# CYCLIC-AMP RECEPTION AND CELL RECOGNITION IN DICTYOSTELIUM DISCOIDEUM

G. Gerisch, D. Malchow, A. Huesgen, V. Nanjundiah, W. Roos, U. Wick

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, 74 Tübingen, Germany [x]

and D. Hülser

Biologisches Institut der Universität Stuttgart, Germany

## Functions of cyclic-AMP in cell aggregation

Single cells of the slime mold, *Dictyostelium discoideum*, aggregate into a multicellular organism in response to cyclic AMP (1, 2), which they detect by binding to cell-surface receptors (3). During the aggregation phase, two different responses to cyclic-AMP are observed. First, the cells orientate by chemotaxis towards the source of a concentration gradient which initially is a group of cells forming an aggregation center. Second, the cells relay pulses which are periodically generated by the centers (4, 5, 6). Relaying of signals can be directly observed because the response to cAMP is associated both with movement steps and with changes of cell shape (7, 8). In a cell layer the zones of activity are distributed in a spatial pattern which consists either of spirals or of concentric waves, propagating from the central area to the periphery of an aggregation territory, at a velocity of 40 µm per minute or more (4, 8, 9). Propagated waves can be induced in pre-aggregative cells by periodic stimulation using a cyclic-AMP electrode; this suggests that cAMP is the transmitter in the relay system (10).

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[x] Present address: Biozentrum der Universität Basel, Klingelbergstraße 70, 4056 Basel, Switzerland.

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The relay mechanism is based on an amplifier system (4) with the following functional cycle: (1) Activation of cell-surface receptors by small pulses of cAMP with an amplitude in the nanomolar range (11, 12, 13). (2) Release of cAMP through the plasma membrane and, possibly, activation of adenylcyclase (14, 15). (3) Stimulation of other cells and (4) inactivation of the extracellular cAMP by phosphodiesterase (16, 17, 18, 19).

## Membrane markers of differentiated cells

Growth-phase cells require several hours to differentiate into aggregation-competent cells. During the interphase they acquire the ability to aggregate into a multicellular body, not only by chemotaxis and signal relaying, but also by cell-to-cell adhesion. In the present paper we discuss the functions and control of three types of cell-surface sites that appear, or increase in activity, concomitantly with the aggregative competence of the cells: (1) cAMP-binding sites that are supposed to function as the chemoreceptors, (2) cAMP-phosphodiesterase, and (3) contact sites A (20, 21). Blockage of the latter by univalent antibody fragments ($F_{ab}$) inhibits cell assembly into streams, a function typical for aggregation-competent cells. It will be shown that the appearance of all these sites is regulated by cAMP-pulses, which means that cAMP-receptors are involved in the control of cell differentiation.

## cAMP-receptors

In aggregating cells, two types of cell-surface sites interact with extracellular cAMP: receptor sites and cyclic-nucleotide phosphodiesterase. These sites can be distinguished by cGMP which has a high affinity to the enzyme (22), but is a weak agonist (2, 23); and also by adenosine-3',5'-cyclic-phosphorothioate (cAMP-S) which, on the contrary, is a good agonist with a weak affinity to the enzyme. Because it preferentially blocks cAMP-hydrolysis, cGMP makes the measurement of cAMP-binding possible. Thus a number of approx. $5 \times 10^5$ cAMP-binding sites at the surface of aggregating cells and an estimate of $2 \times 10^{-7}$ M for the dissociation constant of a receptor-cAMP complex have been found (3). In selected, strongly aggregating clones of the axenic strain Ax-2 significant cAMP-binding can also be obtained in the absence of cGMP.

