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The Cyclic Nucleotide Specificity of Three cAMP Receptors in *Dictyostelium**

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Ronald L. Johnson‡, Peter J. M. Van Haastert§, Alan R. Kimmel¶, Charles L. Saxe III¶‖, Bernd Jastorff, and Peter N. Devreotes‡ ‡‡**

*From the ‡Department of Biological Chemistry, School of Medicine, Johns Hopkins University, Baltimore, Maryland 21205, the §Department of Biochemistry, University of Groningen, 9747AG Groningen, The Netherlands, the ¶Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases (6/B1-12), National Institutes of Health, Bethesda, Maryland 20892, and the **Institute of Organic Chemistry, University of Bremen, Leobenerstrasse, D-280, Bremen, Federal Republic of Germany*

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cAMP receptors mediate signal transduction pathways during development in *Dictyostelium*. A cAMP receptor (cAR1) has been cloned and sequenced (Klein, P., Sun, T. J., Saxe, C. L., Kimmel, A. R., Johnson, R. L., and Devreotes, P. N. (1988) *Science* 241, 1467–1472) and recently several other cAR genes have been identified (Saxe, C. L., Johnson, R., Devreotes, P. N., and Kimmel, A. R. (1991a) *Dev. Genet.* 12, 6–13; Saxe, C. L., Johnson, R. L., Devreotes, P. N., and Kimmel, A. R. (1991b) *Genes Dev.* 5, 1–8). We have expressed three receptor subtypes, cAR1, cAR2, and cAR3, in growing cells and have investigated their affinity and pharmacological specificity in a series of [^{3}H]cAMP binding studies. In phosphate buffer, there were two affinity states of about 30 and 300 nM for cAR1 and 20 and 500 nM for cAR3 but no detectable affinity for cAR2. In the presence of 3 M ammonium sulfate, there was one affinity state of 4 nM for cAR1 and 11 nM for cAR2 and two affinity states of approximately 4 and 200 nM for cAR3. The relative affinities of 14 cyclic nucleotide derivatives were tested for each cAR in ammonium sulfate. These studies suggest a model (Van Haastert, P. J. M., and Kien, E. (1983) *J. Biol. Chem.* 258, 9636–9642) in which cAMP binds to all three receptor subtypes by maintaining hydrogen bond interactions at the N6 and O3′ positions. Interactions at the exocyclic oxygens of cAMP varied between the receptors; cAR2 and cAR3 lacked a stereoselective interaction at the axial oxygen which was present in cAR1. The cleft, which binds the adenine ring of cAMP, was hydrophobic in cAR1 and cAR3 but relatively polar in cAR2. The analog specificity of cAR1 and cAR3 in phosphate buffer was similar to that measured in ammonium sulfate though the derivatives' relative affinity to cAMP was reduced. We conclude that these cAMP receptor subtypes can be distinguished by distinct pharmacological properties which will allow selective activation of each cAR during development.

Extracellular cAMP acts as a primary messenger at several points in the developmental program of *Dictyostelium discoideum*. During early aggregation, intermittent stimulation with cAMP coordinates the accumulation of individual amoebae to form organization centers (Devreotes, 1982) and to regulate the expression of various early genes (reviewed by Kessin (1988)). At the mound stage, induction of prespore and the early stages of prestalk gene expression require persistent exposure to micromolar concentrations of cAMP (reviewed by Gerisch (1987)). Cell differentiation into at least four cell types (Williams *et al.*, 1989) results in the final multicellular structure, the fruiting body.

Cell surface cAMP binding sites, which are present throughout the development cycle of *Dictyostelium*, are most abundant during early aggregation (Schaap and Spek, 1984; Schenk *et al.*, 1991). At this stage, the cell surface receptors are coupled to G-proteins which, when stimulated, activate a variety of effector enzymes. Ligand stimulation of cAMP receptors initiates a signal transduction cascade to cause increases of second messengers, such as intracellular cAMP and cGMP, and permit cell-cell signaling. In addition, cAMP stimulation causes cytoskeletal changes, such as actin polymerization and myosin phosphorylation, which enable chemotaxis (reviewed in Devreotes (1982), Van Haastert (1991)).

A cAMP receptor (cAR1)[1] has been cloned and like other G-protein-coupled receptors found in mammals and yeast, its coding sequence predicts a protein with seven putative transmembrane domains and a cytoplasmic C terminus (Klein *et al.*, 1988). Recently, three additional cAMP receptors (cAR2, cAR3, and cAR4) have been cloned and sequenced. Members of this family of receptor subtypes share approximately 60% identity within their transmembrane and loop regions but have distinct C-terminal domains (Saxe *et al.*, 1991a, 1991b). The developmental regulation of the major mRNA of each cAR is unique, but there is some overlap between each. cAR1 expression is low during growth, peaks during early aggregation, and then subsides (Klein *et al.*, 1987). cAR2 mRNA, which is enriched in prestalk cells, is expressed after 15 h of development while cAR3 mRNA is detected earlier at approximately 10 h of development (Saxe *et al.*, 1991a). Cells which lack cAR1 as a consequence of antisense RNA expression (Klein *et al.*, 1988; Sun *et al.*, 1990) or gene disruption (Sun and Devreotes, 1991) do not enter the developmental program and remain as individual amoebae.

cAMP derivatives have been used to determine the analog

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‖ Present address: Dept. of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, GA 30322.

‡‡ To whom correspondence should be addressed.

[1] The abbreviations used are: cAR, cAMP receptor; PB, phosphate buffer; AS, ammonium sulfate; bp, base pair(s); kb, kilobase(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

specificity of cell surface cAMP receptors (Van Haastert and Kien, 1983) and other cAMP-binding proteins in *Dictyostelium* (De Wit *et al.*, 1982; Van Ments-Cohen and Van Haastert, 1989). These studies have shown that cAMP binds to surface receptors in aggregation stage cells in a manner distinct from that of intracellular cAMP dependent protein kinase and cell surface phosphodiesterase. The pharmacological specificity for chemotaxis (Van Haastert, 1983), activation of guanylate (Van Haastert and Kien, 1983) and adenylate cyclase (Theibert *et al.*, 1986), induction of gene expression (Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Gomer *et al.*, 1986), and cell-type differentiation (Schaap and Van Driel, 1985) have all been demonstrated to match that for surface cAMP receptors.

