspond to those in the actual cell chamber after it has been filled with a cAMP-containing solution and then connected with the reference chamber.

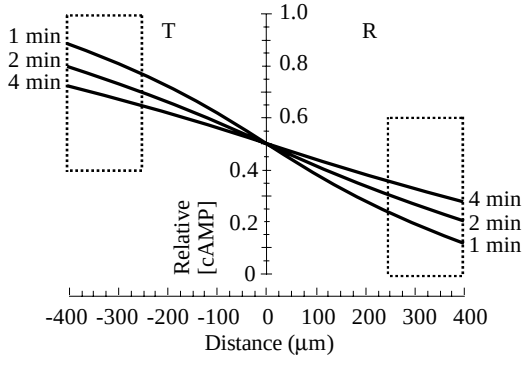

Fig. 2. Computer-simulated temporal and spatial changes in cAMP concentration gradient in two chambers, a chamber containing reference solution, R, and a chamber containing cAMP solution, T. The relative cAMP concentration (with respect to the concentration before the two chambers made contact) was estimated along a line perpendicular to the border between the two chambers 1, 2 and 4 min after the two solutions in these chambers had been connected. The abscissa is the distance from the border (positive, measured towards R; negative, measured towards T). The area in R marked by a dotted rectangle corresponds to Fig. 3A and that in T corresponds to Fig. 3B. See text for further details.

To evaluate the accuracy of computer-estimated cAMP concentration profiles in the cell chamber, a fluorescent analog of cAMP, Mant-cAMP·Na (M_r 484.3), was used instead of cAMP·Na (M_r 351.2). The relative concentration of Mant-cAMP in the actual cell chamber was determined fluorometrically in an area of the cell chamber 240–400 μm away from the border between the reference solution and the Mant-cAMP-containing test solution 1, 2 and 4 min after connecting the solutions. Observations were made at nine successive points along a line perpendicular to the border of the solutions using a fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) and an image analyzer (Luzex III-U, Nireco, Tokyo, Japan).

Fig. 3A shows the Mant-cAMP concentration profiles in this area of the cell chamber when a reference solution was first

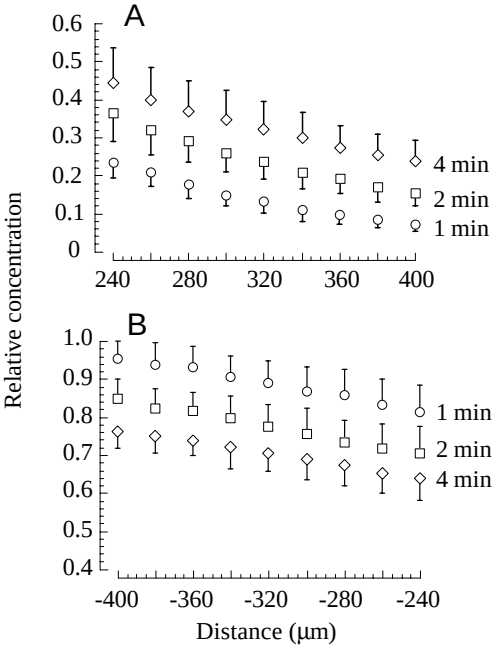

Fig. 3. Fluorometrically determined temporal and spatial changes in the Mant-cAMP concentration gradient in the cell chamber. The relative concentration was determined in an area of the cell chamber 240–400 μm away from the border between the cell chamber and the test chamber or the reference chamber along a line perpendicular to the border 1, 2 and 4 min after the solutions had been connected. The abscissa is the distance from the border (positive, measured towards the reference chamber; negative, measured towards the test chamber). (A) A reference solution was introduced into the cell chamber and then connected with a Mant-cAMP-containing solution in the test chamber. (B) A Mant-cAMP solution was first introduced into the cell chamber then connected with the reference solution in the reference chamber. See text for further details.

introduced into the cell chamber then connected with a Mant-cAMP-containing test solution. These concentration profiles correspond to those shown in the broken rectangle in the R portion of Fig. 2. The measured Mant-cAMP concentration profiles and the simulated cAMP concentration profiles are strikingly similar. The Mant-cAMP concentration gradient changed only slightly in the 4 min after it had been generated in the cell chamber, although the overall Mant-cAMP concentration along the gradient increased with time.

Fig. 3B shows the Mant-cAMP concentration profiles in the area of the cell chamber when a Mant-cAMP-containing test solution was first introduced into the cell chamber and then connected with the reference solution in the reference chamber. These concentration profiles correspond to those shown in the broken rectangle in the T portion of Fig. 2. The Mant-cAMP concentration gradient changed only slightly in the 4 min after it had been generated in the cell chamber, although the overall Mant-cAMP concentration along the gradient decreased with time.

The cAMP concentration gradient is approximately 6.8×10^{-10} mol l^{-1} μm^{-1} at a point in the cell chamber 300 μm away from the border between the reference solution and the 10^{-6} mol l^{-1} cAMP-containing test solution 1 min after connecting the solutions. This approximates to a 1.2×10^{-8} mol l^{-1} difference between the anterior and the posterior ends of an amoeba that is 15 μm long. The rate of increase or decrease in the cAMP concentration at this point in the cell chamber 1 min after connecting the solution approximates to 7.7×10^{-8} mol l^{-1} min^{-1}.

Reversal of the cAMP concentration gradient in the cell chamber

The cAMP concentration gradient generated in the cell chamber could be reversed in less than 30 s in our assay system. As shown in Fig. 4A, a cAMP concentration gradient was generated as described above (see also Fig. 1B). The three coverslips were then gently slid to the right so that amoebae attached to the bottom surface of the cell chamber came to lie under the left edge of the coverslip for the test chamber and the amoebae were, therefore, exposed to the cAMP-containing test solution (Fig. 4B). Coverslips for the reference and the cell chambers were removed together with their respective solutions (Fig. 4C). A coverslip for the extra chamber containing the reference solution (R′), which had been prepared in advance on the left side of the trough, was gently slid towards the test chamber until it made contact with the left edge of the coverslip for the test chamber (Fig. 4D). In this way, the amoebae were exposed to a cAMP concentration gradient in the opposite direction to that to which they had previously been exposed (Fig. 4A).