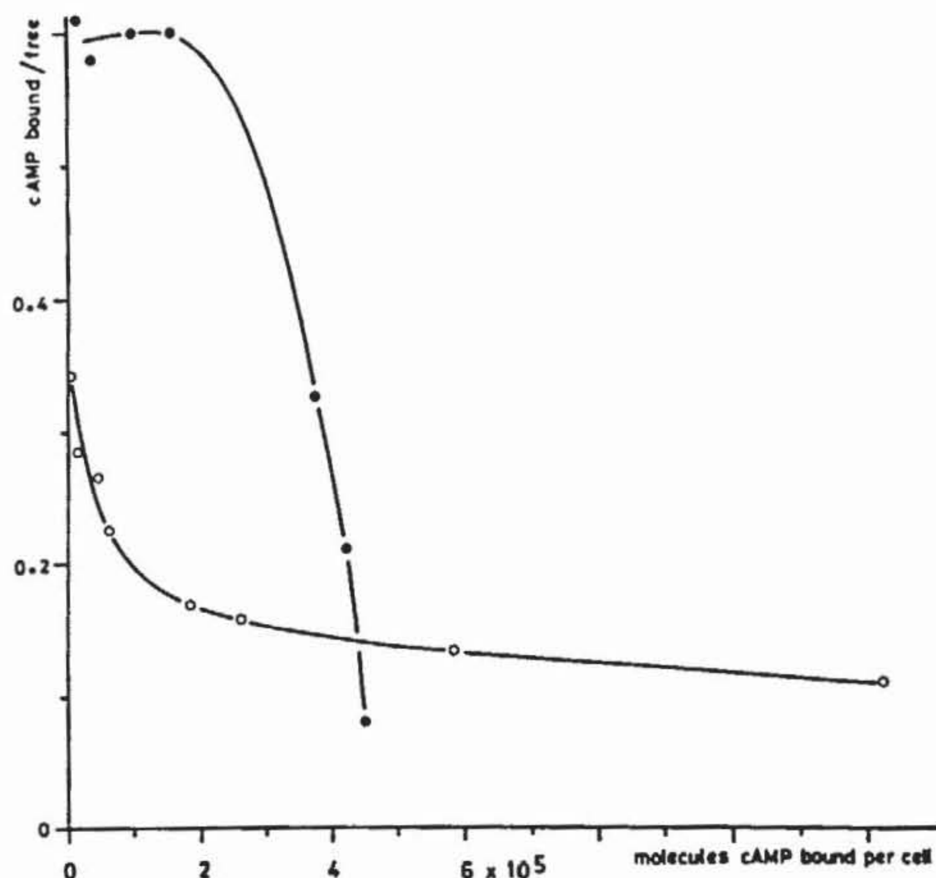


Fig. 1. Scatchard plots of cAMP-binding to Wag-6 cells at 23°C (o) and 5°C (●). Binding was measured in 17 mM phosphate buffer pH 6.0. 5 seconds after cAMP addition the cells were removed by centrifugation and label in the supernatant was counted. Other methods as in (3), except that no cGMP was added for phosphodiesterase inhibition.

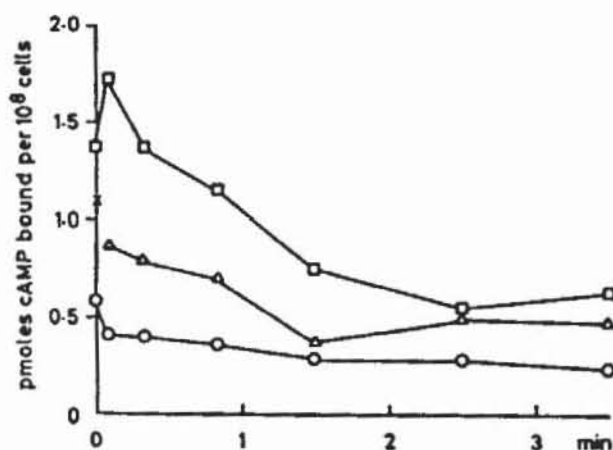


Fig. 2. Binding of cAMP to Wag-6 cells as a function of the time of pre-incubation (abscissa) with cGMP and/or cAMP-S. (□) pre-incubation with 5 x $10^{-4}$M cGMP, (o) with 1 x $10^{-5}$M cAMP-S, (Δ) with both. (x) cAMP added without pre-incubation. Cell concentration was 2 x $10^8$/ml, temperature 23°C, the $^3$H-cAMP added for the assay of binding was 1 x $10^{-8}$M, and cells were removed by centrifugation invariably 5 seconds after cAMP-addition. The synthesis of cAMP-S which has been kindly provided by Dr. Eckstein, is described in (32).

The kinetics of cAMP-binding is investigated more conveniently, however, in a mutant (Wag-6) in which the cell-surface phosphodiesterase activity is low relative to cAMP-binding. At 23°C, the optimal temperature of this organism, binding curves suggesting negative cooperativity with apparent dissociation constants within the limits of 0.2 and 10 μM have been obtained (fig. 1). Curves of similar shape have been found by Green and Newell (24) who used wild-type cells together with dithiothreitol as a phosphodiesterase inhibitor. Their curves, however, tend towards lower concentrations than ours. Complex kinetics like the observed ones possibly account for the ability of a cell to detect concentration differences all the time along its path from low attractant concentrations towards the source of a gradient. At 5°C, Wag-6 showed binding kinetics suggestive of positive cooperativity, indicating a temperature-dependent transition of the binding characteristics (fig. 1).

These results suggest interaction of either identical or different cAMP-binding sites. Other results provide evidence for cyclic-nucleotide binding sites of different specificities. If cGMP is applied to cells simultaneously with $1 \times 10^{-5}$ M cAMP-S, the inhibition of cAMP-binding by the latter is markedly reduced (fig. 2). Thus cGMP does not act supplementary to cAMP-S as an inhibitor of cAMP-binding but, on the contrary, it antagonizes the inhibitory effect of the thioanalogue. It is yet unclear if this cGMP effect is mediated by cell-surface receptors or is due to intracellular action.

