RECIPROCAL PERIODICITY IN CYCLIC AMP BINDING AND PHOSPHORYLATION OF DIFFERENTIATING *DICTYOSTELIUM DISCOIDEUM* CELLS

A. Christie King and William A. Frazier

Departments of Biological Chemistry and Neurobiology
Division of Biology and Biomedical Sciences
Washington University, St. Louis, Mo. 63110

Received August 31,1977

SUMMARY: The binding of [^{3}H]cyclic AMP to cell surface receptors of differentiated *D. discoideum* cells at 25° is an oscillatory process with a periodicity of 2 min. This alternating change in the cells' binding capacity for cyclic AMP may be the basis for the refractory period to cyclic AMP stimulation, an essential feature of the chemotactic system. The incorporation of [^{32}P] by whole cells from [γ^{32}P]ATP is also oscillatory with a periodicity identical to that of [^{3}H]cyclic AMP binding. However, the two processes are inversely related in time such that periods of maximal cyclic AMP binding correspond to periods of minimal cellular phosphorylation. These results suggest a receptor kinase/phosphatase mediated desensitization of the cyclic AMP receptor.

INTRODUCTION: Cyclic AMP (3':5' cyclic adenosine monophosphate) is the extracellular chemotactic transmitter which directs the aggregation of differentiating *Dictyostelium discoideum* cells (1). The nucleotide is released in pulsatile fashion from aggregating cells (2). Nearby cells detect the cyclic AMP through its interaction with cell surface receptors for the nucleotide (3-5). These amoebae respond by movement toward the source of the signal and relay the signal by releasing a pulse of cyclic AMP themselves. Cells which have completed this response are then refractory to further stimulation by cyclic AMP for a short time (2,6). This refractory period prevents cells from moving away from aggregation centers when adjacent outlying cells emit their pulse of cyclic AMP. A possible mechanistic basis for the refractory period is a transient decrease in affinity of the cyclic AMP receptor mediated by cyclic AMP. This nucleotide regulates a great many biological processes through modulation of protein kinase activity, and hence phosphorylation of proteins (7,8). To test the involvement of this type of

ISSN 0006-291X

mechanism in the regulation of cyclic AMP binding we have measured the transfer of the γ-phosphate of [γ^{32}P]ATP to whole slime mold cells under conditions in which their binding capacity for cyclic AMP is varying in a synchronous, periodic manner. Although an apparent periodic binding of cyclic AMP was observed by Klein *et al.* (9), they could not distinguish between variation in binding and variation in isotope dilution by periodic release of the nucleotide from the slime mold cells. The experimental design of the cyclic AMP binding studies reported here rule out the latter explanation and indicate that it is the binding capacity of the cells which varies in a periodic manner. Under these experimental conditions phosphorylation of the cells also occurs with a periodicity such that there is an inverse relationship between degree of phosphorylation and [^{3}H]cyclic AMP binding capacity.

METHODS: Amoebae of the *Dictyostelium discoideum* axenic strain M-3 (from W.F. Loomis) grown on HL-5 medium were harvested at a cell density of 10^7/ml. The cells were washed and resuspended at a density of 10^7/ml in 17 mM phosphate buffer, pH 6.2, containing 50 μg/ml streptomycin sulfate (10). Cells were maintained on a gyratory shaker at room temperature for 5-7 hours. At this time, the cells were washed and resuspended at a density of 1-5 x 10^7/ml in 17 mM phosphate buffer containing 1 mM $MgSO_4$. 5 mM dithiothreitol was added for cyclic AMP binding and carrier ATP for phosphorylation. The suspension was mechanically stirred and the experiment initiated by the addition of [γ^{32}P]ATP (25 Ci/mmole, New England Nuclear) or [^{3}H]cyclic AMP (50 Ci/mmole, New England Nuclear). Duplicate 100 μl samples were removed from the incubation mixture at 30 s intervals and filtered through 0.6 μ pore size polycarbonate membranes (Bio Rad or Nucleopore). The filters were immediately washed with 1 ml of ice cold 17 mM phosphate buffer (cyclic AMP binding) or ice cold 5% TCA (phosphorylation). This procedure requires 5-7 seconds. In some phosphorylation experiments, the aliquot of cells was pipetted directly into 1 ml of ice cold 5% TCA and then immediately filtered. The results of both types of washing procedures were similar. For experiments in which cells were incubated with both nucleotides, cells were washed with 1 ml cold 17 mM phosphate buffer. The extent of cyclic AMP binding or phosphorylation was determined by counting the filters in 10 ml of 3a70 (Research Products, Inc.). Binding to filters was less than 0.003% for [^{3}H]cyclic AMP and less than 0.3% for [γ^{32}P]ATP of the total counts applied. Double label experiments were calculated using the channels ratio method.