Recording the motile activity of the amoebae

Images of amoebae on the bottom surface of the assay chambers were magnified using an inverted phase-contrast microscope (TMN, Nikon, Tokyo, Japan) and recorded through a CCD camera (C3077, Hamamatsu Photonics,

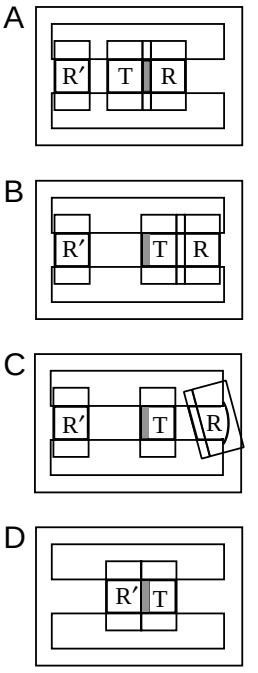

Fig. 4. Procedures for reversing the cAMP concentration gradient in the assay chambers. (A) A cAMP concentration gradient is generated in the cell chamber (thin shaded portion between the test chamber, T, and the reference chamber, R) as shown in Fig. 1B. The second reference chamber (R′) is prepared at the left edge of the trough. (B) The three connected chambers are slid to the right until the amoebae attached to the bottom surface of the cell chamber (shaded area) come to lie under the left edge of T. (C) The reference solution in the cell and reference chambers is pipetted out, and the coverslips for the chambers are then removed from the trough. (D) The second reference chamber, R′, is slid to the right until it comes in contact with T to generate a cAMP concentration gradient in the opposite direction to that generated in A. This procedure was accomplished in 30 s.

Hamamatsu, Japan) on VHS video tape using a video recorder (NV-SF 800, Panasonic, Osaka, Japan). Two objectives of the microscope, 4× and 20×, were alternated in a series of experiments to obtain images at lower magnification to determine the overall distribution of the amoebae in the chambers and at higher magnification to examine the motile activity of individual amoebae.

The assay chambers were kept in a glass-enclosed moist chamber throughout the experiments to prevent evaporation of the solutions in the chambers. All experiments were performed at a room temperature of 20 °C.

Data analysis

Amoebae in an area of the cell chamber 200–400 μm away from the border between the test solution and the reference solution were used for analyses of motile activity. The positions of individual amoebae in the cell chamber after exposure to a cAMP concentration gradient were determined on a monitor screen by displaying images frame by frame. The

direction of movement of each amoeba was defined as the direction in which the major pseudopod pointed.

All the amoebae in the cell chamber are categorized into two groups: (1) ascending amoebae, which move in the direction of a 180° sector facing towards the cAMP-containing test chamber; and (2) descending amoebae, which move in the direction of the 180° sector facing away from the reference chamber. The amoebae were randomly chosen from each group immediately before generating the cAMP concentration gradient, and each individual amoeba was continuously monitored thereafter to determine its direction of movement after the gradient had been generated. When a descending (or ascending) amoeba changed direction so as to move in the ascending (or descending) direction, it was regarded as exhibiting a 'turning' response. The time from the start of generation of the cAMP concentration gradient to the time when each amoeba exhibited turning was determined. The degree of turning for each group of amoebae is expressed as the half-decay time, the time required for half the amoebae that had continued to move in their initial ascending or descending direction to turn. Thus, a shorter half-decay time corresponds to a faster rate of turning. Values are presented as means ± S.D.

Results

Accumulation of the amoebae in the assay chambers

To examine whether some factor other than cAMP causes an unequal distribution of amoebae in the assay system, the reference solution was introduced into both the reference and the test chambers before the distribution of amoebae was examined. As shown in Fig. 5A, the amoebae in the cell chamber migrated into both the reference and the test chambers. The time courses of the increase in the number of amoebae in both chambers are shown in Fig. 6A. No marked difference in the time course was found between the chambers. This implies that the assay system itself does not cause an unequal distribution of amoebae between chambers.

When a solution containing 10^{-6} mol l^{-1} cAMP was introduced into the test chamber, most of the amoebae in the cell chamber migrated into the test chamber (Fig. 5B). The time courses of changes in the number of amoebae in the test and the reference chambers are shown in Fig. 6B. The number in the test chamber increased with time (circles), while that in the reference chamber was maintained at a very low level (squares).

Fig. 5C,D shows the amoebae in the cell chamber at higher magnification, Fig. 5C corresponding to Fig. 5A, while Fig. 5D corresponds to Fig. 5B. The amoebae extended their pseudopods in random directions in the cell chamber before the chamber was connected with the test chamber (time 0). The amoebae extended their pseudopods towards the cAMP-containing test chamber after the cell chamber had been connected with the cAMP-containing test chamber (Fig. 5D, 10 min), while the pseudopod direction remained random when the cell chamber was connected with the test chamber containing reference solution (Fig. 5C, 10 min).

Motile responses of the amoebae in a cAMP concentration gradient with increasing overall cAMP concentration

To examine the chemotactic responses of the amoebae under the influence of a cAMP concentration gradient with increasing overall cAMP concentration, the motile responses of the amoebae were monitored in an area of the cell chamber 240–400 μm away from the border between the reference solution and a cAMP-containing test solution (corresponding to Fig. 3A). The amoebae in the reference solution moved in random directions before exposure to a cAMP concentration gradient. They stopped moving and became rounded for approximately 1 min (54±34 s; N=120) after the cell chamber had been connected with a test chamber containing 10^{-6} mol l^{-1} cAMP solution. This response corresponds to the 'cringing' reported by Futrelle et al. (1982).

