The Cyclic Nucleotide Specificity of Three cAMP Receptors in Dictyostelium*

Ronald L. Johnson‡, Peter J. M. Van Haastert‡, Alan R. Kimmel¶, Charles L. Saxe III∥, Bernd Jastorf***, 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 (R/BI-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

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 [3H]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, 9638–9642) in which each 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) 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 adenylyl 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, 1986) 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 similar 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. [8-3H]cAMP (1.92 TBq/ mmol) was obtained from Amer sham Corp.; [2,5-3H]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 (Upholn Co.); N'-O-cAMP, 3'-NH-cAMP, 5'-NH-cAMP, cBIMP, and PuRMP were synthesized; the synthesis of these analogs has been described previously (Jostorf and Freist, 1974; Morr et al., 1974; Mura yama et al., 1971; Yagura et al., 1980, Bara niak et al., 1979).

Conclusions for Growth and Development—AX-3 cells were main tained 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 KH2PO4/NaHPO4 buffer (PB), pH 6.5. For development, AX-3 cells were shaken in PB for 4 h at 2 × 107 cells/ml as described (Devrootes et al., 1987).

**Construction and Transformation of Expression Vectors**—The construction of cAMP receptor cDNA was 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). This 2-kb clone contains 158 bp of 5'- and about 900 bp of 3'-untranslated sequence and was shuttled into the EcoRI site of Bluescript KS+ (Stratagene). A full-length cAR3 clone, GR-6, was isolated from a partial Sau3A 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 XbaI and Smal. The inserts of cAR2 and cAR3 were filled in with Klenow and ligating them into the BglII 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 cAMP expression by their ability to bind [3H]cAMP. cAR2 and cAR3 expressed cAMP plasmids with pGEM 26-6 (gift of Dr. R. Firtel) in a 1:1 (μg/μg) ratio and selected for growth in unsupplemented HL-5 medium. 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 × 106 cells were added to PB containing 10 mM dithiothreitol, 10 mM [3H]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 0.5 M AS was included in the assay solution, and after adding cells, 50 μl of 10 mg/ml bovine serum albumin was added. Cells were incubated 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 IC50 of each analog was used with data points taken in duplicate. The IC50 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., 1987). The pellet was resuspended with 10 volumes of lysis buffer without CHAPS and centrifuged as above. This pellet was resuspended in Laemmli’s sample buffer (Laemmli, 1970), and 50 μl of sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot ted as described (Klein et al., 1987). The blot was probed with a polyclonal antiserum (1:10,000) raised against the peptide, KREP.FYPCYC, a sequence found in the cytoplasmic loop between the putative transmembrane domains III and IV of all three cARs (Klein et al., 1988).

5 C. Saxe, A. Kimmel, and P. Devroetes, manuscript in preparation.
6 R. Johnson, C. Saxe, A. Kimmel, and P. Devroetes, manuscript in preparation.
7 M. Caterina, J. Kim, and P. Devroetes, manuscript in preparation.
TABLE I
CAMP derivatives used in this study

| No. | Name                          | Abbreviation     |
|-----|-------------------------------|------------------|
| 1.  | Adenosine 3':5'-monophosphate | cAMP             |
| 2.  | Adenosine-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, isomer | (S,)-cAMPS |
| 10. | Adenosine 3':5'-monophosphorothioate, R, isomer | (R,)-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 |

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 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 [3H]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 CAMP receptor 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 CAMP receptors. Cells transformed with either cAR1, cAR2, or cAR3 (lanes 1–4) had an apparent molecular mass of 40, 39, and 62 kDa, respectively. Each of the CAMP receptors 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 CAMP cells. [3H]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

![Fig. 2. Expression of cAR proteins in Dictyostelium cells.](https://example.com/fig2)

![Fig. 3. Scatchard analysis of cAR cells in phosphate buffer.](https://example.com/fig3)
cAMP Receptor Cyclic Nucleotide Specificity in Dictyostelium