The presence of multiple cAR subtypes during development suggests that different cAMP receptors mediate separate physiological responses or signal transduction pathways. The affinity and cyclic nucleotide specificity of each cAR subtype may help to distinguish those functions that each receptor controls. Interactions between cAMP and each cAR subtype may vary and thereby provide insight into how the ligand is oriented in each binding pocket. It also may be possible to identify cAMP analogs which specifically activate or block one receptor subtype.

We have expressed three cAMP receptor subtypes, cAR1, cAR2, and cAR3, in growing *Dictyostelium* cells and examined their biochemical and pharmacological properties. Since there are few endogenous receptors present during growth, each individually expressed cAR can be examined without interference from other receptor subtypes. Cells expressing cAR1 during growth have been described previously and found to have similiar biochemical characteristics to the endogenous receptors in aggregation stage cells (Johnson *et al.*, 1991). In this paper, we demonstrate that cAR1, cAR2, and cAR3 represent a group of similar cAMP-binding proteins which have subtle differences in their interaction with cAMP. Furthermore, it now should be possible to distinguish each receptor subtype during development on the basis of its relative cyclic nucleotide specificity.

EXPERIMENTAL PROCEDURES

Materials—The names and structures of the cAMP derivatives are shown in Fig. 1 and Table I, respectively. [8-^{3}H]cAMP (1.92 TBq/mmol) was obtained from Amersham Corp.; [2,8-^{3}H]cAMP (1.65 TBq/mmol) was obtained from Du Pont. cAMP, 6-Cl-PuRMP, 8-Br-cAMP, 2′-H-cAMP, and cGMP were obtained from Boehringer Mannheim; 7-CH-cAMP was a generous gift of Dr. R. Hanze (Upjohn Co.); N^1-*O*-cAMP, 3′-NH-cAMP, 5′-NH-cAMP, cBIMP, and PuRMP were synthesized; the synthesis of these analogs has been described previously (Jastorff and Freist, 1974; Morr *et al.*, 1974; Murayama *et al.*, 1971; Yagura *et al.*, 1980, Baraniak *et al.*, 1979)

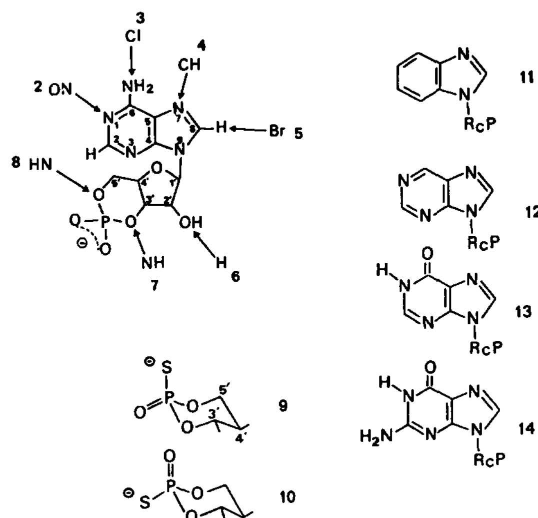

FIG. 1. **Structures of the cAMP derivatives**

Conditions for Growth and Development—AX-3 cells were maintained in HL-5 (Watts and Ashworth, 1970) in shaking culture. Transformants were maintained on Petri dishes in HL-5 with 20 μg/ml G418 but transferred to shaking cultures for experiments. All cells were harvested during late log phase growth and washed once in 10 mM KH_2PO_4/Na_2HPO_4 buffer (PB), pH 6.5. For development, AX-3 cells were shaken in PB for 4 h at 2 × 107 cells/ml as described (Devreotes *et al.*, 1987).

Construction and Transformation of Expression Vectors—The creation of cells overexpressing cAR1 has been described (Johnson *et al.*, 1991). A full-length cAR2 clone was isolated from a sheared, size-selected (2–5-kb range) genomic *Dictyostelium* library (Lambda Zap, Stratagene; gift of Dr. H. Innis).[2] This 2-kb clone contains 158 bp of 5′- and about 900 bp of 3′-untranslated sequence and was shuttled into the *Eco*RI site of Bluescript KS+ (Stratagene). A full-length cAR3 clone, GR-6, was isolated from a partial *Sau*3A *Dictyostelium* genomic library (PAT plasmid, gift of Dr. R. Firtel).[3] This 1.7-kb clone, which contains 35 bp of 5′- and 40 bp of 3′-untranslated sequence, was isolated from the parent plasmid by digesting with *Xba*I and *Sma*I. The inserts of cAR2 and cAR3 were filled in with Klenow, *Bam*HI linkers added, and cloned into the *Bam*HI site of separate Bluescript vectors. cAR2 or cAR3 were then cloned into the expression construct, pB18 (gift of Dr. R. Firtel), by digesting with *Bam*HI and ligating them into the *Bgl*II site of pB18 in the sense orientation. These vectors or the parent construct, pB18, were transformed into AX-3 cells by electroporation as described (Dynes and Firtel, 1989). Stable transformants were selected by resistance to 10 or 20 μg/ml G418 in HL-5. Total transformants (cAR3) or clones (cAR2) were examined for cAR expression by their ability to bind [^{3}H]cAMP. cAR1 Δ208 (thymidine auxotroph) cells (Sun and Devreotes, 1991) were cotransformed with cAR2 or cAR3 expression plasmid with pGEM 26–6 (gift of R. Firtel) in a 3:1 (μg:μg) ratio and selected for growth in unsupplemented HL-5 media. Transformant clones were screened by immunoblot. Cells expressing high levels of cAMP binding sites were used for further experiments.

