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Kinetics and nucleotide specificity of a surface cAMP binding site in *Dictyostelium discoideum*, which is not down-regulated by cAMP

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1. SUMMARY

Dictyostelium cells exhibit four types of kinetically distinct surface cAMP binding sites, the A^H, A^L, B^S, and B^{SS} sites, which are down-regulated during persistent stimulation with cAMP. Although most cAMP-induced responses are subject to desensitization during constant stimulation, some responses, notably the induction of post-aggregative gene expression, require persistent cAMP stimulation. The kinetics and specificity of residual cAMP-binding activity in cells treated for 4 h with micromolar cAMP were investigated. It was found that around 4000 rapidly dissociating binding sites per cell with an affinity of about 300 nM are retained after down-regulation. The nucleotide specificity of the remaining sites was very similar, but not completely identical to the A^H, A^L and B sites, suggesting that these sites belong to the same class of cell surface cAMP receptors and may be utilized to mediate responses requiring continuous cAMP stimulation.

2. INTRODUCTION

Extracellular cAMP regulates major aspects of the *Dictyostelium discoideum* developmental program. It functions as a chemoattractant during aggregation [1] and probably also during multicellular morphogenesis [2]. Furthermore, cAMP regulates the expression of several classes of genes during different stages of development [3]. cAMP interacts with cell surface receptors and elicits the accumulation of several intracellular second messengers such as cAMP, cGMP, inositol-(1,4,5)-triphosphate and Ca^{2+} ions via interaction with GTP-binding proteins [4–9]. These re-

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sponses are transient, because the cells adapt to constant cAMP levels [10,11].

So far, four kinetically distinct receptor forms (A^H, A^L, B^S, and B^{SS}) with different dissociation constants and dissociation rates have been characterized [12–15], which show virtually identical cyclic nucleotide-binding specificity. Besides the cell surface cAMP receptor, three other cAMP-binding proteins have been identified: an intracellular cAMP-dependent protein kinase (CAK) [16], an intracellular cAMP-binding protein of unknown function (CABP1) [17] and a cAMP-phosphodiesterase (cAMP-PDE) [18]. The nucleotide specificity of these cAMP-binding proteins is very different from that of the surface receptors [14,15,19,20]. Cyclic AMP-induced responses such as chemotaxis, cAMP and cGMP accumulation and the induction of post-aggregative gene expression show a nucleotide specificity which is similar to that of the surface cAMP receptor and completely different from the intracellular cAMP binding proteins, suggesting that all these responses are mediated by surface cAMP receptors [21–23].

Persistent stimulation of cells with cAMP induces down-regulation of cAMP binding activity [24–26]. During this process, surface cAMP receptors are sequestered into vesicles and degraded [27]. Most cAMP-induced responses are concomitantly down-regulated, but some responses, such as the induction of post-aggregative gene expression require continuous stimulation with micromolar cAMP concentrations during several hours [23]. In this study the effect of prolonged cAMP stimulation on binding activity on the cell surface was examined. The kinetics and cyclic nucleotide specificity of the remaining binding sites was investigated.

