CORRELATION OF SUBSTRATE SPECIFICITY OF cAMP-PHOSPHODIESTERASE IN *DICTYOSTELIUM DISCOIDEUM* WITH CHEMOTACTIC ACTIVITY OF cAMP-ANALOGUES

Dieter MALCHOW and Janet FUCHILA
Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, 74 Tübingen, W. Germany

and

Bernd JASTORFF*
Max-Planck-Institut für Experimentelle Medizin, 34, Göttingen, W Germany

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1. Introduction

cAMP is thought to be the natural attractant for chemotaxis in amoebae of *Dictyostelium discoideum* [1—3]. During growth, amoebae secrete a cAMP-hydrolyzing phosphodiesterase into the culture medium [4—6] and at the end of growth a macromolecular heatstable phosphodiesterase-inhibitor is also released by the amoebae [6—8]. During differentiation to aggregation—competence a phosphodiesterase is detected on the cell surface, exhibiting its highest activity during aggregation. This cell-bound phosphodiesterase has been suggested to function as part of the chemotactic receptor system [9].

Recently cAMP-analogues have been tested for their chemotactic activities [10—12]. We selected several analogues with different biological activities and determined the analogue specificity of cAMP-phosphodiesterase in order to answer the following questions: 1) Does the analogue specificity of the phosphodiesterase correlate with the chemotactic activity of the analogues; 2) How is the substrate specificity affected by stereochemical alterations of the cAMP molecule and 3) Do the extracellular and the particle-bound phosphodiesterase possess the same substrate specificity?

2. Materials and methods

Culture conditions of *Dictyostelium discoideum* strains Ax-2, mutant aggr 50 and mutant 75 were as described previously [9, 13]. Dialyzed culture supernatant from mutant aggr 50 was used as a source for extracellular phosphodiesterase [8] except for those analogues which were difficult to hydrolyze (fig. 1c, d, f). These were tested with concentrated phosphodiesterase from aggr 75 purified on DEAE-cellulose [8]. Particle-bound phosphodiesterase was prepared from Ax-2 and the phosphodiesterase assays were performed as described elsewhere [9, 13].

Inhibition of cAMP-hydrolysis by analogues was carried out using the assay conditions of particle-bound

* Present address: Universität Bremen, NW1, 28 Bremen 33, W. Germany.

Abbreviations:
cAMP = adenosine-3',5'-cyclic phosphate; cGMP = guanosine-3',5'-cyclic phosphate; 5'-CH_2-AMP = 5'-methylene-5'-deoxy-adenosine 3',5'-cyclic phosphate; 3'-CH_2-cAMP = 3'-methylene-3'-deoxy-adenosine 3',5'-cyclic phosphate; 5'-NH-cAMP = 5'-amino-5'-deoxy-adenosine-3',5'-cyclic phosphate; 5'-NH-cAMPS = 5'-amino-5'-deoxy-adenosine-3',5'-cyclic phosphothioate (diastereomers I and II, the absolute configurations are not known at present); 5'-NCH_3-cAMP = 5'-N-methylamino-5'-deoxy-adenosine-3',5'-cyclic phosphate; 5'-NC_8H_{17}-cAMP = 5'-N-n-octyl-amino-5'-deoxy-adenosine-3',5'-cyclic phosphate; Enzymes: adenosine deaminase (EC 3.5.4.4); alkaline phosphatase (EC 3.1.3.1).

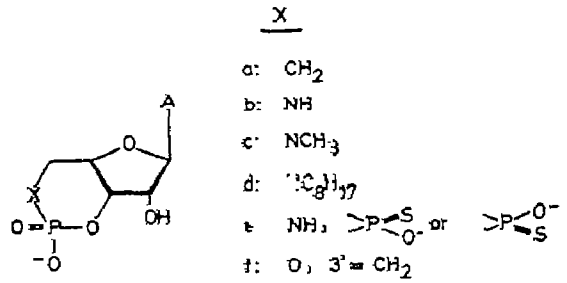

Fig. 1. Chemical structure of cAMP-analogues, modified in the phosphate moiety.

phosphodiesterase [9]. The reaction was started by enzyme.