cAMP Binding Assays—cAMP binding was performed in the absence and presence of ammonium sulfate (AS) as described (Van Haastert, 1985a). In brief, 8 × 10^6 cells were added to PB containing 10 mM dithiothreitol, 10 nM [^{3}H]cAMP, and various concentrations of cAMP or cyclic nucleotide analog in a 100-μl volume at 0 °C. Cells were incubated 1 min and then centrifuged for 2 min at 10,000 × *g*. To determine binding in AS, 850 μl of 3 M AS was included in the above assay, and after adding cells, 50 μl of 10 mg/ml bovine serum albumin was added. Cells were incubated 5–7 min and then centrifuged for 3 min. For both assays, the supernatants were carefully aspirated and the cells resuspended in 80 μl of 0.1 M formic acid. One ml of scintillation fluid (Emulsifier, Packard) was then added and radioactivity determined. Nonspecific binding was determined by adding excess cAMP to the incubation mixture at a final concentration of 1 mM (PB) or 0.1 mM (AS). Scatchard binding curves were best fit using the computer modeling programs, LIGAND (Munson and Rodbard, 1980) and Pfit (Elsevier). For analog studies, 3 different concentrations centering around the IC_{50} of each analog was used with data points taken in duplicate. The IC_{50} of each analog was tested in two to three independent experiments. Correlation matrix values were obtained using linear regression analysis.

Immunoblotting—Membranes were prepared by solubilizing 1 volume of cells with 9 volumes of a lysis buffer containing 1.5% CHAPS and pelleting at 10,000 × *g* for 20 min (Klein *et al.*, 1985). The pellet was resuspended with 10 volumes of lysis buffer without CHAPS and centrifuged as above. This pellet was suspended in Laemmli's sample buffer (Laemmli, 1970), and 50 μl of sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted as described (Klein *et al.*, 1987). The blot was probed with a polyclonal antiserum (1:1000) raised against the peptide, KREPEPERFEKYC,[4] a sequence found in the cytoplasmic loop between the putative transmembrane domains III and IV of all three cARs (Klein *et al.*, 1988).[2,3]

[2] C. Saxe, A. Kimmel, and P. Devreotes, manuscript in preparation.
[3] R. Johnson, C. Saxe, A. Kimmel, and P. Devreotes, manuscript in preparation.
[4] M. Caterina, J. Kim, and P. Devreotes, manuscript in preparation.

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TABLE I
cAMP derivatives used in this study

No.	Name	Abbreviation
1.	Adenosine 3′:5′-monophosphate	cAMP
2.	Adenosine-N^1-oxide 3′:5′-monophosphate	N^1-O-cAMP
3.	6-Chloropurineriboside 3′:5′-monophosphate	6-Cl-PuRMP
4.	7-Deazaadenosine 3′:5′-monophosphate	7-CH-cAMP
5.	8-Bromoadenosine 3′:5′-monophosphate	8-Br-cAMP
6.	2′-Deoxyadenosine 3′:5′-monophosphate	2′-H-cAMP
7.	3′-Deoxy-3′-aminoadenosine 3′:5′-monophosphate	3′-NH-cAMP
8.	5′-Deoxy-5′-aminoadenosine 3′:5′-monophosphate	5′-NH-cAMP
9.	Adenosine 3′:5′-monophosphorothioate, S_p isomer	(S_p)-cAMPS
10.	Adenosine 3′:5′-monophosphorothioate, R_p isomer	(R_p)-cAMPS
11.	Benzimidazoleriboside 3′:5′-monophosphate	cBIMP
12.	Purineriboside 3′:5′-monophosphate	PuRMP
13.	Inosine 3′:5′-monophosphate	cIMP
14.	Guanosine 3′:5′-monophosphate	cGMP

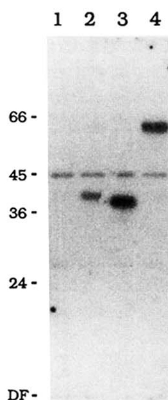

FIG. 2. **Expression of cAR proteins in *Dictyostelium* cells.** Growing cells transformed with either B18, cAR1, cAR2, or cAR3 expression vectors (*lanes 1–4*, respectively) were examined for protein expression by immunoblotting with a cAR common antiserum. cAR1 migrates at 40 kDa, cAR2 at 39 kDa, and cAR3 at 62 kDa.

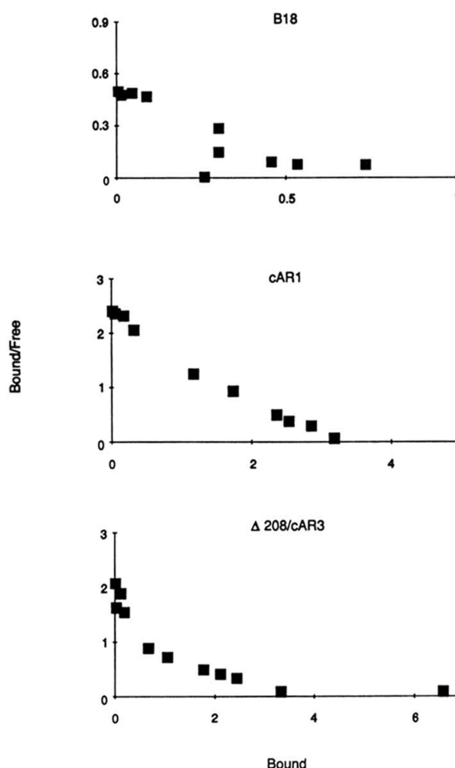

FIG. 3. **Scatchard analysis of cAR cells in phosphate buffer.** Receptor affinity was determined by the binding of [^{3}H]cAMP to cells in phosphate buffer in the presence of increasing amounts of cAMP. The units for bound/free (y-axis) and bound (x-axis) are nM/sites/cell × 1000 and sites/cell × 10^5, respectively. See Table II for binding parameters.

RESULTS

Expression and Affinity of cAMP Receptor Subtypes—Each of the three cAMP receptor subtypes, cAR1, cAR2, and cAR3, were expressed in growing *Dictyostelium* cells. Since at this stage, cells express only a low number of cAMP binding sites (Klein *et al.*, 1987), each individual receptor can be expressed and studied without interference from the endogenous receptors. The expression construct chosen utilizes the actin 15 promoter which is constitutively active during growth and early development (Knecht *et al.*, 1986). Cells overexpressing cAR1 (denoted cAR1 cells) have been previously characterized (Johnson *et al.*, 1991). cAR2 and cAR3 expression constructs were created in a similar fashion and transformed into AX-3 cells. Transformants were selected and screened for their ability to bind [^{3}H]cAMP. One clone (cAR2 cells) or mass culture (cAR3 cells) expressing high levels of cAMP binding sites were examined further.