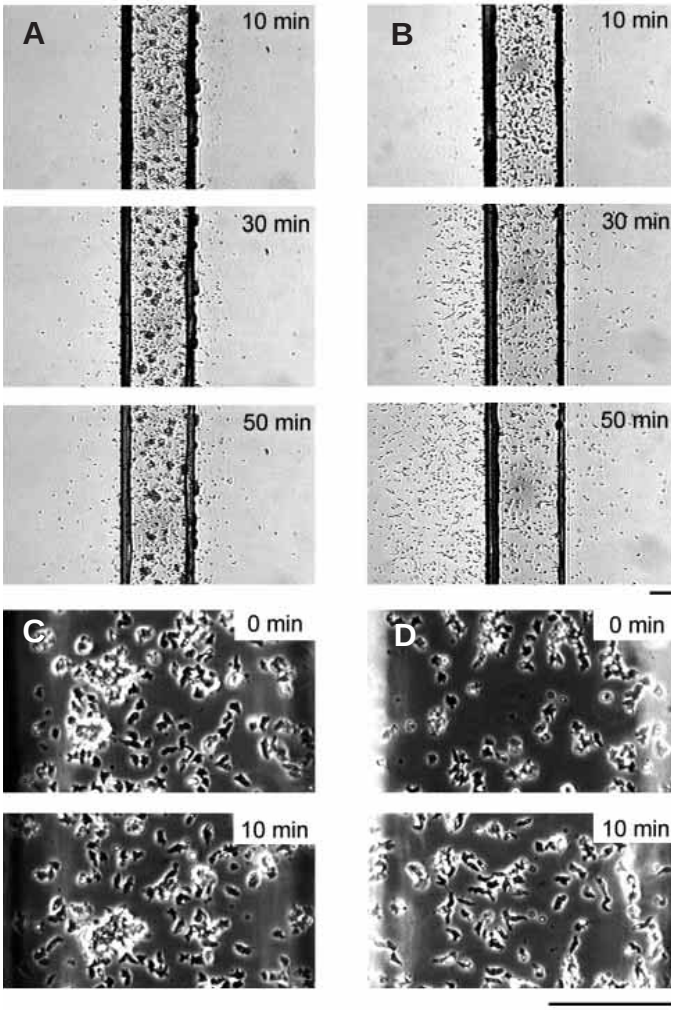

Fig. 5. Photographs showing the distribution of *Dictyostelium discoideum* amoebae in the assay chambers (A,B) and magnified images of the amoebae for examining the locomotion of individual amoebae (C,D). (A,C) A reference solution was introduced into the test chamber, so that no cAMP concentration gradient was generated in the assay chambers. (B,D) A solution containing 10^{-6} mol l^{-1} cAMP was introduced into the test chamber so that a cAMP concentration gradient with increasing overall cAMP concentration was generated in the cell chamber. Scale bars, 100 μm. See text for details.

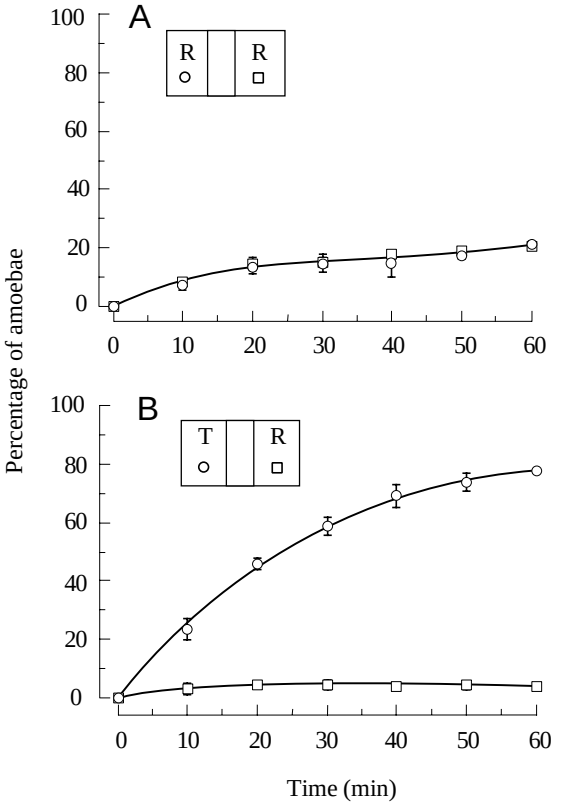

Fig. 6. Changes in the number of *Dictyostelium discoideum* amoebae in both the reference (squares) and the test (circles) chambers after the generation of a cAMP concentration gradient accompanied by an overall increase in cAMP concentration. (A) The reference solution was introduced into the test chamber, so that no cAMP concentration gradient was generated in the chambers. This corresponds to Fig. 5A. (B) A solution containing 10^{-6} mol l^{-1} cAMP was introduced into the test chamber, so that a cAMP concentration gradient was generated in the chambers. This corresponds to Fig. 5B. The number of amoebae is expressed as a percentage of the total number of amoebae examined (N=100) and plotted against the time after the chambers had been connected. Each point is the mean ± S.D. (N=4).

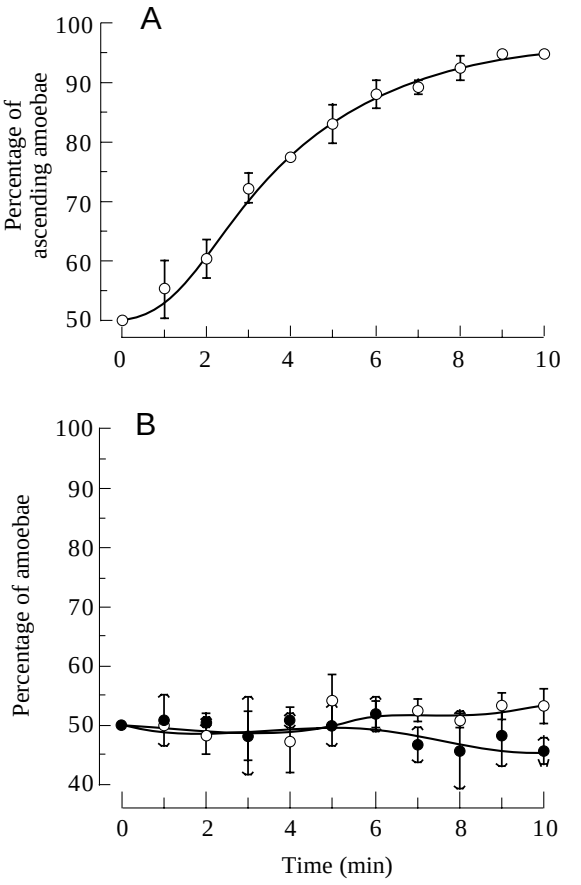

Fig. 7. Time course of the change in the number of *Dictyostelium discoideum* amoebae in the cell chamber that moved towards the test chamber after the contact of solutions. (A) When a cAMP concentration gradient with increasing overall cAMP concentration was generated with a test solution containing 10^{-6} mol l^{-1} cAMP. (B) When no cAMP concentration gradient was generated in the cell chamber. The reference solution was introduced into all three chambers (reference, test and cell chambers) (filled circles) or a solution containing 10^{-6} mol l^{-1} cAMP was introduced into the three chambers (open circles). The number of amoebae is expressed as a percentage of the total number of amoebae examined (N=40) and is plotted against time after the solutions made contact. Each point is the mean ± S.D. (N=4).