Table II

| Cell          | Sites/cell | Kd (nM) | Sites/cell | Kd (nM) |
|---------------|------------|---------|------------|---------|
| Phosphate Buffer |          |         | Ammonium Sulfate |        |
| High     | Low       | High     | Low       |
| B18     | 40 ± 9    | 350 ± 180 | 17 ± 12    | 680 ± 240 |
| cAR1    | 25 ± 8    | 230 ± 45  | 75 ± 38    | 280 ± 34  |
| cAR2    | 47 ± 8    | 680 ± 280 | 16 ± 24    | 210 ± 14  |
| Δ208/cAR3 | 14 ± 3    | 490 ± 23  | 22 ± 3     | 370 ± 4   |
| Arginine Sulfate |          |         | Arginine Sulfate |        |
| High     | Low       | High     | Low       |
| B18     | 1.8 ± 0.3 | —        | 96 ± 2     | —        |
| cAR1    | 3.5 ± 0.3 | —        | 370 ± 6    | —        |
| cAR2    | 11 ± 0.6  | —        | 210 ± 3    | —        |
| Δ208/cAR3 | 4.6 ± 2.3 | 220 ± 81  | 34 ± 11    | 540 ± 110 |
| —        | 2.9 ± 0.6  | 160 ± 37  | 64 ± 7     | 580 ± 76  |

*— binding not detected.

| Cell          | Sites/cell | Kd (nM) | Sites/cell | Kd (nM) | Sites/cell | Kd (nM) |
|---------------|------------|---------|------------|---------|------------|---------|
| Cyclic Nucleotide Specificity of cAR Subtypes—Each of the 14 cAMP derivatives was assayed for the ability to bind to the endogenous receptors in developed wild-type cells (Van Haastert and Kien, 1983). The data are presented as Kd or Kd derivative/Kd cAMP ratios (Table III) and δΔG values (Table IV). δΔG values are derived from the following equation to compare these results to previous studies (Jastorff et al., 1979).

δΔG = RT ln Kd derivative/Kd cAMP

δΔG 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), 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).

The high affinity of 8-Br-cAMP in developed wild-type cells has been attributed to the presence of hydrogen bonds. 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...
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**FIG. 4.** Scatchard analysis of cAR cells in ammonium sulfate. Receptor affinity was determined by the binding of [3H]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 × 100, respectively. See Table II for binding parameters.

**TABLE III**

Specificity of cAR subtypes in ammonium sulfate and phosphate buffer

| Derivative* | WT-D | cAR1 | cAR2 | cAR3 | Δ208/cAR3 | cAR1 | cAR3 |
|-------------|------|------|------|------|-----------|------|------|
| 1. cAMP     | 1    | 1    | 1    | 1    | 1         | 1    | 1    |
| 2. N'-O-cAMP | 58   | 51   | 3    | 21   | —         | 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  | 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 | 7    | 18   | 19   | 23   | —         | —    | —    |
| 9. (S)-cAMPS | 110  | 29   | 5    | 3    | 1         | 68   | 15   |
| 10. (R)-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|

* See Fig. 1 and Table I.
*—, not determined.
* Value differs from that of Van Haastert and Kien (1983).
* Data from one experiment.

phosphate moiety of cAMP. cAR1 bound (S)-cAMP and (R)-cAMP, 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)-cAMP with 6- and 30-fold higher affinity, respectively, than cAR1, and both bound (R)-cAMPs...
with affinities similar to that of cAR1 (Table III). In the Δ208/cAR3 cells, where the low levels of cAR1 were absent, cAR3 bound (S₆)-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 not ionic, since a loss of about 25 kJ/mol binding energy.

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 N⁶-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.

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 comparison 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).

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. 5.** Inhibition of [³H]cAMP binding by cAMP and (S₆)-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 (S₆)-cAMPS (open squares).

