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Analogs of Cyclic AMP as Chemoattractants and Inhibitors of *Dictyostelium* Chemotaxis

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Aggregative amoebae of *Dictyostelium discoideum*, *D. mucoroides*, *D. purpureum*, and *D. rosarium* react chemotactically to cyclic AMP (cAMP). We measured the chemotactic activity of 14 cAMP analogs and found that these four species have a similar sensitivity to chemical modifications of cAMP; this suggests that the cAMP receptor is identical in all of these species. Besides the induction of a chemotactic response, cAMP analogs also may delay or prevent cell aggregation. cAMP analogs like N^1-*O*-cAMP, 2'-H-cAMP, and 5'-NH-cAMP are chemotactically nearly as active as cAMP and induced no, or only a short, delay of cell aggregation. Other cAMP derivatives, such as 6-Cl-cPMP and 8-Br-cAMP, are chemotactically active only at high concentrations and delayed cell aggregation for several hours. Still other cAMP analogs, which do not induce a chemotactic reaction in *D. mucoroides*, *D. purpureum*, and *D. rosarium*, either prevented cell aggregation [cAMPS(S), cAMPS(R), and 3'-NH-cAMP] or had no effect on cell aggregation [cAMPN$(CH_3)_2$(S) and cAMPN$(CH_3)_2$(R)]. cAMP analog 3'-NH-cAMP prevented cell aggregation by the inhibition of chemotaxis, whereas cell locomotion was not affected. Although we cannot provide a satisfactory explanation for these observations, our data suggest that occupation and activation of the cAMP receptors do not always induce a chemotactic response.

Chemotaxis mediates food seeking and cell aggregation of amoebae of the cellular slime molds. The individual cells find their food source by a chemotactic reaction to molecules which are excreted by bacteria. One of these molecules for food sensing is folic acid (19). After the bacterial supply is exhausted, cells pass an interphase, followed by cell aggregation and cell differentiation, ultimately resulting in a fruiting body consisting of stalk cells and spores. Cell aggregation is mediated by a chemotactic reaction to molecules excreted by some cells (2). These chemotactic molecules are called acrasins (2). The only acrasin which has been identified is cAMP (11); it is chemotactically active during the aggregative phase of *D. discoideum*, *D. mucoroides*, *D. purpureum*, and *D. rosarium* (9). These four species have in common that cells form streams, aggregate in a pulsatile manner, and differentiate into large unbranched fruiting bodies.

Chemotaxis is a complex sequence of reactions, consisting of detection and localization of the chemoattractant, transduction of the chemotactic signal across the cell membrane, and processing of the signal followed by pseudopod formation. During the detection of the signal, the chemoattractant binds to cell-surface receptors (13). Binding proteins for cAMP have been shown on the cell surface of *D. discoideum* (5, 6, 13, 15), *D. mucoroides* (15), and *D. rosarium* (15) and for folic acid on *D. discoideum* (23). The second step in a chemotactic reaction should be activation of these chemoreceptors and transduction of the signal to other structures. Recently we obtained evidence that an occupied receptor is not necessarily an active receptor (22). The activity might not be proportional to the fraction of receptors occupied but to the frequency of occupation (22). This could explain why in postvegetative *D. discoideum* cells chemotactically less active analogs induce a higher cGMP accumulation and a higher phosphodiesterase activity than cAMP itself (22).

To obtain more information on the activation mechanism of the cAMP receptor, we measured the chemotactic activity of several cAMP analogs in *D. mucoroides*, *D. purpureum*, and *D. rosarium*. Comparison of their chemotactic activity with the activity in *D. discoideum* cells (14) suggests that the cAMP receptor involved in chemotaxis is identical in these four species. We observed that several cAMP derivatives could act as inhibitors of chemotaxis; they were not