Hydrolysis of analogues was performed at a substrate concentration of 6×10^{-4} M with phosphodiesterase in 50 mM Tris–HCl, pH 7.4, at 36°C (pH 8.4 was used for 5'-NCH_3-cAMP because of the better stability of this analogue at alkaline pH). If possible the enzyme activity was chosen to yield 50% hydrolysis within 1–5 hr of incubation. The extent of hydrolysis was evaluated at various time intervals by thin-layer chromatography of the products on cellulose-F plates (Merck, Darmstadt, W. Germany). Solvents used were 95% ethanol/1 M ammonium acetate 7:3 and n-propanol/ammonia/water 7:1:2. Hydrolysis of 6- and 8-substituted cyclic nucleotides was tested by phosphate liberation using phosphodiesterase in presence of 20 μg/ml alkaline phosphatase (EC 3.1.3.1, Boehringer, Mannheim, W. Germany) and 8 mM $MgSO_4$ in 50 mM Tris–HCl buffer, pH 7.4 at 36°C. Rate of hydrolysis at nucleotide concentrations of 1 and 2 mM was not significantly different which indicates that V_{max} was reached at these concentrations. Inorganic phosphate was assayed according to Ames and Dubin [14]. Control tubes were incubated with alkaline phosphatase only to correct for free 5' nucleotides.

Methylene-cAMP-analogues were kindly provided by Dr. J.G. Moffat (Syntex Research Center, Palo Alto, Calif., USA) and 6- and 8-substituted cyclic nucleotides by Boehringer, Mannheim, W. Germany. The synthesis and characterization of the 5'-amido-analogues of cAMP have been described elsewhere [15, 16].

3. Results

3.1. *Substrate specificity and chemotactic activity of the cAMP-analogues*

The chemical structures of cAMP-analogues modified in the phosphate moiety are shown in fig. 1 and their chemotactic activities as measured by T.M. Konijn [11, 12] are presented in table 1.

Since it is nearly impossible to determine the Michaelis constants for the non-radioactivity labeled analogues, we measured as a rough estimate for binding the inhibition of cAMP hydrolysis using particle-bound

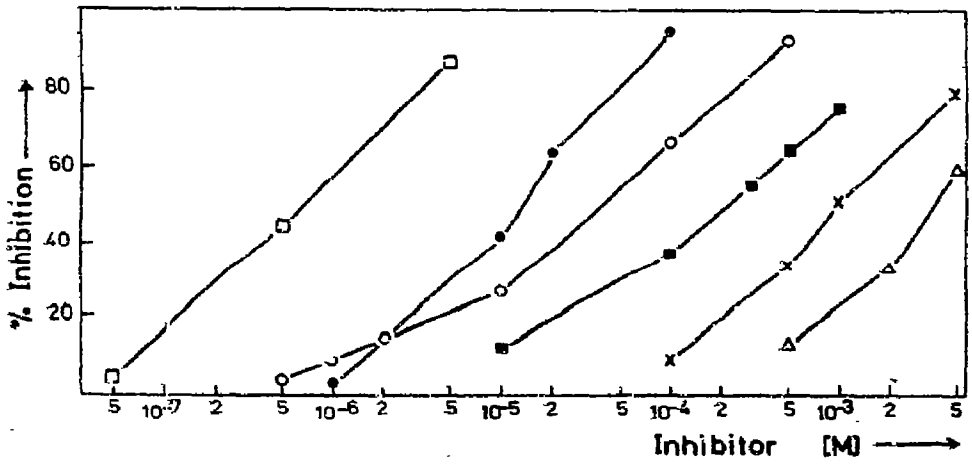

Fig. 2. Inhibition of cAMP-hydrolysis by cAMP-analogues. Hydrolysis was performed at 5×10^{-7} M cAMP using particle-bound phosphodiesterase. □: 5'-NH-cAMP; ○: 5'-CH_2-cAMP; ●: cGMP; ■: 5'-NCH_3-cAMP; ×: 5'-NC_8H_{17}-cAMP; △: 3'-CH_2-cAMP.

Table 1
Rates of hydrolysis of cAMP-analogues by extracellular and particle-bound phosphodiesterase.