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### TABLE IV

| Derivative* | Ammonium sulfate | Phosphate buffer | Polarity |
|-------------|------------------|------------------|----------|
|             | WT-D             | cAR1             | cAR2     | cAR3     | Δ208/cAR3 | cAR1 | cAR3 |          |
| 1. cAMP     | 0.0              | 0.0              | 0.0      | 0.0      | -4.49     | 0.0  | 0.0  | 0.0       |
| 2. N⁶-O-cAMP| 9.2              | 8.9              | 2.5      | 6.9      | -3.69     | 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     | -2.65     | 10.5 | 10.4 | 0.36      |
| 5. 8-Br-cAMP| 11.8             | 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  | -0.48     |
| 7. 3'-NH-cAMP| 15.2             | 13.6             | 13.4     | 11.1     | -2.65     | 14.9 | 16.7 | -0.02     |
| 8. 5'-NH-cAMPd| 4.5              | 2.2              | 2.3      | 2.6      | -2.65     | -    | -    | -1.65     |
| 9. (S₆)-cAMPS| 10.7             | 7.7              | 3.5      | 2.5      | -0.4      | 9.5  | 6.1  | 1.63      |
| 10. (R₆)-cAMPS| 10.8             | 10.4             | 11.8     | 9.2      | -2.65     | 11.8 | 14.1 | 0.66      |
| 11. cBIMP   | 11.5             | 11.1             | 12.4     | 7.5      | -2.65     | 14.4 | 15.5 | 2.75      |
| 12. PuRMP   | 16.0             | 15.7             | 14.7     | 12.0     | -2.65     | 18.4 | 20.5 | -0.46     |
| 13. cIMP    | 21.7             | 21.4             | 17.9     | 21       | -2.65     | 19.5 | 23.6 | -3.41     |
| 14. cGMP    | 22.7             | 22.1             | 17.2     | 22.7     | -2.65     | 20.9 | 23.6 | -3.21     |

* See Fig. 1 and Table I.

**TABLE V**

| 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.” |

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with affinities similar to that of cAR1 (Table III). In the Δ208/cAR3 cells, where the low levels of cAR1 were absent, cAR3 bound (S₆)-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 N⁶-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.

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 comparison 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).

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
distinct from other CAMP-binding proteins in Ments-Cohen and Van Haastert, 1989), cAMP receptors were detected in wild-type NC-4 cells (Van Ments-Cohen and Van Haastert, 1989). As kinase A (CAK) and extracellular phosphodiesterase (ePDE).

The analog specificities of the three cARs were compared with the pharmacological specificity of cAR3. The exposure of aggregation competent cells to UM levels of CAMP for several hours depletes the cells of cAR1 mRNA 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 photolabeled with $^{32}$P-8-N$_3$-cAMP at 6 h of development is cAR1 (Klein et al., 1987). However, (S$_2$)-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).

**TABLE V**

| WT-D | cAR1 | cAR2 | cAR3 | CAK | ePDE |
|------|------|------|------|-----|------|
| WT-D | 1.0  | 0.99 | 0.87 | 0.95| -0.32| 0.21|
| cAR1 | 1.0  | 0.98 | 0.97 | -0.35| 0.22 |
| cAR2 | 1.0  | 0.86 | 0.97 | -0.33| 0.24 |
| cAR3 | 1.0  | 0.67 | 0.97 | -0.21| 0.07 |
| CAK  | 1.0  | 0.21 | 0.97 | 0.01 | 1.0  |
| ePDE | 1.0  | 0.21 | 0.97 | 0.01 | 1.0  |

**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 similar 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 um levels of cAMP for several hours depletes the cells of cAR1 mRNA...
and protein, yet approximately 5 x 10^5 cAMP binding sites still persist. The Kᵦ of these remaining sites is near 700 nM which is similar to the affinity of cAR3. Furthermore, cAR3 is maximally expressed in mound stage cek3. This evidence which is similar to the affinity of cAR3. Furthermore, cAR3 is also a functional receptor since the α208/cAR2 cells display several cAMP-stimulated responses in vivo. 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, Nα-cAMP and (S)-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 Nα-cAMP with about 17- and 7-fold greater affinity relative to cAR1 and cAR3, respectively. cAR3 has a relative affinity for (S)-CAMPS that is 30-fold higher than that of cAR1 (Fig. 5). In addition, cAR3 binds bIMPPα 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, (S)-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)-CAMPS than cAR1. (S)-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 locate a cAMP binding site or account for the differences in analog specificity with the differences in amino acid sequences alone. Each of the cARs has been photoaffinity labeled with [32P]8-N3-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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