RESULTS AND DISCUSSION

Binding of [^{3}H]cyclic AMP. As seen in figure 1A and B, the time course of binding of 10^{-8} M cyclic AMP to *D. discoideum* cells at room temperature is not a simple hyperbolic, steady state process. At the earliest time point measured, significant binding has occurred, and with time, the amount of

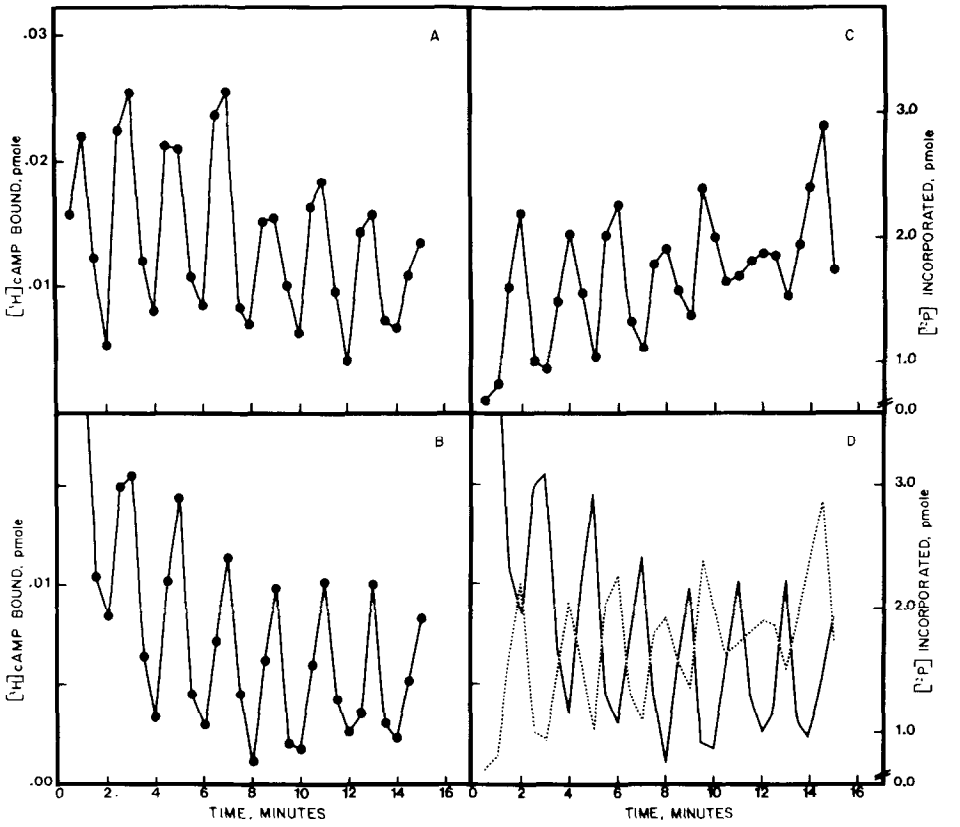

Figure 1(A). Binding of [^{3}H]cyclic AMP by 10^6 differentiated *D. Discoideum* cells as a function of time. The stirred cell suspension contained 10^{-8} M [^{3}H]cyclic AMP and 5 mM DTT. Duplicate samples were removed every 30 s and filtered.

Figure 1(B-D). Binding of [^{3}H]cyclic AMP and the incorporation of [^{32}P] from [γ^{32}P]ATP by 10^6 differentiated *D. discoideum* cells. In a double label experiment, cyclic AMP was present at 10^{-8} M, and the final ATP concentration was 10^{-5} M. DTT was present at a concentration of 5 mM. (B) Time course of [^{3}H] cyclic AMP binding; (C) Time course of [^{32}P] incorporation from [γ^{32}P] ATP; (D) data shown in (B) and (C) displayed on same axis. The solid line is cyclic AMP binding and the dashed line is [^{32}P] incorporation.

