

## Characteristics of the Induction of Phosphodiesterases by Cyclic Adenosine 3',5'-Monophosphate in the Slime Mold, *Dictyostelium discoideum*

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Cyclic adenosine 3',5'-monophosphate (cAMP) stimulated the induction of cAMP-phosphodiesterases of the slime mold, *Dictyostelium discoideum*. The membrane phosphodiesterase was induced by more than  $10^{-5}$  M of cAMP concentration, while the extracellular enzyme was done 3.5-times higher by  $10^{-6}$  M of cAMP compared with free cAMP. The induction of the extracellular phosphodiesterase by cAMP was inhibited by EDTA, *p*-chloromercuribenzenesulfonate (PCMB), concanavalin A (Con A) and progesterone which all are known to cease differentiation of the slime mold. The derivation of the extracellular enzyme was also stopped by dithiothreitol which reversely accelerates development of the cells. On the other hand, Con A and dithiothreitol further stimulated the induction of the membrane phosphodiesterase by cAMP more than by cyclic nucleotide alone.

Cyclic guanosine 3',5'-monophosphate and N<sup>6</sup>,O<sup>2'</sup>-dibutyryl cyclic adenosine 3',5'-monophosphate are known to have much less chemotaxis-characteristics of the slime mold than cAMP. These cAMP-analogs occurred the syntheses of both the membrane and the extracellular phosphodiesterases to same or more extent. When the surface of the slime mold was treated with trypsin, the induction of only extracellular phosphodiesterase by cAMP was suppressed to half.

From these results, it seems that the induction of synthesis of phosphodiesterases by cAMP in the slime mold is independent of the occurrence of chemotaxis.

**Keywords**—slime mold; *Dictyostelium*; phosphodiesterase; cyclic AMP; differentiation; development

Amoebae of the cellular slime mold, *Dictyostelium discoideum*, have a specific life cycle. Under the existence of nutrients, the slime mold can grow as single cells. When the cells exhaust the nutrients, they aggregate one another in response to chemotactic stimulus of cAMP, and finally result in the formation of a fruiting body through the slug.<sup>2)</sup> A cyclic nucleotide phosphodiesterase [EC 3.1.4.17] is found in the cells and the culture medium. The enzyme activities are regulated in chemotaxis with its specific inhibitor during their differentiation.<sup>3-6)</sup> Klein demonstrated that cAMP reacts not only as a chemotactic factor, but also as an inducer of phosphodiesterases.<sup>7)</sup>

The present paper shows the possibility that the induction of phosphodiesterases by cyclic adenosine 3',5'-monophosphate (cAMP) in the slime mold is independent on the occurrence of chemotaxis.

1) Location: 1-1 Keyakidai, Sakado, Saitama 350-02, Japan.

2) T.M. Konijn, J.G.C. Van De Meene, J.T. Bonner, and D.S. Barkley, *Proc. Nat. Acad. Sci. USA*, **58**, 1152 (1967).

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6) A.M. Malkinson and J.M. Ashworth, *Biochem. J.*, **134**, 311 (1973).

7) C. Klein, *J. Biol. Chem.* **250**, 7134 (1975).

## Experimental

**Materials**—Buffer A: One liter of buffer A contained  $\text{KH}_2\text{PO}_4$  (11.55 g),  $\text{K}_2\text{HPO}_4$  (3.8 g) and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (2.5 g). The pH was adjusted to 6.5 with 0.1 N HCl or NaOH. Buffer B: Buffer B was prepared from buffer A by fivefold dilution. Agar A: One liter of agar A solution contained 200 ml of buffer A and 20 g of agar, and was poured into petri dishes of 9 cm diameter. Nutrient agar: Nutrient agar was prepared with agar A supplemented with 0.5 g yeast extract, 5 g proteose peptone and 5 g dextrose per liter.

**Condition of Growth of the Slime Mold**—Cultures of the slime mold, *Dictyostelium discoideum*, strain NC-4 were initiated by plating spores on nutrient agar in addition of *Aerobacter aerogenes*. The incubation was carried out at 23° for 24 hr. The vegetative cells were harvested and washed with chilled buffer B.