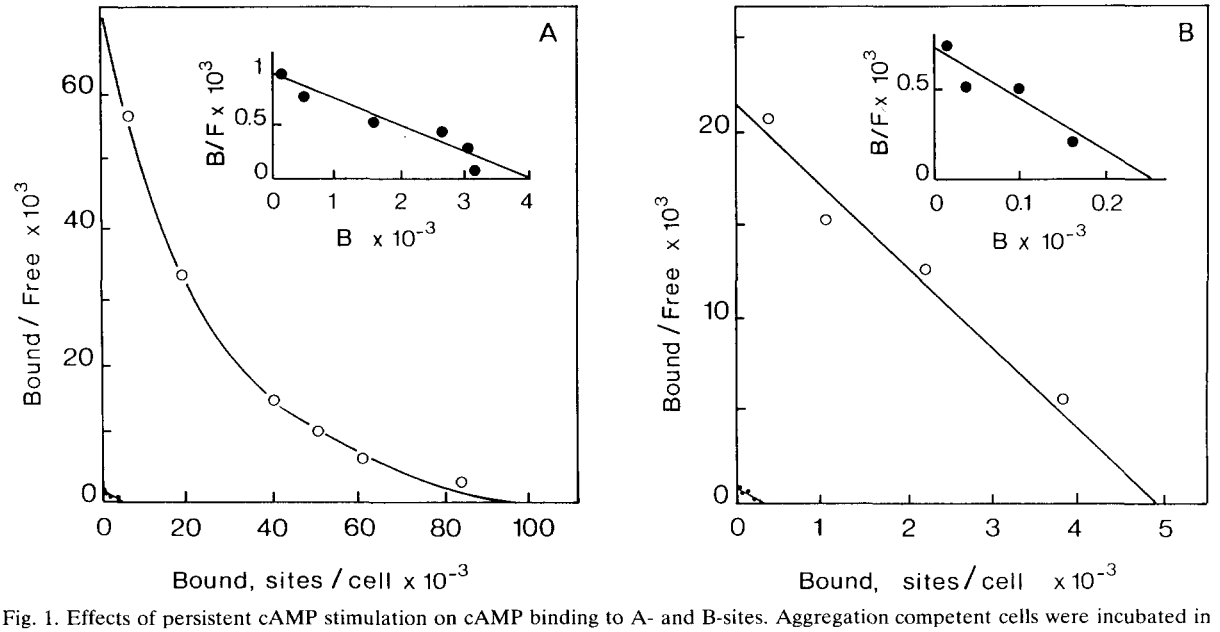

Fig. 1. Effects of persistent cAMP stimulation on cAMP binding to A- and B-sites. Aggregation competent cells were incubated in the absence (○) or presence (●) of 100 μM cAMP. After 4 h, cells were collected and thoroughly washed with PB. To measure total cAMP-binding activity, cells were incubated with 20, 100, 500, 1000, 2000 and 10000 nM [^{3}H]-cAMP for 1 min incubation at 0 °C (**A**). To measure binding to B-sites, cells were first incubated for 1 min at 0 °C with 2, 10, 30, 100 nM [^{3}H]-cAMP and precipitated after a 10-s chase with 100 μM cAMP (**B**). The inserts are enlargements of the main figure with both axes magnified by the same factor (15× in A and 10× in B). The results shown are the means of triplicate determinations of an experiment that was reproduced once.

3. MATERIALS AND METHODS

3.1. Culture and incubation conditions

Dictyostelium discoideum strain NC4 was grown in association with *Escherichia coli* 281 on glucose peptone agar. Cells were freed from bacteria by repeated washings with PB (10 mM Na/K-phosphate buffer pH 6.5), distributed on non-nutrient agar (1.5% agar in PB) and incubated for 16 h at 6 °C to induce aggregation-competence [23].

3.2. Materials

[8-^{3}H]cAMP (1.92 TBq/mmol) was obtained from Amersham (U.K.). The cAMP derivatives 1, 3, 5, 6, 13 and 14 were obtained form Boehringer (F.R.G.); compound 5 was also purchased from Sigma (U.S.A.). Compound 4 was a generous gift of Dr. R. Hanze (The Upjohn Co.). Compounds 2, 7, 8, 11 and 12 were synthesized as previously described [28–32]. Compound 17 was kindly supplied by Dr. D. Shugar (Polish Acad. of Science, Warsaw). Compounds 9, 10, 15, 16, 18 and 19 were synthesized according to the method of Genieser et al. [33,34].

3.3. cAMP binding assays

Scatchard analysis of cAMP binding to A sites was performed by incubating 8×10^6 aggregation competent cells for 1 min at 0 °C with 2–10000 nM [^{3}H]-cAMP and 5 mM dithiothreitol (final concentrations) in a total volume of 100 μl. The cells were subsequently centrifuged through silicone oil and the radioactivity of the pellet was measured [12]. For Scatchard analysis of B sites, 8×10^6 cells were incubated for 1 min at 0 °C with 2–100 nM [^{3}H]-cAMP and 5 mM DTT in a total volume of 100 μl, subsequently the incubation mixture was diluted with 1 ml 100 μM cAMP, and after an additional 10 s the cells were centrifuged through silicon oil.

To measure nucleotide specificity of A^H, A^L and B sites, cells were incubated for 1 min at 0 °C with respectively 3, 100 or 10 nM [^{3}H]-cAMP and increasing concentrations of unlabelled cyclic nucleotides. To assay A^H and A^L sites, cells were immediately centrifuged through silicon oil; to assay B sites, cells were centrifuged after a 10-s chase with 100 μM cAMP. To measure the specificity of sites resistent to down-regulation, aggregation competent cells were pre-incubated for 4 h with 100 μM cAMP, added at 60-min intervals, washed four times with PB, and resuspended to 10^8 cells/ml. Cells were incubated with 10 nM [^{3}H]-cAMP and cAMP derivatives for 1 min at 0 °C and centrifuged through silicon oil. Assay blanks for all assays were obtained by including 100 μM cAMP in the incubation mixture.