The presence of each exogenously expressed cAR in growth stage transformants was verified by an immunoblot (Fig. 2). Membranes were prepared from whole cells and immunoblotted with a polyclonal antiserum developed against a common peptide sequence present in all cARs.[4] Cells transformed with either cAR1, cAR2, or cAR3 (*lanes 2–4*) had an apparent molecular mass of 40, 39, and 62 kDa, respectively. Each of the cAR cells expressed a similar amount of their respective receptor protein, while control cells (*lane 1*) transformed with the parent vector, expressed very low levels of endogenous cAR1 protein and undetectable levels of cAR2 and cAR3. Bands present at 45 and 29 kDa are probably nonspecific proteins and unrelated to the transformed plasmids since they appear in each of the cell lines including the vector control, which contains a low number of cAMP binding sites (Klein *et al.*, 1988).

We determined the affinity and number of cAMP binding sites for each of the cAR cells. [^{3}H]cAMP binding to cells under physiological conditions (phosphate buffer, PB) was determined and the data analyzed by Scatchard plots (Fig. 3 and Table II). Growing cAR1 cells and developed vector control cells each have two binding sites of similar affinities of approximately 30 and 300 nM (Johnson *et al.*, 1991). cAR1 cells expressed over 3 × 10^5 sites/cell, which is about 30-fold higher than growing and 4-fold higher than developed B18

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TABLE II
cAR binding parameters in phosphate buffer and ammonium sulfate
Values for B18 and cAR1 were retrieved from Johnson *et al.* (1991). Values for cAR2 and cAR3 were determined as described in "Experimental Procedures."

Cell	Phosphate Buffer				Ammonium Sulfate			
	K_d		Sites/cell		K_d		Sites/cell	
	High	Low	High	Low	High	Low	High	Low
	nM		*×1000*		*nM*		*×1000*	
B18	40 ± 9	350 ± 180	17 ± 12	58 ± 13	1.8 ± 0.3	—	96 ± 2	—
cAR1	25 ± 8	230 ± 45	75 ± 38	260 ± 34	3.5 ± 0.3	—	370 ± 6	—
cAR2	—[a]	—	—	—	11 ± 0.6	—	210 ± 3	—
cAR3	47 ± 82	680 ± 280	16 ± 24	210 ± 14	4.6 ± 2.3	220 ± 81	34 ± 11	540 ± 110
Δ208/cAR3	14 ± 3	490 ± 23	22 ± 3	370 ± 4	2.9 ± 0.6	160 ± 37	64 ± 7	580 ± 76

[a] —, binding not detected.

cells. cAR3 cells displayed two binding affinities which were lower than cAR1. Of the approximately 2 × 10^5 sites/cell expressed, the majority constituted a low affinity site of about 500 nM while about 5% were of 20 nM.

Surprisingly, little cAMP binding in PB was detected in two independent cAR2 transformants under physiological conditions. The low level of cAMP binding detected did not differ significantly from that of control vector cells, which contained some binding sites derived from endogenous cAR1 expression. 10 mM Ca^{2+}, which has been shown to reveal additional binding sites in aggregation state cells (Juliani and Klein, 1977; Van Haastert, 1985b), did not increase the level of cAMP binding in the cAR2 cells. In addition, filter lysis of cellular membranes did not expose additional cAMP binding sites (data not shown).

The cAMP binding sites in cAR2 cells and the high affinity sites in the cAR3 cells could not be attributed to the endogenous cAR1 present during growth. To demonstrate this, a cAR1 null cell line (designated Δ208, Sun and Devreotes (1991)), was transformed with the cAR2 or cAR3 expression construct.[5] Transformants were screened for protein expression by immunoblot, and clones which expressed high levels of cAR2 or cAR3 were then assayed for cAMP binding. During growth, Δ208 cells have no detectable cAMP binding (Sun and Devreotes, 1991). Since the Δ208 cells transformed with cAR2 (denoted Δ208/cAR2 cells) showed a small amount of cAMP binding (490 ± 370 sites/cell at 10 nM [^{3}H]cAMP), these binding sites must be attributed to the presence of cAR2. The cAR1 null cells expressing cAR3 (denoted Δ208/cAR3 cells) had binding characteristics similiar to that of the cAR3 cells with the wild-type background (Table II). Scatchard analysis of these cells demonstrate that both the high and low affinity components result from cAR3 expression (Fig. 3).

Previous experiments in developed wild-type cells have shown that 3 M AS increases both the affinity and number of detectable cAMP binding sites (Janssens and Van Driel, 1984; Van Haastert, 1985a). The effect of AS on cAMP binding was tested on all three cAR cells and was found to vary for each (Fig. 4 and Table II). We have shown previously that AS enhances receptor affinity in both growing cAR1 and developed B18 cells by over 30-fold (K_d = 4 nM) and increases the number of binding sites (Johnson *et al.*, 1991). For the cAR3 or the Δ208/cAR3 cells, ammonium sulfate enhanced both high and low affinity binding by different extents and increased the number of cAMP binding sites. The high affinity sites increased about 5-fold to 4 nM, while the low affinity sites increased by approximately 3-fold to 200 nM. In the cAR2 cells, AS greatly increased the levels of cAMP binding by exposing over 2 × 10^5 binding sites/cell of a single affinity of 11 nM.

cAMP Derivatives—The 14 cAMP analogs used in this study test various interactions between the ligand and receptor such as hydrogen and ionic bonding and hydrophobic interactions (Fig. 1 and Table I). N^1-*O*-cAMP, 6-Cl-PuRMP, 7-CH-cAMP, 2′-H-cAMP, 3′-NH-cAMP, and 5′-NH-cAMP have modifications which prevent potential hydrogen bonds. To test for stereoselective interactions in the phosphate moiety, (S_p)-cAMPS and (R_p)-cAMPS replace the exocyclic oxygens (axial or equatorial, respectively) with a negatively charged sulfur atom (Frey and Sammons, 1985). In solution, cAMP equally favors either a *syn*- or *anti*- conformation (Hemmes *et al.*, 1976). Since 8-Br-cAMP exists primarily in a *syn*- conformation (Schweizer and Robins, 1973), one may infer the conformation of cAMP when bound to the receptor from its relative affinity. Finally, derivatives cBIMP, PuRMP, cIMP, and cGMP differ in their degree of polarity (cIMP > cGMP > PuRMP > cAMP > cBIMP) (Van Haastert *et al.*, 1983).