**Induction of Phosphodiesterases of the Slime Mold by cAMP and Others**—After washing the slime mold cells, the cells were incubated on the agar A in a density of about  $10^5$  cells/cm<sup>2</sup> for 2 hr (one hr for only Fig. 4) at 23°. The cells were washed with buffer B and suspended in the same buffer to  $10^8$  cells/ml. The aliquote (0.5 ml) of the suspension was mixed with 1 mM cAMP containing or without containing substances. The mixture was incubated at 23° for 2 hr with gentle shaking, and then centrifuged. The resulting pellet (cells) was washed two more times with buffer B and suspended in buffer B. The supernatant was dialyzed against buffer B at 4° for 18 hr for removing cAMP and lower molecular substances. Subsequently the supernatant was concentrated with the use of Amicon PM-10 membrane. The activity of phosphodiesterase in each sample was determined and represents by the enzyme amount which was produced by 1 mg protein of the slime mold cells.

**Determination of Phosphodiesterase Activity**—The reaction mixture (0.7 ml) contained 0.01 unit of 5'-nucleotidase, 0.01 unit of adenosine deaminase, 70 nmol of cAMP, 2.1  $\mu\text{mol}$  of  $\text{MgSO}_4$  and 6  $\mu\text{mol}$  of triethanolamine-HCl (pH 6.5). The incubation was started by adding 0.1 ml of the sample and was carried out at 23° for 5 or 10 min, and then terminated by 0.1 ml of 5%  $\text{ZnSO}_4$  and 0.1 ml of 0.3 N  $\text{Ba}(\text{OH})_2$  in turn. The supernatant was determined at 265 nm after centrifugation. One unit of enzyme activity is defined by the amount of enzyme which can degrade one nmol of cAMP for one min.

**Assay of Protein**—Protein was determined according to the method of Lowry *et al.*<sup>8)</sup>

**Chemicals**—cAMP, cyclic guanosine 3',5'-monophosphate (cGMP), N<sup>6</sup>,O<sup>2'</sup>-dibutyryl cyclic adenosine 3',5'-monophosphate (dibutyl cAMP), adenosine deaminase, *p*-chloromercuribenzenesulfonate (PCMB), concanavalin A (Con A) and progesterone were purchased from Sigma, and yeast extract and proteose peptone; from Difco.

## Results

### Determination of Membrane Phosphodiesterase Activity

While the deproteinizer for the determination of membrane phosphodiesterase activity was being searched, it was found that  $\text{ZnSO}_4$  and  $\text{Ba}(\text{OH})_2$ , so called Somogi-deproteinizer,

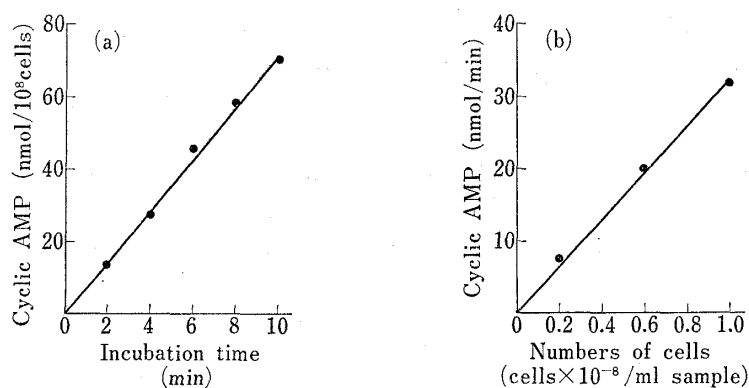


Fig. 1. Determination of Membrane Phosphodiesterase Activity

- (a) Time course; Intact cells ( $10^7$  cells/0.1 ml) were incubated with the reaction mixture (0.7 ml) containing adenosine deaminase, 5' -nucleotidase and cAMP at 23° for 10 min.
- (b) Variation of concentration in samples; One hundred  $\mu\text{l}$  of the various concentrations of the cell suspension were incubated with the reaction mixture (0.7 ml).

The detailed procedure is described in the text.

8) O.H. Lowry, N.J. Rosebrough, A.L. Fae, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

were most proper for this experiment, because the spectra of cAMP and inosine were little affected by the deproteinizing procedure. Fig. 1 shows the time course of membrane phosphodiesterase activity (a), and change of cell concentration (b). It was determined that good linearity for enzyme activity was obtained in this range.