only chemotactically inactive but also prevented a chemotactic reaction to cAMP.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this report are: cAMP, adenosine 3',5'-phosphate; N^1-*O*-cAMP, adenosine N^1-oxide 3',5'-phosphate; 6-Cl-cPMP, 6-chloropurine riboside 3',5'-phosphate; 7-CH-cAMP, 7-deaza-adenosine 3',5'-phosphate; 8-Br-cAMP, 8-bromo-adenosine 3',5'-phosphate; 2'-H-cAMP, 2'-deoxyadenosine 3',5'-phosphate; 2'-THP-cAMP, 2'-deoxy-2'-tetrahydropyranosyl-adenosine 3',5'-phosphate; 3'-NH-cAMP, 3'-deoxy-3'-amino-adenosine 3',5'-phosphate; 5'-NH-cAMP, 5'-deoxy-5'-amino-adenosine 3',5'-phosphate; cAMPS(S), adenosine 3',5'-phosphorothioate, Sp-stereoisomer; cAMPS(R), adenosine 3',5'-phosphorothioate, Rp-stereoisomer; cAMPN$(CH_3)_2$(S), adenosine 3',5'-phosphodimethylamino-amidate, Sp-stereoisomer; cAMPN$(CH_3)_2$(R), adenosine 3',5'-phosphodimethylamino-amidate, Rp-stereoisomer; 5'-AMP, adenosine 5'-phosphate.

Chemicals. cAMP, 5'-AMP, adenosine, 6-Cl-cPMP, 8-Br-cAMP, and 2'-H-cAMP were purchased from Boehringer-Mannheim Corp., New York, N.Y.; 2'-THP-cAMP was a generous gift of Dr. Muehlegger, Boehringer-Mannheim. 7-CH-cAMP was kindly supplied by R. Hanze, The Upjohn Co., Kalamazoo, Mich. cAMPS(S) and -(R) (1) and cAMPN$(CH_3)_2$(S) and -(R) (4) were generous gifts of J. Baraniak and W. J. Stec. The synthesis of N^1-*O*-cAMP, 3'-NH-cAMP, and 5'-NH-cAMP has been described previously (7, 17, 18).

Organisms. *D. mucoroides* (strain WS320), *D. purpureum* (strain 6), and *D. rosarium* were kindly provided by K. B. Raper. Cells were grown in association with *Escherichia coli* B/r on 0.1% lactose-peptone agar for 40 h, harvested by rinsing the petri dishes with a 1% standard salt solution (2), and freed of bacteria by repeated centrifugation at 100 × *g* for 3 min. Cells were resuspended in the same solution at a density of 5 × 10^6 cells per ml.

Chemotactic assay. Chemotaxis was tested with the small population assay (8). About 200 small drops (0.1 μl) of the cell suspension were placed on a hydrophobic surface in a petri dish (diameter, 9.4 cm) containing 20 ml of 0.7% washed agar in standard salt solution. After deposition, the drops had a diameter of approximately 0.7 mm, and the cells did not pass the margins of the drops because of the hydrophobicity of the agar (10). The petri dishes were incubated at room temperature in the light. Cell aggregation started about 2 to 3 h after deposition of the small populations. The chemotactic activity of the test solutions (10^{-3} to 10^{-8} M) was determined by placing small drops (0.1 μl) two times at 5-min intervals close to the small amoebal populations. The chemotactic activity of 18 populations was scored for each cyclic nucleotide concentration. The distribution of cells and their development were monitored with intervals of 10 or more min. A chemotactic reaction was considered positive when the half of the amoebal drop closest to the test solution contained at least twice as many cells as the opposite half. The threshold concentration for chemotaxis is given by two concentrations. At the lower concentration less than 50% of the populations scored positive, and more than 50% scored positive at the higher concentration. The threshold concentrations for cAMP in *D. mucoroides*, *D. purpureum*, and *D. rosarium* varied from 10^{-5} to 10^{-6} M to 10^{-7} to 10^{-8} M with a mean of 10^{-6} to 10^{-7} M. The threshold concentrations of the cAMP derivatives were divided by the threshold concentration of cAMP, which was determined in the same petri dish. In this way the effect of variation in sensitivity and condition of cells should be eliminated. Experiments were carried out at least four times and gave similar relative values.

RESULTS

The structures of the tested cAMP analogs are shown in Fig. 1. cAMP contains several polar atom groups, which can be involved in hydrogen bonding with the surrounding medium (e.g., H_2O or the cAMP receptor), either as hydrogen bond acceptors (N^1, N^3, N^7, $O^{3'}$, $O^{4'}$, $O^{5'}$) or as hydrogen bond donators (N^6N_2, $OH^{2'}$). These hydrogen bonds with the receptor cannot take place at the modified atoms of N^1-*O*-cAMP, 6-Cl-cPMP, 7-CH-cAMP, 2'-H-cAMP, 3'-NH-cAMP, and 5'-NH-cAMP. The *syn-anti* equilibrium, which is 1:1 in cAMP (24), is changed to the *syn* conformation in 8-Br-cAMP (21). The negative charge is fixed in cAMPS(S) and -(R), whereas the electron negativity of sulfur is smaller than that of oxygen. The double-bonded oxygen is fixed in cAMPN$(CH_3)_2$(S) and -(R), whereas the negative charge is removed.