Experiments in which both cGMP and cAMP were applied to cells at intervals of varying lengths, may suggest adaptation processes with time constants in the order of minutes (fig. 2) similar to the desensitization of ACh-receptors in electroplax membranes (25). It is also conceivable, however, that this slow decrease of cAMP binding after cGMP application does not reflect a decrease in receptor sensitivity, but rather an increase of extracellular cAMP which then competes for binding of the added cAMP. In any case, it should be pointed out that in periodic signal generation and relaying, changes of sensitivity either at the receptor level or at a later step of signal processing have to be postulated in order to account for the observed refractory phase after signalling, during which the sensitivity of the signal-generating system to cAMP-stimuli is decreased (23).

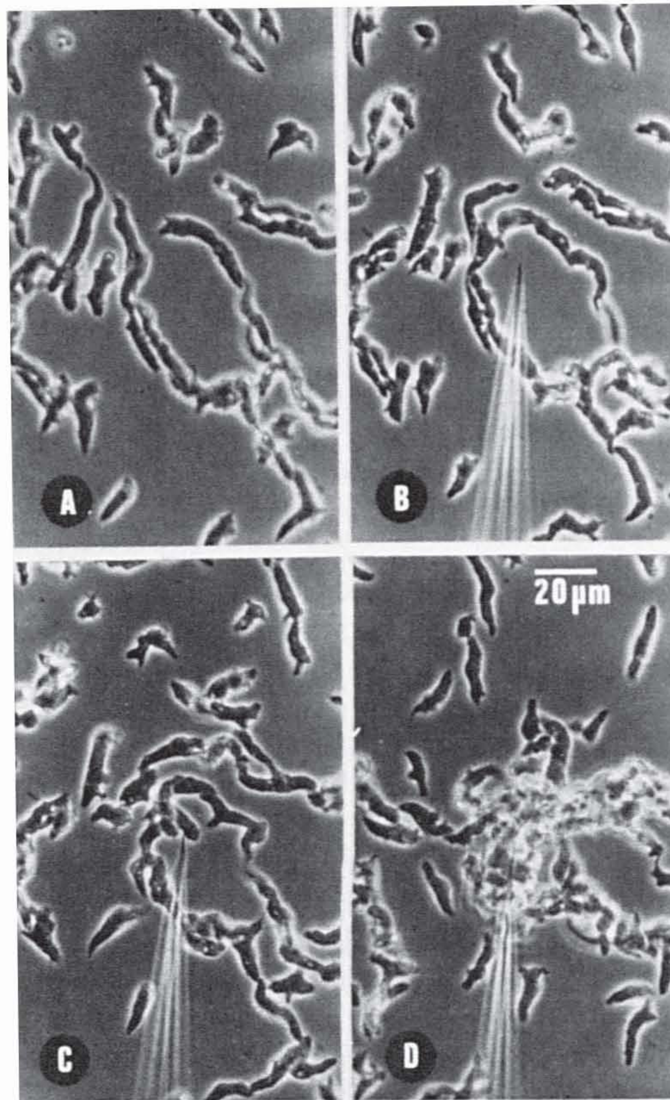


Fig. 3. Chemotaxis towards a cAMP capillary. Times: A, shortly before the capillary was introduced; B, C, D, 1, 2, and 14 minutes later. In C, a cell right of the capillary tip projects a pseudopod towards the capillary, although it still sticks to other cells by its previous front and rear ends. The strain is ga 93, a mutant forming extremely large aggregation territories. The cells were in the early aggregation phase, 5 hours after end of growth. cAMP-concentration was $10^{-6}$M, and release was accelerated by iontophoresis (10). Similar results were obtained purely by diffusion if the capillary was filled with $10^{-4}$M cAMP. The tip diameter was approx. 0.2 µm.

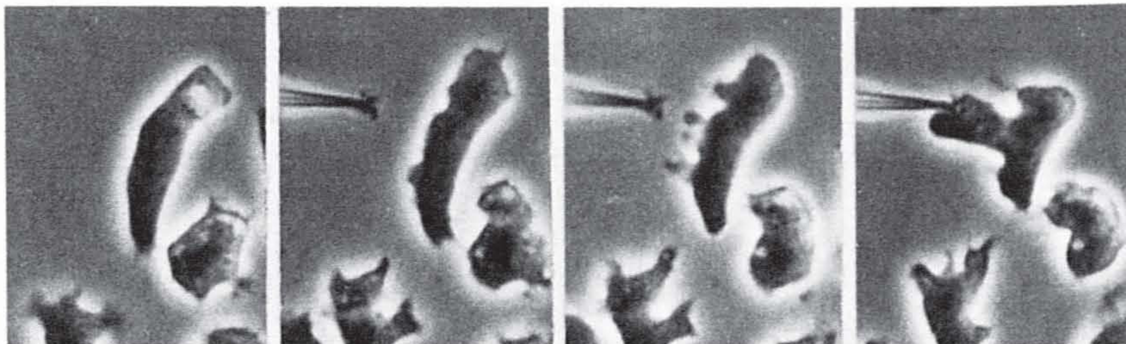

Fig. 4. Local Responses of a cell to cAMP. Conditions were the same as in fig. 3.