When the amoebae resumed locomotion, most (87.5±3.5 %; N=3) moved in the same direction as that in which they had been moving before the cringing response.

The number of ascending amoebae was determined every 1 min after the amoebae in the cell chamber had been exposed to a cAMP concentration gradient. As shown in Fig. 7A, the number began to increase when the amoebae resumed locomotion and reached a plateau in approximately 10 min. The speed of movement of ascending amoebae was 15.0±4.4 μm min^{-1} (N=6) and that of descending amoebae was 12.2±5.0 μm min^{-1} (N=6).

When the reference solution was introduced into the test chamber (both chambers contained the reference solution), the amoebae in the cell chamber moved in random directions as previously mentioned. The number of amoebae moving towards the test chamber remained almost constant at 50 % (Fig. 7B; filled circles). When a test solution containing 10^{-6} mol l^{-1} cAMP was introduced into all three chambers (test, reference and cell chambers), the amoebae, after first showing cringing, moved in random directions as if they were in the reference solution. The number of amoebae moving towards the test chamber again remained almost constant at 50 % (Fig. 7B; open circles).

The turning rate in a cAMP concentration gradient when the overall cAMP concentration is increased

To examine the degree of turning in the amoebae after exposure to a cAMP concentration gradient, the number of amoebae that continued to move in their respective initial ascending or descending directions was determined every 1 min for 10 min. As shown in Fig. 8A, the decrease in the number of amoebae ascending (circles) was markedly slower

than that of amoebae descending (squares) when the test solution contained 10^{-6} mol l^{-1} cAMP. Approximately 80 % (80±3.5 %; *N*=4) of the ascending amoebae continued to ascend even 10 min after exposure to the gradient. The decrease was so slow that the half-decay time could not be determined (more than 30 min). In contrast, only less than 10 % (6.3±2.2 %; *N*=4) of the descending amoebae continued to descend 10 min after exposure to the gradient. The half-decay time was 172±21 s (*N*=4).

When the test solution contained 10^{-8} mol l^{-1} cAMP, so that the cAMP concentration gradient was less steep than in the previous case, the half-decay time for the ascending amoebae (circles) was 306±14 s (*N*=3) and that for the descending amoebae (squares) was 107±41 s (*N*=3) (Fig. 8B). The value for the ascending amoebae was lower than in the previous case when the gradient had been steeper (Fig. 8A; circles), while the value for the descending amoebae was quite similar to that for the descending amoebae in the steeper gradient (Fig. 8A; squares). These observations are consistent with those reported by previous investigators (Varnum and Soll, 1984; Varnum-Finney et al., 1987b; Fisher et al., 1989).

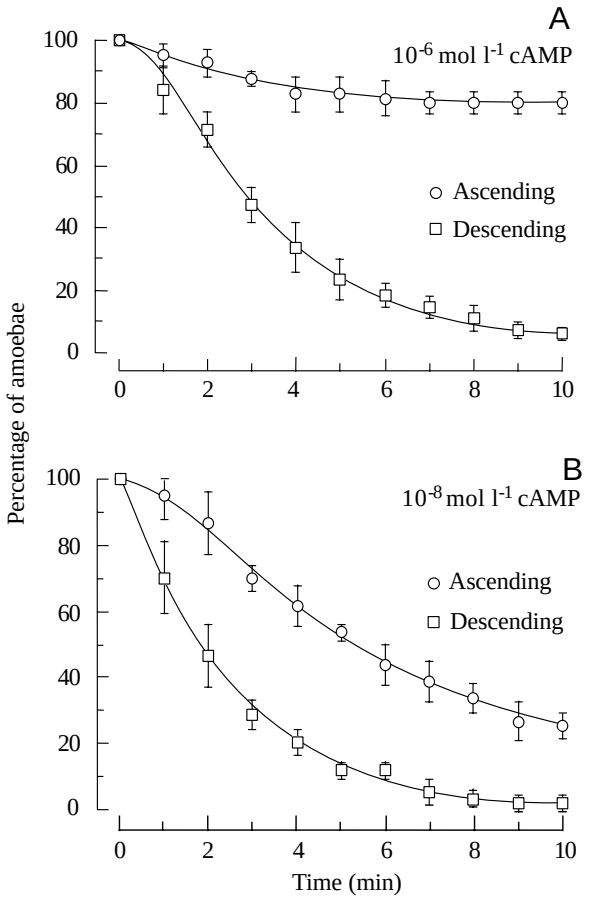

Fig. 8. Responses of *Dictyostelium discoideum* amoebae in a cAMP concentration gradient with an overall increase in cAMP concentration. The time course of the decrease in the number of amoebae that continued to move in the same direction after exposure to the cAMP concentration gradient is shown. Circles, the initial direction of movement was ascending; squares, the initial direction of movement was descending. The number of amoebae is expressed as a percentage of the total number of amoebae examined (*N*=20). (A) A test solution containing 10^{-6} mol l^{-1} cAMP was used to generate a cAMP concentration gradient. (B) A test solution containing 10^{-8} mol l^{-1} cAMP was used to generate a cAMP concentration gradient. Each point is the mean ± S.D. (*N*=4).