Response of small populations of *D. mucoroides*, *D. purpureum*, and *D. rosarium* to cAMP analogs. cAMP did not induce a positive response in *D. mucoroides*, *D. purpureum*, and *D. rosarium* at a concentration of 10^{-8} M. Cell aggregation started within 15 min after deposition of the cyclic nucleotide. Less than 50% of the populations reacted chemotactically positively to 10^{-7} M cAMP, and more than 50% of the populations reacted chemotactically positively to 10^{-6} M cAMP. The positive reaction was most pronounced at 10 to 20 min after deposition of the cAMP. After about 30 to 40 min, the positive reaction was not visible any longer, and cells were mostly aggregating. Cells reacted to 10^{-5} M cAMP by pressing themselves to the edge closest to the cAMP drop. The reaction was optimal after about 30 min. Sometimes this positive reaction was followed by a radial outward movement by which the cells became situated at the border of the population, whereas the center was relatively empty. Cell aggregation started about 40 min after deposition of the higher cAMP concentration, while the distribution of cells was still unequal. At 10^{-4} M cAMP, cells first reacted positively and after 30 to 40 min moved radially outward. While cells were still distributed along the edge, cell aggregation started about 60 min after deposition of

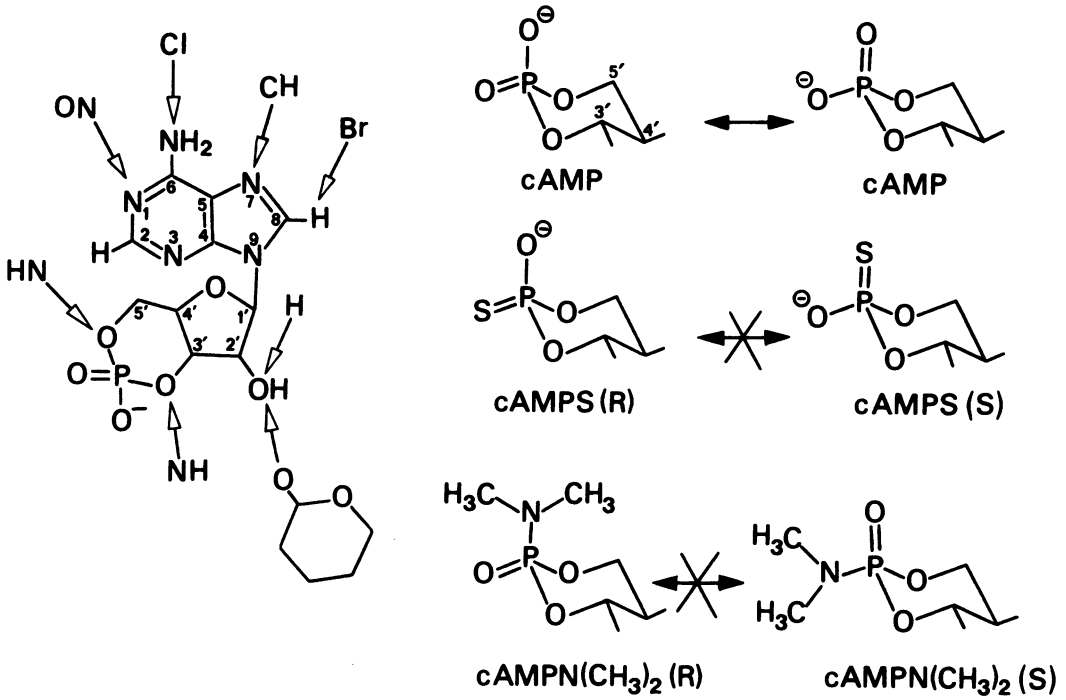

FIG. 1. Structure and conformation of the cAMP derivatives.