### Effect of External cAMP on Induction of Phosphodiesterases of the Slime Mold

Figure 2 shows the results of the induction of the membrane and extracellular phosphodiesterases by various concentration of cAMP. The induction of extracellular phosphodiesterase was enhanced even by  $10^{-6}$  M of cAMP. The membrane enzyme was, however, induced by a higher concentration than  $10^{-5}$  M cAMP. The extracellular activities at  $10^{-6}$  and  $10^{-5}$  M of cAMP were 4-fold and 9-fold respectively higher than that at free cAMP, while the membrane phosphodiesterase was approximately two times higher at  $10^{-5}$  M cAMP than that at free cAMP.

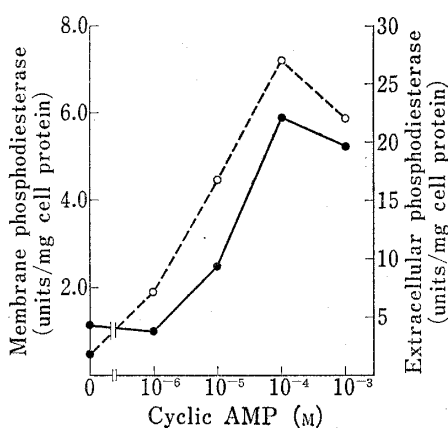


Fig. 2. Influence of cAMP-Concentration on the Inductions of Phosphodiesterases of the Slime Mold

Washed cells ( $0.5 \times 10^8$  cells in 0.5 ml) were incubated with various concentration of cAMP for 2 hr at  $23^\circ$ . After incubation, the activities of membrane (●—●) and extracellular (○—○) phosphodiesterases were determined. The detailed procedure is described in the text.

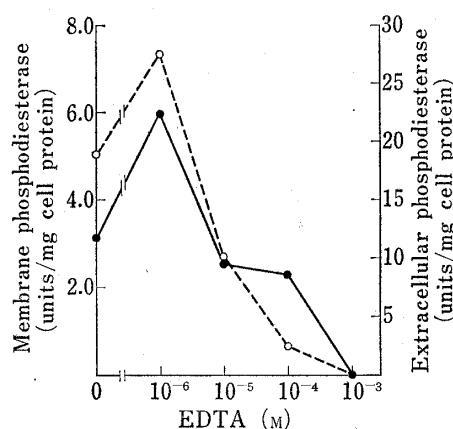


Fig. 3. Effect of EDTA on Induction of Phosphodiesterases of the Slime Mold by cAMP

After the cells were collected, the cells were washed with  $\text{MgSO}_4$  free buffer B four times. Then the cells were incubated with 1 mM cAMP in  $\text{MgSO}_4$ -free buffer B containing various concentration of EDTA for 2 hr at  $23^\circ$ . The detailed procedure is described in the text. Membrane (●—●) and extracellular phosphodiesterase (○—○) activities were determined.

### Influence of EDTA on Synthesis of Phosphodiesterases

The effect of EDTA on induction of phosphodiesterases by 1 mM cAMP was investigated and is shown in Fig. 3. One  $\mu\text{M}$  of EDTA stimulated the induction of both the membrane and the extracellular enzymes, which were both suppressed by a higher concentration of EDTA than  $10^{-5}$  M. The stimulation of both enzyme syntheses by cAMP was completely inhibited by 1 mM EDTA. These results resemble the report by Klein and Bachet in which it showed that the activities of intracellular and extracellular phosphodiesterases decreased to one sixth and one third respectively when the slime mold was incubated in 1 mM EDTA not containing cAMP for 7 hr.<sup>9)</sup>

### Effects of Dithiothreitol, PCMB, Con A and Progesterone on Induction of the Phosphodiesterases

As shown in Table I, dithiothreitol and Con A enhanced the induction of the membrane phosphodiesterase to 1.8-fold in the presence of cAMP. PCMB, however, reduced the induc-

9) C. Klein and P. Bachet, *Nature*, 254, 432 (1975).

