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Possible Involvement of Calmodulin in the Induction of Phosphodiesterase by Cyclic Adenosine 3',5'-Monophosphate in *Dictyostelium discoideum*

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We reported previously that Ca^{2+} participates in the induction of phosphodiesterase [EC 3.1.4.17] (PDE) by cyclic adenosine 3',5'-monophosphate in *Dictyostelium discoideum* [F. Yamasaki and H. Hayashi, *J. Biochem. (Tokyo)*, **92**, 1911 (1982)]. The results reported here raise the possibility that the action of Ca^{2+} in the induction of PDE may be mediated by calmodulin. Cells were incubated with cyclic adenosine 3',5'-monophosphate in the presence of various calmodulin inhibitors, and the cellular and extracellular PDE activities were determined. All calmodulin inhibitors reduced the inductions of both cellular and extracellular PDEs, and the order of effectiveness was trifluoperazine, chlorpromazine, dibucaine, tetracaine and W-7. It was confirmed that these calmodulin inhibitors neither decrease the cell viability, nor reduce PDE activity directly. The inhibition of PDE induction by dibucaine was not overcome by addition of phospholipid and CaCl_2 , ruling out the participation of Ca^{2+} in membrane phospholipid. We also ruled out any involvement of Ca^{2+} -activated phospholipid-dependent protein kinase, which is reported to be inhibited by calmodulin inhibitors, because this activity was not found in the slime mold even in the absence of the inhibitors.

On the basis of the above results, calmodulin may be considered to play an important role in the induction of PDE by cAMP in the slime mold.

Keywords—*Dictyostelium*; slime mold; phosphodiesterase; cAMP; calcium; calmodulin

Amoebae of the cellular slime mold *Dictyostelium discoideum* are very useful experimental materials, since *Dictyostelium* cells differentiate only into spore and stalk cells. The first step of the differentiation is cell aggregation. Before and during the aggregation the cells release phosphodiesterase (PDE) as a result of stimulation by cyclic adenosine 3',5'-monophosphate (cAMP).¹⁾ We as well as Klein reported that PDE is induced by cAMP.^{2,3)} Although the mechanism of PDE induction is not yet fully understood,⁴⁻⁷⁾ it is known that PDE induction by cAMP requires Ca^{2+} .⁷⁾ In this paper, we propose possibility that the action of Ca^{2+} in the induction of PDE by cAMP may be mediated by calmodulin.

Experimental

Stimulation of PDE Synthesis—Cells of *Dictyostelium discoideum* amoebae, strain NC-4, were used in all experiments. The slime mold cells were harvested as reported previously²⁾ and the aggregation competent cells were suspended in 20 mM phosphate buffer (pH 6.5) containing 2 mM MgSO_4 (buffer) at a density of 2.5×10^8 cells/ml. An aliquot (0.4 ml) of the suspension was incubated with 1 mM cAMP in 4 ml of buffer at 23 °C with gentle shaking in the presence of various calmodulin inhibitors. After centrifugation of the incubation mixture, the resulting supernatant was dialyzed against buffer and the precipitate was washed to remove cAMP and the inhibitors as previously⁷⁾; and they were used as preparations of extracellular and cellular PDE, respectively.

Assays of Enzyme Activities and Protein Content—The PDE activity was determined at 35 °C by the method described previously.²⁾ The activity of Ca^{2+} -activated phospholipid-dependent protein kinase (protein kinase C) was assayed according to the method of Mori *et al.* using CaCl_2 , phosphatidylserine, diolein, H1 histone and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (8.1 Ci/mmol).⁸⁾ Protein was measured according to the method of Lowry *et al.*⁹⁾

Preparation of Phospholipid—Lipid was extracted from rat erythrocytes by the method of Turner and

Rouser.¹⁰⁾ Phospholipid was partially purified by thin layer chromatography.¹¹⁾ Phospholipid phosphorus was determined by the method of Bartlett.¹²⁾

Materials—Trifluoperazine, chlorpromazine, dibucaine, tetracaine, phosphatidylserine, diolein and H1 histone were purchased from Sigma Chemicals (U.S.A.), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was from Rikaken Co. (Nagoya, Japan) and [γ -³²P]ATP (8.1 Ci/mmol) was from New England Nuclear (U.S.A.).

Results and Discussion

Inhibition of PDE Induction by Various Calmodulin Inhibitors

The slime mold cells contain calmodulin, the properties of which have been partly elucidated.^{13–16)} However, its function is not yet clear. It has been shown¹³⁾ that this calmodulin does not itself affect the PDE activity of the slime mold. The purpose of the present experiment was to determine whether or not calmodulin is involved in PDE induction. For this purpose, the cells were incubated with 1 mM cAMP in the presence of various calmodulin inhibitors. All the calmodulin inhibitors reduced the inductions of both cellular