The turning rate in the reference solution and in a cAMP-containing test solution in the absence of a cAMP concentration gradient

The half-decay time was determined when a reference solution or a solution containing 10^{-6} mol l^{-1} cAMP was introduced into all three chambers so that no cAMP concentration gradient was generated in the cell chamber. As shown in Fig. 9 (Fig. 9A, reference solution; Fig. 9B, solution containing 10^{-6} mol l^{-1} cAMP), there was no marked difference in the time course of decrease in the number of

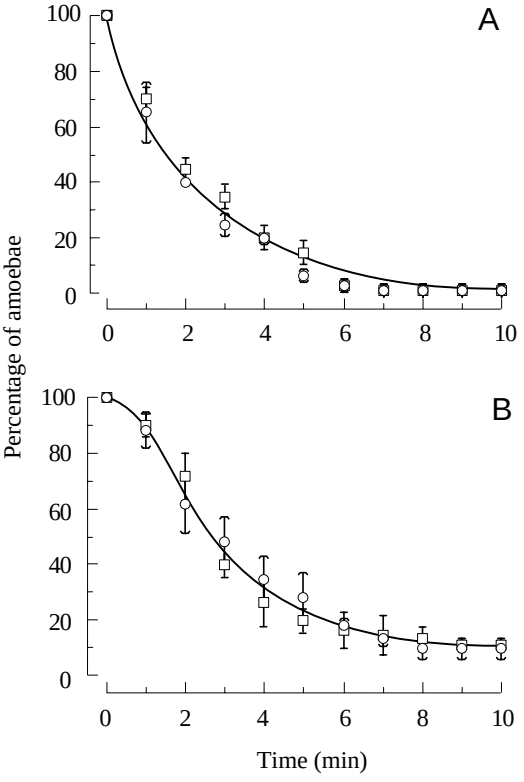

Fig. 9. Responses of *Dictyostelium discoideum* amoebae in the reference and cAMP-containing solution in the absence of a cAMP concentration gradient. The time course of the decrease in the number of amoebae that continued to move in the same direction was determined. Circles, the initial direction was towards the test chamber; squares, the initial direction was towards the reference chamber. The number of amoebae is expressed as a percentage of the total number of amoebae examined (*N*=20). (A) The reference solution was introduced into all three chambers (reference, test and cell chambers). (B) A solution containing 10^{-6} mol l^{-1} cAMP was introduced into all three chambers (reference, test and cell chambers) so that the amoebae in the cell chamber were bathed in this solution without being exposed to a cAMP concentration gradient. Each point is the mean ± S.D. (*N*=3).

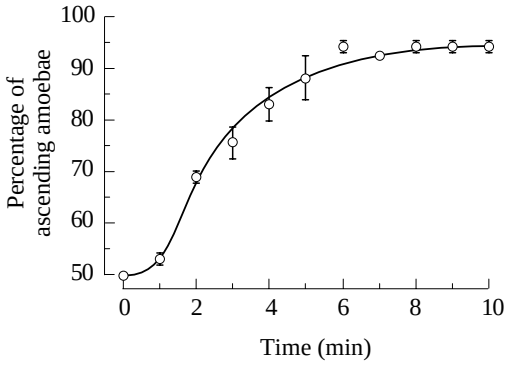

Fig. 10. Motile responses of *Dictyostelium discoideum* amoebae to a cAMP concentration gradient with an overall decrease in cAMP concentration. The time course of the increase in the number of ascending amoebae in the cell chamber after exposure to the cAMP concentration gradient was determined. The number of amoebae is expressed as a percentage of the total number of amoebae examined (N=18). Each point is the mean ± S.D. (N=3).

amoebae between the amoebae moving towards the test chamber (corresponding to ascending amoebae; circles) and those moving towards the reference chamber (corresponding to descending amoebae; squares) with the exception that, in the case of the solution containing 10^{-6} mol l^{-1} cAMP, the amoebae exhibited cringing for approximately 1 min after they had been exposed to the cAMP-containing solution. This can be observed in Fig. 9B as an initial delay in the decrease in the number for approximately 1 min. The half-decay time was 88±17 s (N=6) in the reference solution and 152±50 s (N=6) in the solution containing 10^{-6} mol l^{-1} cAMP. These two values are similar when the time spent cringing (approximately 1 min) is taken into account.

Motile responses of amoeba in a cAMP concentration gradient when the overall cAMP concentration is decreased

To examine the chemotactic responses of amoebae under the influence of a cAMP concentration gradient with decreasing overall cAMP concentration, the reference solution in the cell chamber was first replaced by the test solution containing 10^{-6} mol l^{-1} cAMP. The cell chamber was then connected with the test and reference chambers within 30 s of the first replacement of the solutions in the cell chamber. An examination of the motile responses was carried out within the area corresponding to that in shown Fig. 3B. As shown in Fig. 10, the number of ascending amoebae increased with time after exposure to the cAMP concentration gradient. More than 90 % of the amoebae in the cell chamber were ascending 6 min after they had been exposed to this gradient. The velocity was 14.3±2.6 μm min^{-1} (N=6) for the ascending amoebae and 11.0±3.5 μm min^{-1} (N=6) for the descending amoebae.

The number of amoebae that continued to move in their respective initial ascending or descending directions was also determined for 10 min at 1 min interval after exposure to the cAMP concentration gradient. The number of ascending amoebae decreased so slowly that approximately 80 % (76.7±6.7 %; N=3) were still ascending 10 min after exposure to the gradient (Fig. 11; circles). Although the half-decay time could not be determined, it was assumed to be more than 20 min. In contrast, the number of descending amoebae decreased so fast that less than 5 % of the amoebae continued to descend 10 min after exposure to the gradient (Fig. 11; squares). The half-decay time was 137±14 s (N=3).

Effect of a prior cAMP treatment on the turning rate

To test the effect of prior treatment with cAMP on the turning rate, amoebae attached to the bottom surface of the cell chamber were first exposed to a homogeneous 10^{-6} mol l^{-1} cAMP solution for 1, 2 or 8 min and then to a cAMP concentration gradient with an overall decreased cAMP concentration. As shown in Fig. 12, the number of ascending amoebae in these three different groups was determined every 1 min after exposure to the gradient. The half-decay times were 421±157 s for the 1 min pretreated group, 276±67 s for the 2 min pretreated group and 190±19 s for the 8 min pretreated group (N=3). The half-decay time decreased (turning rate increased) in amoebae treated for longer times with the cAMP solution (analysis of variance, $F_{2,6}$=4.177, P=0.073). No conspicuous chemoaccumulation was observed for the amoebae treated with the cAMP solution for 8 min (data not shown).