[^{3}H]cyclic AMP bound varies in a periodic oscillatory way. Binding maxima occur at 2 min intervals at levels 2 to 3 fold above the minimum points. Although the absolute levels of binding observed vary somewhat in experiments with different batches of cells, the 2 min periodicity is always observed (20 experiments). The range of binding capacities observed is similar to that

reported by others (4,5,9). During the time course of the experiment, a downward trend in the level of binding maxima is seen, presumably due to isotope dilution by unlabeled cyclic AMP released from cells. This release, although itself periodic (2), cannot contribute directly to the oscillatory nature of the binding since the labelled nucleotide is continuously present in the rapidly stirred cell suspension. The on and off rates of the cyclic AMP binding process are sufficiently rapid to allow for the oscillations observed (A.C. King, unpublished observations).

Periodic changes in [^{3}H]cyclic AMP binding have been reported (9) to correlate with oscillations in light scattering of cell suspensions which are thought to reflect the periodic morphogenetic movements and concomitant cell shape changes which occur during chemotaxis (11). Klein *et al.* (9) report that variation in cyclic AMP secretion by the cells may have caused the apparent change in binding capacity observed. Our results indicate that periodic oscillations in the binding capacity of cells do occur. These changes reflect a rapid alteration in cyclic AMP receptor affinity and/or number. Studies in several hormone receptor systems (12-14) suggest that changes in receptor affinity can occur on a more rapid time scale than changes in receptor number, which appear to involve protein turnover (12,14).

Phosphorylation of Slime Mold Cells. A mechanism by which the binding properties of cyclic AMP receptors might be rapidly regulated is that of alternating phosphorylation and dephosphorylation. Protein kinase activity toward exogenous substrates has previously been identified on intact slime mold cells (15) and these cells were found to be labelled with [γ^{32}P]ATP (16,17). Since cyclic AMP is known to regulate protein kinase activity in many systems (7,8), an investigation of the potential relationship between cyclic AMP binding and phosphorylation of slime mold cells was undertaken.

Figure 2 shows the time course of incorporation of radioactivity from [γ^{32}P]ATP by intact slime mold cells at three concentrations of ATP (2A-10^{-5} M, 2B-10^{-7} M and 2C-10^{-9} M). Although more variability was

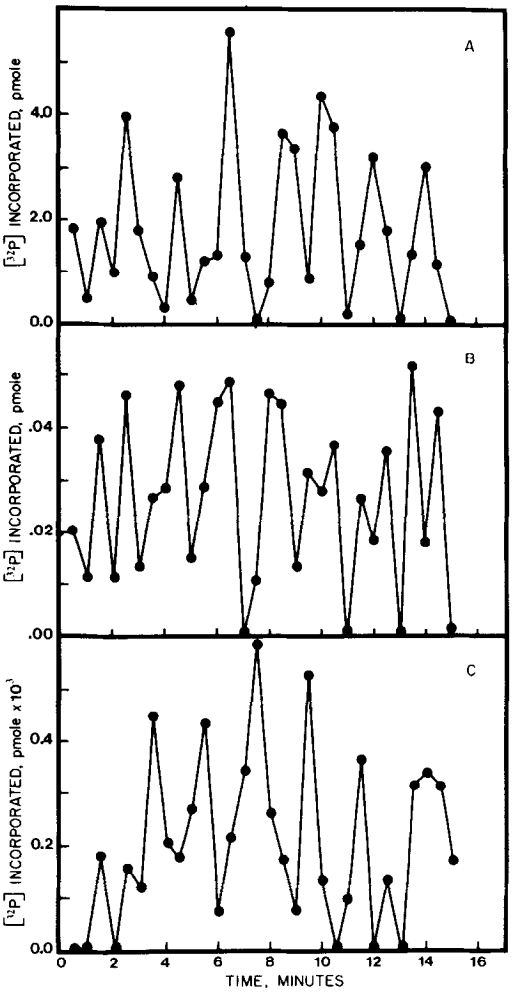

Figure 2. Incorporation of [^{32}P] from [γ^{32}P] ATP by 10^6 differentiated *D. discoideum* cells. Stirred cell suspensions were incubated with [γ^{32}P]ATP and unlabelled ATP at a final concentration of: (A) 10^{-5} M; (B) 10^{-7} M; (C) 10^{-9}M. Duplicate samples were removed and filtered every 30 s.