cAMP. With 10^{-3} M cAMP, the positive reaction was visible for only a short period, directly followed by a radial response. Aggregation started within 2 h, while cells were still located at the edge. cAMP derivatives N^1-*O*-cAMP, 2'-H-cAMP, and 5'-NH-cAMP induced the same type and pace of reactions as cAMP, except that a 10-fold higher concentration was required. The derivatives 2'-THP-cAMP and 7-CH-cAMP induced a positive chemotactic response at 10^{-4} to 10^{-5} M. An outward response always occurred at a concentration of 10^{-3} M, and often at 10^{-4} M. After the positive and radially outward response disappeared, cell aggregation did not always occur immediately; the delay varied from 0 to 1 h. The derivatives 6-Cl-cPMP and 8-Br-cAMP induced a positive reaction at a concentration of 10^{-4} M; 10^{-3} M first evoked a positive reaction, followed by a peripheral distribution of cells. Within 2 h after deposition of 10^{-3} M test solutions, the distribution of cells was homogeneous, but cells failed to aggregate for some hours. The derivatives 3'-NH-cAMP, cAMPS(S), and cAMPS(R) did not disturb the homogeneous distribution of cells, not even at 10^{-3} M, but cell aggregation was prevented. The derivatives cAMPN$(CH_3)_2$(S) and -(R) and 5'-AMP and adenosine had no effect, either on cell distribution or on the time of cell aggregation. The reactions of *D. purpureum* cells to cAMP (10^{-4} M and 10^{-6} M), 8-Br-cAMP (10^{-3} M), 3'-NH-cAMP (10^{-3} M), and 5'-AMP (10^{-3} M) are shown in Fig. 2.

Cyclic nucleotide specificity for chemotaxis and delay of cell aggregation. The threshold concentrations for a chemotactic reaction to cAMP and cAMP derivatives are shown in Table 1. The effect of chemical modification of cAMP on the increase of threshold concentration was about the same in all four of these species, which suggests that the same structures are involved in the detection of cAMP. Only cAMPS(S) seemed to be an exception. This compound was chemotactically active in *D. discoideum* at 100 times higher concentration than cyclic AMP, whereas in the other species at least 10,000 times higher concentrations were necessary.

Since a compound which induces a chemotactic reaction in aggregative cells also induces a delay of cell aggregation, we introduce the term specific delay of cell aggregation for the time interval between the disappearance of the non-homogeneous distribution and the beginning of cell aggregation. A specific delay of cell aggregation of more than 30 min was evoked in *D. mucoroides*, *D. purpureum*, and *D. rosarium* by the same cyclic nucleotides: 10^{-3} M 6-Cl-cAMP, 7-CH-cAMP, 8-Br-cAMP, 3'-NH-cAMP, and cAMPS(R); and 10^{-3}, 10^{-4}, and 10^{-5} M cAMPS(S). Table 2 shows that only weak chemoattractants were inhibitors of cell aggregation. The strongest inhibitors, 3'-NH-