Motile responses after reversal of the cAMP concentration gradient

Amoebae in the cell chamber were first exposed to a cAMP concentration gradient for 1 min, and the gradient was then reversed (Fig. 4). The number of ascending amoebae that continued to move in their initial direction was determined every 1 min after the gradient had been reversed. The number did not decrease in the first 2 min after the gradient reversal,

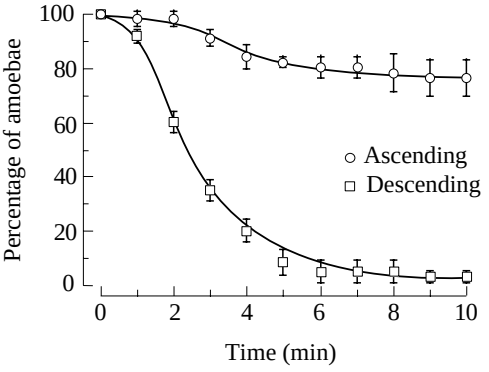

Fig. 11. Responses of *Dictyostelium discoideum* amoebae in a cAMP concentration gradient with an overall decrease in cAMP concentration. The time course of the decrease in the number of amoebae that continued to move in their initial direction of movement after being exposed to the cAMP concentration gradient (circles, ascending amoebae; squares, descending amoebae) was determined. The number of amoebae is expressed as a percentage of the total number of amoebae examined (N=18). Each point is the mean ± S.D. (N=3).

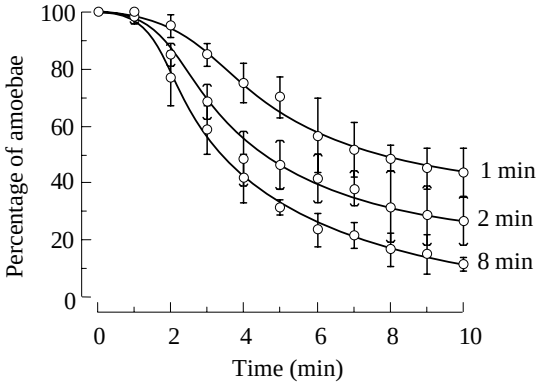

Fig. 12. The effect of pretreatment of *Dictyostelium discoideum* amoebae with a solution containing 10^{-6} mol l^{-1} cAMP on the turning rate in a cAMP concentration gradient with an overall decrease in cAMP concentration. The time course of the decrease in the number of ascending amoebae that continued to move in the same direction was determined after exposure to the concentration gradient. The duration of the pretreatment was 1, 2 or 8 min. The number of amoebae is expressed as a percentage of the total number of amoebae examined (N=20). Each point is the mean ± S.D. (N=3).

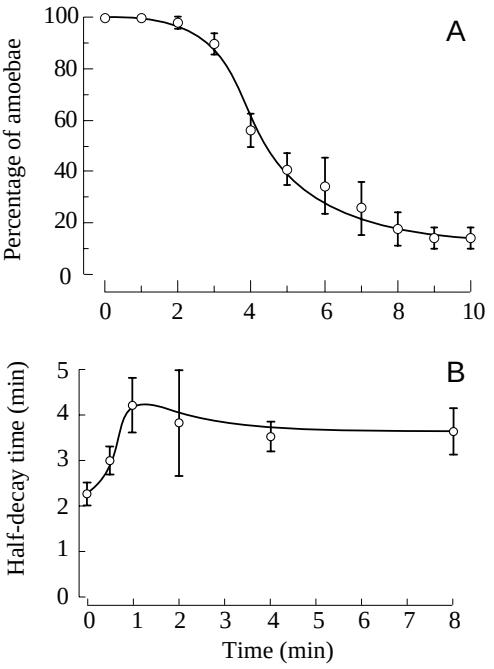

Fig. 13. The effect of a reversal of the cAMP concentration gradient on the turning rate of *Dictyostelium discoideum* amoebae. (A) The time course of the decrease in the number of ascending amoebae that continued to move in the same direction after the cAMP concentration gradient had been reversed was determined. The duration of exposure to the initial concentration gradient was 1 min. The number of amoebae is expressed as a percentage of the total number of amoebae examined (N=20). Each point is the mean ± S.D. (N=3). (B) The effect of the time of exposure of the amoebae to the cAMP concentration gradient on the half-decay time of the number of ascending amoebae that continued to move in the same direction after the cAMP concentration gradient had been reversed. The half-decay time, the time required for half the amoebae that had continued to move in the same initial ascending direction to turn after the gradient had been reversed, is plotted against the time of pre-exposure to cAMP. Each point is the mean ± S.D. (N=3).

but began to decrease rapidly 3 min after the reversal (Fig. 13A). The half-decay time for the decrease was 252±35 s (N=3).

The effect of the period of exposure of the amoebae to the initial cAMP concentration gradient on the half-decay time, the time required for half the ascending amoebae that had kept their initial direction of movement (against the initial gradient) to turn in the reversed gradient, was examined (Fig. 13B). The half-decay time varied depending on the period of exposure to the initial cAMP concentration gradient (analysis of variance, $F_{5,12}$=4.217, P=0.019) and reached its maximum of approximately 4.2±0.6 min at an exposure time of 1 min (Tukey *post-hoc* test for the values at 0 min exposure and 1 min exposure, P=0.012). The half-decay time did not change further when the initial exposure was longer than 1 min (Tukey *post-hoc* test, P>0.05).

Discussion

Motile responses and chemoaccumulation

Amoebae of *D. discoideum* in the cell chamber moved towards a cAMP-containing test chamber and accumulated there (Figs 5B, 6B). The number of ascending amoebae increased with time after exposure to the gradient (Fig. 7A). This ascending movement is the major cause for the accumulation of amoebae in the test chamber. The turning rate was much lower in the ascending amoebae than in the descending ones as indicated by the longer half-decay time for the decrease in the number of amoebae that continued their initial movement in the ascending direction compared with that for the descending ones (Fig. 8A). The lower turning rate of the ascending amoebae is the major cause for the increase in the number of ascending amoebae in the cell chamber (Fig. 7A). These results are identical with those obtained by previous investigators who employed assay systems that differ from ours (Varnum and Soll, 1984; Varnum-Finney et al., 1987b; Fisher et al., 1989).

Which factor is involved in sensing a cAMP source, a temporal gradient or a spatial gradient?