Cyclic Nucleotide Specificity of cAR Subtypes—Each of the cAR cells was tested in ammonium sulfate for their ability to bind 14 cAMP derivatives. $K_{0.5}$ values were determined by measuring the concentration of derivative which inhibited 50% of [^{3}H]cAMP binding to the receptors. The data are presented as $K_{0.5}$ derivative/$K_{0.5}$ cAMP ratios (Table III) and $\delta\Delta G$ values (Table IV). $\delta\Delta G$ values are derived from the following equation to compare these results to previous studies (Jastorff *et al.*, 1979).

$$\delta\Delta G = RT \ln K_{0.5} \text{ derivative}/K_{0.5} \text{ cAMP}$$

$\delta\Delta G$ values are expressed in kJ/mol and represent the derivative's reduction of binding energy when compared with the binding of cAMP.

The interactions of all three cARs with cAMP share some common features which have been previously noted in studies on the endogenous receptors in developed wild-type cells (Van Haastert and Kien, 1983). The low affinity of 6-Cl-PuRMP and 3′-NH-cAMP indicates that hydrogen bonds are formed between the receptor and cAMP at the O3′ position in the ribose ring and the N6 position in the adenine moiety in all three cARs. In addition, since 8-Br-cAMP is primarily in the *syn*- conformation (Schweizer and Robins, 1973), the greatly reduced affinity of this derivative suggests that cAMP is in an *anti*- conformation when bound to the receptors. However, the poor affinity of this analog may result from the bromine's effect on the electron distribution in the purine ring or steric hindrance as well.

Receptor interactions varied at the exocyclic oxygens in the

[5] R. Johnson, R. Gundersen, J. Milne, S. Turgendreich, and P. Devreotes, manuscript in preparation.

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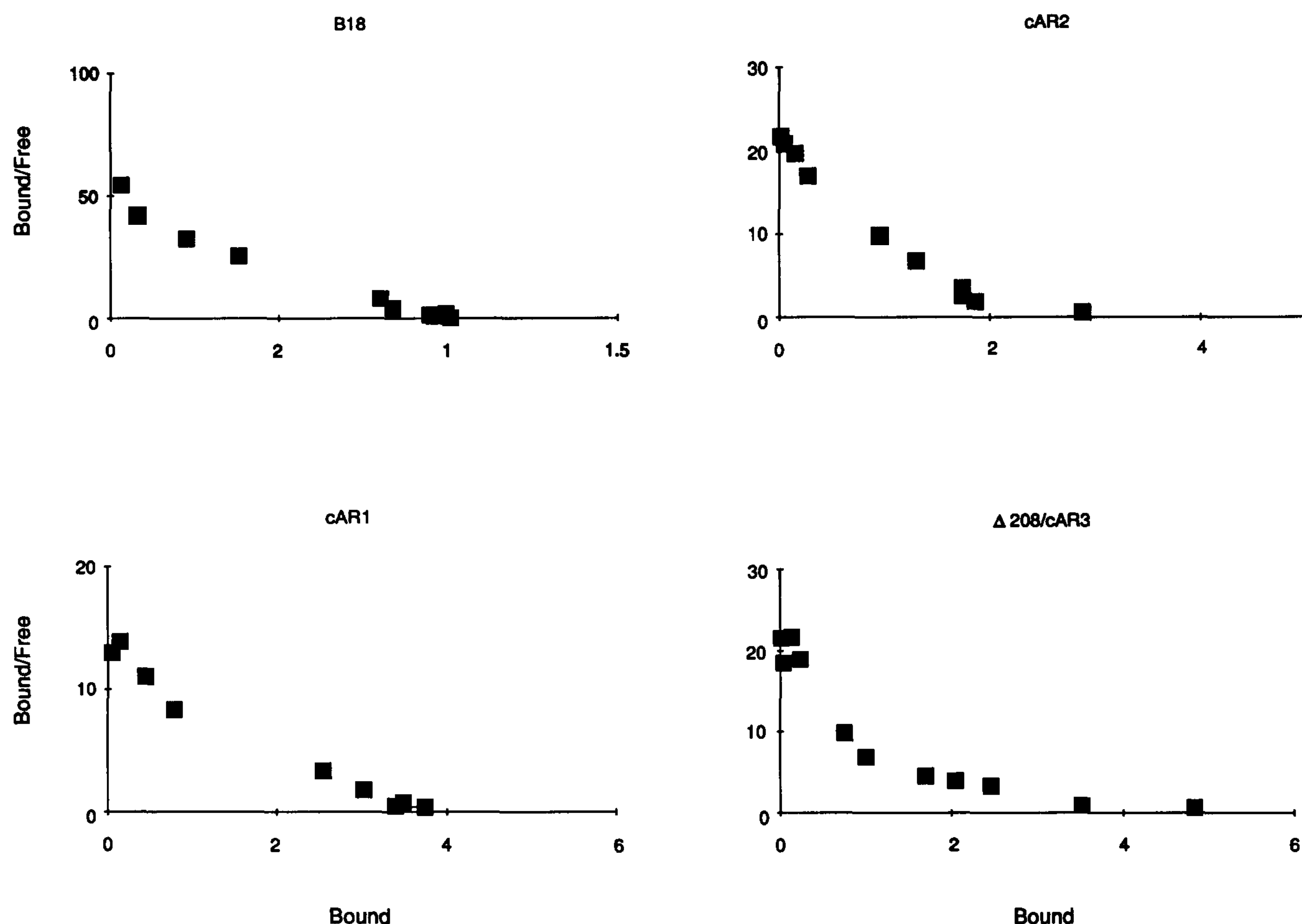

FIG. 4. **Scatchard analysis of cAR cells in ammonium sulfate.** Receptor affinity was determined by the binding of [^{3}H]cAMP to cells in 3 M ammonium sulfate in the presence of increasing amounts of cAMP. The units for bound/free (y-axis) and bound (x-axis) are nM/sites/cell × 1000 and sites/cell × 10^5, respectively. See Table II for binding parameters.