encountered in phosphorylation time courses than in [^{3}H]cyclic AMP binding experiments, these data clearly indicate the periodic nature of the phosphorylation process. That the [^{32}P] bound to the cells represents phosphorylation rather than noncovalent binding of ATP is suggested by the following: (a) the bound radioactivity is stable to TCA precipitation; (b) bound [^{3}H]cyclic AMP is removed by TCA precipitation; (c) under identical

incubation conditions, [^{32}P] can be found covalently attached to membrane proteins after SDS gel electrophoresis (A.C. King, unpublished experiments). Parish *et al.* (17) have also reported the phosphorylation of *D. discoideum* membrane proteins. The period of oscillations in [^{32}P] incorporation seen in these experiments (figure 2) is approximately 2 min, as is the periodicity in [^{3}H]cyclic AMP binding. To determine the temporal relationship of these two phenomena, slime mold cells were incubated with both [^{3}H]cyclic AMP (10^{-8} M) and [γ^{32}P]ATP (10^{-5} M),and the binding of the two radioactive labels was followed with time. The binding of [^{3}H]cyclic AMP in this experiment is shown in figure 1B and the incorporation of [^{32}P]phosphate is shown in figure 1C. Again the oscillatory nature of both processes is apparent. When the double label experiment (in figures 1B and 1C) is displayed on the same time scale (figure 1D),it is seen that the binding of cyclic AMP and the phosphorylation process are related in a precisely inverse manner. That is, at times of highest cyclic AMP binding, cell phosphorylation is at a minimum.

Although, at present, this correlation is only circumstantial, it is sufficiently striking to warrant consideration of phosphorylation of the cyclic AMP receptor as a mechanism by which it could be rapidly converted from a high affinity to a low affinity state. Since these data suggest that removal of incorporated phosphate from cells occurs as the receptor returns to a higher affinity state, we suggest that a phosphatase acting sequentially with a receptor kinase could produce the alternating states of high and low affinity. This mechanism could cause the rapid "desensitization" of the cyclic AMP receptor thus accounting for the refractory period to cyclic AMP of chemotaxing cells. The binding data presented here suggest that the refractory period to cyclic AMP is mediated directly at the level of the receptor. Klein and Juliani (18) have reported a cyclic AMP-dependent decrease in binding capacity for cyclic AMP of slime mold cells. The properties of this effect appear to be more compatible with the enzymatic

mechanism suggested here than with turnover of receptors. All the component steps of such a cyclic AMP-dependent regulation of cell membrane receptors by phosphorylation have been identified in a variety of unrelated systems (8). The chemotactic system of *D. discoideum* provides an advantageous model for the detailed study of a potentially widespread mechanism of receptor regulation.

Acknowledgements We thank Dr. Luis Glaser for helpful discussion and Sandy K. Lemmon who contributed to the inception of these experiments. This work was supported by NS-13269 and by Cancer Center Support Grant 1P01CA16217.

REFERENCES

1. Bonner, J.T., Barkley, D.S., Hall, E.M., Konijn, T.M., Mason, J.W., O'Keefe, G. and Wolfe, P.B. (1969) Devel. Biol. 20, 72-87.
2. Shaffer, B.M. (1975) Nature 255, 549-552.
3. Malchow, D. and Gerisch, G. (1974) Proc. Nat. Acad. Sci. USA 71, 2423-2427.
4. Green, A.A. and Newell, P.C. (1975) Cell 6, 129-136.
5. Henderson, E.J. (1975) J. Biol. Chem. 250, 4730-4736.
6. Cohen, M.H. and Robertson, A. (1971) J. Theoret. Biol. 31, 101-118.
7. Robinson, G.A., Sutherland, W.E. and Butcher, E. (1971) Cyclic AMP, Academic Press, New York.
8. Greengard, P. (1976) Nature 260, 101-108.
9. Klein, C., Brachet, P. and Darmon, M. (1977) FEBS Lett. 76, 145-147.
10. Loomis, W.F. (1975) *Dictyostelium discoideum*: A Developmental System, Academic Press, New York.
11. Gerisch, G. and Hess, B. (1974) Proc. Nat. Acad. Sci. USA 71, 2118-2122.
12. Bradshaw, R.A. and Frazier, W.A. (1977) Current Topics in Cellular Regulation, 12, 1-37.
13. De Meyts, P. (1976) J. Supramol. Struct. 4, 241-258.
14. Lesniak, M.A. and Roth, J. (1976) J. Biol. Chem. 251, 3720-3729.
15. Weinstein, B.I. and Koritz, S.B. (1973) Devel. Biol. 34, 159-162.
16. Mato, J.M. and Konijn, T.M. (1976) Exp. Cell Res. 99, 328-332.
17. Parish, R.W., Müller, U. and Schmidlin, S. (1977) FEBS Lett. 79, 393-395.
18. Klein, C. and Juliani, M.H. (1977) Cell 10, 329-335.