## Chemotaxis showing distribution of cAMP-receptors over the total cell surface

Localized cAMP-stimuli were applied to different parts of the cell surface by microcapillaries (26, 27, 10), and extrusion of pseudopods from any part of the surface was observed within a few seconds (fig. 3 and 4). To exclude redistribution of receptors in response to the signal, cells were alternatively stimulated from two opposite sides with intervals of less than 10 seconds. New pseudopods were formed after as little as 5 seconds, even before the former pseudopods pointing in the opposite direction had been retracted. These results show that any part of the surface is a potential moving front able to sense cAMP and to perform a local response, and they confirm those of Alcantara and Monk (8) demonstrating reorientation of cells within no more than 12 seconds.

## Cellular responses to cAMP-pulses

Cellular responses to cAMP which, at least in part, reflect changes of cell shape can be recorded by the measurement of light scattering in cell suspensions (fig. 7). These responses are evoked by the rapid increase of the extracellular cAMP level by concentration steps in the nanomolar to micromolar range (23). The decline of the responses with time is independent of the continued presence of the stimulating cyclic nucleotide in the extracellular medium, and is also independent of its continued binding to cell-surface receptors (11, 12). We have postulated, therefore, that the signal is transformed into a derivative of cAMP-concentration at a step of signal processing later than the primary interaction of cAMP with receptors and, consequently, that the optimal stimuli for the chemoreceptor system are cAMP-pulses rather than steady concentrations.

The stimulating effect of cAMP-pulses is obvious in signal relaying. Pulsing is also the way cAMP stimulates cell differentiation, as will be shown below. Recognition of stationary spatial gradients by detectors responding to changes of concentration with time has been demonstrated for chemotaxis in bacteria (30, 31) and has been discussed for chemotaxis in the ameboid cells of *Dictyostelium* (11, 12).

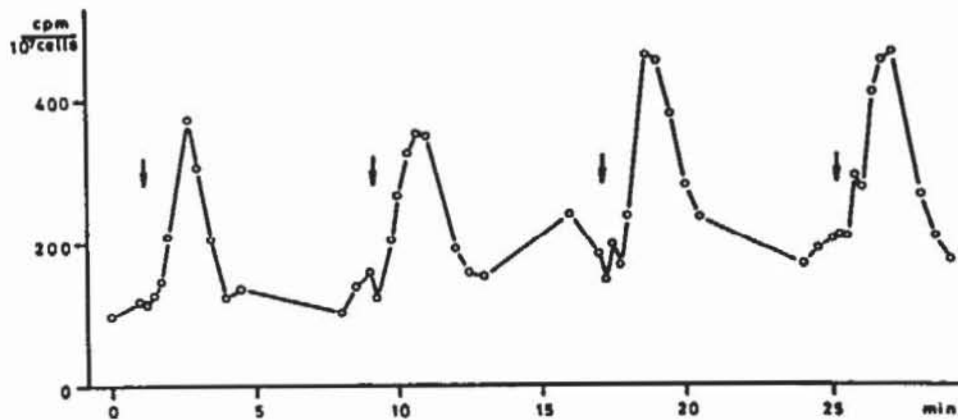


Fig. 5. cAMP-release stimulated by cAMP-pulses. Cells were pre-labelled with 2-$^3$H-adenine and washed. At the times indicated by arrows, 6 x $10^{-8}$M unlabelled cAMP was added to a stirred suspension of 2 x $10^8$ Ax-2 cells per ml, and at intervals the cells were rapidly separated from the supernatant. The ordinate indicates label in the extracellular cAMP fraction after chromatographic separation. Methods as described in (13).