TABLE I. Effects of Dithiothreitol, PCMB, Con A and Progesterone on Induction of Membrane and Extracellular Phosphodiesterases of the Slime Mold

Substances	Concentrations	cAMP (1 mM)	Phosphodiesterase activities			
			Membrane		Extracellular	
			(units/mg cell protein)	(%)	(units/mg cell protein)	(%)
None		+	4.01	(100)	22.3	(100)
		-	0.656	(16.4)	5.29	(23.7)
Dithiothreitol	1 mM	+	7.13	(178)	6.82	(30.6)
		-	1.98	(49.4)	3.98	(17.8)
PCMB	1 mM	+	0.752	(18.8)	4.87	(21.8)
		-	0.770	(19.2)	1.68	(7.53)
Con A	100 µg/ml	+	7.18	(179)	4.97	(22.3)
		-	2.04	(50.9)	1.40	(6.28)
Progesterone	30 µM	+	4.37	(109)	7.78	(34.9)
		-	0.809	(20.2)	2.48	(11.1)

The cell suspension ( $5 \times 10^7$  cells/0.5 ml) was incubated in 25 ml of buffer B containing 1 mM cAMP and additionally containing either of dithiothreitol, PCMB, Con A or progesterone. After 2 hr of the incubation at 23°, the cell suspensions and supernatants were prepared as described in the text.

tion to one fifth of the activity of membrane enzyme in the slime mold. The membrane enzyme activity was increased only by dithiothreitol or Con A in the absence of cAMP when compared with the activity of the slime mold which was incubated in only buffer B. The increases of these activities were approximately about three times respectively.

With the extracellular phosphodiesterase, the formation of the enzyme was inhibited by any reagents described in Table I. The range of the inhibition in the induction was from one fifth to one third of the activity when incubated with 1 mM of cAMP.

### The Induction of Phosphodiesterases by cAMP-Analogs

In regard to the specific induction of phosphodiesterases, it was reported by Klein that dibutyryl-cAMP has only one half of the inductive effect of the enzyme when compared with the induction of the same concentration of cAMP; furthermore cGMP did not act as the inducer.<sup>7)</sup> However, in the present study cGMP produced 83% of the induction of both the membrane and extracellular enzymes when compared with cAMP. Dibutyryl-cAMP increased both the membrane and extracellular phosphodiesterases to approximately 130 and 140% of the activities respectively of cAMP (Table II).

TABLE II. Effect of cAMP-Analogs on Synthesis of Membrane and Extracellular Phosphodiesterases of the Slime Mold

Substances	Phosphodiesterase			
	Membrane		Extracellular	
	units/mg cell protein	(%)	units/mg cell protein	(%)
cAMP	3.63	(100)	20.2	(100)
cGMP	3.02	(83.2)	16.8	(83.2)
Dibutyryl-cAMP	4.65	(128)	28.2	(140)
None	1.10	(30.3)	2.65	(13.1)

The density of the cell suspension was adjusted to  $10^8$  cells/ml with buffer B. The suspension (0.5 ml) was incubated with 25 ml of either 1 mM cAMP, cGMP or dibutyryl-cAMP.

### Synthesis of Phosphodiesterases by Trypsin-treated Slime Mold

It is shown in Fig. 4 that the induction of the membrane phosphodiesterase in trypsin-treated cells by cAMP was slightly higher than that in intact cells, while the induction of the extracellular enzyme was suppressed considerably by the trypsin-treatment. Although not illustrated in the figure, the aggregation and the formation of the fruiting bodies of the trypsin-treated cells were prolonged than that of the intact cells.

### Discussion

Somogi's deproteinizer using  $ZnSO_4$  and  $Ba(OH)_2$  is found not to have an effect of the ultraviolet spectra of cAMP and inosine around 265 nm. The membrane phosphodiesterase of the slime mold can be easily determined by the utilization of this deproteinizer as shown in Fig. 1.

In this experiment, it appears that membrane phosphodiesterase of the slime mold as well as extracellular one could be induced by cAMP. The induction of the membrane and extracellular phosphodiesterases was affected by EDTA (Fig. 3), suggesting that the inductions by cAMP were associated with divalent metal ion. Pannbacker and Bravard showed that chemotactic response of the amoebae to cAMP was stimulated by dithiothreitol.<sup>5)</sup> Henderson also demonstrated that dithiothreitol enhanced the binding of cAMP to the aggregation-competent amoebae.<sup>10)</sup> In spite of these results, the induction of extracellular phosphodiesterase was inhibited by this material as shown in Table I.