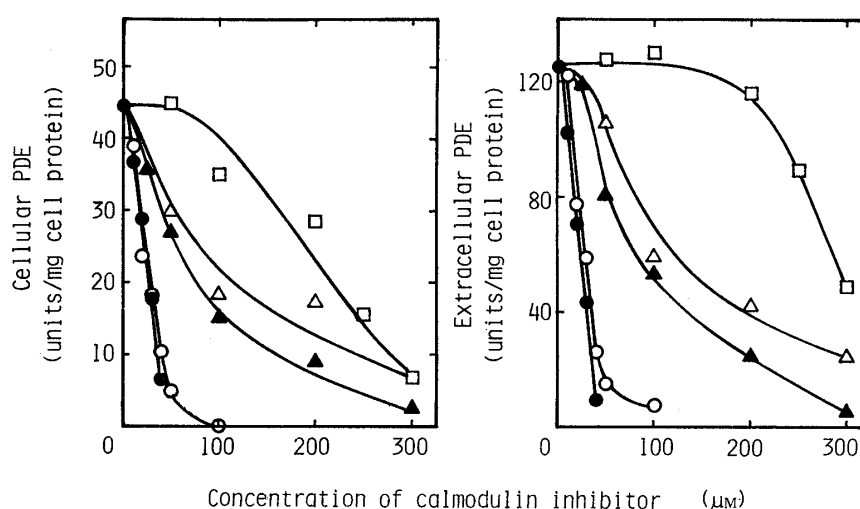


Fig. 1. Effects of Various Calmodulin Inhibitors on PDE Induction by cAMP

The cells were incubated with various concentrations of trifluoperazine (●), chlorpromazine (○), W-7 (□), dibucaine (▲) or tetracaine (△) in the presence of 1 mM cAMP for 2 h, then the mixture was centrifuged. The activities of cellular and extracellular PDE were determined.

and extracellular PDEs (Fig. 1). Cellular PDE was inhibited to the same extents as extracellular PDE. The induction was most strongly inhibited by trifluoperazine and chlorpromazine, which almost completely inhibited the induction at 40–50 μ M. The next strongest inhibitors were dibucaine and tetracaine (70–80% inhibition at 200 μ M), while 200 μ M W-7 inhibited the induction of cellular PDE by ca. 50%, but hardly affected that of extracellular PDE. Trifluoperazine has been reported to inhibit completely the activation of bovine brain PDE by *Dictyostelium* calmodulin at 30 μ M.¹⁴⁾ In this experiment, approximately the same concentration of trifluoperazine inhibited PDE induction.

When these calmodulin inhibitors were added to the incubation mixture after PDEs had been already induced by cAMP, the activities of PDEs were not influenced by the inhibitors (data not shown), supporting the results of Clarke *et al.*¹³⁾

Survival of the Cells after Treatment with Calmodulin Inhibitors

It is possible that the apparent reduction of PDE induction by the calmodulin inhibitors simply occurred owing to the toxicity of the inhibitors, resulting in a lower cell population. To test this possibility, the cells were treated with trifluoperazine (40 μ M), chlorpromazine (50 μ M),

TABLE I. Effects of Phospholipid and Calcium on PDE Induction Inhibited by Dibucaine

Agents	Cellular PDE		Extracellular PDE	
	units/mg cell protein	(%)	units/mg cell protein	(%)
None	4.33	(100)	33.4	(100)
156 $\mu\text{g/ml}$ phospholipid	3.01	(69.5)	22.3	(66.8)
312 $\mu\text{g/ml}$ phospholipid	2.34	(54.0)	21.1	(63.2)
1 mM CaCl_2	5.02	(116)	30.9	(92.5)
156 $\mu\text{g/ml}$ phospholipid and 1 mM CaCl_2	3.28	(75.8)	35.0	(105)

The cells were incubated with 300 μM dibucaine and 1 mM cAMP for 1 h at 23°C, then the incubation mixture was supplemented with phospholipid and/or CaCl_2 , and further incubated for 3 h.

W-7 (300 μM), dibucaine (300 μM) or tetracaine (500 μM) for 1 h, then the inhibitors were removed by washing, and the PDE activities that developed in 3 h were determined. In all cases, the cellular and extracellular PDE activities of washed cells were both significantly higher than those of unwashed cells (data not shown). Furthermore, essentially all the washed cells could form fruiting bodies on gel plates within 24 h (data not shown). These data suggest that the calmodulin inhibitors were not toxic to the cells.

Effects of Phospholipid and Calcium on PDE Induction in Cells Pretreated with Dibucaine

Dibucaine at a high concentration influences membrane phospholipid, and displaces Ca^{2+} from the membrane.^{17,18)} If the inhibition of PDE induction by dibucaine occurred as a result of some effect on membrane phospholipid, the induction should recover upon the addition of phospholipid and CaCl_2 . However, no increase of PDE activity was found after the addition of phospholipid and CaCl_2 (Table I). This result may suggest that membrane phospholipid is not directly involved in PDE induction.

Activity of Protein Kinase C

The above calmodulin inhibitors also suppress the activation of protein kinase C, which requires Ca^{2+} and phospholipid.⁸⁾ Therefore, it remains possible that the inhibition of PDE induction by calmodulin inhibitors is associated with protein kinase C. However, we could not detect protein kinase C activity in the slime mold (data not shown), thus eliminating the possibility that the result obtained in Fig. 1 reflects some action of protein kinase C.

Conclusion

We showed previously that Ca^{2+} participates in PDE induction by cAMP.¹²⁾ The present results indicate that the action of Ca^{2+} in the PDE induction by cAMP is probably mediated by calmodulin.

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