The fact that the ascending amoebae reduce their turning rate implies that the amoebae somehow detect a cAMP concentration gradient around them. In an ascending amoeba, the cAMP concentration is always higher at its anterior end. This spatial difference in the cAMP concentration could be detectable by the amoeba and be a factor in reducing the turning rate. In addition, amoebae are always exposed to an increase in cAMP concentration when they ascend a stationary cAMP concentration gradient. This temporal increase in the cAMP concentration around the amoeba could also be detectable by the amoeba and could therefore be a factor in reducing the turning rate.

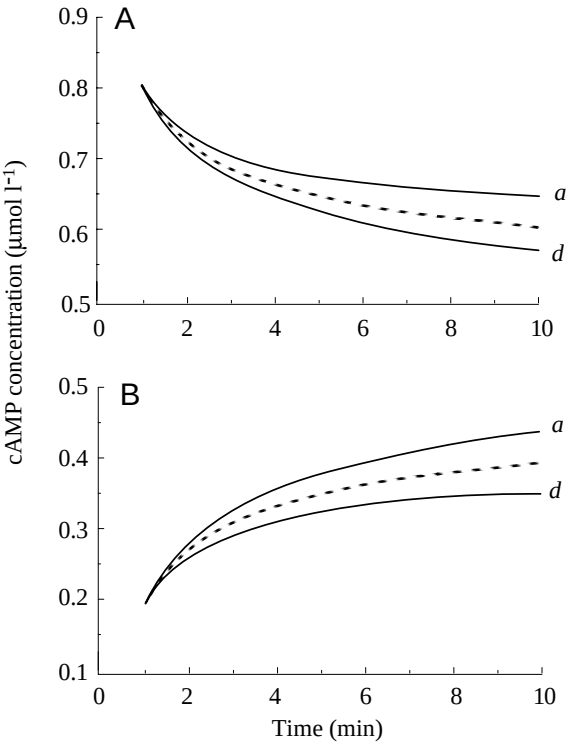

Fig. 14. Computer-simulated temporal changes in cAMP concentration around an amoeba moving along a cAMP concentration gradient in the cell chamber. The amoeba starts from a point in the cell chamber 300 μm away from the border between the reference solution and a test solution. (A) The overall cAMP concentration in the cell chamber decreases with time. (B) The overall cAMP concentration in the cell chamber increases with time. Broken lines, the amoeba remains immotile (corresponding to the change in cAMP concentration at one point). Lines labeled *a* are for amoebae ascending the gradient. Lines labeled *d* are for amoebae descending the gradient. The simulation was performed on the basis of the following variables: concentration of cAMP in the test solution, 10^{-6} mol l^{-1}; diffusion constant of cAMP, 10^{-7} m s^{-1}; velocity of the amoeba, 14.3 μm min^{-1} for A*a*, 11.0 μm min^{-1} for A*d*, 15.0 μm min^{-1} for B*a* and 12.2 μm min^{-1} for B*d*. See text for further details.

To provide an answer to the question of which of these two factors is involved in cAMP-sensing by the amoebae, we compared the rate of turning between two groups of ascending amoebae, one exposed to a temporal increase in the overall cAMP concentration and the other to a temporal decrease in the overall cAMP concentration. It was found that the rate of turning was essentially identical for these two groups (compare Fig. 8A with Fig. 11) and that there was no significant difference in the half-decay time of the number of amoebae that continued their initial descending movement (*t*-test, t=2.498, d.f.=5, P=0.055). The time course of the increase in the number of ascending amoebae in the cell chamber was also very similar between these two groups (compare Fig. 7A with Fig. 10). These observations strongly support the idea that *D. discoideum* amoebae sense the cAMP concentration gradient around them independently of any temporal change in the surrounding cAMP concentration and this leads them to reduce their turning rate. This, in turn, causes their ascending movement against the cAMP concentration gradient.

We should consider here the possibility that a temporal change in cAMP concentration around an amoeba caused by its movement diminishes or even overcomes a temporal change in cAMP concentration caused by diffusion. To do this, we calculated the time course of the change in cAMP concentration around an ascending amoeba after the generation of a cAMP concentration gradient with decreasing overall concentration in the cell chamber based on a diffusion constant of 10^{-7} m s^{-1} for cAMP and on a mean velocity of 14.3 μm min^{-1} for the movement of the amoeba. As clearly shown by the line labeled *a* in Fig. 14A, the temporal change in cAMP concentration around the ascending amoeba, which corresponds to the slope of the cAMP concentration/time plot, is always negative (decreasing) in spite of the ascending movement of the amoeba, although the slope was always slightly less steep than that for an amoeba that did not move (shown as a dotted line in Fig. 14A).

The temporal change in cAMP concentration around a descending amoeba was also estimated as shown in Fig. 14A (line labeled *d*). The slope of the plot was, of course, negative and always steeper than that for a non-motile amoeba, since the descending movement itself caused a temporal decrease in cAMP concentration.

Similarly, temporal changes in cAMP concentration around an ascending and a descending amoeba after they had been exposed to a cAMP concentration gradient with increasing overall cAMP concentration were estimated. The slope of the cAMP concentration/time plot was always positive (increasing) in both ascending (Fig. 14B, line *a*) and descending (Fig. 14B, line *d*) amoebae, although the slope was steeper in the ascending amoeba and less steep in the descending amoeba than in a non-motile one (Fig. 14B; dotted line). It may, therefore, be said that all the amoebae examined in the case of Fig. 8A are exposed to a temporal increase in cAMP concentration irrespective of their direction of movement. Thus, the temporal change of cAMP concentration experienced by the amoebae has little effect on the mechanism that detects the direction of the cAMP source.

If an amoeba that is ascending a cAMP concentration gradient with decreasing overall cAMP concentration can extend its leading pseudopod (in which a hypothetical cAMP-sensing mechanism is present) fast enough to overcome the temporal decrease in cAMP concentration around it, it is possible that the amoeba can sense a temporal increase in the cAMP concentration around it and use this to establish chemotactic behavior. If this is the case, the speed of pseudopod extension should exceed 100 μm min^{-1} (8–10 times as fast as the mean velocity of the amoeba), from estimates based on the speed of movement of the amoeba and the rate of temporal change in the cAMP concentration (see Fig. 14). Precise examination of pseudopod extension in our assay system is needed to evaluate this possibility.