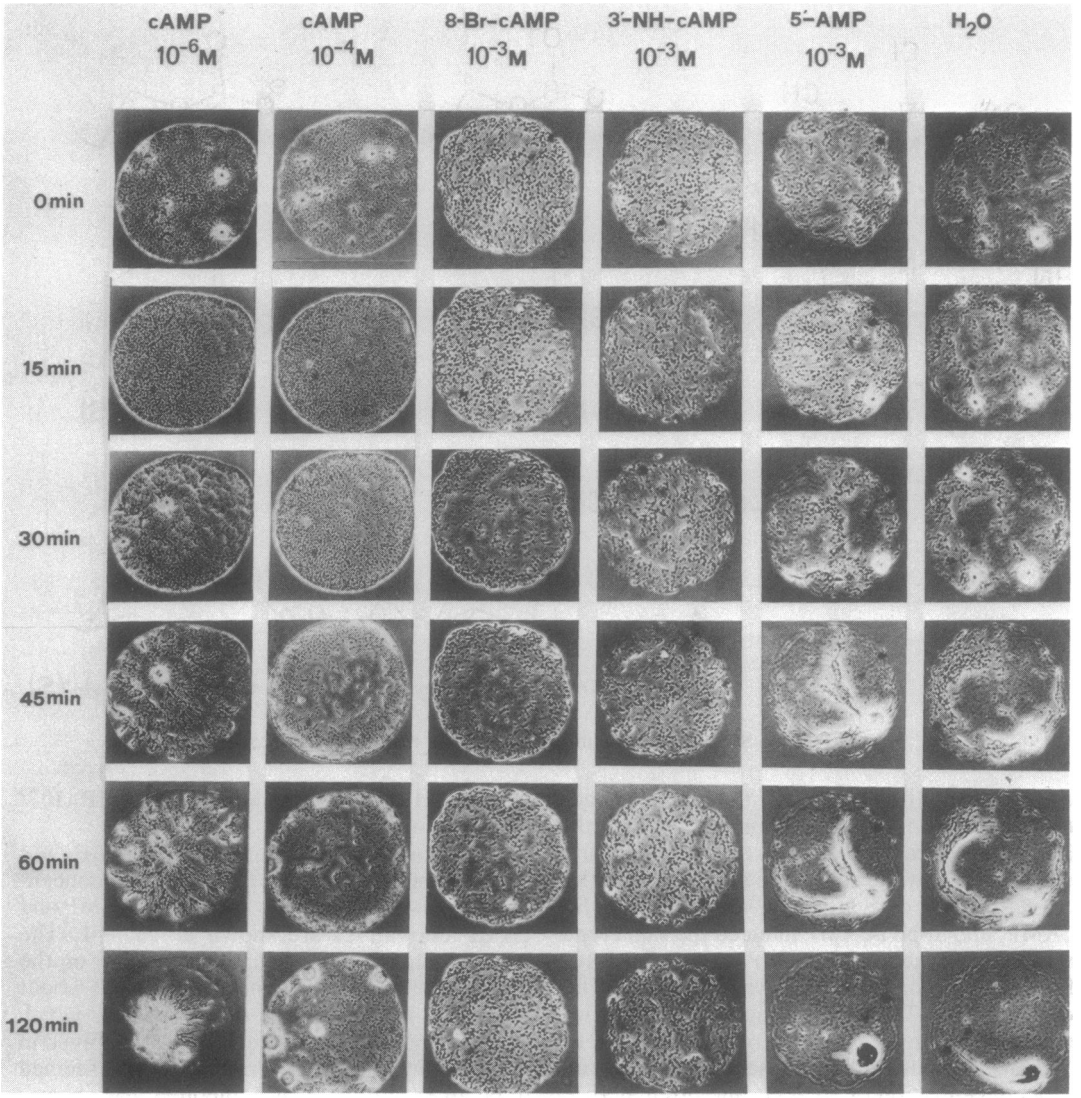

FIG. 2. cAMP derivatives may induce a positive chemotactic reaction, a radial reaction, and a delay of cell aggregation in aggregative *D. purpureum* cells. Small drops of different nucleotides were deposited twice at 5-min intervals at the left side of small populations of aggregative *D. purpureum* cells. Pictures were taken at different times after the deposition of the last droplet.

cAMP, cAMPS(S), and cAMPS(R), blocked cell aggregation for at least 20 h and were chemotactically inactive.

Reasons for specific delay of cell aggregation. cAMP derivatives may interfere with cell aggregation because of (i) inhibition of the secretion of cAMP by the cells, (ii) desensitization of the cells to cAMP, (iii) diminishment of cell adhesion, or (iv) impairment of cell movement. Table 3 shows that 10^{-3} M 3'-NH-cAMP desensitized cells to cAMP. 3'-NH-cAMP prevented the appearance of aggregation centers (experiment D) and when aggregation centers were present, they disappeared after the addition of 3'-NH-cAMP (experiment E). The addition of 3'-NH-cAMP antagonized a positive reaction of the cells to cAMP when cAMP was placed earlier (experiment F), and prevented a positive reaction to cAMP when placed simultaneously on the agar (experiment G). Time-lapse films of a small population of *D. purpureum* were recorded from the early interphase to investigate the effect of 3'-NH-cAMP on cell aggregation and cell locomotion. One drop (0.1 μl) containing 10^{-3} M 3'-NH-cAMP was placed close to a small population at the moment that about 50%