Cyclic nucleotide	Chemotactic activity[1] 50% of populations react positively between: (M)	50% Hydrolysis[2] by phosphodiesterase	
		extracellular (hr)	particle-bound (hr)
cAMP	10^{-8}–10^{-9}	1	1
5'-CH_2-cAMP	10^{-8}–10^{-9}	4	4
5'-NH-cAMP	10^{-8}–10^{-9}	6	6
5'-NH-cAMPS I	10^{-6}–10^{-7}	8	8
5'-NH-cAMPS II	10^{-5}–10^{-6}	8	8
cGMP	10^{-5}–10^{-6}	1	1
5'-NCH_3-cAMP	10^{-4}–10^{-5}	2000	> 150[3]
5'-NC_8H_{17}-cAMP	10^{-3}–10^{-4}	12000[4]	> 150[3]
3'-CH_2-cAMP	10^{-2}–10^{-3}	> 18000[5]	> 150[5]

1) Data for chemotactic activities were taken from Konijn et al. [10, 12]. 2) Hydrolysis was evaluated by thin layer chromatography of the reaction products. Relative rates are shown. 50% Hydrolysis of cAMP was set to 1 hr. 3) The specific activity of particle-bound phosphodiesterase was too low to obtain hydrolysis within reasonable incubation times. 4) The time for 50% hydrolysis was calculated from the time required for 25% hydrolysis assuming a linear relationship. 5) No hydrolysis was observed for both enzymes with 3'-CH_2-cAMP.

phosphodiesterase (fig. 2). Hydrolysis was performed at 5×10^{-7} M cAMP which corresponds to the lowest Michaelis constant measured for particle-bound phosphodiesterase. This enzyme shows an anomalous kinetic indicating either negative cooperativity or a set of enzymes with different K_m-values (Malchow et al., in preparation). In addition we determined the time required for 50% hydrolysis of the analogous relative to cAMP hydrolysis using extracellular phosphodiesterase and particle-bound phosphodiesterase at substrate concentrations which were in the range of V_{max} for cAMP (table 1).

Fig. 2 shows that 5'-NH-cAMP (fig. 1b) was a powerful inhibitor of cAMP-hydrolysis yielding a 45% inhibition at a 1:1 analogue/cAMP ratio. Substitution of the amino group by a methylamino- (fig. 1c) or an *n*-octylamino-group (fig. 1d) shifted the 50% inhibition to an analogue/cAMP ratio of 400 or 2000:1. 5'-CH_2-cAMP (fig. 1a) was a 100-fold better inhibitor than 3'-CH_2-cAMP (fig. 1f) yielding a 50% inhibition at an 80:1 analogue/cAMP ratio.

As shown in table 1 all cAMP-analogues were hydrolyzed by the phosphodiesterase except 3'-CH_2-cAMP. The weakly inhibiting analogues 5'-NCH_3-cAMP and 5'-NC_8H_{17}-cAMP were slowly hydrolyzed and were relatively inactive in chemotaxis whereas the strong inhibiting 5'-NH-cAMP was easily hydrolyzed having the same biological activity as cAMP.

Another analogue which is as active as cAMP in chemotaxis, 5'-CH_2-cAMP is 10^6-fold more active than its isomer 3'-CH_2-cAMP. This large difference may be mainly due to the fact that the 3'-compound is not hydrolyzed, since the weaker binding of 3'-CH_2-cAMP alone could not account for the drastic difference in the biological activities of the two analogues.

The rate of hydrolysis of the cyclic nucleotides at $V_{max\ cAMP}$ did not in each case correspond to the threshold concentrations for chemotactic activity (table 1). Because at low concentrations binding rather than hydrolysis would be expected to be the limiting factor for chemotactic activity, the latter should be reflected more accurately by the K_m values. To some extent this is the case for cGMP which is far less active in chemotaxis than cAMP but is hydrolyzed at the same rate as cAMP. The K_m value of extracellular phosphodiesterase for cGMP is 10-fold higher than for cAMP [8]. Unfortunately, radio-labeled analogues to determine their K_m values are not available.