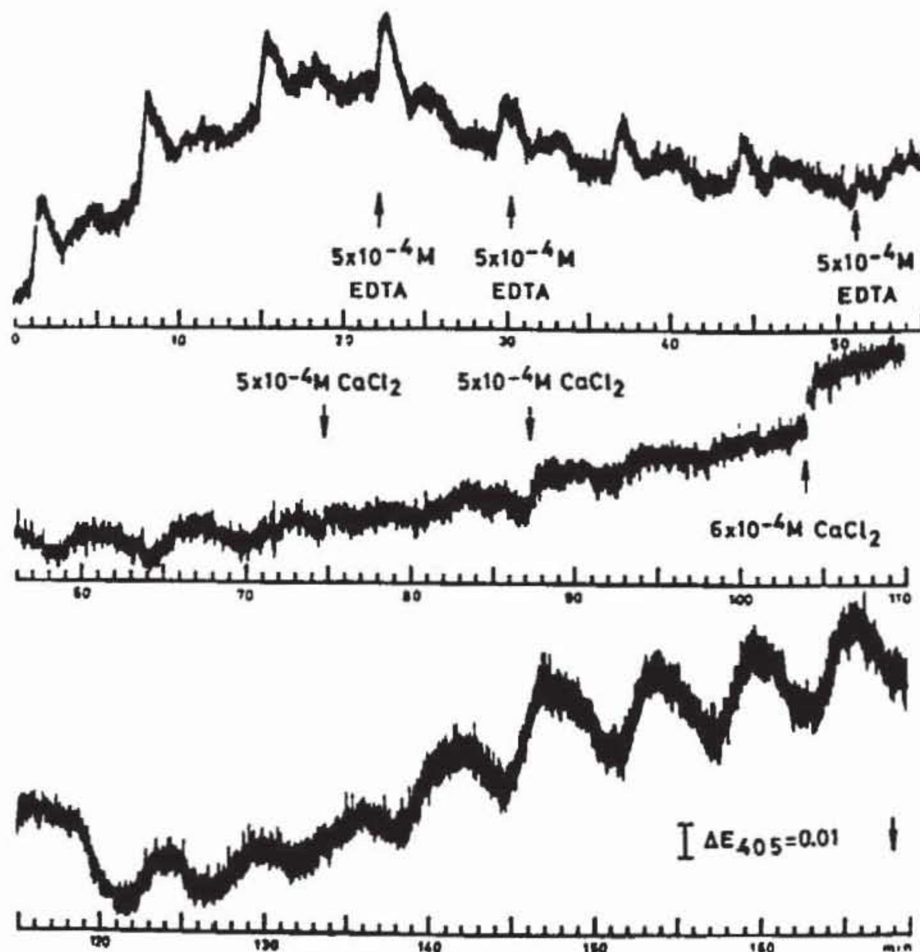


Fig. 6. Inhibition of periodic signalling by EDTA, and recovery therefrom by $Ca^{++}$. Periodic cellular activities were recorded by light scattering; methods as in (23).

## Amplification of cAMP-pulses

Signal amplification is a prerequisite for the action of cAMP as a transmitter in the relay system by which waves of chemotactic activity are propagated in aggregation territories. Fig. 5 shows that cAMP-pulses stimulate cells to release pulses of cAMP. Mean amplification factors of 6 to 10 in terms of output/input concentration changes have been found, both using the protein-binding assay of Gilman and prelabelling of cells by $^{3}$H-adenine for measuring the amplitude of the evoked response (12, 13). If hydrolysis by cell-surface phosphodiesterase during the period of cAMP-release is taken into account, a ratio in the order of 1:40 has been obtained for the number of cAMP-molecules given as stimulant, compared to those released in response to the signal (13). On the same basis, an approximate number of $6 \times 10^{6}$ cAMP-molecules released per cell per pulse has been calculated.

## Periodic signal generation

Spontaneous onset of periodic cellular activities, indicated by changes in light scattering, has been observed in actively stirred cell suspensions; and under the same conditions sustained oscillations of these activities have been recorded (23). Periodic signal generation is normally a function of aggregation centers, and the spontaneous oscillations observed in cell suspensions can be used as a model for investigating the processes underlying the centers' activity. First of all, oscillation of light scattering is accompanied, and supposedly caused, by periodic changes of the extracellular cAMP-concentration with amplitudes similar to those observed in response to experimental cAMP-pulses (12). These activities, therefore, indicate that the cAMP-amplifier system is spontaneously activated by rhythmic triggers, which presumably are small pulses of released cAMP.

Both extracellular cAMP and $Ca^{++}$ influence the signal generating system. Their effects, however, are different. cAMP-pulses result in phase shifts, indicating that the cAMP-receptors are functionally connected to the oscillator controlling cAMP-release (23). The involvement of $Ca^{++}$ in cell aggregation of D.discoideum has been concluded from a series of previous experiments (33, 34, 35). Under our conditions, $Ca^{++}$ is a cofactor for signalling, not for the