TABLE III
Specificity of cAR subtypes in ammonium sulfate and phosphate buffer
K_{50} derivative/K_{50} cAMP ratios for WT-D were derived from Van Haastert and Kien (1983). Ratios for cARs were determined as described under "Experimental Procedures."

Derivative[a]	Ammonium Sulfate					Phosphate buffer	
	WT-D	cAR1	cAR2	cAR3	Δ208/cAR3	cAR1	cAR3
1. cAMP	1	1	1	1	1	1	1
2. N^1-O-cAMP	58	51	3	21	—[b]	68	140
3. 6-Cl-PuRMP	620	370	950	160	70	1100	3300
4. 7-CH-cAMP	350	230	390	110	—	100	98
5. 8-Br-cAMP	180[c]	180	50	44	20	220	270
6. 2′-H-cAMP	12	6	3	2	3	21	31
7. 3′-NH-cAMP	810	400	360	130	—	720	1500
8. 5′-NH-cAMP[d]	7	18	19	23	—	—	—
9. (S_p)-cAMPS	110	29	5	3	1	68	15
10. (R_p)-cAMPS	120	97	180	58	—	180	500
11. cBIMP	160	130	230	28	—	580	930
12. cPuRMP	1200	1000	660	200	—	3400	8300
13. cIMP	14000	>10000	2700	>10000	—	5500	11000
14. cGMP	22000	>10000	2000	>10000	—	10000	30000

[a] See Fig. 1 and Table I.
[b] —, not determined.
[c] Value differs from that of Van Haastert and Kien (1983).
[d] Data from one experiment.

phosphate moiety of cAMP. cAR1 bound (S_p)-cAMPS and (R_p)-cAMPS, which replace an axial or equatorial oxygen respectively with a sulfur atom, with approximately equal affinity. The loss of 8–10 kJ/mol binding energy for these two derivatives relative to cAMP suggests that there are important interactions at both exocyclic oxygens. In contrast, both cAR2 and cAR3 bound (S_p)-cAMPS with 6- and 30-fold higher affinity, respectively, than cAR1, and both bound (R_p)cAMPS

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TABLE IV

Specificity of cAR subtypes in ammonium sulfate and phosphate buffer

$\delta\Delta G$ values for WT-D and polarity were derived from Van Haastert and Kien (1983). $\delta\Delta G$ values for cARs were determined as described in "Experimental Procedures."

Derivative[a]	Ammonium sulfate					Phosphate buffer		Polarity
	WT-D	cAR1	cAR2	cAR3	Δ208/cAR3	cAR1	cAR3	
1. cAMP	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2. N^1-*O*-cAMP	9.2	8.9	2.5	6.9	—[b]	9.6	11.2	−4.49
3. 6-Cl-PuRMP	14.6	13.4	15.6	11.6	9.6	15.8	18.3	2.06
4. 7-CH-cAMP	13.3	12.4	13.5	10.8	—	10.5	10.4	0.36
5. 8-Br-cAMP	11.8[c]	11.7	8.9	8.6	6.9	12.2	12.7	1.98
6. 2′-H-cAMP	5.6	4.2	2.6	2.0	2.6	7.0	7.8	−0.46
7. 3′-NH-cAMP	15.2	13.6	13.4	11.1	—	14.9	16.7	−0.02
8. 5′-NH-cAMP[d]	4.5	2.2	2.3	2.8	—	—	—	−1.65
9. (S_p)-cAMPS	10.7	7.7	3.5	2.5	−0.4	9.5	6.1	1.63
10. (R_p)-cAMPS	10.8	10.4	11.8	9.2	—	11.8	14.1	0.66
11. cBIMP	11.5	11.1	12.4	7.5	—	14.4	15.5	2.75
12. PuRMP	16.0	15.7	14.7	12.0	—	18.4	20.5	−0.46
13. cIMP	21.7	21.4	17.9	21	—	19.5	23.6	−3.41
14. cGMP	22.7	22.1	17.2	22.7	—	20.9	23.6	−3.21

[a] See Fig. 1 and Table I.
[b] —, not determined.
[c] $\delta\Delta G$ value differs from that of Van Haastert and Kien (1983).
[d] Data from one experiment.

with affinities similar to that of cAR1 (Table III). In the Δ208/cAR3 cells, where the low levels of cAR1 were absent, cAR3 bound (S_p)-cAMPS as well as or better than cAMP (Fig. 5). This suggests that both cAR2 and cAR3 lack a stereoselective interaction at the axial exocyclic oxygen that is present in cAR1. The nature of this interaction is probably not ionic, since a loss of about 25 kJ/mol binding energy would be expected. Steric disruption is a more likely explanation since a thio-substitution of an exocyclic oxygen would occupy more space.

The hydrophobic cleft which binds the adenine ring (Van Haastert and Kien, 1983) varies in hydrophobicity among the three receptors. Fig. 6 plots the relative binding energy of five derivatives (cIMP, cGMP, PuRMP, 6-Cl-PuRMP, cBIMP) as a function of their relative polarity in comparison with cAMP. As shown previously in developed wild-type cells, the polarity of these derivatives is negatively correlated with binding energy. In addition, these compounds are missing the N6 amino group in the adenine ring. The loss of this amino group raises the binding energy by about 15 kJ/mol relative to cAMP. When this energy increment is subtracted away, the binding energy of N^1-*O*-cAMP, which has the N6 amino group but is very polar, fits this correlation well. Hence the adenine ring is thought to rest in a hydrophobic pocket.