4. RESULTS AND DISCUSSION

4.1. Scatchard analysis of down-regulated cells

Kinetic studies showed that 96% cAMP-binding activity in aggregation competent cells consists of rapidly dissociating A sites (off-rate ap-

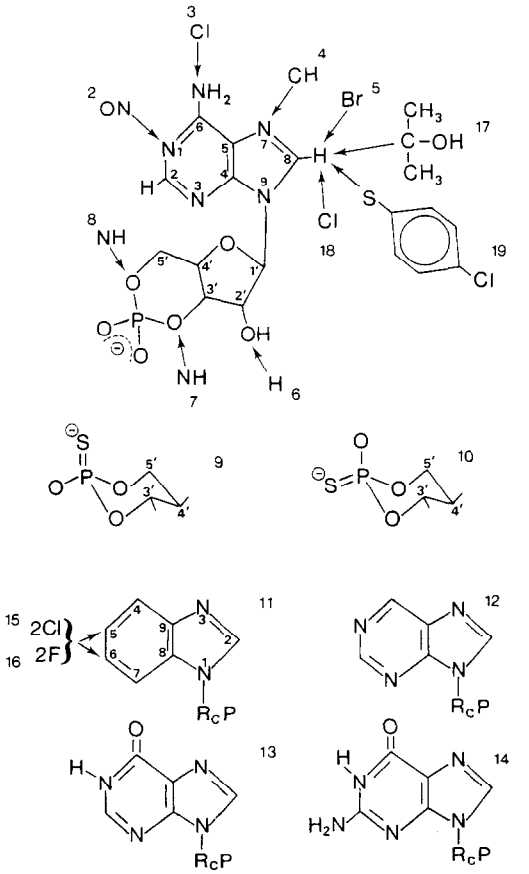

Fig. 2. Structures of cAMP derivatives.

prox. 1.5 s), which exist in a high (A^H) and a low (A^L) affinity form. The remaining 4% binding activity represent the slow (off-rate approx. 15 s) and superslow (off-rate approx. 150 s) dissociating B^S and B^{ss} sites [12,13]. A short (15 min) treatment of cells with cAMP induces a 90% reduction in the number of A sites. The number of B sites is unaffected, but their affinity decreases 10-fold [25,26].

The fate of A and B sites, after 4 h of incubation with 100 μM cAMP was investigated (supersaturating stimuli are required during prolonged stimulation, because due to the presence of cAMP phosphodiesterases, cAMP is rapidly degraded). Figs. 1A and B show Scatchard plots of [^{3}H]-cAMP binding to aggregation competent cells incubated for 4 h in the presence and absence of constant cAMP stimulation. At binding equilibrium (Fig. 1A), both A and B sites are measured, but since the B-sites comprise only 4% of total binding activity, binding at equilibrium mainly represents the A sites. Binding of [^{3}H]-cAMP to B sites is measured 10 s after addition of excess cAMP (Fig. 1B), in which case binding to the fast dissociating A sites is lost and binding to the slow dissociating B sites persists.

Equilibrium binding to control cells revealed about 100000 A sites which were composed of a high-affinity (K_d approx 45 nM) and a low-affinity (K_d approx. 450 nM) component. Binding, 10 s after dissociation (B sites), showed in control cells about 4800 sites/cell with a K_d of approx. 40 nM. After treatment of cells with cAMP for 4 h, a 95% reduction of equilibrium binding had occurred; the residual 4000 rapidly dissociating sites appear to belong to a single class with a K_d