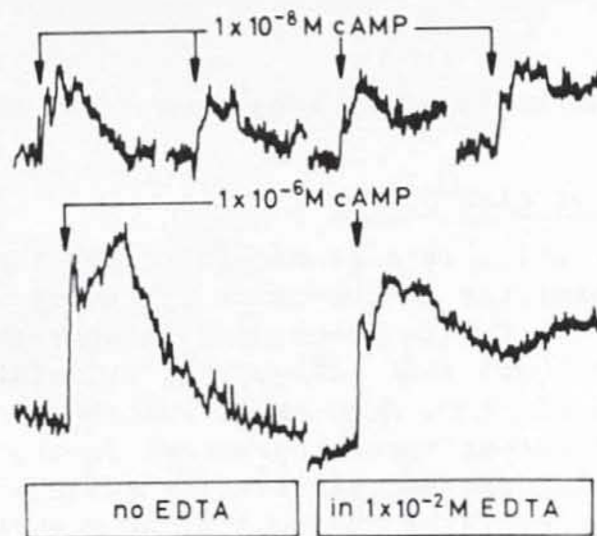


Fig. 7. Light scattering responses to cAMP-pulses in the presence and absence of EDTA. In addition to the finding that the cells are able to bind cAMP in the presence of EDTA (3), the results shown indicate that they are also able to respond to cAMP with almost normal sensitivity. In conclusion, the EDTA-effect shown in fig. 6 is presumably due to disturbance of periodic signalling rather than to inhibition of the response.

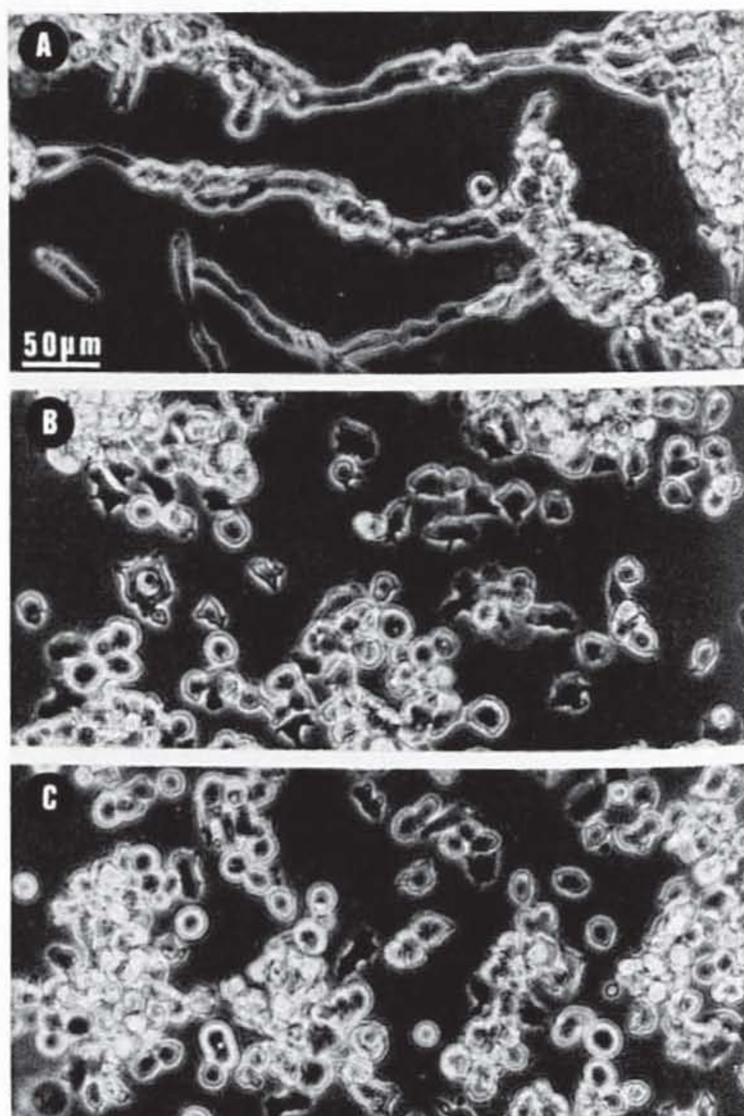


Fig. 8. Stationary-phase cells stimulated by cAMP-pulses (A), continuous cAMP-flow (B), and control (C). Only in the pulsed culture (A) cells were elongated and connected end-to-end, as is typical for aggregation-competent cells. Pulse size in (A) was 5 nM, the period of pulsing 6.5 minutes. Average rate of cAMP-injection in (A) and (B) were the same. Cells were incubated in shaken suspensions containing $1 \times 10^7$ cells/ml. After 6 hours of stimulation, cells were washed and allowed to aggregate for 1 hour in the absence of any further stimulation.

response to cAMP which remains essentially unchanged in the presence of EDTA (fig. 7). EDTA and also EGTA decrease the amplitude of the signals down to undetectability, and $Ca^{\cdot\cdot}$ restores them (fig. 6). Neither phase shifts nor any considerable effects on frequency have been observed in response to Ca-pulses. According to our results, the $Ca^{\cdot\cdot}$-requirement of a cell-surface site that controls cAMP-release is a most probable function of $Ca^{\cdot\cdot}$ in information transfer among aggregating D.discoideum cells.