Fisher et al. (1989) and Vicker (1994) examined the chemotaxis of *D. discoideum* amoebae in an assay system in which the cAMP concentration gradient was held constant

while the overall cAMP concentration was increased or decreased. They found that the amoebae did not show chemotactic behavior when the overall cAMP concentration was decreased during the time of exposure to a cAMP concentration gradient. They therefore concluded that a temporal increase in the cAMP concentration was more important in evoking cAMP-mediated chemoaccumulation of amoebae than a spatial difference in the cAMP concentration.

Van Haastert (1983) examined chemoaccumulation of *D. discoideum* amoebae under the influence of a cAMP concentration gradient with a temporal decrease in the cAMP concentration, which was generated using phosphodiesterase. He found that chemoaccumulation of the amoebae was not observed under these conditions and suggested that adaptation of the amoebae to cAMP before exposure to a cAMP concentration gradient could be a cause of the disappearance of chemoaccumulation. In the present paper, we have demonstrated that inhibition of the turning of ascending amoebae was reduced when amoebae were treated with a homogeneous cAMP solution prior to exposure to a cAMP concentration gradient (Fig. 12). This implies that the amoebae lose their sensitivity to the cAMP concentration gradient with decreasing overall cAMP concentration during pretreatment with cAMP. Amoebae in the assay systems of Fisher et al. (1989) or Vicker (1994) were exposed to a cAMP solution for approximately 30 min before exposure to a cAMP gradient with decreasing overall cAMP concentration. The failure of these studies to demonstrate chemotaxis could, therefore, be due to desensitization of the amoebae. Further examination of the cAMP desensitization process is needed to understand its mechanism and physiological significance.

Korohoda et al. (1997) clearly demonstrated chemotactic locomotion of *Amoeba proteus* along an H^+ gradient generated in their pocket-like assay chamber. The amoebae migrated towards the solution with a higher H^+ concentration in the pH range 5.75–7.75, irrespective of the temporal change in H^+ concentration around the cell. Our present findings on the chemotactic migration of *D. discoideum* towards cAMP are similar to these observations on *Amoeba proteus* moving in an H^+ gradient.

Varnum et al. (1985) and Varnum-Finney et al. (1987a) examined the locomotory behavior of individual amoebae of *D. discoideum* under the influence of a temporal increase or decrease in the cAMP concentration in the absence of a spatial cAMP concentration gradient. Contrary to our observations, they found that the turning rate of the amoebae was inhibited by a temporal increase in the cAMP concentration. The rate of temporal increase in the cAMP concentration in our assay system was essentially identical to that of their assay system (approximately 10^{-7} mol l^{-1} min^{-1}). At present, we do not know the cause for the discrepancy between these two sets of results.

Is the turning rate increased in the descending amoebae?

As mentioned above, turning rate is reduced when the amoebae ascend against a cAMP concentration gradient. It was also interesting to examine whether the turning rate was enhanced when the amoebae descended the gradient. The half-decay time of the descending amoebae under the influence of a temporal increase in cAMP concentration (172±21 s, N=4; Fig. 8A) was the same as that of amoebae after immersion in a homogeneous 10^{-6} mol l^{-1} cAMP solution (152±50 s, N=6; Fig. 9B) (t-test, t=0.853, d.f.=8, P=0.418). This implies that neither a descending orientation of the amoeba nor a temporal change in cAMP concentration around the amoeba affects the rate of turning of the amoeba after the cessation of its initial cringing response to an abrupt increase in cAMP concentration.

Moreover, the half-decay time of the amoebae was approximately 90 s (88±17 s; N=6) in the solution lacking cAMP, while it was approximately 150 s in a homogeneous 10^{-6} mol l^{-1} cAMP-containing solution (Fig. 9). The amoebae showed cringing for approximately 60 s (55±34 s, N=120) after they had been exposed to the cAMP. The half-decay time of the amoebae in the cAMP-containing solution includes this 60 s cringing time. The half-decay time of the amoebae that resumed locomotion after cringing in the cAMP-containing solution can be estimated to be approximately 90 s (the value obtained by subtracting the cringing time of 60 s from the half-decay time of 150 s). This value approximates the half-decay time of the amoebae in the reference solution. On the basis of these estimates of the half-decay time of the amoebae under various conditions, we conclude that the turning rate of the descending amoebae is the same as that of the amoebae in the reference solution, i.e. that no increase in turning rate is observed in the descending amoebae. In other words, the turning rate of *D. discoideum* amoebae is modified (reduced) only when the amoebae ascend against a cAMP concentration gradient.

Effect of a simulated cAMP wave on the ascending amoebae

Our novel technique of reversing the cAMP concentration gradient in the assay chambers made it possible to mimic the cAMP concentration change experienced by an amoeba when a cAMP wave originating from the accumulation center passes over the amoeba. We found that amoebae that had ascended a cAMP concentration gradient for 1 min continued to move in the same direction for a few minutes even after the gradient had been reversed (Fig. 13A). In other words, the lower rate of turning caused by exposure of the amoebae to the initial cAMP concentration gradient remained unchanged for 1–2 min after the gradient had been reversed. Our finding supports the idea that a change in the direction of the gradient around the amoeba during the passage of a cAMP wave does not negate migration of the amoeba towards the accumulation center (Tomchik and Devreotes, 1981; Devreotes et al., 1983; Wessels et al., 1992).

Differences in pseudopod formation after exposure to a spatial chemical gradient between Dictyostelium discoideum *amoebae and leukocytes*

We found that the direction of movement of a *D. discoideum* amoeba after the cringing response to cAMP had subsided was identical to that before exposure to a cAMP concentration gradient. That is, the direction in which a *D. discoideum* amoeba

extends a pseudopod was not altered by exposure to a cAMP concentration gradient during cringing. In contrast, horse leukocytes extended a pseudopod towards the source of γ-globulin when they resumed locomotion after the cringing response to γ-globulin had subsided (Zigmond, 1974). The question of whether this difference reflects diverse mechanisms of cell polarization between these two types of cell or results from the need for a longer latency for establishing cell polarity in *D. discoideum* than in horse leukocytes remains unanswered.

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