Con A produced only one fifth of the induction of the extracellular phosphodiesterase of the slime mold in the presence of cAMP (Table I). Con A also inhibited the reaction of the amoebae to cAMP and retarded its aggregation time.<sup>11,12)</sup> However, the membrane enzyme was reversely increased by Con A as well as dithiothreitol.

Progesterone causes the slime mold to remain dissociated and not to exhibit any of the morphological changes observed during starvation, even after 18 hr of incubation.<sup>9,13)</sup> Progesterone also inhibited the induction of the extracellular phosphodiesterase by cAMP (Table I). This steroid probably affected the membrane surface of the amoebae, and therefore, inhibited the response of the amoebae to cAMP.

cGMP is a good substrate for phosphodiesterase, and a potent inhibitor of cAMP hydrolysis by these enzymes. However, its chemotactic and other biological activities are 1000-fold lower than those of cAMP.<sup>14,15)</sup> In spite of these findings cGMP produced 83% of induction in both the membrane and the extracellular phosphodiesterases when compared with cAMP

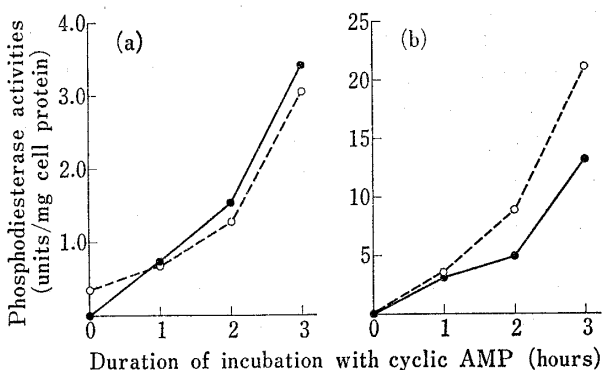


Fig. 4. Induction of Phosphodiesterases by the Trypsin-treated Slime Mold

The slime mold cells were starved for one hr on agar A. The cells were washed with 20 mM phosphate buffer (pH 6.5) containing 1 mM EDTA and suspended in same buffer to  $10^8$  cells/ml. One ml aliquote of the suspension was incubated with 2 ml of the above buffer containing or without 1 mg of trypsin at 23° for 40 min. The cells were washed with buffer B and resuspended in buffer B to  $10^8$  cells/ml. Aliquotes (0.5 ml) of the suspensions were added into 25 ml of buffer B containing 1 mM cAMP. After the incubation of the mixtures at 23° for duration described in the figure, cell suspensions and supernatants were prepared as the procedure described in the text. (a) Membrane phosphodiesterase activities, (b) Extracellular enzyme activities. The samples are trypsin-treated cells (●—●) and control cells (○---○).

10) E.J. Henderson, *J. Biol. Chem.*, **250**, 4730 (1975).

11) M.U. Gillette and J.F. Filosa, *Biochem. Biophys. Res. Commun.* **53**, 1159 (1973).

12) M. Darmon and C. Klein, *Biochem. J.*, **154**, 743 (1976).

13) P.H. Brachet and C. Klein, *Exp. Cell Res.*, **93**, 159 (1975).

14) G. Gerisch and B. Hess, *Proc. Nat. Acad. Sci. USA*, **71**, 2118 (1974).

15) D. Malchow and G. Gerisch, *Proc. Nat. Acad. Sci. USA*, **71**, 2432 (1975).

(Table II). Dibutyryl-cAMP is also an inhibitor of phosphodiesterase activity and has a very low chemotactic activity.<sup>16)</sup> The induction of synthesis of the membrane and the extracellular enzymes also occurred by this cAMP analog. Their inductions by these analogs were probably carried out by the same mechanism as that by cAMP.

When the surface of the slime mold was treated with proteolytic enzyme, trypsin, the induction of extracellular phosphodiesterase by cAMP was decreased to one half (Fig. 4). Therefore, the induction of the enzyme seems to be associated with surface protein.

Judging from the above results cAMP-binding sites for the induction of the phosphodiesterases may possibly exist on the cell membrane, which may be different characteristics from the sites for chemotaxis.

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16) T.M. Konijn, "Antibiotics and Chemotherapy," Vol. 19, ed. by Karger, S. Separatum, Basel, 1974, pp. 96—110.