TABLE 1. Threshold concentrations for a positive chemotactic reaction

Compound	Relative threshold concn[a] for:			
	D. discoideum[b]	*D. mucoroides*	*D. purpureum*	*D. rosarium*
cAMP	1	1	1	1
N^1-*O*-cAMP	10	10–100	10–100	10–100
6-Cl-cPMP	1,000	100–1,000	1,000	1,000
7-CH-cAMP	100–1,000	100	100	100
8-Br-cAMP	1,000	100–1,000	100–1,000	1,000
2′-H-cAMP	10	10	10	10
2′-THP-cAMP	100–1,000	100	100	100
3′-NH-cAMP	10,000	≥10,000	≥10,000	≥10,000
5′-NH-cAMP	1–10	10	10	10
cAMPS(S)	100	≥10,000	≥10,000	≥10,000
cAMPS(R)	10,000	≥10,000	≥10,000	≥10,000
cAMPN$(CH_3)_2$(S)	10,000	≥10,000	≥10,000	≥10,000
cAMPN$(CH_3)_2$(R)	10,000	≥10,000	≥10,000	≥10,000
5′-AMP	≥1,000,000	≥10,000	≥10,000	≥10,000
Adenosine	≥1,000,000	≥10,000	≥10,000	≥10,000
Absolute threshold concn of cAMP (M)	10^{-8}–10^{-9}	10^{-6}–10^{-7}	10^{-6}–10^{-7}	10^{-6}–10^{-7}

[a] The threshold concentrations of the cAMP analogs were divided by the threshold concentration of cAMP.
[b] Relative threshold concentrations were, with slight modifications, partly derived from reference 14.

of the cells were taken up in the aggregation center. The addition of 3′-NH-cAMP caused almost immediate cessation of the pulsatile cell aggregation. The aggregation center dissolved, and amoebae moved at random as during the interphase. 3′-NH-cAMP did not induce positive chemotaxis, and although the amoebae seemed to slow down for a short period, there was no drastic change in the rate of cell locomotion. After 20 h cells still moved at random, although at a reduced speed. Aggregative *D. discoideum* cells reacted chemotactically to cAMP with a threshold concentration of 10^{-8} to 10^{-9} M (8, 14); 3′-NH-cAMP was chemotactically active at high concentrations in these aggregative cells (14). Postvegetative *D. discoideum* cells (cells starved for 3 h) reacted positively to cAMP with a threshold concentration of 10^{-6} to 10^{-7} M (3). In these postvegetative cells 3′-NH-cAMP was inactive and inhibited a chemotactic reaction to cAMP (data not shown). Thus, depending on the sensitivity of the cells, 3′-NH-cAMP can act both as an effector and as an inhibitor of chemotaxis in *D. discoideum*.

DISCUSSION

Cyclic nucleotides induced three types of reactions in the small population assay for chemotaxis in *Dictyostclium*: (i) attraction of the amoebae (positive chemotaxis), (ii) a radially outward movement of the amoebae (Fig. 2, 10^{-4} M

TABLE 2. Relation between positive chemotaxis, radial reaction, and delay of aggregation by cAMP derivatives in *Dictyostelium*[a]

Compound	Concn (M) for positive chemotaxis[b]	Threshold concn (M) for radial reaction[c]	Specific delay (h) of cell aggregation[d] by 10^{-3} M
cAMP	10^{-6}–10^{-7}	$10^{-4}/10^{-5}$	No delay
N^1-*O*-cAMP, 2′-H-cAMP, and 5′-NH-cAMP	10^{-5}–10^{-6}	10^{-4}	No delay
2′-THP-cAMP and 7-CH-cAMP	10^{-4}–10^{-5}	$10^{-3}/10^{-4}$	0–1
6-Cl-cPMP and 8-Br-cAMP	10^{-3}–10^{-4}	10^{-3}	2–5
cAMPS(S), cAMPS(R), and 3′-NH-cAMP	No reaction	No reaction	>20
cAMPN$(CH_3)_2$(S), cAMPN$(CH_3)_2$(R), 5′-AMP, and adenosine	No reaction	No reaction	No delay

[a] Means for *D. mucoroides*, *D. purpureum*, and *D. rosarium*.
[b] Concentrations at which 50% of the population reacted positively.
[c] In a radial reaction the cells are localized at the edge of the small population (Fig. 2; 10^{-4} M cAMP, 45 min).
[d] Specific delay of cell aggregation is defined as the time period between the recovery from a nonhomogeneous distribution of cells and the onset of cell aggregation.