Table 1

Binding specificity of cAMP binding sites in *D. discoideum*

No.	Derivative	$\delta\Delta G$ (kJ/mol)						
		Surface cAMP binding sites				Intracellular binding protein		
		C	A^H	A^L	B	CABP1	CAK	PDE
1.	cAMP	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.	N^1O-cAMP	10.6 ± 1.7	8.5 *	11.0 *	9.9 *	3.9	4.7	3.2
3.	6Cl-cPuMP	16.5 ± 1.9	17.8 *	17.5 *	16.4 *	−0.2	1.8	2.4
4.	7CH-cAMP	9.7 ± 0.2	12.4 *	10.7 *	13.5 *	−0.72	0.7	0.0
5.	8Br-cAMP	9.8 ± 1.2	12.2 ± 1.0	11.5 ± 0.6	11.1 ± 0.8	−0.5	−2.6	5.4
6.	2′H-cAMP	4.7 ± 0.8	6.2 *	6.6 *	5.6 *	19.0	22.0	4.4
7.	3′NH-cAMP	15.7	15.2 *	13.5 *	15.0 *	17.1	13.0	≥ 16.0
8.	5′NH-cAMP	2.7 ± 0.4	3.4 *	3.7 *	4.0 *	16.9	17.5	−6.0
9.	(Sp)-cAMPS	13.6 ± 4.4	11.9 *	11.3 *	13.3 *	6.9	4.5	10.2
10.	(Rp)-cAMPS	10.9 ± 3.1	14.6 *	13.3 *	14.9 *	17.0	12.0	≥ 16.0
11.	cBIMP	13.5	14.6 *	13.8 *	15.8 *	7.2	6.0	8.3
12.	cPuMP	≥ 18	–	–	–	2.2	3.9	8.3
13.	cIMP	≥ 18	–	–	–	3.4	3.9	4.5
14.	cGMP	≥ 18	–	–	–	12.3	13.9	4.4
15.	5,6-Cl_2-cBIMP	12.9 ± 0.0	12.9	–	–	–	–	–
16.	5,6-F_2-cBIMP	≥ 15	18.5	–	–	–	–	–
17.	8OHiP-cAMP	7.5 ± 1.4	9.7 ± 0.8	–	–	–	–	–
18.	8Cl-cAMP	10.5	10.0	–	–	–	–	–
19.	8pCPT-cAMP	8.3 ± 2.4	12.8 ± 0.8	12.5 ± 1.4	12.3 ± 1.3	–	–	–

Binding to A^H, A^L, B and C sites was measured as described in MATERIALS AND METHODS. Data were standardized using the following equation: $\delta\Delta G = RT \ln K_{0.5}$ derivative$/K_{0.5}$ cAMP [35]. The $K_{0.5}$ represents the concentration of derivative that induces half-maximal inhibition of [^{3}H]-cAMP binding. Means and SD of 3–6 individual experiments performed in triplicate are presented. Some derivatives were tested once. The $\delta\Delta G$ values for the binding of cAMP derivatives to A^H, A^L, B-sites, marked * and to CABP1 were retrieved from Van Ments-Cohen and Van Haastert [15]; The $\delta\Delta G$ values for binding of cAMP derivatives to CAK and PDE are derived from De Wit et al. [19] and Van Haastert et al. [20], respectively. –, not determined.

of approx. 300 nM. Binding to slowly dissociating sites was reduced after cAMP treatment to about 300 sites with a K_d of about 70 nM.

4.2. Specificity of down-regulation resistant cAMP binding activity

To establish whether the cAMP-binding sites, which can be detected after 4-h stimulation with micromolar cAMP concentrations (further called 'C' sites), represented cell surface cAMP receptors or other *Dictyostelium* cAMP-binding proteins, the nucleotide specificity of these sites were compared with the specificity of different forms of surface cAMP receptors and with cAMP-PDE, CAK and CABP1 using 18 different cAMP derivatives (Fig. 2).

The data given in Table 1 show that the specificity of the C sites was completely different from that of the intracellular cAMP-binding proteins CABP1 and CAK. For instance, N^1O-cAMP, 6Cl-cPuMP and 7CH-cAMP, which are good ligands for the intracellular cAMP-binding proteins, are poor ligands for the C sites. 2'H-cAMP, which is a poor CABP1 and CAK ligand, binds well to the C sites. The specificity of the C sites also did not resemble the binding specificity of cAMP-PDE.

Binding to A^H, A^L and B sites showed virtually identical specificity. Binding to C sites was very similar to A^H, A^L and B sites when comparing the derivatives 2, 3, 4, 7, 8, 11, 15, 16, and 18. However, there appeared to be a bias for derivatives with bulky substitutions at the C^8-position, such as compounds 5, 17 and 19 to bind better to C sites than to the other surface cAMP binding sites. The $\delta\Delta G$ values for 8Br-cAMP binding to A^H, A^L and B sites were previously reported to be around 16 kJ/mol [14,15]. Using 8Br-cAMP preparations from different sources, values around 11 kJ/mol were consistently found during the present study.

The data presented here indicate that after prolonged cAMP stimulation of cells, a class of low-affinity receptors remains present with similar, but not completely identical nucleotide specificity to the A and B sites. The down-regulation-resistant binding sites may be involved in responses, such as cAMP-induced post-aggregative gene expression, which require sustained exposure to micromolar cAMP concentrations. However, the dissimilarity in nucleotide specificity is not sufficiently pronounced to conclude that down-regulation-resistant sites represent a different class of receptors, or to correlate specificity of post-agregative gene expression with specific subclasses of surface cAMP-binding sites.

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