## Function of cell-surface phosphodiesterase

The spatiotemporal pattern of cellular activities produced by periodic signal generation and relay depends on the detectability of cAMP-pulses and thus on the fast inactivation of the released cAMP. As a developmentally controlled enzyme, the cell-surface phosphodiesterase shows highest activity during the aggregation phase. Its kinetics may be interpreted as negative cooperativity, which has the effect of more rapidly destroying cAMP signals down to the noise level of the receptors than would be the case with a Michaelis-Menten enzyme (19).

## cAMP-pulses stimulating cell differentiation

If exponentially growing cells of the axenic strain Ax-2 (36) of D.discoideum are washed free of nutrient, they differentiate within 7 to 9 hours to aggregation-competent cells both in shaken suspensions (18) and in cell layers on millipore filters (28). Since prior to full aggregation-competence these cells are able to generate periodic cAMP-pulses, the possibility exists that they interact by means of these pulses, thus synchronizing their development. If harvested at the stationary phase, the cells show only rudimentary differentiation in our suspension cultures (fig. 8C). Their differentiation can be drastically stimulated, however, by pulses of cAMP (fig. 8A). In contrast, continuous cAMP-flow of the same average rate has only a slight, if any, effect (29) (fig. 8B). These results show that the potency of differentiation can be fully restored in stationary-phase cells by cAMP-pulses of 5 nM amplitude and a period of about 6 minutes.

Assays of contact-sites A revealed that the expression of these sites at the cell surface is not only accelerated by cAMP-pulses, but also stimulated up to a maximum con-

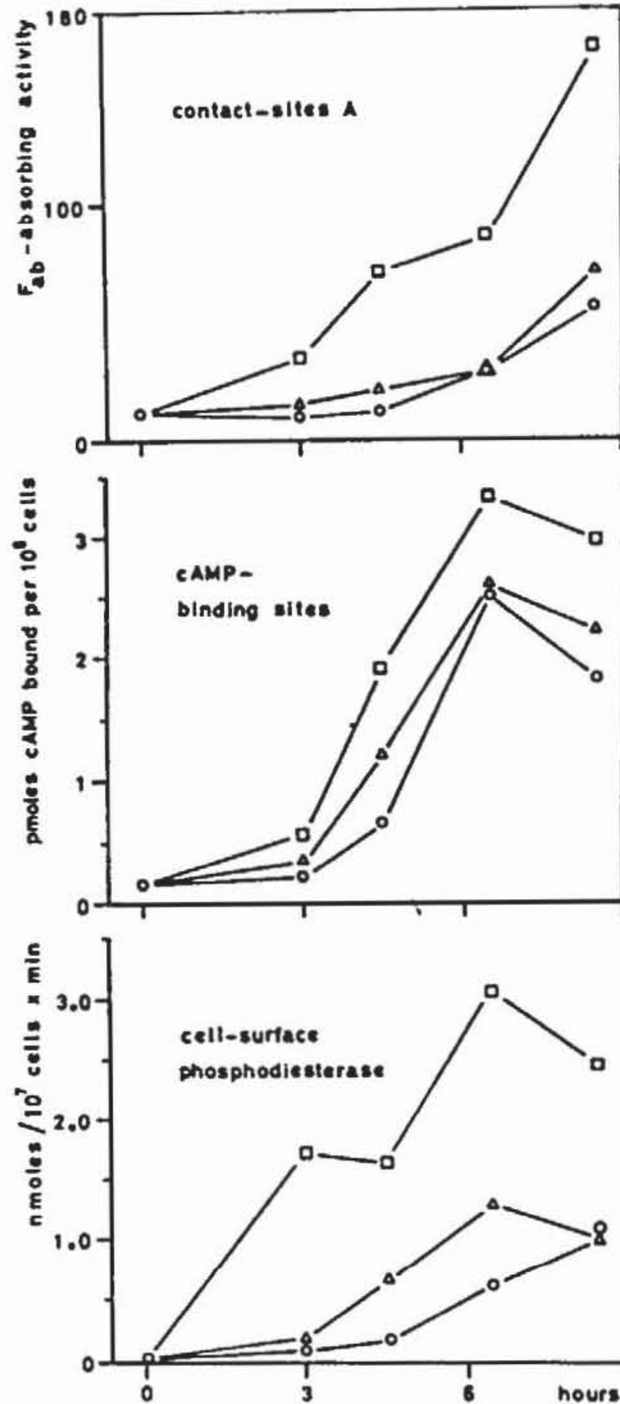


Fig. 9. Stimulation of cell-surface sites by cAMP-pulses (□) compared to continuous cAMP-flow (△) and a non-stimulated control (o). Conditions were similar to those of fig. 8. Abscissa: time after washing of the cells free of nutrient. Stimulation began 1 hour later. Ordinate on top: A $F_{ab}$-absorbing activity of 100 corresponds to the activity of aggregation-competent, bacterial grown wild-type cells. Living cells were used for the $F_{ab}$-absorption assay as described in (20) and (21). cAMP-binding (middle) was measured by incubation for 20 seconds at 5°C in $1 \times 10^{-8}$M $^3$H-cAMP plus $5 \times 10^{-4}$M cGMP (3). Phosphodiesterase was assayed using living cells according to (3).

siderably higher than normal (fig. 9A). The conclusion is that signal inputs to the cAMP-receptors regulate the expression of other types of cell-surface sites, in this case one related to cell adhesion.