TABLE 3. Effect of 3'-NH-cAMP on the chemotactic response of *D. purpureum* to cyclic AMP[a]

Expt	Time of cAMP deposit (min)	Time of 3'-NH-cAMP deposit (min)	Chemotactic response and development[b] monitored at (min):								
			10	20	40	47	60	85	105	120	7 h
A	—[c]	—	−/s	−/a	−/a	−/a	−/a	−/a	−/a	ac	ec
B	0	—	−/s	+/s	++/s	++/s	++/a	±/a	−/a	ac	ec
C	30	—	−/s	−/a	−/a	+/s	++/s	++/(a)	−/a	a	ec
D	—	0	−/s	−/s	−/s	−/s	−/s	−/s	−/s	−/s	−/s
E	—	30	−/s	−/a	−/(a)	−/s	−/s	−/s	−/s	−/s	−/s
F	0	30	−/s	+/s	±/s	−/s	−/s	−/s	−/s	−/s	−/s
G	30	30	−/s	−/a	−/(a)	−/s	−/s	−/s	−/s	−/s	−/s

[a] Small populations of *D. purpureum* were deposited on hydrophobic agar. After about 2 h, aggregation started (t = 0 min). A droplet of 10^{-5} M cAMP or a droplet of 10^{-3} M 3'-NH-cAMP or both was deposited close to the cell population, and the chemotactic response and development were monitored at different times before and after the deposition of the cyclic nucleotides.

[b] Chemotaxis: −, no chemotactic response; ±, positive reaction but in less than 50% of the populations; +, positive reaction in more than 50% of the populations; ++, strong positive reaction in all populations. Development: s, single cells; (a), small aggregate or aggregates in less than 20% of the populations; a, aggregates in more than 50% of the populations; ac, aggregation has been completed; ec, early culmination.

[c] —, Not done.

cAMP, 45 min), and (iii) a delay of cell aggregation. The cyclic nucleotide specificities for positive chemotaxis in aggregative cells of *D. mucoroides*, *D. purpureum*, and *D. rosarium* were nearly identical and similar to the specificity for chemotaxis in aggregative *D. discoideum* cells (Table 1). The specificities for a radially outward reaction and delay of cell aggregation were also nearly identical (Table 2). The same specificity was found for the cAMP-mediated cGMP accumulation in aggregative *D. discoideum* cells (16) and phosphodiesterase induction in postvegetative *D. discoideum* cells (22). This suggests that the same cAMP receptors are involved in the reception of the cAMP signals for these responses.

cAMP derivatives which induced only a weak chemotactic response delayed or prevented cell aggregation. This was probably due to a blockade of the detection of cAMP by the amoebae (Table 3).

To explain these observations we have to consider the cAMP analogs do not only act as effectors of chemotaxis (e.g., 2'-H-cAMP) or as inhibitors of chemotaxis (e.g., 3'-NH-cAMP), but can also act as effectors and inhibitors of chemotaxis (e.g., 8-Br-cAMP, which is chemotactically active and delays cell aggregation). Furthermore, 3'-NH-cAMP is an effector and an inhibitor of chemotaxis in *D. discoideum*, depending on the developmental stage of the cells. Time-lapse films show that the effect of 3'-NH-cAMP on the cessation of aggregation is virtually immediate (within a few minutes) and lasts for more than 20 h. Finally, we have to consider that 3'-NH-cAMP is an inhibitor of chemotaxis in postvegetative *D. discoideum* cells, and simultaneously it is a more potent effector than cAMP in producing a cGMP response. The different hydrolysis rates of cAMP analogs by cell-surface cyclic nucleotide phosphodiesterase of *D. discoideum* (12, 20, and unpublished data) probably cannot account for these ambivalent effects. 2'-H-cAMP, 8-Br-cAMP, 6-Cl-cPMP, and 7-CH-cAMP were hydrolyzed at high concentrations at approximately the same rate as cAMP, whereas 5'-NH-cAMP and cAMPS(S) were hydrolyzed 10 and 100 times, respectively, more slowly than cAMP. 3'-NH-cAMP (0.25 mM) did not inhibit the hydrolysis of cAMP (0.1 μM); 3'-NH-cAMP was hydrolyzed more than 100 times more slowly than cAMP at 0.1 mM concentrations.

We have to conclude that the characteristics of the interaction between cAMP analogs and the cAMP receptor are responsible for the seemingly paradoxical effects of cAMP analogs during chemotaxis.

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