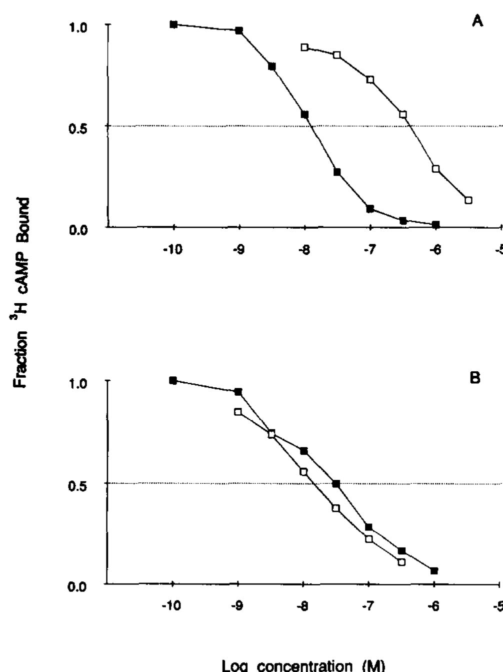

FIG. 5. **Inhibition of [³H]cAMP binding by cAMP and (S_p)-cAMPS on cAR1 and cAR3/Δ208 cells in ammonium sulfate.** The binding of [³H]cAMP to cAR1 (*A*) or cAR3/Δ208 (*B*) cells was inhibited by increasing concentrations of cAMP (*filled squares*) or (S_p)-cAMPS (*open squares*).

All three cARs bound these analogs similarly in that the loss of the N6 amino group contributed an increase of about 15 kJ/mol in binding energy. However, the nature of the hydrophobic cleft differed for each cAR as reflected in the slope of the lines. Both cAR1 and cAR3 have large negative slopes (−1.78 and −1.96, respectively), whereas cAR2 has a slope that is 3 times smaller (−0.622). These data suggest that the adenine moiety is bound in a cleft of the receptor which is more hydrophobic for cAR1 and cAR3 than for cAR2. The loss in hydrophobicity in cAR2 may be caused by changes of amino acid residues in the cleft from a nonpolar to polar nature.

The analog specificity of cAR1 and cAR3 was also examined in phosphate buffer (Tables III and IV). cAR2 cells were not included in these studies because of the low number of cAMP binding sites detected in PB. In comparision with specificity studies performed in ammonium sulfate, both cAR1 and cAR3 maintain the general order of analog specificity in phosphate buffer with two exceptions. 7-CH-cAMP showed increased affinity, while cBIMP had reduced affinity relative to the other cAMP analogs. Interestingly, the relative binding affinities of the derivatives were not influenced by ammonium sulfate for cAR1 but were enhanced for cAR3. In addition, while adenine ring polarity negatively correlated with binding affinity, the slopes were less steep (data not shown). N^1-*O*-cAMP, however, does not fit this correlation as well; its high polarity cannot account for all of the loss in binding energy.

Some analogs were also tested on the Δ208/cAR3 cells which lack cAR1 (Tables III and IV). Since cAR3 is a low affinity receptor, the low levels of the endogenous, higher

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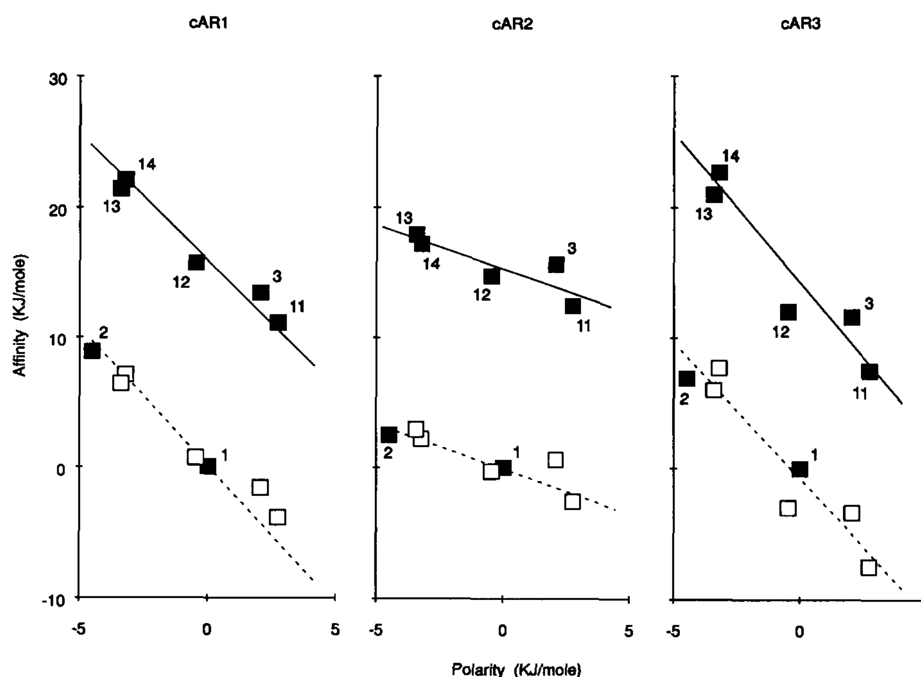

FIG. 6. **The dependence of binding affinity on the polarity of some cAMP derivatives varies between cAR subtypes.** For each graph, *filled squares* represent binding data in ammonium sulfate from Table IV for N^1-*O*-cAMP (*2*), cIMP (*13*), cGMP (*14*), PuRMP (*12*), cAMP (*1*), 6-Cl-PuRMP (*3*), cBIMP (*11*) in order from *left* to *right*. Linear regression analysis of the *top solid line* shows the strong influence of polarity on binding affinity for cAR1 and cAR3 (slope = −1.67, r = −0 .986 and slope = −2.17, r = −0.95, respectively) but less so for cAR2 (slope = −0.652, r = −0.86). These derivatives are all missing the N6 amino group which contributes about 16, 15.3, and 14 kJ/mol, respectively, to the binding energy of cAR1, cAR2, and cAR3. Subtraction of this binding increment yields the *open squares*. Linear regression analysis of the *bottom dashed line* (which includes N^1-*O*-cAMP) gives a similar slope for each cAR (cAR1, slope = −1.78, r = −0.988; cAR2, slope = −0.622, r = −0.887; cAR3, slope = 1.96, r = −0.946).

TABLE V

Correlation matrix of the δΔG values of derivatives of cAMP binding proteins in Dictyostelium

δΔ*G* values for WT-D, CAK, and ePDE were derived from Van Ments-Cohen and Van Haastert (1989).