3.2. *Structure dependency of phosphodiesterase-hydrolysis*

Exchange of the oxygen atom at the 5' position of the cAMP molecule by NH- or CH_2-groups did not significantly alter the rates of hydrolysis and 5'-NH-cAMP proved to be a powerful inhibitor (table 1, fig. 2). However, substitution by protruding groups within the phosphate ring, drastically reduced the rates of hydrolysis (table 1) and similarly decreased the amount of inhibition (fig. 2).

Exchange of the oxygen atom at the 3' position by a CH_2-group completely abolished hydrolysis by phosphodiesterase and inhibition of cAMP-hydrolysis occurred only at high analogue concentrations. It seems therefore that not only for hydrolysis of the analogue but also for good competition of cAMP-hydrolysis, the cyclic ring must be chemically reactive at the 3' position.

6- Or 8-substitution of cyclic nucleotides by protruding groups did not greatly affect the rates of

Table 2
Rates of hydrolysis of, and inhibition of cAMP-hydrolysis by 6- and 8-substituted cyclic nucleotides.

Cyclic nucleotide	Hydrolysis by phosphodiesterase[1]		Inhibition of cAMP-hydrolysis[2]
	extracellular (%)	particle-bound (%)	(%)
cAMP	100	100	
cAMP-derivatives			
6-Benzoyl	104	97	72
6-Benzyl	102	115	67
8-Bromo	138	137	52
8-Piperidino	60	59	20
cGMP-derivatives			
8-Benzylamino	151	162	19
8-Bromo	106	128	32
cIMP-derivatives			
8-Benzylamino	83	90	11
8-Bromo	147	150	28

1) The rate of hydrolysis was determined at 1 and 2 mM nucleotide concentrations by phosphate liberation in presence of alkaline phosphatase. Data are presented as percent hydrolysis of cAMP (= 100%). 2) Inhibition of cAMP-hydrolysis using extracellular phosphodiesterase was assayed at 7×10^{-5} M cAMP and 2×10^{-4} M cyclic nucleotide concentration.

hydrolysis at $V_{max\ cAMP}$ (table 2). Inhibition of cAMP hydrolysis revealed that 8-substitution reduced the inhibiting activity much more than 6-substitution (table 2).

3.3. *Substrate-specificity of extracellular and particle-bound phosphodiesterase*

The rates of hydrolysis of cyclic nucleotides and their analogues, were found to be the same for extracellular and particle-bound phosphodiesterase (tables 1 and 2).

4. Conclusions

In this communication, we have shown that binding of cAMP-analogues to particle-bound phosphodiesterase – as measured by inhibition of cAMP-hydrolysis – was fairly well correlated with the ability of these analogues to trigger a chemotactic response in amoebae. Additionally, all analogues were hydrolyzed by phosphodiesterase except 3′-CH_2-cAMP. This analogue however, is 10^6-fold less active in chemotaxis than 5′-CH_2-cAMP [10]. Thus, the results are in accord with the idea that for adequate biological function the cyclic nucleotide has to be hydrolyzed to ensure a continuous measurement of its concentration and hence, a correct orientation of the cell within the morphogenetic field [9].

Konijn et al., using their biological assay system, found that the cAMP receptor was highly sensitive to stereochemical modifications at the 5′ position of the cyclic phosphate ring [12]. This sensitivity to chemical alterations by protruding groups at the 5′ position was also observed for the rate of hydrolysis and for the inhibition of cAMP-hydrolysis. Furthermore, we have shown that 8-substituted cyclic nucleotides were less inhibiting than the 6-substituted ones, indicating a stronger steric hindrance for binding of the 8-substituted analogues. In agreement with this observation, Michal et al. found that 8-substituted cyclic nucleotides were worse substrates for bovine heart muscle phosphodiesterase than 6-substituted ones [17].

One pertinent question in the phosphodiesterase regulation is whether the cell-bound phosphodiesterase and the extracellular one are modifications of one and the same enzyme. Though this question will be solved only by solubilization and purification of the particle-bound phosphodiesterase, the finding that both enzymes exhibited the same substrate specificity at $V_{max\ cAMP}$ with all analogues tested may favour the idea of their identity.

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