Both the receptors themselves and cell-surface phosphodiesterase are also under the control of cAMP-pulses, indicating positive as well as negative feedback controls in the cAMP-system of D.discoideum (fig. 9B and C). In general, interdependence of the differentiation markers at the cell surface is obvious, which is underlined by the result that in most of the aggregation-deficient mutants we have screened, multiple defects of differentiation markers have been found.

## Acknowledgements

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## References

1. Bonner, J.T., Barkley, D.S., Hall, E.M., Konijn, T.M., Mason, J.W., O'Keefe, G. and Wolfe, P.B. Develop. Biol. 20, 72 (1969).
2. Konijn, T.M. Advan. Cyclic Nucleo. Res. 1, 17 (1972).
3. Malchow, D. and Gerisch, G. Proc. Nat. Ac. Sci. USA 71, 2423 (1974).
4. Shaffer, B.M. Advan. Morphogenesis 2, 109 (1962).
5. Cohen, M.H. and Robertson, A. J. Theoret. Biol. 31, 101 (1971).
6. Gerisch, G. In: Current Topics in Developmental Biology (A. Monroy and A.A. Moscona, Eds.). Academic Press, New York. Vol.3, 1968.
7. Gerisch, G. Naturwissenschaften 58, 430 (1971).
8. Alcantara, F. and Monk, M. J. Gen. Microbiol. 85, 321 (1974).
9. Gerisch, G. Roux' Arch.Entwicklungsmechanik 156, 127 (1965).
10. Robertson, A., Drage, D.J. and Cohen, M.H. Science 175, 333 (1972).
11. Gerisch, G., Malchow, D. and Hess, B. In: Biochemistry of Sensory Functions. Mosbacher Kolloquium 1974 (L. Jaenicke, Ed.). Springer Verlag, Heidelberg.

12. Gerisch, G., Hülser, D., Malchow, D. and Wick, U. Transactions of the Royal Society (B), in press (1975).
13. Roos, W., Nanjundiah, V., Malchow, D. and Gerisch, G. FEBS-Letters, in press (1975).
14. Rossomando, E.F. and Sussman, M. Proc. Nat. Ac. Sci. USA 70, 1254 (1973).
15. Goldbeter, A. Nature 253, 540 (1975).
16. Chang, Y.Y. Science 160, 57 (1968).
17. Pannbacker, R.G. and Bravard, L.J. Science 175, 1014 (1972).
18. Malchow, D., Nägele, B., Schwarz, H. and Gerisch, G. Eur. J. Biochem. 28, 136 (1972).
19. Malchow, D., Fuchila, J. and Nanjundiah, V. Biochem. Biophys. Acta, in press (1975).
20. Beug, H., Katz, F.E. and Gerisch, G. J. Cell Biol. 56, 647 (1973).
21. Beug, H., Katz, F.E., Stein, A. and Gerisch, G. Proc. Nat. Ac. Sci. USA, 70, 3150 (1973).
22. Malchow, D., Fuchila, J. and Jastorff, B. FEBS-Letters 34, 5 (1973).
23. Gerisch, G. and Hess, B. Proc. Nat. Ac. Sci. USA 71, 2118 (1974).
24. Cited in: Newell, P.C. Microbiology, in press (1975).
25. Popot, J.L., Sugiyama, H. and Changeux, J.P. To be published in C.R. Acad. Sci. Paris, Série D (1974).
26. Pfeffer, W. Untersuch. Botan. Inst. Tübingen 1, 363 (1884).
27. Adler, J. Science 166, 1588 (1969).
28. Malkinson, A.M. and Ashworth, J.M. Biochem. J. 134, 311 (1973).
29. Fromm, H. Diplomarbeit, Universität Tübingen (1974).
30. Macnab, R.M. and Koshland, D.E. Proc. Nat. Ac. Sci. USA 69, 2509 (1972).
31. Berg, H.C. and Brown, D.A. Nature 239, 500 (1972).
32. Eckstein, F., Simonson, L.-P. and Baer, H.P. Biochemistry 13, 3806 (1974).
33. Mason, J.W., Rasmussen, H. and Dibella, F. Exp. Cell Res. 67, 156 (1971).
34. Chi, Y.-Y. and Francis, D. J. Cell. Physiol. 77, 169 (1971).
35. Maeda, Y. and Maeda, M. Exp. Cell Res. 82, 125 (1973).
36. Watts, D.J. and Ashworth, J.M. Biochem. J. 119, 171 (1970).