	WT-D	cAR1	cAR2	cAR3	CAK	ePDE
WT-D	1.0	0.99	0.87	0.95	−0.32	0.21
cAR1		1.0	0.89	0.97	−0.35	0.22
cAR2			1.0	0.86	−0.33	0.24
cAR3				1.0	−0.21	0.07
CAK					1.0	0.01
ePDE						1.0

affinity cAR1 present in growing wild-type cells may affect the measurement of the true nucleotide specificity of cAR3. In AS, the relative nucleotide specificity of cAR3 appears similiar in both sets of cells, but because the IC_{50} for cAMP is larger in the Δ208/cAR3 cells, the relative δΔ*G* values are smaller. These same analogs were tested on the Δ208/cAR3 cells in phosphate buffer and gave similiar results to the cAR3 cells (data not shown).

Comparison of cARs with Other cAMP-binding Proteins—The analog specificities of the three cARs were compared with each other, the endogenous receptors in aggregation stage cells (WT-D) and two cAMP-binding proteins in *Dictyostelium*: the regulatory subunit of cAMP dependent protein kinase A (CAK) and extracellular phosphodiesterase (ePDE). As shown in Table V, the pharmacological specificity of the three cAR subtypes were highly correlated and formed a group of cAMP-binding proteins. Similar to previous results (Van Ments-Cohen and Van Haastert, 1989), cAMP receptors were distinct from other cAMP-binding proteins in *Dictyostelium* since there was no correlation with either CAK or ePDE. As expected, cAR1 was most similar to the endogenous receptors in developed wild-type cells. The developmental expression of cAR1 protein correlates with the increase of cAMP binding sites during early development and the major band photoaffinity labeled with ^{32}P-8-N_3-cAMP at 6 h of development is cAR1 (Klein *et al.*, 1987). However, (S_p)-cAMPS was of higher affinity in cAR1 cells than in developed wild-type cells. In addition, our studies showed 8-Br-cAMP to have 3.3 kJ/mol less binding energy in developed wild-type cells than previously reported by Van Haastert and Kien (1983) (Table IV).

DISCUSSION

We have examined the affinity and cyclic nucleotide specificity of three cAMP receptor subtypes by expressing each individually in growing *Dictyostelium* cells. Each receptor has a different affinity for cAMP in phosphate buffer and the binding parameters of each is uniquely influenced by ammonium sulfate. The cyclic nucleotide specificity indicates that all three receptors comprise a family of cAMP-binding proteins, but each cAR interacts with cAMP in a slightly different manner.

The affinity of the three cAR subtypes in phosphate buffer vary greatly. cAR1 affinity is similiar to the endogenous receptors in developed AX-3 cells (Johnson *et al.*, 1991). The majority of cAR3 binding, however, has an affinity of approximately 500 nM that is about 2-fold lower than cAR1. cAMP binding sites of low affinity, termed C sites, have been detected in wild-type NC-4 cells (Van Ments-Cohen *et al.*, 1991). The exposure of aggregation competent cells to μM levels of cAMP for several hours depletes the cells of cAR1 mRNA

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and protein, yet approximately 5 × 10^3 cAMP binding sites still persist. The K_d of these remaining sites is near 700 nM which is similar to the affinity of cAR3. Furthermore, cAR3 is maximally expressed in mound stage cells.[3] This evidence suggests that C sites are cAR3.

The very low level of cAMP binding in cAR2 cells under physiological conditions (PB) is intriguing. It is possible that cAR2 has a very low affinity for cAMP. Our binding assay in PB would be unable to detect affinities above 5 μM because of the high levels of nonspecific binding. Indeed, cAR2 is a functional receptor since the Δ208/cAR2 cells display several cAMP-stimulated responses *in vivo*.[5] This suggests that cAR2 is on the cell surface and at least a fraction of the receptors are in a functional state. Upon exposure to ammonium sulfate, all of the cAR2 sites appear to be converted to a high affinity form. The origin of this phenomenon can now be examined by creating cAR1/cAR2 chimeric proteins to determine which domains of cAR2 dictate this unusual behavior.

Since each cAR may mediate different signal transduction pathways during development, it would be useful to stimulate one receptor subtype selectively and examine subsequent responses. Two analogs, N^1-*O*-cAMP and (S_p)-cAMPS, are sufficient to distinguish the receptor subtypes. cAR1 has a lower affinity for both of these derivatives relative to the other two cARs. cAR2 binds N^1-*O*-cAMP with about 17- and 7-fold greater relative affinity than cAR1 and cAR3, respectively. cAR3 has a relative affinity for (S_p)-cAMPS that is 30-fold higher than that of cAR1 (Fig. 5). In addition, cAR3 binds cBIMP by approximately 10-fold higher relative affinity than cAR2. Therefore to examine a response in, for instance, mound stage cells where both cAR1 and cAR3 are present,[3] (S_p)-cAMPS could be used to discriminate between the two subtypes. cAR3 has a 2-fold lower affinity for cAMP but a 30-fold higher affinity for (S_p)-cAMPS than cAR1. (S_p)-cAMPS should activate cAR3 at concentrations 15-fold lower than for cAR1.

A recent report (Ma and Siu, 1990) has suggested that a cAMP receptor different from cAR1 mediates the expression of gp80 in *Dictyostelium*. The cyclic nucleotide specificity of the three cAR subtypes do not match that of this different receptor. However, the authors' experiments were performed on dense suspensions of cells, and no precautions were taken to control either self-amplification or phosphodiesterase activity. More analogs will need to be tested to determine whether gp80 expression is mediated by an unidentified cAMP receptor.

While the three cARs have approximately 60% sequence identity within the putative transmembrane and loop regions (Saxe *et al.*, 1991a), we are unable to localize a cAMP binding site or account for the differences in analog specificity by the differences in amino acid sequences alone. Each of the cARs has been photoaffinity labeled with [^{32}P]8-N_3-cAMP (Theibert *et al.*, 1984, data not shown), but the labeled residue(s) has not been identified. We are now attempting to isolate labeled peptides from the cARs and determine their sequence. Furthermore, random mutagenesis of each cAR may determine regions within the receptors that are critical for binding.

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